



**Long term Efficacy of a Pre-erythrocytic malaria vaccine and correlates of
protection in children residing in a malaria endemic country**

A thesis submitted for degree of Doctor of Philosophy.

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iv. Author's contribution

Prior to my D. Phil studies, I participated as a Co-principal investigator, in setting up the original randomized controlled trial to assess the safety and efficacy of RTS,S/AS01E against clinical malaria by *P. falciparum* in young children aged 5-17 months residing in Kilifi, Kenya, and in Korogwe, Tanzania. This trial was sponsored by GlaxoSmithKline Biologicals (GSK Biologicals) and ended in November 2008. I then led an investigator-led extension of follow-up beyond November 2008, which was completed in November 2012. I registered in October 2009 for my D Phil. The extended follow up in the field conducted between October 2009 and November 2012, in conjunction with a series of epidemiological analyses form the basis of this D Phil thesis. I detail my exact contributions by chapter below.

Chapter 2: Comparison of Active and Passive Surveillance Methods

Data were provided from active surveillance of the RTS,S cohort (for which I supervised the field work), and from passive surveillance at Pingilikani dispensary (which I was not involved in). I planned and conducted the analysis that I present below.

Chapter 3: Exposure Index.

Data were provided from longitudinal surveillance of three cohorts (Junju, Chonyi and Ngerenya) over a 12 year period. I planned and conducted the analysis presented below.

Chapter 4: Long term efficacy of RTS,S/AS01

I participated in the protocol writing, training study staff and supervising research assistants and project manager in the field. I also supervised all the field work activities. I wrote the statistical analysis plan, and conducted the analysis including further exploratory analysis which I present below.

Chapter 5: Correlate of protection: Anti-CS protein antibody

I wrote the statistical analysis plan and conducted the analysis of the data. I designed and supervised collection and shipment samples from the field. Measurement of antibody titres was done by research scientists at GSK Biologicals Laboratory.

Chapter 6: Correlate of protection: T cell immune responses

I supervised collection of the samples from the field and participated in the separation of peripheral blood mononuclear cells from the whole blood, but I did not conduct the CMI assays. I conducted the analysis that I present below.

Chapter 7: Anti-CS protein antibody avidity and protection

I conducted the avidity assays at Centre for Vaccinology (CEVAC), Ghent University and analysed the data.

The literature review presented in chapter 1, presentation of results and interpretation, and concluding remarks in chapter 8 are my own work.

Some of the work presented in this thesis has also been published in peer review journals as follows:

1. Chapter 2: **Olotu A**, Fegan G, Williams TN et al. *Defining clinical malaria: the specificity and incidence of endpoints from active and passive surveillance of children in rural Kenya*. PLoS One. 2010 Dec 16;5(12)
2. Chapter 3: **Olotu A**, Fegan G, Wambua J, Nyangweso G, Ogada E, Drakeley C, Marsh K, Bejon P. *Estimating individual exposure to malaria using local prevalence of malaria infection in the field*. PLoS One. 2012;7(3)
3. Chapter 4: **Olotu A** et al, *Four-year efficacy of RTS,S/AS01E and its interaction with malaria exposure*, NEJM. 2013 Mar 21
4. Chapter 5: **Olotu A**, Lusingu J, Leach A et al. *Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-CS protein antibody titres and protection in children aged 5-17 months in Kenya and Tanzania: a randomised controlled trial*. Lancet Infect Dis. 2011 Feb;11(2):102-9
5. Chapter 6: **Olotu AI**, Fegan G, Bejon P. *Further analysis of correlates of protection from a phase 2a trial of the falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naïve adults*. J Infect Dis. 2010 Mar 15;201(6):970-1.
6. Chapter 7: **Olotu A**, Moris P, Mwacharo J, et al. *Circumsporozoite-specific T cell responses in children vaccinated with RTS,S/AS01E and protection against P falciparum clinical malaria*. PLoS One. 2011;6(10)

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v. Abstract

Malaria remains an important cause of morbidity and mortality among children in sub-Saharan Africa despite recent reductions in malaria incidence in some parts of Africa. Current control tools face threats such as the emergence of drug resistant parasites and insecticide resistant mosquitoes. A malaria vaccine is needed to complement and/or replace existing tools in order to achieve better malaria control and eventually eliminate the disease.

RTS,S/AS01E is the most clinically advanced pre-erythrocytic malaria vaccine candidate and is currently being tested in a phase III trial. The short-term efficacy of RTS,S/AS01E is known but the duration of protection is unknown. Furthermore, although RTS,S is protective, it is unclear which immunological assays predict efficacy: hence there are no known correlates of vaccine-induced protection against clinical malaria.

In a randomized controlled trial, I assessed the efficacy of RTS,S/AS01E in children (5-17 months old) residing in Kilifi, Kenya, over 4 years of follow-up and determined the correlates of protection against clinical malaria. In order to examine the effect of variations in malaria exposure on vaccine efficacy, I developed an individual marker of malaria exposure calculated as distance-weighted prevalence of malaria infection within 1 km radius of every child.

Over 4 years of follow-up, RTS,S/AS01E had an efficacy of 29.9% (95%CI: 10.3% to 45.3%, $p=0.005$) and 16.8% (95%CI: -8.6% to 36.3% $p=0.18$) against first and all malaria episodes, respectively (by intention to treat analysis). Vaccine efficacy waned over time and with increasing malaria exposure. RTS,S/AS01E efficacy was 43.6% (95% CI, 15.5 to 62.3) in the first year but was -0.4% (95% CI, -32.1 to 45.3) in the fourth year. Vaccine efficacy was 45.1% (95%CI 11.3% to 66.0%) among children with lower than average malaria exposure index, but 15.9% (95%CI -11.0 to 36.4%) among children with higher than average malaria exposure index. Despite waning in efficacy, RTS,S/AS01E averted 65 cases of malaria per 100 vaccinated children, with more cases averted among the children in the higher malaria-exposure cohort (78 cases per 100 vaccinated children) than those the low exposure cohort (62 cases per 100 vaccinated children).

RTS,S/AS01E induced high titres of anti-CS protein antibodies and CD4⁺ T cell but not CD8⁺ T cell responses. Anti-CS antibody titres and the frequency of TNF- α producing CD4⁺ T cell responses were independently associated with protection from clinical malaria, and the combination of both anti-CS titers and TNF- α producing CD4⁺ T cell response satisfied the Prentice criteria for surrogate markers of protection.

There was no association between avidity of RTS,S-induced anti-CS protein antibodies and protection from clinical malaria.

Conclusions:

RTS,S/AS01E efficacy against all episodes is 16.8% over the 4 years of follow-up. The vaccine efficacy wanes over time and with increasing malaria exposure. RTS,S/AS01E-induced TNF- α producing CD4 T cell and anti-CS protein antibody responses were independently associated with protection from clinical malaria. Anti-CS avidity did not predict protection from clinical malaria. Long-term follow-ups of malaria vaccine trials are essential in the evaluation of the longevity of vaccine efficacy.

1 Introduction

1.1 Burden of malaria

Malaria is one of the most important causes of mortality and morbidity in the developing world. Estimates from the World Health Organization are 261 million cases in the year 2010, with 650,000 cases resulting in death [1]. Other estimates suggest more than 1,000,000 deaths in 2010 [2]. The estimates are highly influenced by the surveillance methods used [3].

The highest burden is in children below the age of five years (>85% of the burden). In addition to the immediate morbidity and mortality risk, malaria may inflict long-term effects with negative consequences on the health and quality of life. For instance, children who suffer from severe malaria are at increased risk of epilepsy [4,5], chronic neurological and cognitive impairments [6].

Malaria causes significant economic hardships and disproportionately affects the poor [7]. In endemic countries, more than 1% loss in gross domestic product is attributable to malaria [8] [9].

1.2 Current malaria interventions

The main interventions for malaria control include insecticide treated bed-nets (ITNs), treatment with artemisinin based combination therapies (ACTs), intermittent presumptive treatment (IPT) and indoor residual spraying (IRS).

1.2.1 Insecticide treated bed nets (ITNs)

ITNs are effective in reducing malaria transmission [10] and their use is associated with up to a 40% reduction in all-cause child mortality [11,12]. Their use has increased over the years in line with the Roll Back Malaria and UN Millennium development goals programmes [13,14,15]. In Kenya, bed net usage in children has increased from 7.1% in 2004 to more than 65% in 2006 [16]. The increase in use among the poor has largely been attributed to the free bed net distribution programme [16,17]. The emergence of mosquitoes that are resistant to pyrethroids and others with altered biting behaviour (towards outdoor biting) is of concern, since these developments could undermine ITN effectiveness [18,19].

1.2.2 Antimalarial drugs

Artemisinin based combination therapies (ACTs) are the recommended first line treatment for clinical malaria. They include combinations such as artemether-lumefantrine, artesunate-amodiaquine, and dihydroartemisinin-piperaquine. ACTs are effective against both mild and severe forms of malaria [20,21], and have a good safety profile [22,23]. In addition, they inhibit gametocytes [24] and therefore may play a role

in protecting the community against further transmission [25]. The development of drug resistance in the parasite threatens their effectiveness [26,27,28].

1.2.3 Intermittent presumptive treatment

Intermittent presumptive treatment (IPT) involves administration of a full therapeutic course of antimalarial to individuals who are at risk regardless of their malaria status at the time of administration. The aim of the IPT is to reduce subsequent episodes of clinical malaria. It is a recommended approach to the prevention of malaria in pregnancy (IPTp) and infant (IPTi). IPTp protects pregnant women from malaria infection and anaemia [29] and when given in the background of ITN use, leads to substantial reductions in neonatal mortality and low birth weight outcomes [30,31].

IPTi is safe and provide significant protection against clinical malaria and all-cause mortality in infants [32]. Intermittent presumptive treatment in children (IPTc) is a recent IPT strategy which has also shown promising results, with efficacy of 82% (95%CI 75%-87%) against clinical malaria in children during the immediate malaria transmission season and 57% reduction in all-cause mortality in children[33]. Malaria rebound as the result of delayed immunity acquisition appears to be a risk in chemoprophylaxis, but has not been consistently identified following IPT [33]. The development of antimalarial drug resistance poses a challenge to the IPT strategy [34,35].

1.2.4 Indoor residual spraying

Indoor residual spraying (IRS) has been shown to be effective in reducing malaria transmission in areas of different malaria transmission intensities [36]. Since the World Health Organization (WHO) reaffirmed the importance of IRS in the fight against malaria, several countries have reintroduced it in their malaria control armoury [37]. It is however more effective against endophilic than exophilic vectors [38]. Like ITNs, changes in the vector's behaviour and the development of resistance against insecticides are imminent challenges facing its effectiveness [39,40].

Hence the development of resistance in either the vector or parasite threatens current control measures and might lead to the resurgence of malaria in areas where malaria has been controlled.

1.3 Changing malaria epidemiology

There are encouraging reports showing a decline in malaria from several parts of Africa [41,42,43,44]. In Kenya, malaria admissions in children in the three major district hospitals in the coast have dropped significantly between 1999 and 2007 [45]. Time-series models suggested that the observed decline in malaria admissions correlated with malaria-specific control efforts in the hospital catchment's areas. However, this trend is not consistent everywhere. Some areas have reported sustained or even an increase in the burden of malaria [46,47]. Temporal relationships with the control interventions described above are seen in some cases [42] but are not so clear in other cases [45]. Some changes in transmission intensity may be attributed to improved living standards and/or be part of natural variations [48].

Hence, while progress has been made in controlling malaria using the interventions described above, this progress may be threatened by resistance in the parasite and in the vector, and in any case these interventions do not appear sufficient to eliminate malaria in high transmission settings in Africa [49].

1.4 Role of malaria vaccines

Childhood immunization against communicable diseases is one of the most cost-effective public health interventions [50,51]. The global health success of eradicating smallpox was the result of a widespread deployment of smallpox vaccine [52]. Furthermore, significant inroads have been made in the fight against other major childhood infectious diseases such as polio, *Haemophilus influenzae* and measles through immunization [53,54,55]. The success of vaccines is based not only on their ability to offer protection to the individual recipients, but also to the community by reducing the transmission through herd immunity [56,57].

Given the challenges facing current interventions and healthcare systems with limited provision for prompt diagnosis and treatment [58], a vaccine would be an important addition to the existing control methods in the fight against malaria.

There are three types of malaria vaccines based on the stages of the *Plasmodium* life cycle at which they act. They include pre-erythrocytic, blood stage and transmission-blocking vaccines. They are further described below.

1.4.1 Pre-erythrocytic vaccines

Pre-erythrocytic vaccines target sporozoites and liver-stage parasites, before they infect erythrocytes. They are based on the parasite antigens present on either the surface of the sporozoite or expressed on the surface of infected hepatocytes. They work by inducing antibodies that neutralize sporozoites, block sporozoite invasion of hepatocytes, or by provoking cytotoxic cellular responses against infected hepatocytes.

The most clinically advanced pre-erythrocytic candidate vaccine is RTS,S which is based on Circumsporozoite protein (CSP)[59]. The candidate vaccine has demonstrated a partial protection against clinical malaria in children residing in a malaria endemic country over a short period of follow-up and the long term outcome of RTS,S vaccination is the subject of this thesis. The clinical development of RTS,S is reviewed in greater detail in section 1.5 below.

Thrombospondin-related adhesion protein (TRAP) fused to a multi-epitope (ME) is another candidate pre-erythrocytic vaccines currently in phase II trials. This antigen is delivered using a prime-boost strategy where Fowlpox strain 9 virus or a chimpanzee adenovirus expressing the ME-TRAP construct is used to prime the immune system and modified vaccinia virus Ankara (MVA) expressing ME-TRAP is used to boost the responses. However, despite encouraging protection against experimental malaria challenge in malaria naïve adults [60] the approach of priming with the Fowlpox strain FP9 then boosting with MVA was not protective in malaria endemic areas [61]. Recent studies using the chimpanzee adenovirus ChAd63 as the priming vector in place of FP9 have shown superior immunogenicity both in malaria naïve and semi-immune adults [62,63].

Sanaria is developing a live irradiated whole sporozoite vaccine for human immunization [64]. The candidate irradiated sporozoite vaccine is in early development but preliminary data have shown inadequate levels of cellular immune responses in malaria naïve adults who received the vaccine by the subcutaneous route [65]. Studies are ongoing to optimize the dose and route of inoculation to facilitate adequate delivery of attenuated sporozoites to the hepatocytes, which is required to provoke a protective immunological response.

It has also been shown that immunisation using multiple bites of infected mosquitoes under cover of simultaneous chloroquine prophylaxis to prevent blood-stage infection confers sterile protection against homologous malaria challenge [66,67,68]. This strategy provides a platform for studying the pre-erythrocytic and erythrocytic mechanisms of malaria protective immunity [69] and open new avenues for the development of next-generation vaccination strategies that completely prevent malaria.

1.4.2 Blood stage vaccines

Most blood stage vaccines in development aim at mimicking and improving the natural immunity to blood stage parasites. Rationale for the development of blood stage vaccines came from two sources; a) natural immunity to clinical malaria develops after repeated attacks of malaria and adults are relatively resistant to severe forms of disease compared to young children and b) in 1961 it was shown that passive transfer of immunoglobulin from immune African adults to young children can control parasite growth and malaria symptoms [70]. In 1991 it was shown that passive transfer of immunoglobulin from African adults cleared malaria parasitaemia and clinical symptoms in acutely infected Thai adults [71]. Most of the research on blood stage vaccines has focused on the

antigens which are critical in the host-parasite interactions and expressed on the merozoite.

There are many blood stage antigens expressed on the merozoites which have been intensively investigated as potential malaria vaccine candidates. They include merozoite surface protein 1 (MSP-1)[72], MSP-2 [73], MSP-3 [74,75], apical membrane antigen 1 (AMA-1)[76], erythrocyte-binding antigen-175 (EBA-175) [77], glutamate-rich protein (GLURP) [75,78]), and serine repeat antigen 5 (SERA5)[79]. None of these candidate vaccines have been progressed to phase III trials because of limited efficacy.

Although the safety and immunogenicity of many blood stage candidate vaccines have been promising, the efficacy has not been. For example, a three-component blood stage vaccine consisting of MSP-1, (MSP-2) and ring-infected erythrocytic surface antigen (RESA) showed modest strain-specific reduction of parasite density in Papua New Guinea but had no effect on clinical malaria episodes [73]. Similarly, (AMA-1) failed to provide protection against clinical malaria in Malian children. However, the authors showed strain-specific protection against clinical malaria caused by parasites with AMA-1 genotypes similar to that used in the vaccine [80].

High rates of genetic polymorphism among blood stage antigens presents a major challenge for the development of blood stage vaccines [81]. This implies that a combination of multiple antigens in one construct is necessary to achieve significant protection.

Recently *P. falciparum* reticulocyte-binding protein homologue 5 (PfRH5) has been identified as potential erythrocytic candidate malaria antigen with less polymorphisms

and the ability to induce cross neutralizing antibodies against heterologous parasite strains [82] . The antigen is still in pre-clinical stages of development.

1.4.3 Transmission blocking vaccines (TBV)

A transmission blocking vaccine targets the sexual stages of parasite growth, preventing fertilization and the development of malaria parasites in the mosquito midgut. When deployed in a sufficient proportion of population, it can reduce the intensity of malaria transmission. Transmission blocking vaccines will not protect individual vaccinees, but will protect the community from malaria. Therefore to achieve significant impact they need to be given to a high proportion of the population.

The target antigens under development include those expressed by the gametocytes within the human host such as Pfs45/48 and Pfs230 and those expressed in the zygote and ookinete within the mosquito gut such as Pfs25 and Pfs28. Antibodies to these antigens can completely block transmission of *P falciparum* to mosquitoes [83,84], and early phase I trials with Pfs25 and Pvs25 have demonstrated a correlation between levels of anti-Pfs25 and Pvs28 and transmission blocking activity [85,86].

By preventing transmission of breakthrough infections in individuals who have received other types of malaria vaccines or antimalarial drugs, it is proposed that TBVs can be used to prolong the protective efficacy of other malaria vaccines and antimalarial drugs respectively [87]. Challenges facing development of TBVs include a) high antibody levels required for inhibition of transmission as predicted from mathematical models [88] b) Poor immunogenicity of the antigens; c) lack of natural boosting for antigens

expressed by the ookinete [89]. Transmission blocking vaccines are still in early stages of development with no field trials yet and only a handful of phase I trials in preparation.

An alternative vaccine strategy for transmission blocking involves targeting mosquito's proteins for immunization to induce transmission blocking immune responses that would affect the mosquito's gut susceptibility to ookinete invasion [90]. Studies have shown the ability of monoclonal antibodies to several midgut molecules to block the development of plasmodium parasite (including *P. falciparum* and *P. vivax*) in multiple species of *Anopheles* mosquitoes [91,92,93]. This is an ongoing field of research.

1.5 Introduction to RTS,S: a candidate pre-erythrocytic malaria vaccine

1.5.1 Circumsporozoite protein (CS protein)

CS protein (CSP) is the major component of the surface of the plasmodium sporozoite. It is also located intracellularly within vesicular structures resembling micronemes within the sporozoites [94]. Its expression starts at the oocyst stage in the mosquito and persists throughout intra-hepatic stages of plasmodium development, but declines as hepatic schizonts mature[94,95].

A single copy of *P. falciparum* CS gene encodes 412 amino acids and has a similar molecular structure to CS gene of other *Plasmodium* species. The protein contain 41

tandem repeat amino acids of which 37 are NANP (N, Asparagine; A, Alanine; P, Proline) and 4 are NVDP (N; Asparagine, V; Valine, D; Aspartic acid, P; Proline)[96]. The protein has a molecular size of 58 kD.

CS protein contain three main regions a) polymorphic N terminus that binds heparin sulfate proteoglycans b) a conserved central region consisting of tetrapeptide repeats (NANP), and c) a polymorphic C terminus that contains a thrombospondin-like type I repeat (TSR) domain[97]. The central conserved region is termed the R region while the polymorphic N and C termini are referred to as RI and RII-plus respectively. The R region is considered a dominant B cell epitope while RI and RII contain both B and T cell epitopes.

The CS protein plays an important role in the motility of the sporozoites and their interaction with the sulphated glycoconjugate receptors on the hepatocyte surface, which is crucial for the invasion process. The N-terminus region is believed to be important for the sporozoite invasion of mosquito salivary glands [98,99] while the C-terminus region is involved in motility of sporozoites and their invasion of the mosquito salivary gland and human hepatocytes [100]. Expression of CS peptides on the surface of the infected hepatocyte can initiate Major Histocompatibility Complex (MHC) restricted, and antigen specific killing of the *P. falciparum* infected hepatocyte following recognition by the T-cells [101].

1.5.2 Natural anti-CS protein antibodies

Natural exposure to malaria induces detectable antibodies to CS protein albeit at relatively lower levels compared to those induced by inoculation by irradiated sporozoites or CS based subunit vaccines. Their level reflects the magnitude of exposure to *P. falciparum* sporozoites providing a useful measure of malaria transmission [102]. No consistent correlation has been found between anti-CS protein occurring under natural condition and immunity to infections [103,104] although antibodies against multiple pre-erythrocytic antigens may be a correlate of protection [105]. Given the protection that can be achieved with CSP based vaccination regimens (see below), the lack of protection with naturally acquired anti-CSP antibodies is likely to reflect the low titre of these antibodies.

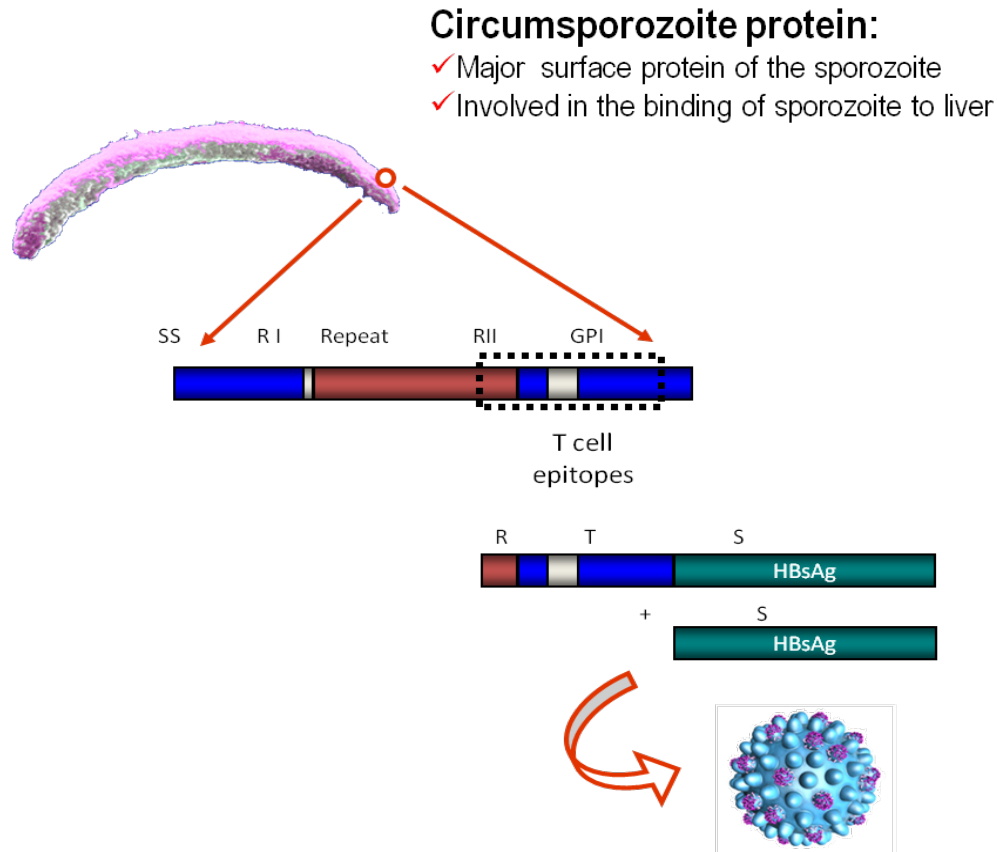
1.5.3 RTS,S antigen

RTS,S is a recombinant protein produced in *Saccharomyces cerevisiae* (yeast) cells by GlaxoSmithKline Biologicals (GSKBiologicals). It is a 51 kDa (kilodalton) hybrid protein which consists of central repeat and C-terminal flanking regions of CS protein (CSP) from the NF54 strain of *P. falciparum* fused with the hepatitis B surface (S) antigen (HBsAg). The antigen is co-expressed with unfused 24 kDa native Hepatitis B surface antigen (HBsAg). When produced, the proteins spontaneously assemble into virus like particle (Figure 1:1: RTS,S particles). Each particle is estimated to contain 100 polypeptides.

RTS,S has been given with either of two different proprietary adjuvants to boost its immunogenicity; AS02 (an oil-in-water emulsion formulated with MPL® and Stimulon® QS21 immunostimulants) or AS01 (a liposomal formulation of the same immunostimulants, MPL and QS21). Monophosphoryl lipid A (MPL) molecule is a detoxified version of the parent highly toxin lipopolysaccharide from Gram-negative bacterium *Salmonella minnesota*. MPL is a strong stimulant of both humoral and cellular immune responses. QS21 is a natural saponin purified from the bark of the tree *Quillaja saponaria* which induces strong Th1 type and cytotoxic T lymphocyte cell-mediated immune responses.

The nomenclature for the various formulations of RTS,S is as follows; a) "RTS,S" describes the protein as described above, "AS01" or "AS02" after a "/" indicates the Adjuvant System that is combined with the particle, and a letter suffix "A" or "B" indicates an adult dose formulation (i.e. 50 µg of lyophilized RTS,S reconstituted with 500 µL of either AS02 or AS01, respectively) and "D" or "E" indicates a paediatric dose formulation (i.e. 25 µg of RTS,S with 500 µL of either AS02 or AS01, respectively). Hence, for example, RTS,S/AS01E indicates the RTS,S protein, formulated with AS01 as a paediatric dose.

Figure 1:1: RTS,S particles; Source (GSK Biologicals RTS,S power point 2012)



1.5.4 RTS,S development: Initial proof of concept

The demonstration that immunization with radiation-attenuated sporozoites confers sterile protection against experimental malaria challenge in mice and humans was an important milestone in the quest for a malaria vaccine [106,107]. Irradiated sporozoites induced anti-CS protein antibodies a thousand-fold higher than those induced from natural exposure, and those antibodies correlated with protection from malaria challenge [108]. However, unavailability of purified aseptic sporozoites and safety and logistical challenges complicated the development of whole sporozoites as a deployable vaccine.

The discovery that CS is the major component of *P falciparum* sporozoite surface [109] and that monoclonal antibodies against this particular protein correlated with the protection against malaria challenge [110] [111] paved the way for subunit vaccine development. DNA recombinant technology revolutionized the development of subunit malaria vaccine and enabled the expression of CS protein in a prokaryotic system [112].

It was later demonstrated that several CS repeat constructs expressed in *Escherichia coli* were highly immunogenic in mice, reacted with CS protein on live sporozoite and blocked the sporozoite invasion of human hepatoma cells [112]. R32tet₃₂ which consisted of 30 (Asn-Ala-Asn-Pro) tetrapeptide repeats and 2 (Asn-Val-Asp-Pro) tetrapeptide repeats fused to 32 amino acids derived from the tetracycline resistance gene was found to be the most immunogenic construct producing sustained level of antibody levels when given with alum[113].

In the early 1980's the Walter Reed Army Institute of Research (WRAIR) and GSK Biologicals initiated a collaboration to develop a malaria vaccine based on this CS protein construct after evidence supported their role as target for functional antibodies [114]. The original objective for WRAIR was to develop a malaria vaccine for USA military while GSK Biologicals aimed to develop a vaccine for tourists.

1.5.5 Phase 1 and 2a trials

Early phase I trials in malaria naïve adults showed that immunization with the CS repeat antigen combined with the first 32 amino acids from a tetracycline resistance gene (R32tet₃₂) produced detectable antibody responses to CS protein repeat region and the antibody levels correlated with protection from homologous experimental malaria

challenge [115]. However, subsequent trials showed minimal protection (0-20%) and low antibody titres [116,117,118].

Two modifications were then done on the CS repeat antigen a) Inclusion of T cell epitopes in the C terminus part to include more potent T cell epitopes and b) utilize hepatitis B surface antigen as the carrier matrix to improve antigen presentation. This resulted into RTS,S which consisted of 19 NANP repeats and the carboxy terminus (amino acids 210-398) of the circumsporozoite (CS) antigen co-expressed in yeast with hepatitis B surface antigen. The result was improved cellular and humoral immunogenicity in malaria naive adults [119]. However protection against experimental malaria challenges was still limited (6 out of 8 vaccinated-individuals got malaria after challenge).

It was hypothesized that use of more potent adjuvant could improve efficacy of RTS,S. Through series of studies conducted in non-human primates, two potent adjuvant systems developed by GSK Biologicals were selected to be tested with RTS,S in humans [120,121]. In a landmark study involving malaria naive adults from United States three formulations of RTST,S were assessed for safety, immunogenicity and efficacy against experimental challenge. RTS,S given with AS02 provided an efficacy of 85% against homologous experimental challenge. [122]. This was the initial proof of concept for RTS,S and prompted further clinical development of this antigen as a candidate malaria vaccine.

1.5.6 Phase 2b trials

Following the success of the RTS,S/AS02 in malaria naïve adults [122] a small phase I study was undertaken to assess the safety and immunogenicity of RTS,S/AS02 in semi-immune adults in Gambia. The results showed that RTS,S/AS02 vaccination had good safety profile and induced higher antibody titres against CS protein than normally acquired from natural exposure to infected mosquito bites [123]. Later, a larger phase IIb trial of RTS,S/AS02 in the same population demonstrated 71% efficacy against malaria infection during the first nine weeks of follow-up, but efficacy declined to 0% in the last 6 weeks of follow-up [124]. Booster RTS,S doses given 1 year later induced even higher antibody responses than acquired after the primary schedule and individuals were further protected against infection in the subsequent malaria season with vaccine efficacy of 47% (95%CI: 4% to 71%, $p=0.037$). Although the results were considered promising, the partial protection meant that the vaccine could not qualify as a vaccine for the USA military or for tourists.

1.5.7 Paediatric vaccine development

Paediatric development of RTS,S was undertaken despite short term efficacy. The following were the justifications for the paediatric development of RTS,S; a) although the efficacy against infection in Gambian adults was partial and short-lived, [125], it was anticipated that vaccination of children who suffer the most from malaria would result in a significant reduction in frequency and severity of clinical malaria and; b) because other

vaccines had raised better immunological responses in children compared with adults [126,127].

The first proof of concept study of RTS,S given with adjuvant AS02 was conducted in Mozambican children following a series of age de-escalation and dose finding studies. The study consisted of two cohorts. In the first larger cohort, older children (1-4 years) were recruited to assess efficacy against mild and severe clinical malaria. In the second smaller cohort, young infants (6-12 weeks) were recruited to assess safety, immunogenicity and efficacy of RTS,S against malaria infection. In the smaller cohort the adjusted efficacy against infection was 35.4% (95% CI 4.5% to 56.3%; $p = 0.029$) over 6 months and 9.0% (95% Confidence Interval (CI) -30.6% to 36.6%; $p = 0.609$) over subsequent 12 months showing evidence of waning over time[128]. In the larger cohort adjusted efficacy was at 32.9% (95% CI 11.0% to 44.8%; $p=0.004$) and 57.7% (95% CI 16.2% to 80.6%; $p=0.019$) against clinical episodes and severe malaria, respectively, over eight months of surveillance [129].

These first trials in children used the AS02 adjuvant, but parallel preclinical and clinical trials in animals and naive adults had demonstrated higher immunogenicity and similar safety profile for the adjuvant AS01 [130,131]. Subsequently the focus shifted to AS01 as a more promising adjuvant for consideration in clinical development of RTS,S.

The first evidence of field efficacy for RTS,S given with AS01 was obtained in Kilifi, Kenya, and Korogwe, Tanzania in children aged 5-17months. The estimated efficacy against clinical episodes of malaria was 53 % (95% CI 28% to 69%) over 8 months follow up [132]. In a separate study, co-administration of RTS,S/AS01E with Extended

Program of Immunization (EPI) vaccines was safe and did not reduce the frequency of protective immune responses to the other EPI vaccines [133].

1.5.8 Long term efficacy

The long-term efficacy of RTS,S/AS01E is not known. Only one study in Mozambican children has documented the long-term efficacy of RTS,S given with a less immunogenic adjuvant, AS02. Over four years of follow-up adjusted efficacy of RTS,S/AS02 against clinical malaria was 25.6% (95% CI, 13.4% to 36.0%; $P < 0.001$) with no evidence of waning and adjusted efficacy against severe malaria was 38.3% (95% CI: 3.4% to 61.3%; $P < 0.045$)[134]. However the long term follow-up study in Mozambique used passive surveillance to identify cases of clinical malaria and observed relatively few clinical episodes of malaria during the last two years of follow-up (230 episodes compared to 664 episodes in the first two years). This reduced the power of the study to identify waning during the later years of follow-up. Furthermore, since efficacy was relatively modest initially (32.9%), the power to detect variation over time was reduced further.

1.6 Vaccine-induced immune responses

Vaccines are designed to induce protective long-term immunological memory capable of clearing infection or minimizing the severity of the disease once a recipient encounters the pathogen. Effector mechanisms include antibodies, cytotoxic CD8⁺ T lymphocytes (CTL) and CD4⁺ T helper cells.

1.6.1 Vaccine-induced antibody responses

Following vaccination, inflammatory cells such as local monocytes, immature dendritic cells and neutrophils are recruited to the site of inoculation. Vaccine antigens are then phagocytosed by immature dendritic cells which undergo activation and migration to the nearby draining lymph node. At the same time, antigens which are not phagocytosed may also find their way to the local lymph nodes through the lymphatic system.

At the local lymph node, naive B lymphocytes in extra-follicular zones recognize these vaccine antigens and in the absence of CD4 T helper cells undergo differentiation and proliferation into short-lived plasma cells which produce antigen-specific antibodies. These antibodies which appear within a few days following (primary immune response) vaccination are mainly of IgM isotype and of low affinity and titre.

It is the interaction between activated dendritic cells and antigen-specific T lymphocytes that stimulate B cells to produce higher avidity antibody. The interaction between dendritic cells and T cells activates antigen-specific T lymphocytes which in turn triggers antigen-specific B lymphocytes to migrate to the follicular zone and initiate germinal centre reaction (GC). Within GCs, B lymphocytes receive activation from follicular T lymphocytes resulting in massive clonal proliferation, hypermutation of their immunoglobulin genes, isotype switching (from IgM to IgG isotypes) and differentiation into plasma secreting cells. Plasma cells producing antibodies of low affinity undergo apoptosis while those producing high affinity differentiate further into memory B cells and long-lived plasma cells producing antibody of increasing affinity (affinity maturation) [135,136,137].

Long-lived plasma cells then migrate to the bone marrow niches where they can survive for a long time [138]. Upon re-encounter with the pathogen, memory B cells are activated and differentiate into more antibody-secreting plasma cells that may deploy protective antibodies in time to provide immediate protection from disease. Additionally, memory B cells are replenished following re-activation.

Short-lived plasma cells are responsible for the immediate antibody responses that occur within few days after vaccination while long-lived plasma cells (located in the bone marrow) mediate long-term maintenance of antibody levels. The maintenance of long-lived plasma cells is thought to be through stimulation of memory B cells to differentiate into long-lived plasma cells [139] while recurrent or chronic encounter with antigens and continuous polyclonal activation play a role in maintaining memory B cells [140].

1.6.2 Vaccine-induced T cell responses

Within the local lymph node, activated mature dendritic cells present the processed vaccine antigens to T lymphocytes in association with major histocompatibility complex (MHC) molecules. MHC molecules are glycoproteins encoded by a large gene family that is represented in all vertebrates. They are responsible for the appropriate lymphocyte recognition and subsequent "antigen presentation". Classically, there are two types of MHC molecules; MCH class I and MCH class II. MCH class I molecules display antigens that are processed within infected cells while MHC class II molecules display antigens that have been processed within antigen-presenting cells such as dendritic cells.

Antigen-specific CD4⁺ T cells recognize vaccine peptides displayed by class II MHC molecules whereas CD8⁺ T cells recognize peptides displayed by class I MHC-peptide

complexes. The activated antigen-specific CD4⁺ T cells then differentiate into two distinct cell types; Th1 or Th2 cytokine-producing CD4 T cells. The Th1 CD4 T cells produce cytokines such as IL-2, IL-5, IFN- γ and TNF- α while Th2 CD4⁺ T cells produce cytokines such as IL-4, IL-6 and IL-10.

Th2 CD4⁺ T cell supports the differentiation of antigen-specific B lymphocytes into antibody-producing cells and are also directly involved in protection against extracellular pathogens through production of IL-4, IL-5 and IL-13 [141]. Th1 CD4⁺ T cells are responsible for destruction of intracellular pathogens either directly or through the release of cytokines such as IFN- γ and TNF- α [142]. Furthermore Th1 CD4⁺ T cells induce the differentiation of CD8⁺ T cells to cytotoxic T lymphocytes capable of destroying infected cells [143].

Activation of antigen-specific CD8⁺ T cells causes them to differentiate into cytotoxic effector cells capable of killing infected cells or pathogens. Live attenuated viral or bacterial vaccines are strong inducers of CD8⁺ T cell responses since they present the vaccine antigens within cells facilitating interaction through MCH class I molecules [144,145].

Effector T cell responses last for a short time (i.e. a few days) and are replaced by memory T cells. Two types of memory T cells are generated, namely effector memory T cells (Tem) and central memory T cells (Tcm) [146]. Effector memory T cells circulate through non-lymphoid tissues and once they encounter pathogens, they are quickly stimulated to kill invading pathogen through their powerful cytotoxic armament. Central memory T cells on the other hand patrol the lymph nodes and bone marrow, and despite

their low cytotoxic capacity they can undergo dramatic proliferation and produce massive numbers of effector T cells upon re-encounter with dendritic cells presenting respective specific antigens [147].

The persistence of antigen appears to be a key determinant of effector T cells responses while early antigen clearance skew the response towards central memory T cells [148]. For non-replicating vaccines, use of adjuvants such as Alum provides a platform for the slow release of antigen to favour development of effector memory T cell response. Lifetime persistence of memory T cells may not require persistent exposure to antigen and is mediated by cytokines such as IL-15 and IL-17 [149,150].

In addition to the CD4 and CD8 T cells responses, the antigen encounter leads to the production of antigen-specific regulatory T cells such as CD4⁺CD25⁺ Treg cells and type 1 regulatory T (Tr1) cells [151]. These cells selectively inhibit the proliferation of IFN γ -producing CD4⁺ and CD8⁺ T cells through inhibition of IL-2 production ensuring protection from excessive detrimental effects of immune responses.

1.6.3 Measuring vaccine-induced responses

Some of the vaccine-induced immune responses may be correlates of protection and as such could be useful tools in the development of new vaccines or optimization of immunization strategies[152].

1.6.3.1 *Measuring antibody levels*

Measurement of antibody levels is usually done by Enzyme-linked immunosorbent assay (ELISA). The assay employs an antigen molecule, specific to the antibody to be measured, which is immobilised on the bottom of the wells of a microtitre plate, before the sample containing the antibodies of interest is added. Anti-human antibodies labelled with an enzyme which bind the test-antibodies fixed on the immobilised antigen are subsequently added. Thereafter, the wells are washed and incubated with chromogen/substrate solution, which in a presence of the enzyme (e.g. peroxidases) conjugated anti-human antibody produces colouration whose intensity is proportional to the amount of antibody in the test sample. ELISA techniques can be easily standardized and used to assess the concentrations of vaccine-induced antibodies.

ELISA can also be modified to assess the avidity (i.e. the “average binding strength”) of polyclonal antibodies to their respective epitopes. To determine the avidity two sets of ELISA test are conducted in parallel. In one set the ELISA is carried out in the usual way, while in the other a chaotropic agent is added after incubation with the test serum and before peroxidase-labelled anti-human IgG is added. The chaotropic agent causes dissociation of the antibodies from their epitopes immobilised on the bottom of the microtitre plate-well. The amount of antibody dissociated is proportional to the strength of the interactions between antibodies and their respective epitopes (measure of avidity). A relative avidity index is then calculated from the measured values with and without chaotropic incubation.

1.6.3.2 Measuring cellular responses

Measuring T cell responses is complicated by the need to use live cells and lack of standardization across different quantitative assays for T cell function[153].

Enzyme-linked immunospot assay (ELISPOT) and polychromatic flow cytometry technology are among the techniques which measure the ex vivo frequencies of cytokine-secreting cells following short or long term *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) or isolated cell-phenotypes with the peptides (antigen) of interest. An ELISPOT assay is an adaption of ELISA. Specific monoclonal or polyclonal antibodies, which bind the cytokine of interest, are immobilised on a membrane at the bottom of the wells of a filtration microplate. The stimulated PBMC containing cytokine-secreting cells are then added and incubated for a specified period of time. The cytokine of interest is captured by the anti-cytokine (e.g. IFN- γ or IL-2) capture antibody immobilised on the bottom of the microplate.

After washing the plate, a biotinylated polyclonal antibody specific for the captured cytokine is added to the wells. Streptavidin conjugated to an enzyme (eg alkaline-phosphatase) is then added, which binds to biotin. The detected cytokine is visualized following incubation with a substrate, which produces coloured spots in the presence of the enzyme conjugating the detection antibody (e.g. alkaline-phosphatase). Coloured spots representing individual cytokine producing cells are then counted using an automated ELISPOT reader or manually, using a stereomicroscope.

Polychromatic flow cytometry is a more robust technique with the ability to study several subpopulations of T cells, revealing their stage of activation and proliferation, effector function (cytokine production, degranulation and lytic potential) as well as their homing potential simultaneously. Intra-cellular cytokine staining (ICS) is a flow cytometry based assay that can be used to examine the cytokine-producing functional properties of T cells. After a brief period of exposure to the peptides (antigen) of interest, the specific T-cells are activated and begin to synthesize cytokines. The cytokines are then trapped inside the cells following addition of brefeldin A which blocks protein secretory pathways. Specific anti-cytokine fluorescent antibody conjugates are then added following permeabilization of the cell membrane allowing specific interactions with the immobilised intracellular cytokines. Additionally, extracellular surface receptors that identify the cell phenotypes of interest are stained for by adding specific fluorescent monoclonal antibodies. Data acquisition is performed by multi-parameter Flow Assisted Cell Sorting (FACS).

Unlike ICS, ELISPOT assays such as IFN- γ ELISPOT are validated and have been used widely in vaccine trials [148] . Despite challenges facing these cell mediated immunity (CMI) assays, their use in measuring the T cell responses in vaccine trials is important in order to ascertain the role of different vaccine-induced T cells effectors in protection.

1.6.4 RTS,S-induced anti-CS protein antibodies

RTS,S with AS01 and AS02 administration induces high levels of antibodies to CS protein in both adults and children [123,124,154] with mean titres higher in children than in adults [59]. Although lower titres have recently been reported when RTS,S is co-

administered with EPI vaccines, the titres were significantly higher than in those who received the control vaccine [133]. These antibodies, measurable by standardized ELISA, peak after the last dose of vaccination and decline over time with exponential decay.

In experimental challenge studies, anti-CS protein titres correlate with protection [131] but no protective threshold titres have been established [155]. In infected individuals anti-CS protein titre is associated with delayed time to infection. The anti-CS protein titres in children and adults residing in malaria endemic countries correlate with protection from malaria infection but not clinical disease [156,157].

1.6.4.1 Anti-CS protein antibody function

Most of the information on anti-CS protein function comes from animal studies and challenge studies in malaria naïve adults after immunization with CS protein. The ability of anti-CS protein antibodies to opsonise CSP has been demonstrated in sera of malaria naïve adults immunized with RTS,S/AS02. The human monocyte-like cells from pre-monocyte THP-1 cell-line (TIB 202, American Type Culture Collection, Rockville, MD) expressing receptors recognizing N and C termini of CS protein were able to phagocytose fluorescent anti-CS protein complexes [158]. In a subset of sera the authors also demonstrated the ability of these monocyte-like cells to phagocytise live sporozoites in the presence of sera from individuals immunized with RTS,S/AS02. This opsonisation may play an important role in preventing subsequent invasion of the hepatocyte. The sera

used were those taken on the day of challenge and the duration of this effect was not described.

Anti-CS protein antibodies produced following immunization with irradiated sporozoites and CS protein based subunit vaccines can inhibit hepatocyte invasion by sporozoites [159,160]. The hepatocyte migration assay offers an alternative way of studying the effects of anti-CS protein. This assay is based on the fact that sporozoites traverse through various hepatocytes before establishing infection in the final hepatocyte, causing membrane damage that can be detected by the membrane non-permeable fluorescent molecular tracer dextran in the process [161]. In a study of virosomal CS protein malaria peptide vaccine, it was shown that anti-CS protein antibodies inhibited sporozoite migration through hepatocytes [160]. The inhibition of sporozoite migration correlated well with hepatocyte invasion assays. Anti-CS protein antibodies can also disrupt the sporozoite surface structure and block posterior translocation of the CS protein [162] leading to the immobilization of sporozoites and inhibition of hepatocyte infection [163].

1.6.4.2 Anti-CS protein antibody subtypes

In endemic areas, IgG1 and IgG3 are the more predominant anti-CS protein antibody isotypes [105,164]. In malaria naive adults, RTS,S induces predominantly IgG1 and IgG2 with minor levels of IgG3 and IgG4 isotypes [160]. Antibody isotypes are important determinants of protection against malaria infection [165]. For instance IgG3 antibodies are thought to be the most important in the protection against erythrocytic stages of

malaria [166]. In mice immunized with CS protein DNA vaccine, the protection against sporozoite challenge is associated with higher IgG1:IgG2b ratio than lower IgG1:IgG2b ratio [167]. However other studies have shown antibody isotype to be less important in mediating the protection conferred by CS subunit vaccines [168].

1.6.4.3 Anti-CS protein antibody affinity

In addition to the quantity of antibody, quality of antibodies also plays an important role in the protection conferred by vaccination against infectious diseases [169]. Early antibody secreting cells usually produce antibodies of low affinity. These cells then proliferate within germinal centres where somatic hypermutation of V(D)J immunoglobulin gene and antigen-driven selection of high affinity antibody-producing B cells occurs. Affinity is the strength of the interaction between a single antigen-binding site on the antibody and its specific antigen epitope. Antibody affinity is difficult to measure since it requires monoclonal antibodies, their specific purified epitopes and must be carried out under strict chemical conditions. However antibody avidity can be used as the surrogate marker for affinity following vaccination [170]. Avidity is defined as the antigen binding capacity resulting from the addition of all epitope specific affinities antibodies in a serum. High avidity antibodies appear important in the protection conferred by Haemophilus influenza type b vaccine, Hepatitis B vaccine and Pneumococcal conjugate vaccine [171,172,173]. The avidity of anti-CS protein antibody may determine protection against malaria in a mouse model [174]. To date, no study has

investigated the role of avidity of RTS,S-induced anti-CS protein antibodies in protection against malaria infection among RTS,S vaccinees in the field.

1.6.5 RTS,S-induced cell mediated immune response

RTS,S-induced cell mediated immune responses have been assessed using proliferation assays, cytokine production on cell culture, intracellular cytokine staining and flow-cytometry, and *ex-vivo* and cultured ELISPOT assays [175,176].

1.6.5.1 Studies in adults

RTS,S/AS02 immunization induces a CD4⁺ T cell response but little or no detectable CD8⁺ T cell response [131,155,177,178,179]. Sun et al observed IFN γ -producing CD8⁺ T cells, but only after cells were stimulated for 10-14 days *in vitro* [180].

The frequency of poly-functional CD4⁺ T cells identified by intracellular cytokine staining (ICS) correlate with protection from *P. falciparum* infection after experimental challenge in adults [131,181]. In a field study, Reece et al reported a correlation between protection against re-infection and cultured IFN γ ELISPOT assays using a single conserved T cell epitope from the CS protein [178]. However, this analysis was not adjusted for anti-CS protein antibody titres, and did not include ICS studies.

1.6.5.2 Studies in children

Few studies have studied the cell mediated immune responses elicited by RTS,S in young children. Barbosa et al reported vaccine-specific CD8⁺ T cell responses among RTS,S/AS02 vaccinees after 42 hours of *in vitro* stimulation of PBMC at 10 but not at 4 weeks post immunization in children less than one year old [182]. They also reported low but significant frequencies of CS protein specific CD4 T cell responses among the vaccinees. Both single ICS cytokine CD4⁺ T cell and CD8⁺ T cell responses showed borderline correlation with protection from *P. falciparum* infection [182].

The relative contribution of cell mediated immune responses and anti-CS protein antibody titres in mediating pre-erythrocytic immunity following vaccination with RTS,S/AS01E in young children is unclear. This means there are no defined correlates of protection. This lack of established RTS,S/AS01E induced correlates of protection has meant the candidate had to be empirically tested for its clinical efficacy. Furthermore, efficacy must be confirmed in a variety of different transmission settings and populations to be sure it is a generalizable observation.

1.7 Malaria surveillance

Malaria vaccine trials normally use passive case detection, active case detection or a combination of the two for surveillance of clinical episodes or infections. In active case detection, participants are followed once every specified period of time (daily, monthly, weekly or fortnight) and assessed for the presence of malaria parasites associated with

axillary fever, or a history of fever in the last 24 hours. But it is argued that active case surveillance is likely to misclassify the occurrence coincidental parasitaemia in subjects with fever of other causes as if it represented a true malaria case [183].

A modification of this method involves taking blood slides only if the child has documented fever (defined as axillary temperature $\geq 37.5^{\circ}\text{C}$). A child with a history of fever but no confirmed fever (temperature $\geq 37.5^{\circ}\text{C}$) is followed up in the next specified hours (6-12 hours) to confirm or exclude an objective fever [61]. Applying a parasite threshold may further improve the specificity of the case definition. [184]. Studies assessing children with high vulnerability to malaria[185], genetic and environmental risk factors of malaria [186] and correlates of malaria immunity [187] have successfully used this form of active surveillance.

Conventional passive case surveillance involves health facilities where study participants attend when ill. This method measures public health burden in a real life situation and is believed to be less prone to misclassification of coincidental parasitaemia with fever as true malaria cases [188]. Although the public health burden of malaria is felt in terms of presentations to routine health facilities, the attendance to these facilities during passive surveillance depends on economical geographical and social factors [189,190,191] which may in turn reduce the number episodes identified through this method.

An alternative form of passive case detection involves trained resident field workers or community health workers. These workers act as points of contact in the community, perform malaria tests and offer antimalarial medication after consultation with the clinicians. Referral to the health facility is done when necessary. This form of passive

case surveillance differs from the conventional passive case surveillance at health facilities and it is not clear how the combination of the two may affect the quality and frequency of clinical malaria episodes compared to passive case surveillance at health facilities alone. Combination of passive and active surveillance can also be deployed in a study in order to improve the identification of cases especially when both infection and clinical disease are the main endpoints of the study.

1.8 Methods of analysing malaria vaccine efficacy

In the context of a randomized controlled trial, vaccine efficacy is defined as the reduction in the risk of disease among vaccinated individuals compared with unvaccinated individuals. It is expressed as percentage efficacy using the formula $1-RR$, where RR is the relative risk of the disease in vaccinated compared to unvaccinated group. Vaccination can have different effects both at individual and population levels. Consequentially the choice of analysis method will depend on what effect one is interested in and the kind of data available.

At an individual level, vaccination reduces one's susceptibility to infection or reduces the degree or duration of infectiousness to others. The assessment of the effect of vaccination is therefore conditional on the knowledge of level of exposure (or whether an individual is exposed or not). In other words we can only assess the effect on susceptibility to infection when exposure has occurred. Similarly we can only assess the effect on infectiousness when optimal contact between infectious and susceptible individuals has

occurred. The efficacy is therefore estimated from transmission probabilities or secondary attack rates. To accurately calculate these parameters, data on infection status, degree of exposure and types and frequency of infectious contacts is required. Early phase 1b malaria vaccine trials which use experimental challenge with infected mosquitoes fall under this category since the amount of malaria exposure can be accurately or reasonably quantified to assess the transmission probabilities.

Vaccine trials which use disease as the primary endpoint or infection without knowledge of malaria exposure usually estimate unconditional estimates of vaccine efficacy since they do not take into account either exposure to infection or type of infectious contact for each individual. In a randomized controlled trial the assumption is that the number of potential infective contacts is the same between the two groups and the vaccine efficacy estimated from relative risk measures will be similar to the efficacy estimated from transmission probabilities. Phase 2 and 3 malaria vaccine trials fall under this category. They generally provide an estimate of the average direct effect of the vaccine.

At the population level, vaccination can have an indirect effect on unvaccinated individuals through its action of reducing infectiousness. Historically, the primary interest in phase 1 and 2 vaccine trials has been to assess the direct individual effect of the vaccines in protection against infection or disease with indirect effects considered of secondary interest, and may be estimated in Phase 4 studies.

1.8.1 Parameters used in vaccine efficacy analysis

Halloran et al. have reviewed various hierarchies of vaccine efficacy parameters depending on the level of information required to estimate them [192]. Parameters that estimate biological effects of vaccines are at the highest level since they require more information (conditional estimates) (Level I). Such parameters include transmission probability and secondary attack rate. Phase II and III malaria vaccine trials provide unconditional estimates of malaria vaccine efficacy and fall under Hallorans Levels II, III and IV. The parameters used under these levels include cumulative incidence rate, incidence rate and hazard rate ratios.

Cumulative incidence rate ratios require the least information (Level IV). Only information on whether individuals have experienced the endpoint of interest is required. The method assumes all individuals were at risk during the entire follow-up of the study and produces crude estimates of malaria efficacy. It does not allow for adjustment of covariates. Vaccine efficacy for the direct effect of vaccine is given by:

$$VE = 1 - \frac{\text{Cumulative incidence rate in vaccinated group}}{\text{Cumulative incidence rate in unvaccinated group}}$$

Incidence rate ratios are considered to be at Level II and require knowledge of the frequency of endpoint in each group and individual times at risk. Clinical trials of candidate malaria vaccine SPf66 used this method to determine the efficacy [193,194]. The method is applicable in vaccine trials where there is staggered recruitment and/or

loss of follow-up during the study. Like the previous method, it does not allow for the adjustment of covariates and only produces crude estimates of malaria vaccine efficacy.

Incidence rates can alternatively be modelled using Poisson distributions. The Poisson regression models scale incidence rates by person-time at risk. The number of cases is given as:

$$\log(\mu) = \beta_0 + \beta_i X_i + \log(\text{follow up time})$$

where μ is mean number of cases, β_i is the estimated coefficients and $i=1 \dots p$ for the p number of covariates considered, β_0 is the mean number of cases when all the covariate values are zero and X is the value of the covariate. The advantage of this method is that it allows one to adjust for covariates of interest. Several clinical trials evaluating the efficacy of candidate malaria vaccine against multiple episodes have used this method [61,194].

The third approach uses the hazard rate ratio as the relative risk measure (Level III). Information required is similar to that of incidence rate. This is the most common method used to evaluate efficacy of candidate malaria vaccines. The hazard rate is defined as an instantaneous risk of having an outcome of interest at specified time t given that the subject has not suffered from the outcome up to time t . The hazard rate ratio describes how likely will the vaccinated subject suffer from outcome of interest [195]. The hazard rate ($h(t)$) is modelled by Cox regression as:

$$h(t; x) = \lambda_0(t) \exp(X\beta)$$

where $X\beta = X_1\beta_1 + X_2\beta_2 + \dots + X_p\beta_p$. The function $\lambda_0(t)$ is called the baseline hazard rate and refers to the hazard when all X_p 's are 0. This method allows for adjustment of covariates. Although the model allows for the variation in the baseline hazard rate, it assumes that the hazard rate ratio is constant throughout the follow-up.

1.8.2 Measures of vaccine impact

Vaccine efficacy estimated in terms of relative risk (also known as relative risk reduction) provides information on the proportional reduction of malaria events by a vaccine. In randomized controlled trials vaccine efficacy from relative risk reduction provides estimates similar to those from transmission probabilities [196,197]. All evaluations of biological effect of the vaccine and their licensure are based on relative risk reduction. In addition, the uptake of the vaccine in the community is highly influenced by its perceived risk and beneficial effect to the individual which can be inferred from relative risk reduction [198].

From a public health point of view, the absolute effect of the vaccine is however more important. When a vaccine introduction results in a reduction of malaria events in the population, it may be more intuitive to determine the absolute risk reduction due to vaccination. This is usually estimated as the difference between malaria events rate in the control and vaccine group. When there are more malaria events in the control than

vaccine group, we have a positive absolute risk reduction implying the vaccine is beneficial. Conversely, a negative absolute risk reduction means there are more malaria events in the vaccine than control group and vaccine is harmful. Absolute risk reduction is also referred to as number of cases averted or vaccine-attributable reduction. Such information provides a measure of impact if such a vaccine were to be introduced in the population taking into account the baseline risk. It allows analysis of cost-effectiveness of the vaccine and can help inform decisions on efficient allocation of resources to achieve maximum public health benefit.

In many instances the benefit of an intervention as measured by the relative risk reduction is relatively constant over a wide range of baseline risk. In such a situation the relative risk reduction has an advantage in that a single measure can be used to describe the benefit of the intervention under variable levels of risk to the disease. However the risk of malaria varies depending on the malaria transmission and studies have shown that malaria transmission can vary widely over short distances and even within a single settlement [199,200]. Therefore it is important to consider the baseline risk in control groups in the assessment of the malaria vaccine benefit since for a given relative risk reduction, the expected absolute risk reduction could vary considerably as the transmission changes.

Both relative risk and vaccine-attributable reductions are important measures of vaccine effect. The former reflects its biological effect which is critical for licensure, whereas the latter provides a measure of its public health utility. The choice of the measure needs to consider the stage of clinical development (early versus later development) and the objective of the study. In early stages of development (phase 1 and 2) the relative risk

measure is usually preferred. It is recommended that both measures be evaluated especially in phase 3 trials to facilitate assessment of a public health utility of candidate malaria vaccines which is critical in informing decision on their introduction.

1.8.3 Decision making on vaccine introduction

Both the effectiveness (number of malaria cases or death averted) and cost effectiveness of vaccination are important factors to inform policy makers on the introduction of a vaccine. Cost effectiveness of the vaccine is normally estimated from Cost Effectiveness Analysis (CEA) which is an economic evaluation that assesses both the cost and health outcomes of alternative intervention strategies. Comparison is then made by using expected incremental cost effectiveness ratio (ICER) which measures additional cost per additional unit of health gain produced by one intervention relative to the other intervention. This can be expressed as cost per QALY (Quality Adjusted Life Years) or DALY (Disability Adjusted Life Years) gained. The ICER is subsequently compared to the cost effectiveness threshold which represents society's willingness to pay for an additional unit of health gain (e.g. QALY). This implies if ICER for a new malaria vaccine is less than cost effectiveness threshold, then the society will be ready to fund its introduction. On other hand if ICER is more than cost-effectiveness threshold, the society is likely not to accept the new intervention[201]. The cost-effectiveness threshold is expressed as the opportunity cost of current intervention(s) as the result of displacement by new intervention. However the selection of the threshold is not completely objective and is a subject of contention [202]. The cost of RTS,S is currently not known.

1.9 Challenges in estimating efficacy of malaria vaccines

Estimating accurate malaria vaccine efficacy is essential in order to inform policy decisions on introduction of a malaria vaccine. There are numerous challenges in estimating the efficacy estimates in malaria vaccine trial and the following sections describe main key areas where statistical issues arise.

1.9.1 Vaccine efficacy from time to first event analysis

The majority of malaria vaccine trials have used time to first event as the primary analysis for the estimation of malaria vaccine efficacy [124,129,203]. They use proportional Cox regression models to estimate the hazard rate ratio for clinical malaria between vaccinees and controls. However, such analysis precludes the contribution of subsequent episodes in estimating efficacy.

Malaria is a complex disease in which immunity takes time to develop and individuals can experience repeated infections during follow-up. Analysis of all events gives a better insight of the public health utility of the vaccine. Furthermore including all events provides more power to the analysis of efficacy estimates and allows analysis to explore interactions between vaccination and other covariates of public health importance. At the time of commencing my DPhil work, only two clinical trials on RTS,S had reported efficacy against multiple episodes i.e. a phase 2b trial on RTS,S/AS02 in Mozambican

children and a phase 2b trial on RTS,S/AS01 in Tanzanian and Kenyan children [132,157].

1.9.2 Heterogeneity in malaria exposure

Spatial heterogeneity in malaria exposure has been described at a micro-epidemiological level at varying transmission settings [200,204] and accounts for substantial variation in the degree of exposure to malaria from person to person. It is responsible for variations in disease risk within a small area and is evidenced by geographical clustering of malaria infections. Smith et al showed that approximately 80% of transmission occurs within 20% of the population [205,206]. It has been attributed to factors such as varying local density of malaria vectors [207], the pattern of contact between human host and vectors and intrinsic human host factors [208,209].

Heterogeneity in malaria exposure can give rise to variable pre-erythrocytic vaccine efficacies as a result of a) its true biological effect and b) as an artefact of statistical analysis in the time to event analysis. In the former, high malaria exposure may overcome the vaccine-induced protection and produce lower efficacy estimates or differential acquisition of natural immunity to blood stage parasite between the control and vaccinated group during the follow-up can lead to higher risk of clinical malaria in vaccinated group over time. In the later case underestimation of vaccine efficacy occur as the result of differential removal of susceptible in the control versus vaccinated group in the time to event analysis

1.9.2.1 Effect on time to event analysis

Heterogeneity in malaria exposure may bias estimates of malaria vaccine efficacy over time in longitudinal studies [210,211]. This is predicted by simulations of populations under heterogeneous malaria exposure, where vaccine efficacy is underestimated as a consequence of heterogeneity and apparent waning of efficacy over time is seen even if vaccine protection is maintained [134]. This phenomenon, well known to demographers was first described by Vaupel et al. who sought to explain the apparent but counter-intuitive deceleration in human mortality rates in old age [212].

Although a randomized controlled trial may ensure equal distributions of malaria exposure between vaccine and control group at the start of the trial, unequal rates of depletion of susceptible individuals between the two groups in the presence of an effective vaccine will upset this balance over time. In any cohort consisting of individuals with variable levels of susceptibility to clinical malaria, the more highly susceptible individuals will experience earlier clinical malaria episodes. In time to first event analysis, individuals are removed from the “at risk” set once they suffer from infection. Subsequently, the malaria incidence will decline over time as less susceptible individuals remain.

In a randomized controlled trial, this phenomenon occurs in both vaccine and control group. However the depletion of susceptible individuals will be more rapid in the control group (assuming the vaccine is protective). As the result, the vaccine group will be compared with a control group consisting of progressively less susceptible individuals as more time since vaccination elapses. The vaccine efficacy against first event will

therefore appear to wane overtime despite sustained biological protection of the vaccine (Figure 1:2) [210,211]. This effect is more marked as time since randomization increases and more events occur.

1.9.2.2 Measuring individual malaria exposure

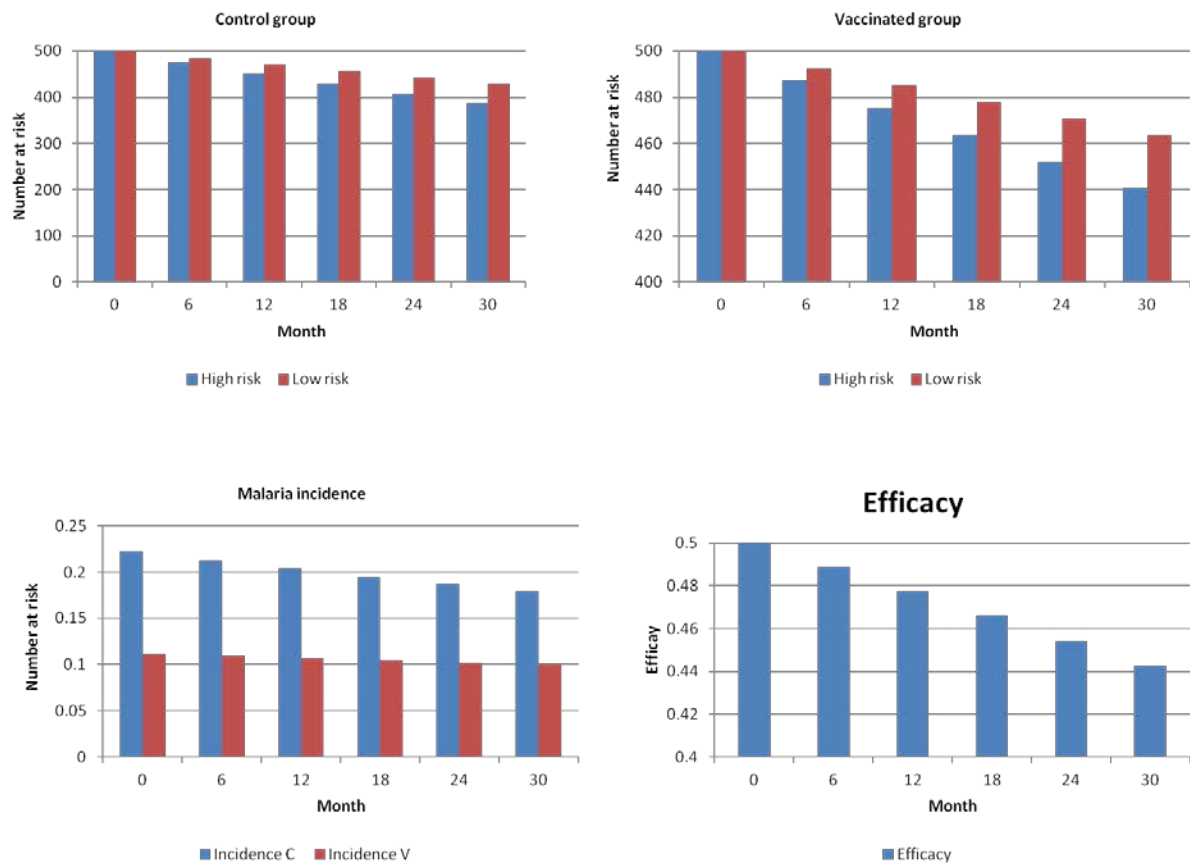
Knowledge of the individual malaria exposure status would allow one to estimate vaccine efficacy stratified by level of exposure or adjust for the individual level of malaria exposure in the analysis. It is however not clear how to estimate an individual's level of exposure in the field. Entomological Inoculations Rates, parasite rates and infant conversion rates have frequently been used to describe exposure at the level of population, but are not readily applied to individuals. Some studies have used individual antibodies to schizonts extracts as a marker of exposure [187,213] or other recombinant malaria antigens[214]. This approach is validated as a marker of exposure at a population level [215], but at an individual level is complicated by variations in an individual's capacity to make antibodies to specific antigens and saturation effects of antibody responses [216,217].

Individuals who develop neither a febrile episode nor asymptomatic parasitaemia during follow up might be considered as unexposed. Exclusion of these unexposed individuals from the analysis strengthens the ascertainment of the effects of immunity, transmission intensity and age [218]. However the choice of an individual marker of exposure remains a challenge. Using cross-sectional surveys for parasitaemia could misclassify those whose parasitaemia had been cleared by anti-malaria drugs or immunity. Furthermore this approach could only categorize individuals into 2 groups (exposed and non-exposed)

and doesn't take into account the underlying variability in level of malaria exposure at an individual level.

There are several statistical approaches that have been proposed to address heterogeneity of exposure [211,219], but most are difficult to implement and interpret, and make assumptions on the distribution of the malaria exposure within the population.

Figure 1:2: Simulation data showing effect of heterogeneity in malaria exposure on efficacy estimates



The Figure shows deterministic simulation data depicting two groups in a randomized controlled trial (Vaccine versus control). As time progresses more children in the susceptible group will get malaria than in less susceptible group and subsequently will be removed from the pool of susceptible individual. Because the susceptible group contributes the most to the overall incidence and as the pool of susceptible is depleted, the incidence will decline with time, and this will occur faster in control than in RTS,S group. Consequently the vaccine efficacy will appear to wane as time progresses because the difference in incidence between the two groups becomes smaller with time. The simulation was conducted in excel assuming a constant efficacy of 50% with the control group having a 6 monthly malaria risk of 0.05 and 0.03 for the high and low risk individual respectively. Both vaccine and control group started with 1000 individuals 500 in each high and low risk group. Incidence C=malaria incidence in control group and Incidence V=Malaria incidence in vaccinated group

1.9.2.3 *Frailty model*

In a frailty model, the observed variability in survival times is assumed to be a result of systematic selection of individuals who are less frail from a heterogeneous population. This heterogeneity is therefore an unobserved variable which can explain some of the variability in survival times.

The frailty model modifies the Cox proportional hazard model such that the hazard of each individual depends in addition to unobservable variable Z which has multiplicative effect on baseline hazard function μ .

$$\mu(t, Z, X) = Z\mu_0(t)\exp(\beta^T X)$$

where $\mu_0(t)$ is the baseline hazard function, β the vector of regression coefficients, X is the vector of observed covariates and Z now is the frailty variable. The frailty Z is a random variable varying over the population and can either reduce ($Z < 1$) or increase ($Z > 1$) individual hazard rate.

However, the estimates by frailty model depend heavily on the assumptions made on the distribution of Z and therefore the accuracy of the results is heavily dependent on how realistic the chosen distribution is.

1.9.3 Multiple episode analysis

Multiple event analysis takes into account all events in an individual. The event can either be of the same type (recurrent clinical malaria episodes) or different type (type of fractures). The strength of multiple event analysis lies in its greater efficiency in estimating the effect of covariates compared to time to event analysis [220].

1.9.3.1 Poisson regression

Poisson regression models are normally used to report the efficacy of malaria vaccine against all clinical malaria episodes. They model the number of events per unit time of follow-up using a Poisson distribution. The log of the incidence rate is expressed as a linear function of covariates of interest allowing one to adjust for multiple covariates. The limiting factor of Poisson distribution is its assumption of equality of the mean and variance which limits its ability to model wider range of event data. For instance clinical malaria events do not always fit Poisson distributions, as demonstrated in the field [185]. Alternatively, a negative binomial distribution model can be used when the Poisson assumptions are not met. In the negative binomial distribution, the mean and variance are independent of each other making it more flexible in modelling frequency patterns of observed event data.

Poisson regression assumes that observed events within individuals are independent. However, prior malaria episodes may negatively or positively influence the risk to subsequent episodes. On the one hand frequency of previous malaria episodes may be a marker of increased exposure to malaria and could predict more subsequent malaria

episodes. On the other hand with each clinical malaria episode a child develops a natural immunity which reduces the risk of subsequent malaria episodes. This implies that multiple events within the same individual are unlikely to be independent.

1.9.3.2 Generalized estimating equation models

Generalized Estimating Equation (GEE) models can be used to account for lack of independence of events within individuals. They are an extension of generalized linear models (GLM) which estimate the population averaged estimates. GEE is used for data within individuals in longitudinal studies where there are repeated measures on the same individual over time. The GEE model incorporates a robust estimation of the variance in order to adjust for the dependency between events.

To further improve efficiency, the method also allows for incorporation of a correlation structure describing the type of correlation between observations within the same individual. There are four types of commonly used working correlations namely independent, exchangeable, autoregressive and unstructured working correlations[221]. With independent working correlation, one assumes correlation between events is zero. An exchangeable working correlation assumes uniform correlations across time and one can swap the order of two observations without affecting correlating structure. Autoregressive working correlation assumes that correlations between observations decrease with time with closer events being more correlated than distant events. An unstructured working correlation does not assume any particular form of correlation between events and is the most general form of correlation structure. However even if the

assumed working correlation is mis-specified, GEE can still provide consistent estimators of the regression coefficients and of their robust variances[222].

1.9.3.3 Modified Cox regression models

There are various modifications of Cox regression models which are available for analyzing multiple/recurrent events data in which events may not be independent. These models use different approaches to account for the within-individual correlation between events. The first is the conditional approach which introduces a time-varying covariate in the model such as number of previous events which captures dependence structure among recurrence times. The second is the marginal approach which uses sandwich robust standard error to take into account multiple events within individuals. Lastly is the random effect approach (frailty) which introduces a random covariate in the model in order to induce dependence among recurrent event times.

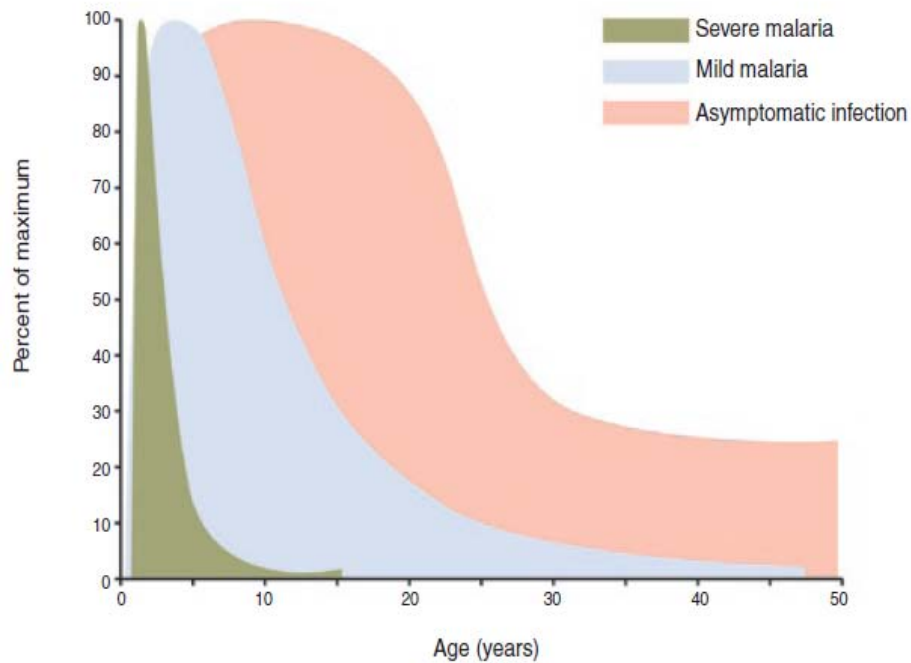
The Andersen and Gill (AG) model is one form of modified Cox regression model which uses a counting process as the risk interval and restrictive risk set (individuals are considered for the k^{th} event only if they have experienced $(k-1)$ events). All events are assumed to have the same underlying baseline hazard. It uses robust sandwich standard errors to take into account correlation of multiple events within individuals.

1.9.4 Duration of protective efficacy

Duration of efficacy is an important determinant of a vaccine's public health utility. A vaccine with long duration of protection would avert more malaria cases and deaths especially when given before the age at peak incidence of severe malaria. The malaria vaccine technology roadmap has set a strategic goal of having a malaria vaccine whose efficacy lasts for more than four years. For young infants in an endemic area, this represents the period of maximum risk of severe malaria and death (Figure 1:3).

Reduction in malaria transmission is associated with changes in the mean age for severe malaria [223]. Therefore understanding the duration of vaccine efficacy and its interaction with the level of malaria transmission is important in order to inform public health policymakers on the expected utility of a vaccine under different transmission settings.

Figure 1:3: Proportion of different presentations of malaria by age (from Langhorne et al [224])



Knowledge of the duration of protection is also important in order to determine the need and if required, timing of a booster dose. In the presence of reliable immunological correlates or surrogate markers of protection, the duration of vaccine efficacy can be inferred from the variation of correlates of protection over time and hence the best time at which a booster dose should be given can be determined. However in absence of such markers, long term empirical estimation of clinical efficacy of vaccine is the only available method.

1.9.4.1 Measurement of duration of vaccine efficacy

Assessment of proportionality of hazard rate over time using Schoenfeld's residuals and models with time-dependent covariates has been used to infer sustained vaccine efficacy over time and/or lack of waning. However, if there is heterogeneity in vaccine action within individuals, even if the true vaccine efficacy wanes, it can appear from an analysis of incidence of first event, that efficacy is sustained or even increasing over time[225]. Furthermore, as explained in section 1.9.2, time to first event analysis can give misleading results in terms of waning of vaccine efficacy given its inherent characteristics of removing infected individuals from the "at risk" set during the follow-up.

Dividing the follow-up time into number of equally spaced periods and performing time to event analysis is another alternative approach of determining the duration of vaccine efficacy [134]. This is called "resetting the clock" as everyone regardless of whether they had a malaria episode is considered at risk at the start of every new period. For a disease which confers some immunity after exposure, a vaccine which reduces exposure to malaria will result in differential acquisition of blood stage immunity between vaccinees and controls. Therefore the control groups at each time period will be acquiring immunity at different rates. This will complicate the interpretation of efficacy estimates at each time period [226].

Data on all malaria events in a person reflects the entire clinical malaria experience of an individual and if measured over many time intervals may give a better insight about changes of vaccine efficacy over time. Poisson and/or negative binomial regression can

therefore be used to determine the duration of vaccine efficacy. Analysis stratified by year of follow-up can give an insight into the variation of efficacy over time. The dependence of events within individuals can be accounted by use of GEE models. Alternatively the Andersen Gill Cox regression model which takes into account all malaria episodes can be used to assess duration of efficacy by assessing proportionality of hazard rate over time.

1.10 Potential for rebound malaria in RTS,S vaccinees

RTS,S is a pre-erythrocytic vaccine, and so its mechanism of protection may be similar in some respects to using an insecticide treated net (ITN). Both interventions reduce exposure to pre-erythrocytic stages of malaria parasites and as such RTS,S may be thought of as a biological bed net. Fears of potential for rebound malaria were expressed with Insecticide Treated Nets (ITN's) [227,228]. ITN's exert their protective effect by several mechanisms. First, ITN's use prevents mosquito bites by acting as a physical barrier between the mosquitoes and humans. Secondly, the impregnated insecticide also repels and kills mosquitoes before they have time to bite. This leads to fewer bites and subsequently reduction of the load of infectious sporozoites invading hepatocytes.

This protection against pre-erythrocytic stages of the malaria might modulate the development of malaria-specific immunity and as such may potentially lead to decreased immunity later in life. Therefore fewer infections in early life might be followed by more infections in later life (rebound). This is different from resurgence of malaria which is

modulated by the increase in the overall transmission following a period of low transmission that leads to a highly vulnerable population with low malaria immunity. However no evidence of rebound malaria in later life has been reported on prolonged use of ITN's [229,230,231]. One possible explanation for absence of rebound may be due to reduction of mosquito's survival and overall transmission with widespread use of ITN's. When used by a majority of the population, ITN's not only protect the individual users but the community at large including those not sleeping under the ITN's [12,232]. This indirect effect of ITN may offset the reductions in immunity and thus lead to positive public health benefits for long-term ITN use [233].

RTS,S on the other hand does not affect mosquitoes survival, and is unlikely to significantly reduce transmission unless given to the entire population (including adults) for a sustained period [234]. It not is clear what will be the consequence of waning vaccine efficacy on the life time experience of malaria in vaccinees. By blocking infection by sporozoites, RTS,S may prevent exposure to blood stage antigens and delay acquisition of natural immunity. Declining efficacy may subsequently predispose vaccinees to rebound malaria.

Alternatively RTS,S may have the opposite effect. Rather than an "all or nothing" prevention of exposure, it may partially reduce the number of infected hepatocytes resulting in lower inoculums of blood stage parasites [235]. This exposure to lower numbers of parasites might actually enhance blood stage immunity, avoiding potential for rebound malaria in the long run [236]. This underlines the importance of longer follow-up of RTS,S children in order to identify possible rebound malaria.

1.11 Summary

The above literature review highlights three major areas for further research that I address in my thesis. The first area pertains to the duration of malaria vaccine efficacy. The most clinically advanced candidate malaria vaccine RTS,S has entered phase III and although data on the short term efficacy against clinical malaria in young children exist, the duration of efficacy is still unknown. Informed decisions on vaccine introduction cannot be taken without this data. The second area relates to the challenges facing the evaluation of a malaria vaccine candidate. Time to event analysis ignores the subsequent malaria episodes and can underestimate vaccine efficacy due to the effect of heterogeneity in malaria exposure. Furthermore, there may be genuine biological variation in efficacy due to heterogeneity in malaria exposure, apart from the artifactual variation described above. In my thesis, I consider a means of estimating individual variation in exposure so it can be included directly in models of vaccine efficacy. It has been perceived that active surveillance and passive surveillance may give distinct malaria case definitions and hence different estimates of malaria vaccine efficacy from the same population. My thesis investigates this possibility by comparative analysis of active and passive surveillance of malaria in the field. The third area relates to the lack of reliable correlates of vaccine protection. This has meant experimental approaches to vaccine development have been mostly empirical slowing the progress of vaccine development significantly. RTS,S induced antibodies correlated with protection from malaria infection but not disease. In addition quality of antibodies induced by RTS,S has not been investigated in the field and therefore its role in the protection against clinical malaria is not known.

My thesis is therefore an attempt to address these gaps in knowledge as far as RTS,S is concerned and provide solutions to some methodological challenges in the evaluation of malaria vaccine candidates.

1.12 Objectives

The main objective of my thesis work was to determine the duration of protection of RTS,S/AS01E candidate pre-erythrocytic vaccine and identify its immunological correlates of protection in young children residing in a country endemic to malaria.

Specific objectives were:

1. To determine whether there was a difference between active and passive malaria surveillance regarding specificity and sensitivity of malaria case definitions. (This was relevant since I rely on active case detection in my trial, but much of the literature describes passive case detection).
2. To examine a means of measuring individual malaria exposure in the field in order to account for the effect of heterogeneity in malaria exposure on efficacy estimates.
3. To determine four-year efficacy of RTS,S/AS01E against *P. falciparum* clinical malaria episodes in young children aged 5-17 months residing in a country endemic to malaria, and to describe variations in efficacy over time and by intensity of malaria exposure.

4. To describe the dynamics of humoral and cellular immunogenicity of RTS,S/AS01E in young children aged 5-17 months residing in a malaria endemic country.
5. To assess the correlations between RTS,S-induced anti-CS protein antibodies and/or RTS,S-induced T cell responses and protection from *P. falciparum* clinical malaria in young children aged 5-17 months residing in a malaria endemic country.
6. To determine if RTS,S-induced anti-CS protein antibodies and T cell immune responses meet Prentice criteria for surrogate marker of protection from *P. falciparum* clinical malaria in young children aged 5-17 months residing in a malaria endemic country.
7. To determine the avidity of RTS,S/AS01E induced anti-CS protein IgG antibodies in sera of children immunized with RTS,S and their association with protection from clinical malaria by *P. falciparum* in young children aged 5-17 months residing in a malaria endemic country.

2 Comparison of Surveillance methodologies

2.1 Background

In this chapter I describe work conducted to determine if malaria case definitions derived from active and passive surveillance differ in their specificity and compare the sensitivity of each method in identifying malaria cases.

Defining clinical malaria in endemic countries is difficult because individuals may carry parasites without symptoms, and coincidental febrile episodes may have etiologies other than malaria. The malaria-attributable fraction method uses population data to estimate the frequency of true febrile malaria among all febrile cases by fitting the risk of fever as a function of parasite density using a logistic regression model[237]. This method has been widely used under different malaria transmission intensities, and has become a standard approach for deriving parasite density thresholds to optimize sensitivity and specificity [184,238,239,240,241,242].

So which type of the surveillance is most appropriate in a malaria clinical trial? The choice of the surveillance method in malaria vaccine trials is dependent on the type of the endpoint (infection versus disease), objective of the study (effectiveness versus efficacy), sensitivity and specificity of each method in identifying most relevant clinical malaria cases and the proportion of total number of cases identified by each methods. The selected surveillance method should produce case definitions with high specificity and sensitivity. However there has been no attempt to assess if there is any difference

between active and passive surveillances in terms of sensitivity and specificity of clinical malaria case definitions. Comparison of the two methodologies of surveillance and their variations and/or combinations would provide supportive evidence for the choice of more appropriate surveillance method in malaria vaccine trials.

Both active case detection and passive case detection using trained fieldworkers alters the dynamics of the health seeking behaviour by providing easier access to healthcare than would have normally been the case. Studies combining both active and passive surveillance may therefore report less severe forms malaria [243].

Regardless of the surveillance method used, cases are often defined by malaria parasitaemia in association with either an objective fever (i.e. temperature ≥ 37.5 °C) or a subjective fever. Use of subjective fever in the case definition may lack specificity, and has been avoided in some studies [238], but the specificity of this endpoint has not been formally calculated.

There are no analyses that examine the sensitivity and specificity of febrile malaria case definitions identified by passive case detection alone, yet this is critical to the accuracy of clinical trials and public health surveillance.

When both active and passive surveillance are combined, the likelihood of presentation between active visits rises as results of frequent contacts [244]. For this reason, and also to avoid confusion, I have described the use of the combination of methods as “active surveillance”. The alternative, which I refer to as passive surveillance, relies only on individuals visiting at health care facilities where malaria is identified.

2.2 Methods

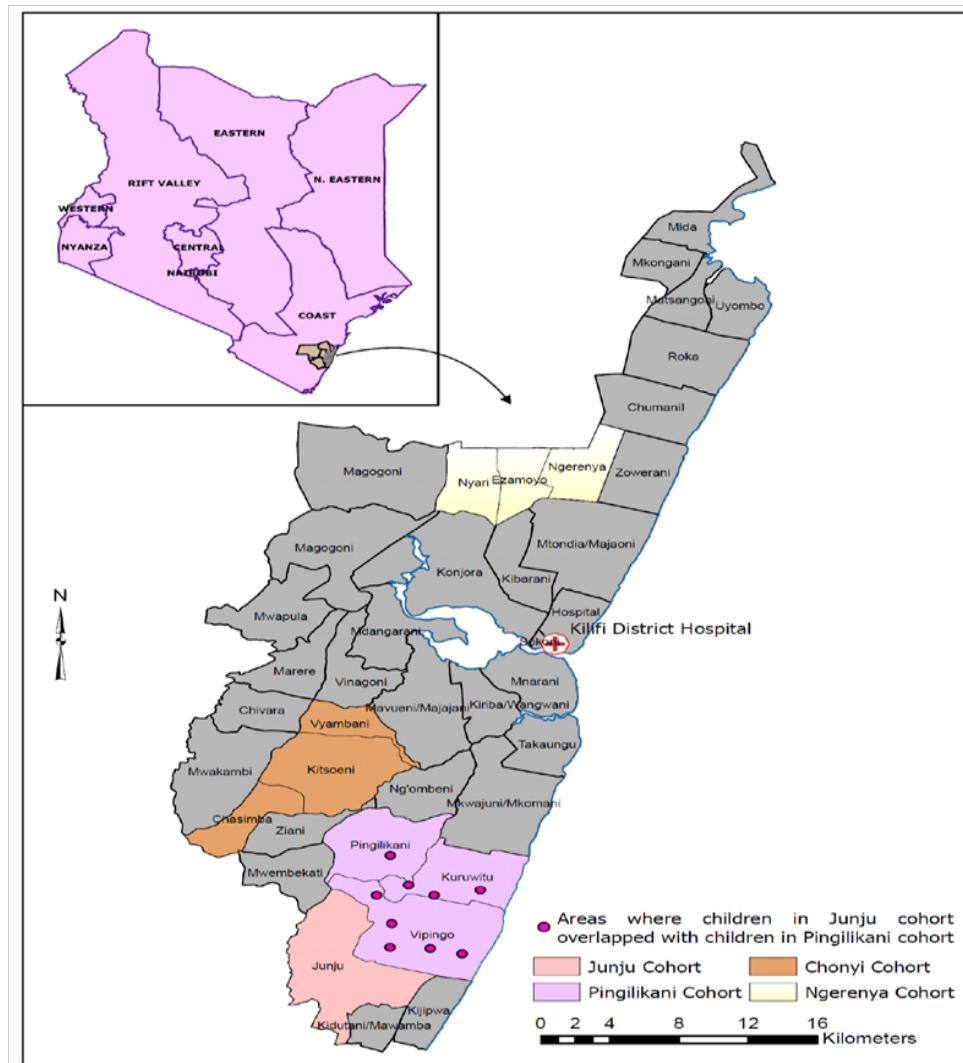
2.2.1 Study cohorts and surveillance

I analyzed data from four cohorts which underwent different surveillance methods for mild *P. falciparum* malaria, in order to describe the sensitivity and specificity of case definitions within cohorts and compare the specificity and incidences of the endpoints from active and passive surveillance. The cohorts were located in Chonyi, Ngerenya, Junju and Pingilikani sub-locations of Kilifi District, on the coast of Kenya between January 1998 and June 2009 (Figure 2:1). The Junju cohort included children located both in Junju and Pingilikani sub-locations. All cohorts were nested within the wider demographic surveillance system (DSS) which covers an area of about 891 km² around Kilifi District Hospital and involves at least every six monthly re-enumeration visits to about 25,000 households. Junju and Pingilikani have generally been high transmission areas, with moderate transmission in Chonyi, and low transmission in Ngerenya, as evidenced by entomological studies [245] and parasite rates [184,238]. However, transmission has been falling throughout the period of study [223,246]. For the purpose of this study I categorized the transmission intensity based on the concurrent parasite prevalence in each cohort.

Different follow up and blood slide evaluation protocols were used in each cohort. In the Chonyi and Ngerenya cohorts, clinical malaria episodes were detected using weekly active surveillance implemented over the entire study period [184]. Children with subjective or objective fever (axillary temperature $\geq 37.5^{\circ}\text{C}$) had blood samples taken to estimate the parasite density from blood smears. In contrast, in Junju blood smears were

only done in children with an objective fever (axillary temp $\geq 37.5^{\circ}\text{C}$) and children with subjective fever without elevated temperature were followed 6-12 hours later, and the temperature measurement repeated. Blood smears were made if objective fever was confirmed at this measurement. Clinicians reviewed all children who were unwell but without objective fever. In addition while the parents of the children in Chonyi and Ngerenya were instructed to report to Kilifi District Hospital (20 km away) whenever the child was sick, in Junju, dispensaries were located within 5 km and trained field workers were available at all times in the villages for passive surveillance. The Pingilikani cohort was monitored purely by passive surveillance at the Pingilikani dispensary, where blood smears were done for all children presenting with a complaint of fever (both objective and subjective fever). A cross sectional blood smear was done before long rains in all individuals within Junju, Chonyi and Ngerenya cohorts regardless of the fever. Sulfadoxine-pyrimethamine was the first line anti-malarial drug used until early 2006 when the artemisinin-based combination therapy (artemether-lumefantrine) was introduced throughout Kenya.

Figure 2:1: Location of cohorts used in the study



The map shows the location of the cohorts used in the analysis. Coloured regions represent the locations where the cohorts were located. The sub-locations within each location are also shown, some of which have the same name as the cohort.

2.2.2 Ethics statement

The details of consent procedures have been published elsewhere [61,184]. Briefly, in Chonyi and Ngerenya, written informed consent was obtained from parents/guardians of young children and adults from randomly selected homesteads using an approved consent form. In Junju written informed consent was obtained from parents/guardians of children

who earlier participated in a malaria vaccine trial that was non-efficacious. Subsequently the consent was sought for all the newborns from these homesteads. The Pingilikani dispensary cohort is nested within the wider Demographic Surveillance system with established recruitment process. The clinical records of children in the cohort were obtained by matching their personal identification numbers from anonymised Demographic Surveillance System and dispensary database.

The approval for human participation in these cohorts was given by the Kenya Medical Research Institute Scientific Committee and National Review and the Ethical Committee of the Kenya Medical Research Institute.

2.2.3 Laboratory investigations

Malaria parasitaemia was determined by examination of blood smears stained with 2% Giemsa solution. For Junju, Pingilikani dispensary, Chonyi and Ngerenya cohorts, the number of asexual-stage parasites/200 leukocytes was counted, and parasitaemia was estimated on the basis of an assumed uniform white cell count; 8,000 leukocytes/ μ L. For members of the Junju cohort located in the Pingilikani sub-location, parasitaemia was estimated on the basis of actual leukocyte count measured for each blood smear. Regression models for the determination of Malaria Attributable Fraction used parasite densities calculated from a single method. Only *Plasmodium falciparum* parasitaemia was included in the analysis.

2.2.4 Calculation of Malaria Attributable Fraction

I used the Malaria Attributable Fractions to estimate the specificity of the malaria endpoints detected by different surveillance methods. Cases were febrile children at the time of surveillance visit. Control cases were afebrile healthy children seen at the cross sectional bleeds during the follow up period. For the Pingilikani dispensary cohort, I used controls from Junju cross sectional bleed. Junju is contiguous with and immediately next to the south of Pingilikani. The Malaria Attributable Fraction was determined by using logistic regression to model the risk of fever as a continuous function of parasite density as developed by Smith et al [237].

$$\text{Log}\left(\frac{p}{1-p}\right) = \alpha + \beta x^\pi$$

In the above equation, p is the probability that a subject with x parasite density has fever and π is the power function of parasite density. The power function maximizes likelihood estimation for the different age groups, cohorts and surveillance methods and was used to model the relationship between fever and parasite density as a continuous function. The power function was re-optimized for each fit of the model; hence the shape of the curve relating parasite density to the probability of fever was allowed to vary for different populations. Confidence intervals were calculated by the bootstrap method using 1000 repeat samplings. Analysis was performed using Stata software (version 9.0; Stata Corp). Only children aged 0-5 years old were included in the analysis of general MAF. Age specific MAF were estimated to investigate variation of MAF with age for each cohort.

Children who had objective fever and also were reported to have subjective fever were included in the sub-analysis of “objective fever” but not “subjective fever”.

2.2.5 Calculation of Incidence rate

Active surveillance in the Junju cohort located in Pingilikani sub-location overlapped with passive surveillance at Pingilikani dispensary. This overlap applied to the 105 children aged 5-17 months who were under active surveillance between May 2007 and April 2008. In order to define a comparable cohort under passive surveillance at Pingilikani dispensary, I used the demographic surveillance system to identify age- and location-matched children during the same period. However, I did not include children sharing a homestead with a child under active surveillance in this matched cohort, to avoid a possible contaminating effect of the homestead being visited. Incidence rates of malaria clinical episodes were calculated by counting the number of clinical episodes of malaria divided by the total time at risk expressed as total person years at risk.

2.3 Results

The detailed characteristics of each cohort are shown in

Table 2-1. In total there were 7,606 children and 299,189 surveillance visits across all four cohorts. Fever was documented in 19,462 surveillance visits and clinical malaria was diagnosed in 9,219 surveillance visits.

Table 2-1 Baseline characteristics of cohort used in the analysis

Number of children	Chonyi cohort 315	Ngerenya cohort 575	Pingilikani cohort 6123	Junju cohort 488
Follow up period analyzed	1998-2001	1998- June 2006	2003- June 2009	2006- March 2009
Median age in years (IQR) at the start of follow up*	1.8 (3.3)	0.5(2.5)	1.6 (2.1)	2.5 (2.3)
Female %	153 (49%)	292 (51%)	2760 (45%)	202 (41%)
Transmission intensity	High	Low	Moderate	Moderate
Time at risk (years)	480.4	1930.4	25,144.1	864.8
Incidence rate	1.16[1.07-1.26]	0.63[0.6-0.67]	0.16[0.15-0.16]	0.93 [0.87-1.0]
Parasite prevalence#	30.8%	8.8%	18.7%	18.7%
Surveillance methods	Weekly active surveillance by field worker	Weekly active surveillance by field worker	Passive visits at dispensary	Weekly active surveillance by field worker
	Passive surveillance at district hospital	Passive surveillance at district hospital		Passive surveillance by field workers and at dispensary
Indication for blood smear	Axillary temperature ≥ 37.5 or history of fever in the last 24 hours.	Axillary temperature ≥ 37.5 or history of fever in the last 24 hours.	Axillary temperature ≥ 37.5 or history of fever in the last 24 hours.	Axillary temperature ≥ 37.5 only
Number of total contact	29,353	178,292	28,384	59,604
# =Parasitaemia prevalence for all cohorts is summed up across the entire follow up period. *= Only children less than 5 years old are included in the analysis				

2.3.1 MAFs of clinical malaria with objective or subjective fever

Using a subjective history i.e. reported by mother or guardian, of fever in the case definition was associated with a consistently lower MAF than objectively elevated temperature (axillary temperature $\geq 37.5^{\circ}\text{C}$) independent of age. This difference was more marked in the higher transmission cohorts (Chonyi and Pingilikani) Table 2-2. As expected, using a threshold of >2500 parasite/ μl increased the MAF for all case definitions. However, among children with subjective fever in Chonyi the MAF was 59% even after applying this threshold (Table 2-2).

In the Junju cohort, where blood smears were only made on objectively febrile children, on only 8 out of 532 occasions (0.02%) were children with a history of fever but no elevated temperature found to have febrile malaria on a return visit conducted 6-12 hours later. The children who had no objective fever at 6-12 hours follow-up had a similar risk of a subsequent malaria episode as the children (matched by season and age group) who had no history of fever (HR: 0.9 95%CI 0.7-1.5, p value=0.9).

Older children (2.5-5 years) had lower MAF than younger children (0-2.5 years) which increased after using a parasite threshold of $>2500/\mu\text{L}$ (data not shown). However this difference was less marked in a low transmission cohort (Ngerenya).

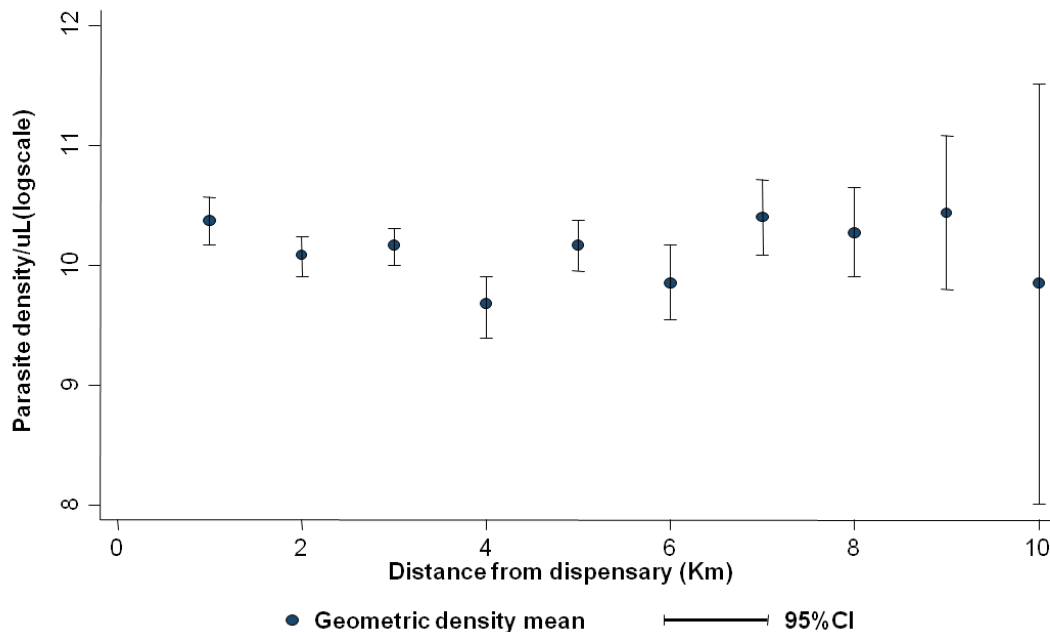
Table 2-2 Malaria attributable fractions of malaria case definitions for any parasitaemia and >2500/uL in the four cohorts

	Using objective fever(temp $\geq$ 37.5 C)	Using subjective <i>but not</i> objective fever (temp<37.5 C)	Using both case definitions
<i>Chonyi cohort; active surveillance cohort</i>			
MAF for density >0/ μ L (95%CI)	68% (63%-71%)	44% (38%-50%)	56% (52%-61%)
Number of cases	558	1105	1663
MAF for density>2500/ μ L(95%CI)	81% (78%-84%)	59% (53%-64%)	70% (66%-74%)
Number of cases	456	671	1127
<i>Ngerenya cohort ; active surveillance cohort</i>			
MAF for density >0/ μ L (95%CI)	76% (73%-78%)	67% (65%-69%)	74% (72%-76%)
Number of cases	1225	1802	3028
MAF for density>2500/ μ L(95%CI)	83% (81%-85%)	81% (79%-83%)	83% (81%-85%)
Number of cases	1060	1253	2313
<i>Pingilikani cohort; passive surveillance only cohort</i>			
MAF for density >0/ μ L (95%CI)	75% (74%-76%)	40% (39%-41%)	61% (60%-61%)
Number of cases	3954	3209	7163
MAF for density>2500/ μ L(95%CI)	85% (84%-86%)	54% (53%-55%)	73% (72%-74%)
Number of cases	2433	1609	4042
<i>Junju cohort; active surveillance package cohort</i>			
MAF for density >0/ μ L (95%CI)	72% (69%-76%)	NA	NA
Number of cases	809	NA	NA
MAF for density>2500/ μ L(95%CI)	85% (83%-88%)	NA	NA
Number of cases	636		

2.3.2 Parasite densities

Children under passive surveillance in Pingilikani had a significantly higher geometric mean parasite density than children under active surveillance in Junju [10,300/ μ L:95% CI: 9,800-10,800 versus 4,775/ μ L:95%CI: 3,900-5,800]. Furthermore, there was no variation in geometric mean parasite density with distance from dispensary for the Pingilikani dispensary cohort within 10 kilometres of the dispensary suggesting that distance to healthcare was not a factor that determined the severity of disease at presentation (Figure 2:2).

Figure 2:2 Variation of geometric parasite density mean with distance from Pingilikani dispensary



Variation of geometric mean parasite densities with distance from Pingilikani dispensary. Parasite densities have been converted into log₁₀ scale.

2.3.3 Comparison of active and passive surveillance

Part of the Junju active cohort and the Pingilikani dispensary-based cohort overlapped geographically (Figure 1). Among an age-, time- and location-matched subgroups of these cohorts the Malaria Attributable Fractions were similar (Table 2-3). However, the incidence of clinical malaria identified by active surveillance was over three times the incidence by passive surveillance. The mean febrile temperature for passive surveillance at dispensary cohort was 38.6°C [95%CI: 38.6-38.7] compared with 38.3°C [95%CI: 38.2-38.4] on active surveillance, although the geometric mean parasitaemia were not statistically significantly different (Table 2-3).

Table 2-3 Comparison between passive surveillance and active surveillance during follow-up from May 2007 to May 2008 in a subset of children aged 5-17 months

Cohort location	Passive surveillance# Pingilikani	Active surveillance package* Junju-Pingilikani
Total number of children	561	105
Total number of contacts	932	3394
Total time at risk (person years)	562.6	91.6
Total malaria episodes ^θ	58	34
Median contacts (Range)	1.6 (1-9)	32 (2-48)
Geometric mean parasite density (/μL) (95% CI)	14,700(8,700-24,900)	41,000(18,300-92,000)
Children with at least one malaria episode	33	14
Incidence of clinical malaria ^θ	0.10 [95%CI: 0.08-0.13]	0.37 [95% CI: 0.27- 0.52]
Malaria Attributable Fraction >0/μL	92% (89%-95%)	88% (81%-96%)
Malaria Attributable Fraction >2500/μL	98% (95%-100%)	94% (87%-100%)

*= The active surveillance package consisted of passive surveillance at dispensary, passive surveillance at community by trained field worker with supervision from study clinician and active weekly surveillance by trained field workers. θ= Clinical malaria defined as fever (axillary temperature $\geq 37.5^{\circ}\text{C}$ plus parasitaemia of any density. #= Passive surveillance package consisted of Passive surveillance at dispensary only. Numbers in brackets are 95% confidence interval unless indicated otherwise.

2.4 Discussion

In a subset of concurrent location- and age-matched cohorts, I found that purely passive surveillance without specific prompts for visits detected about one third of the episodes that would have been identified by active surveillance yet MAFs were similar. Furthermore, cases with subjective fever but no objective evidence (temperature < 37.5°C) had persistently lower Malaria Attributable Fractions, even when a parasite density threshold was used.

It was surprising that the difference between rates for active and passive case detection in matched subset of children was not larger. Pingilikani dispensary serves a population of 4000 children under 5, and there had been no contact at the homestead or village level to encourage attendance. In contrast, active surveillance required one local fieldworker per 30-40 children monitored with frequent contact at the homestead level (compared to 1 field worker per >1,000 children in passive surveillance). The large numbers of children using Pingilikani dispensary could be attributed to the availability of adequate staff seven days a week, good malaria diagnostic facilities and a constant antimalarial drug supply in contrast to frequent supply problems reported elsewhere in Kenya [247].

Nevertheless, I identified fewer cases of malaria at the dispensary than would have been seen on active surveillance, and in comparison children brought to the dispensary had significantly higher mean temperatures when they were febrile but similar symptomatic blood stage parasitaemia. This suggests that parents were inclined to postpone presentation at the dispensary until more acute overt illness. This was not simply the parents' inability to identify illness, since when field workers were made highly

accessible in the subset of Junju cohort children located in Pingilikani sub-location, the majority of malaria episodes were identified by assessments initiated by the mothers between regular weekly visits. There was no gradient of increasing parasite density with distance from the dispensary, suggesting that the distance parents had to travel with their children did not delay their treatment seeking. I conclude that, in our setting, there is a barrier to approaching medical staff in a health facility that does not exist with more familiar, local field workers. Cases of febrile malaria not presenting to the local dispensary may resolve without treatment or be treated by anti-malarials bought by the parents from shops[248].

Passive surveillance in health care facilities has been reported to be insensitive outside Africa [249,250] and globally [3]. As malaria incidence falls it becomes increasingly important to identify the majority of infections to sustain progress in control [251]. Novel approaches, for instance passive surveillance operated by fieldworkers stationed locally, deserve further consideration and evaluation.

The proportion of fevers attributable to malaria was persistently lower in children with subjective fever than in children with objective fever. Fever diagnosis by parents is unreliable [252,253]. Almost half of the cases with reported but not objective fever were classified as not “true malaria” (i.e. they represented asymptomatic parasitaemia in febrile patients with other aetiologies). Based on my results, the inclusion of reported fever cases in the endpoint of a clinical trial, or in measuring the public health burden of malaria is questionable.

Other factors may be relevant in defining the endpoint of a study. Although the main outcome for most Phase IIb vaccine trials in children would be clinical malaria, cluster randomized trials of transmission blocking vaccines might appropriately include all parasitaemic cases identified regardless of the definition of clinical malaria.

However, approximately half of all cases presenting had only subjective fever, and so the loss in power of the study must be balanced against the need to include endpoints of high specificity. As an illustration, consider a hypothetical example where the incidences of subjective and objective febrile malaria were both 10 per 100 children, and the respective MAFs were 54% and 85% (using a threshold of 2500 parasites/ μ L). A study would have 90% power to detect 50% vaccine efficacy on including 1800 children using objective fever alone as the endpoint, falling to 1300 children using objective plus subjective fever. However, the estimate of efficacy would be 43% using objective fever, but 35% using objective fever plus subjective fever.

When I looked at 532 children with subjective fever in Junju cohort who were followed 6-12 hours later, I found only 4% (19/532) developed an objective fever despite not receiving treatment and only 8 (0.02%) had parasitaemia. Furthermore, children who remained afebrile at 6-12 hours of follow-up had a similar risk of subsequent malaria episodes and other adverse events as other children matched by age and time of follow up. This was done in a clinical trial where we carefully monitored children and ensured 24 hour access to health care, and would not be practical for routine healthcare or larger studies, but demonstrates that subjective fever alone does not identify children at a high risk of serious disease.

When estimating MAFs in the Pingilikani cohort, cross-sectional surveys from neighbouring Junju were used. Since malaria transmission can vary significantly within short distances [204] using controls from a different area could have biased our MAF estimates. Furthermore controls were from a closely monitored cohort which could underestimate the true population prevalence of asymptomatic parasitaemia. However, these two directly adjacent areas are similar in soil-type, housing and Entomological Inoculation Rate (EIR) [245]. A potential estimation error is likely to non-differentially affect both MAF estimates, and thus, is expected not to bias incidence estimates.

My results of the comparative analysis between passive surveillance and active surveillance apply in the context of our transmission patterns (seasonal/low to moderate) and age group compared, and extrapolation to other transmission patterns and age groups must be done with caution. Ngerenya and Chonyi cohorts operated identical surveillance methods, but the difference between objective fever and subjective fever was greater in Chonyi (at moderate transmission intensity) than in Ngerenya (at low transmission intensity). I have avoided comparisons between surveillance in Junju and Ngerenya/Chonyi because both the surveillance methodology and transmission intensity are different. Another limitation of our study is its retrospective nature which is prone to unmeasured confounding and bias.

2.5 Conclusion

In conclusion, the Malaria Attributable Fractions are similar between active and passive surveillance, and passive surveillance at the dispensary underestimates malaria occurrence substantially. Using passive case detection at dispensaries alone in a phase II

trial would have only picked a small proportion of clinical malaria cases and undermined the efficacy estimates. Cases of malaria identified on the basis of reported fever alone should not be included in the primary endpoint of a clinical trial since the specificity of the endpoint is low.

3 Malaria exposure index

3.1 Background

Heterogeneity in malaria exposure can bias the estimates of malaria vaccine efficacy, as reviewed in detail in Chapter 1 section 1.8.2. Furthermore vaccine efficacy may vary according to the intensity of exposure [254] and therefore the utility of a malaria vaccine can be different in different transmission settings.

Knowledge of individual malaria exposure would allow analysis of vaccine efficacy to take into account the intensity of malaria exposure. Adjusting for the individual malaria exposure would a) minimize the bias of heterogeneity in malaria exposure on efficacy estimates b) allow one to disentangle the effects of acquisition of blood stage immunity from the effect of malaria vaccine and c) allow study of interaction between vaccination and malaria exposure. I therefore conducted an analysis to develop an individual marker of malaria exposure in the field.

3.2 Methods

3.2.1 Cohort population and data

I used cohort data from Chonyi, Junju and Ngerenya sub-locations located within Kilifi Health and Demographic Surveillance System (HDSS) [55]. The data were prospectively collected between 1999 and 2001 for Chonyi, 1998 and 2010 for Ngerenya and 2006 and 2010 for the Junju cohort.

Surveillance methods and detailed information on the cohorts have been described in section 2:2:1 and previously published [184,255]. In 291 children aged 5 to 17 months from the Junju cohort, a venous blood sample was obtained at a single cross sectional bleed and tested for anti-merozoite surface protein-1 (MSP-1₄₂) and anti-apical membrane antigen-1 (AMA-1) human immunoglobulin G (IgG) antibodies by enzyme-linked immunosorbent assay as described previously[256]. Additional data collected included individual homestead locations (latitude and longitude coordinates).

For the purpose of this study, malaria infection was defined as any *P. falciparum* positive blood smears (i.e. either asymptomatic parasitaemia or an episode of febrile malaria). I also determined if each individual was living within a malaria hotspot [257]. A malaria hotspot was defined, independent of this current work, as an area where the observed incidence of febrile malaria or asymptomatic parasitaemia was higher than would be expected if cases were evenly distributed, as defined using the spatial scan statistic at $p < 0.05$, including a maximum of 30% of the population in a hotspot[258]. Chonyi has

been considered as a relatively high malaria transmission area with Junju and Ngerenya regarded as moderate and low malaria transmission areas respectively [245]. However since 1999 malaria has been declining in the overall study area [259].

3.2.2 Relationship between malaria infection and proximity to infected case

I computed distances (in Kilometres) from each individual to all others in each of the cohorts. The proximity of the index child to the next nearest infected child and next nearest uninfected child was calculated. This was done separately for two time windows; four months and one year time intervals. To derive the best powers for transforming distances, we fitted a set of power functions of distance as a function of malaria infection status in logistic regression models to optimize the log likelihood. This allowed for a nonlinear relationship to be fitted. The power functions that maximized the log likelihood fit were then used to transform absolute distances, and subsequently used in modified Poisson regression models to assess the effect of proximity to infected/uninfected children on the risk of malaria infection in the index child.

3.2.3 Calculation of weighted local prevalence of malaria infection

The weighted local prevalence was calculated as distance-weighted proportions of malaria infected children within an area of specified radius and over specified time intervals. Two time intervals were used with the fourth month interval reflecting three distinct seasons with varying malaria transmission [260] whilst the one year time interval

was selected as a convenient annual summary. I used inverse distance weighting to give the children nearest to the index more weight in determining the local prevalence [261].

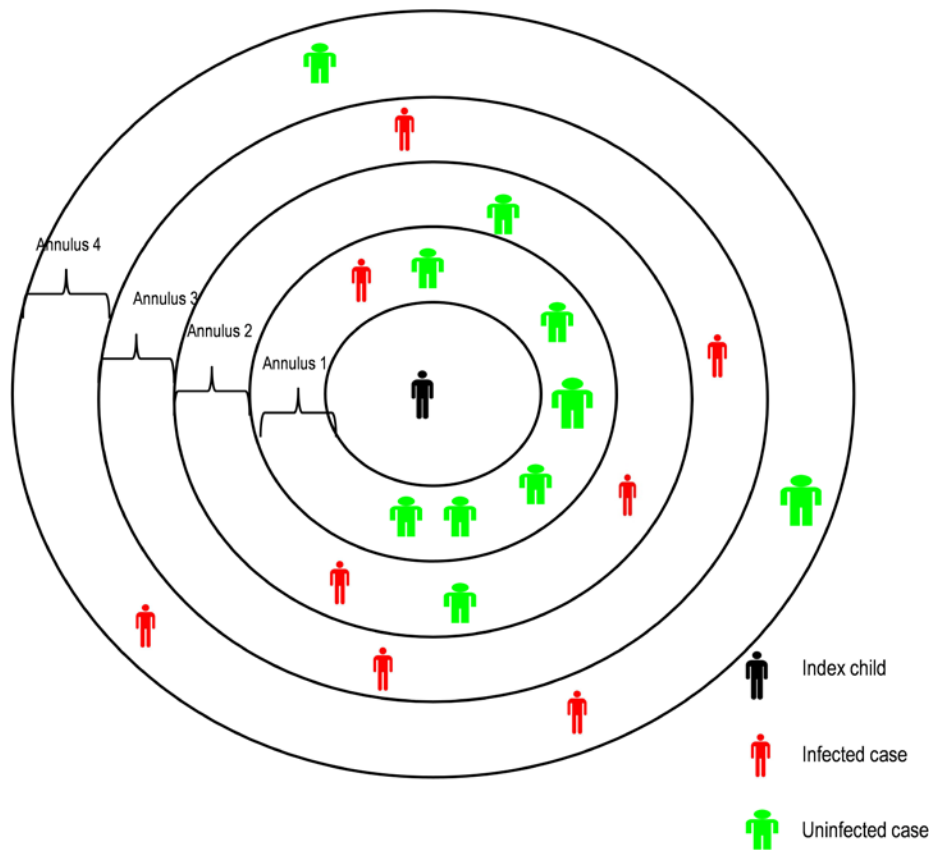
$$x = \frac{\sum_{i=1}^N Zi \div Di}{\sum_{i=1}^N (1 \div Di)}$$

(Where x is the interpolated weighted local malaria prevalence for the index individual, Zi is the known infection status of the surrounding child (0: for uninfected and 1: for infected), Di is the distance from the index individual to the surrounding child. The weighted local prevalence was expressed as a proportion with values between 0 and 1. I also calculated an unweighted local malaria prevalence as the simple proportion of infected children within 1 km.

3.2.4 Selection of best radius

To determine the best radius over which the weighted local prevalence should be calculated, I grouped children around each index child in the cohort into annuli at ≤ 0.2 km, > 0.2 km to ≤ 0.5 km, > 0.5 km to ≤ 1 km and > 1 km to ≤ 2 km (Figure 3:1). I then determined how well the calculated weighted local prevalence from these annuli predicted the risk of individual malaria infection. The annuli analyses allowed me to determine if the individuals in the outer zones had any additional impact in the risk prediction. The cut-off point for the radius was based on the last distance beyond which the weighted local prevalence didn't predict risk of infection.

3:1 Annuli around index child



3.2.5 Univariate analysis

The outcome measure was binary; malaria infection (i.e. either asymptomatic parasitaemia or at least one febrile malaria episode) or no malaria infection (i.e. no asymptomatic parasitaemia and no febrile malaria episodes) within four months or within one year time intervals. I investigated the effect of the following variables; weighted local malaria prevalence, distance to the next nearest infected and uninfected children and age. Residence in malaria hotspot as a binary variable was also included in the analysis because of prior report of its effect on risk of malaria infection [257]. In 291 children

from Junju, the effect of log transformed AMA1 and MSP1 antibody levels on malaria infection were also assessed. The effect of each variable was assessed by modified Poisson regression analyses with a robust error variance [262].

Multiplicative interaction models were used to assess interactions between proximity to the infected and uninfected children on the risk of malaria infection in the index child. Adjustments were made for the multiple observations per individual with a fixed effect for the time period and random effect term for individual. Risk Ratios (RR) and 95% confidence intervals (95% CIs) were estimated. To visualize the relationship between risk of malaria infection in the index child and proximity to other infected and uninfected children I differentiated the modified Poisson equation for the effect of distance and plotted the rate of change in risk over the first 1 km.

3.2.6 Multivariable analysis and model calibration

A multivariable modified Poisson regression model was used to evaluate the independent role of each variable to predict malaria infection in the index child by including all significant variables ($p < 0.05$) from the univariate analysis.

I also used causal directed acyclic graph (DAG) as described before [263] to assess the suitability of our covariates for use in the final multivariable model. The aim was to minimize the magnitude of bias for the estimates of local malaria prevalence on the risk of malaria infection.

I determined the area under the receiver operating characteristic (ROC) curve in order to evaluate the discriminatory ability of weighted local malaria prevalence and log

transformed AMA1 and MSP1 antibody levels for malaria infection in the index child [264]. I then compared the discriminatory power of individual models with the model consisting of both anti-merozoite antibody levels and weighted local prevalence. Analyses were done using STATA version 11 software (Stata Corp., College Station, TX).

3.2.7 Ethical considerations

Written informed consent was obtained from the adults enrolled and from parents/guardians of the children enrolled using an approved consent form. The approval for human participation in three cohorts was given by the Kenya Medical Research Institute (KEMRI) National Ethical Review committee [61,184].

3.3 Results

A total of 2,425 participants were included in the final analysis constituting 7,166 person years of follow up. The age of participants ranged between 0 to 81 years (median 15, IQR; 0-76.2). There were 10,304 confirmed malaria infections of which 6,377 (62%) were asymptomatic. The demographic, parasitological characteristics and duration of follow-up for the three cohorts is shown in Table 3-1.

Table 3-1 Demographic and parasitological characteristic of the cohort used in the analysis

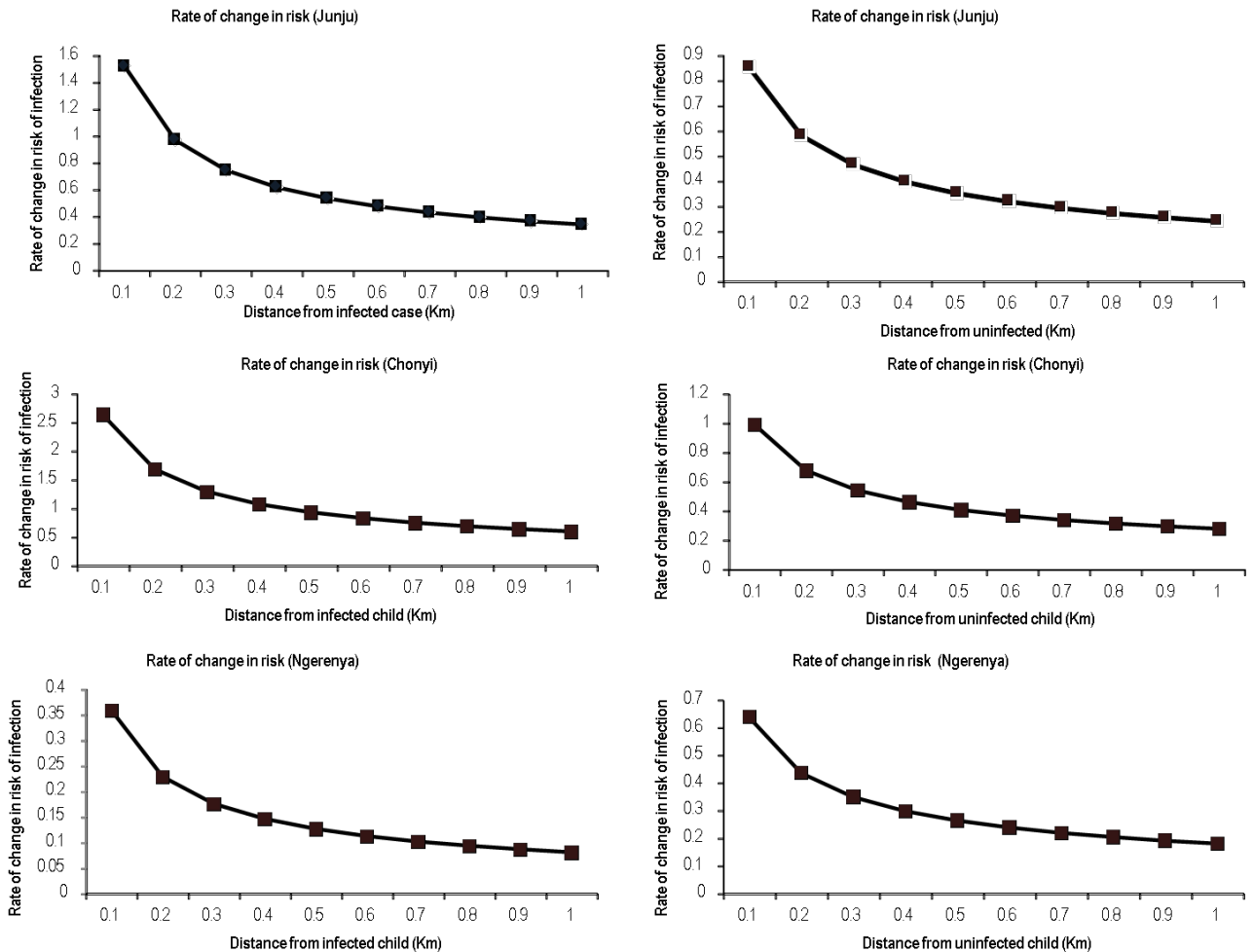
Cohorts	Junju	Chonyi	Ngerenya
Follow up period	2006 to 2010	1999-2000	1998-2010
Age (median, IQR)	3.1(0.1-6.4)	15.6 (0.1-78.9)	14.5 (0-80)
Number of all participants	620 (100)	874 (61.6)	931(66.9)
(Percentage below 10 years)			
Female %	48.4%	58.7%	56%
Total number of malaria infections	2109	3283	4912
Asymptomatic infection	408	2480	3489
Total surveillance visits	83,566	90,437	200,074
Mode of surveillance	Active surveillance	Active surveillance	Active surveillance

3.3.1 Risk of malaria as a function of proximity to the infected case

Increasing distance to the next nearest infected child was associated with a reduced risk of malaria infection in the index child in all three cohorts (Risk ratio (RR) = 0.37, 95%CI: 0.28-0.50 for Junju, RR=0.18, 95%CI 0.03-0.84 for Chonyi and RR=0.52, 95%CI 0.42-0.66 for Ngerenya). The rate of change in risk was highest within 1 km (Figure 3:2).

In contrast increasing distance to the next nearest uninfected child was associated with an increased risk of malaria infection in the index child; RR of 1.88 (95%CI: 1.30-2.72), 1.72 (95%CI: 1.48-2.0) and 1.49 (95%CI: 1.35- 1.65) in Chonyi, Junju and Ngerenya respectively. The rate of change in risk was similarly highest within the first 1km (Figure 3:2). I identified no interaction between the effects of distance to infected and uninfected children on the risk of malaria infection in the index child.

Figure 3:2 Rate of change in the risk of malaria infection over distance to infected and uninfected case within first kilometer



Y axis represents a change in risk coefficient per unit increase in kilometer from infected or uninfected case (Only the first 1km is included). As the distance from both infected and uninfected case increases, the marginal change in risk (increase or decrease in risk) to infection decreases. The marginal changes in risk are minimal beyond 1km.

3.3.2 Risk of malaria as a function of the weighted local malaria prevalence within a 1 km radius

The values of weighted local prevalence range between 0 and 1 and their distributions are shown in Figure 3:3, Figure 3:4 and Figure 3:5. The distributions of weighted local prevalence were related to the malaria transmission intensity and the trend in mean weighted local prevalence over time in years mirrors the changes in transmission (see box 1). In Junju and Chonyi, weighted local malaria prevalence estimated from participants within ≤ 0.2 km, >0.2 km to ≤ 0.5 km, >0.5 km to ≤ 1 km but not those within > 1 km to ≤ 2 km zones were predictive of malaria infection in the index child. In Ngerenya weighted local malaria prevalence estimated from participants within all four annuli were predictive of malaria infection in the index child).

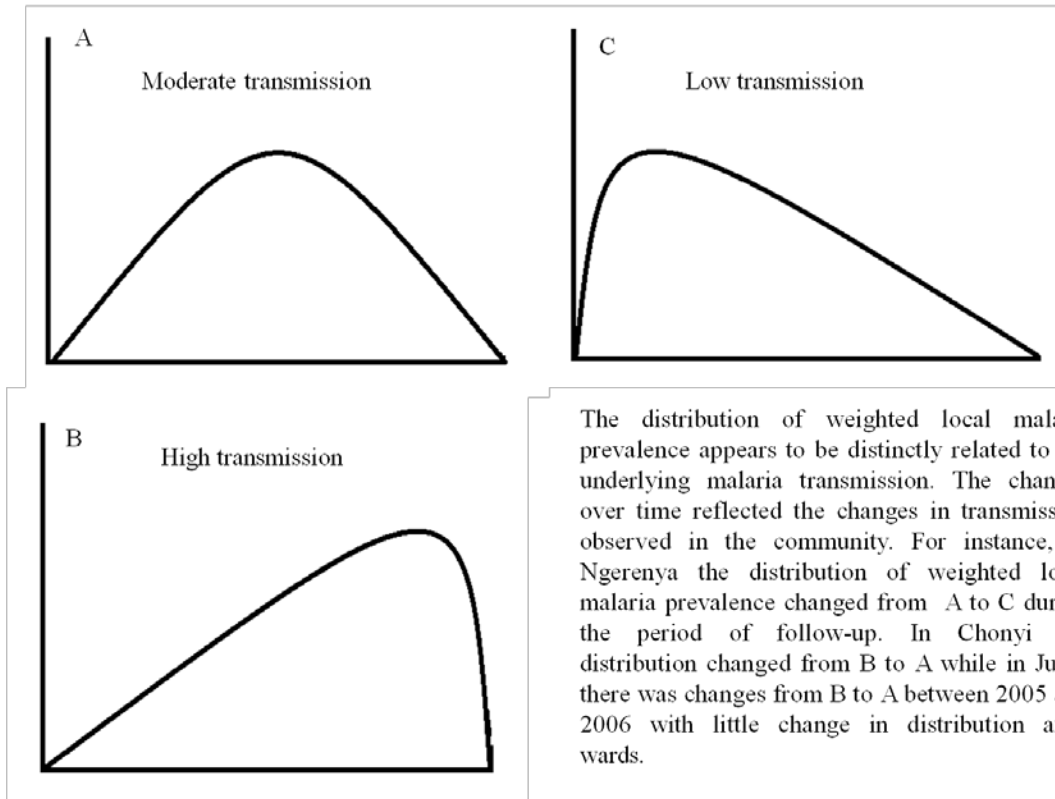
I reasoned that because there was an inconsistent effect on the risk of malaria infection by the weighted local malaria prevalence beyond 1km, but a consistent effect for the three zones examined within 1km, that the optimal measure of exposure would be the distance-weighted proportion of malaria infections within 1 km radius. Consistently the plots of rate of change in risk of malaria infection versus proximity to infected case showed only a marginal effect beyond 1km in all three cohorts (Figure 3:2).

In univariate analysis, weighted local malaria prevalence within 1 km was a strong predictor of risk of malaria infection in the index child in all three cohorts. An increase of 10% in weighted local malaria prevalence resulted in malaria infection RR of

1.99(95%CI: 1.75-2.26), 2.19(95%CI: 1.77-2.70) and 2.25 (95%CI: 1.90-2.67) in Junju, Chonyi and Ngerenya cohort respectively. Areas under the ROC curve for the univariate weighted local malaria prevalence models were 0.72(95%CI: 0.66-0.73), 0.71(95%CI: 0.69-0.73), and 0.82 (95%CI: 0.80-0.83) for Chonyi, Junju, and Ngerenya respectively.

The effect of unweighted local malaria prevalence was similar to weighted local prevalence with a tendency towards higher areas under ROC curve for distance-weighted than unweighted local malaria prevalence).

I also examined the effects of weighted local malaria prevalence estimated from four monthly follow up data. These did not differ significantly from those estimated from yearly follow up data in Junju and Chonyi but was significantly higher in Ngerenya cohort (Table 3-3). The Areas under ROC curve were similar to those of yearly follow up in all the cohorts.

Box 1

The distribution of weighted local malaria prevalence appears to be distinctly related to the underlying malaria transmission. The changes over time reflected the changes in transmission observed in the community. For instance, in Ngerenya the distribution of weighted local malaria prevalence changed from A to C during the period of follow-up. In Chonyi the distribution changed from B to A while in Junju there was changes from B to A between 2005 and 2006 with little change in distribution afterwards.

Figure 3:3 Local distance-weighted malaria prevalences in Junju cohort

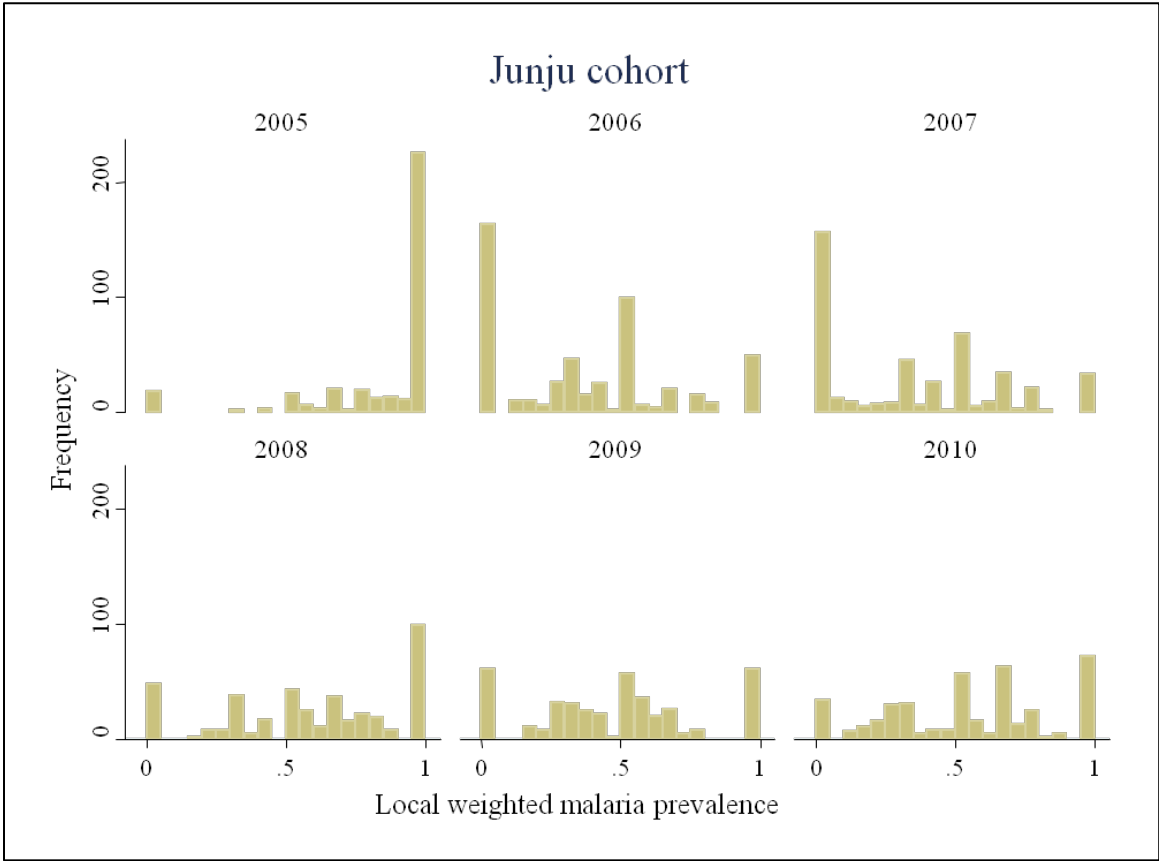


Figure 3:4 Local distance-weighted malaria prevalences in Chonyi cohort

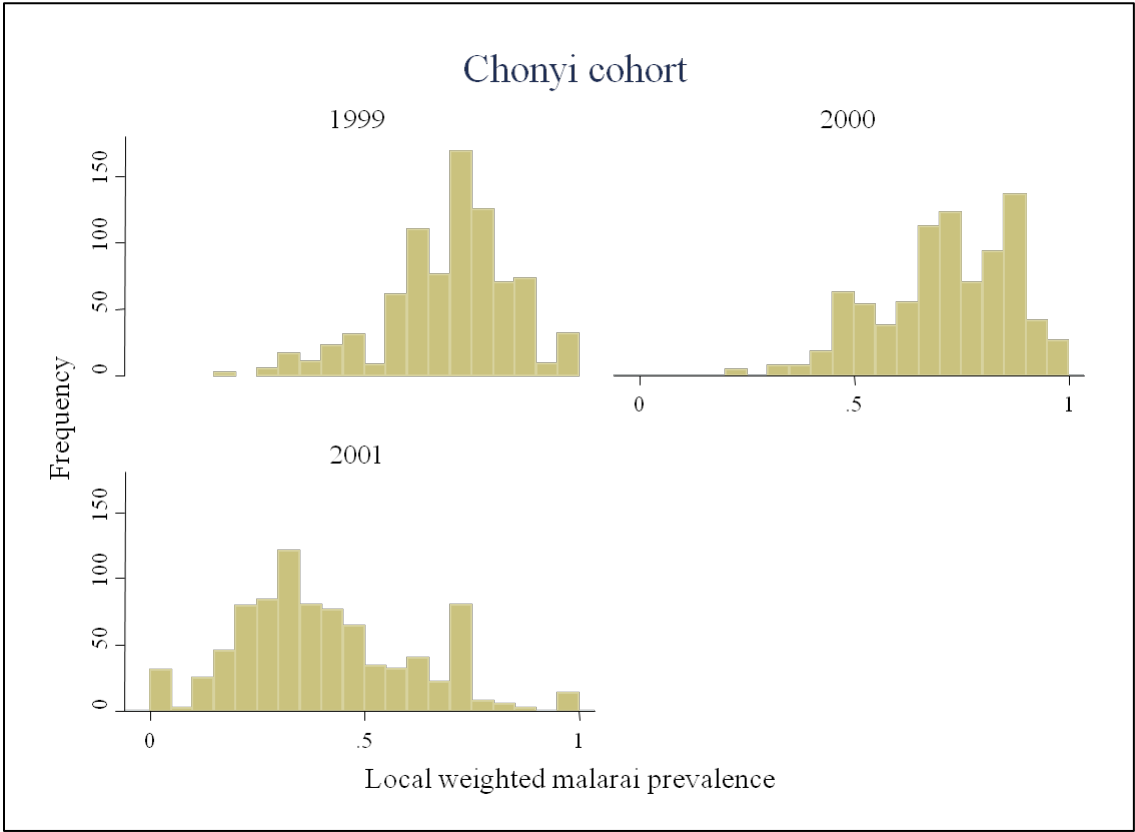


Figure 3:5 Local distance-weighted malaria prevalences in Ngerenya cohort

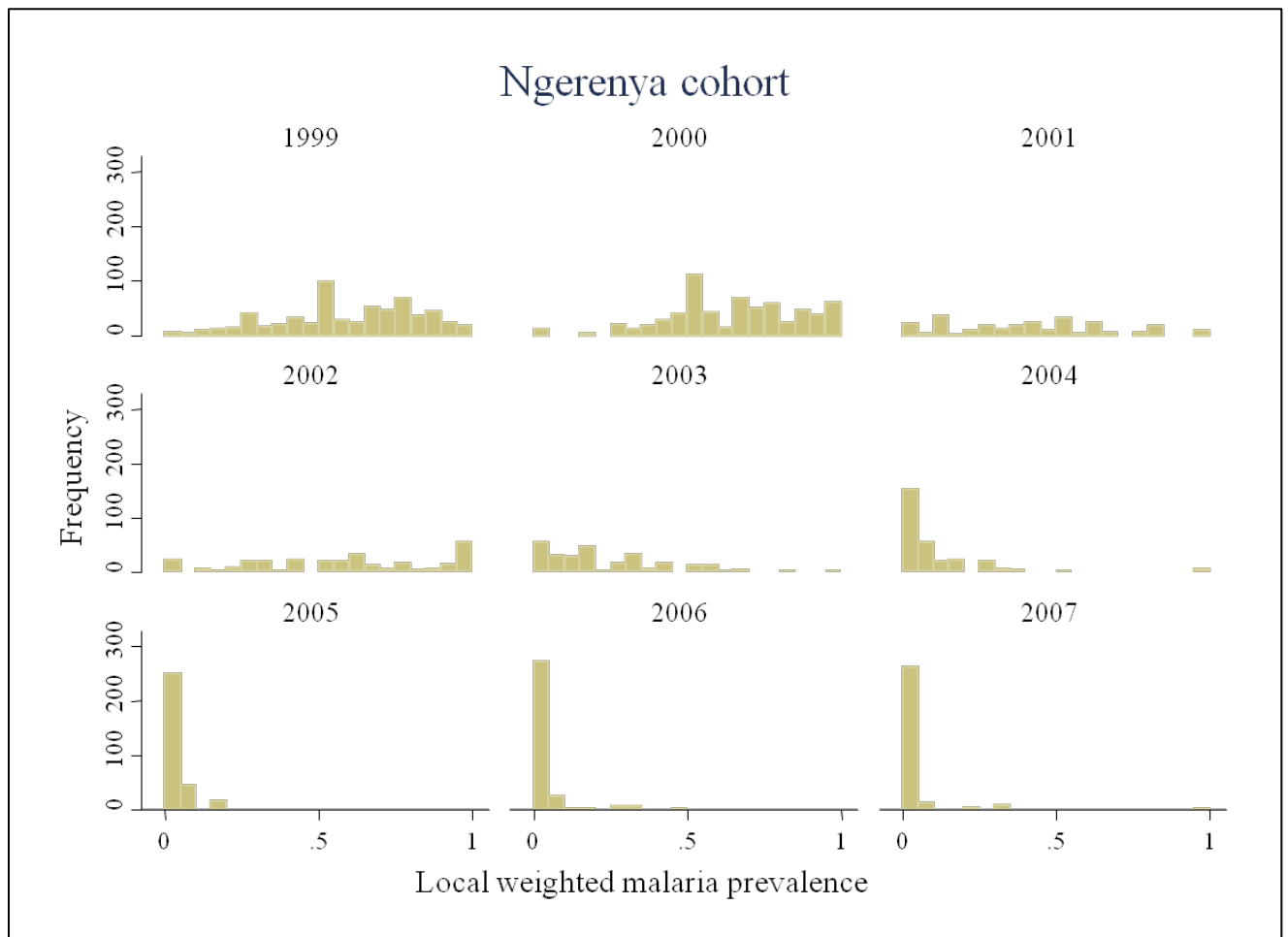


Table 3-2 Effect of weighted local prevalence of malaria infection from four annuli around each individual on risk of malaria infection

	RR(95%CI)	P value	AUC
<i>Chonyi cohort</i>			
Weighted local malaria prevalence(<0.2km)	2.19(1.78-2.70)	<0.001	0.68
Weighted local malaria prevalence (>0.2-0.5km)	2.23 (1.66-3.02)	<0.001	0.68
Weighted local malaria prevalence (>0.5- 1km)	1.80 (1.279-2.55)	0.001	0.67
Weighted local malaria prevalence (>1-2km)	1.49 (0.95-2.33)	0.079	NA
Unweighted local malaria prevalence<1km	3.36 (1.66- 6.77)	0.001	0.65
Weighted local malaria prevalence <1km	2.19 (1.78- 2.70)	<0.001	0.68
<i>Junju cohort</i>			
Weighted local malaria prevalence(<0.2km)	1.95 (1.71-2.22)	<0.001	0.71
Weighted local malaria prevalence (>0.2-0.5km)	1.42 (1.15-1.74)	0.001	0.68
Weighted local malaria prevalence (>0.5- 1km)	1.99 (1.22-3.24)	0.005	0.68
Weighted local malaria prevalence (>1-2km)	0.71 (0.33-1.52)	0.383	NA
Unweighted local malaria prevalence<1km	1.54 (1.18-2.01)	0.001	0.67
Weighted local malaria prevalence <1km	1.99 (1.75-2.26)	<0.001	0.71
<i>Ngerenya cohort</i>			
Weighted local malaria prevalence(<0.2km)	2.25 (1.90-2.67)	<0.001	0.82
Weighted local malaria prevalence (>0.2-0.5km)	0.97 (0.69-1.36)	0.887	NA
Weighted local malaria prevalence (>0.5- 1km)	1.81 (1.44-2.27)	<0.001	0.81
Weighted local malaria prevalence (>1-2km)	1.52 (1.13-2.04)	0.005	0.79
Unweighted local malaria prevalence<1km	3.38 (2.58- 4.42)	<0.001	0.80
Weighted local malaria prevalence <1km	2.25 (1.90- 2.67)	<0.001	0.82
AUC: Area under the curve, RR: Risk Ratio			

Table 3-3 Weighted local prevalence of malaria infection for four monthly follow-up data

	Univariate analysis		Multivariable analysis	
	RR (95%CI)	P value	RR (95%CI)	P value
<i>Ngerenya cohort</i>				
Weighted mean incidence (radius 1km)	4.55(3.76- 5.51)	<0.001	2.59(2.11-3.18)	<0.001
Distance to nearest infected case	0.46(0.38-0.54)	<0.001	0.73(0.6-0.90)	0.003
Distance to the nearest uninfected case	2.19(1.91-2.53)	<0.001	1.17(0.98- 1.41)	0.080
Residence in malaria hotspot	1.89(1.74 -2.06)	<0.001	1.58(1.43-1.73)	<0.001
Age*	NA	<0.001	NA	<0.001
<i>Chonyi cohort</i>				
Weighted mean incidence (radius 1km)	2.48(2.06- 2.98)	<0.001	1.55(1.31-1.84)	<0.001
Distance to nearest infected case	0.20(0.11- 0.38)	<0.001	0.3(0.16- 0.57)	<0.001
Distance to the nearest uninfected case	2.54(1.77- 3.61)	<0.001	1.68(1.31- 2.14)	<0.001
Residence in malaria hotspot	1.47(1.36- 1.59)	<0.001	1.34(1.25-1.44)	<0.001
Age*	NA	<0.001	NA	<0.001
<i>Junju cohort</i>				
Weighted mean incidence (radius 1km)	2.09(1.84- 2.36)	<0.001	1.70(1.32- 2.18)	<0.001
Distance to nearest infected case	0.43(0.35-0.52)	<0.001	0.66(0.49-0.89)	0.007
Distance to the nearest uninfected case	1.5(1.21-1.86)	<0.001	0.86(0.61-1.20)	0.369
Residence in malaria hotspot	1.37(1.25-1.51)	<0.001	1.29(1.18-1.41)	<0.001
Age*	NA	0.37	NA	0.39
* Age has non-linear association with risk of clinical malaria (see Figure 3:6), RR: Risk ratio				

3.3.3 Effect of malaria hotspot and age on the risk of malaria infection

Residence in a malaria hotspot was associated with an increased risk of malaria infection in the index child. The effect was more pronounced in the lowest transmission area; Ngerenya (RR: 1.45, 95%CI: 1.35-1.55) than in areas of moderate to high malaria

transmission; Junju (RR; 1.29, 95%CI: 1.19-1.41) and Chonyi (RR: 1.23, 95%CI: 1.15-1.32) respectively. Age had a statistically significant non-linear effect on malaria infection in the index child. In all three cohorts risk of malaria infection increased with age and peaked at 5 years before starting a slow decline (Figure 3:6).

3.3.4 Multivariable models for predicting risk of malaria infection

Multivariable models were separately developed for the three cohorts to assess the independent role of predictors of malaria infection in the index child and to determine the overall discrimination achieved with the multivariable model. The final multivariable model incorporated the weighted local malaria prevalence within a 1 km radius, distance to the next nearest infected child, distance to the next nearest uninfected child, age and whether resident in a malaria hotspot. Using the DAG approach we confirmed that all selected covariates were plausible confounders and their inclusion in the final model would minimize the magnitude of the bias in the estimate of effect of local malaria prevalence on the risk of malaria infection (Figure 3:7).

Weighted local malaria prevalence, location within a malaria hotspot and age remained significant predictors of malaria infection in the multivariable model (Table 3-4). Proximity to the nearest infected child was predictive in Chonyi but not in Junju and Ngerenya. The areas under the ROC curve for the multivariable prediction models were 0.74 (95%CI: 0.72-0.76) 0.72 (95%CI: 0.70-0.74), and 0.84 (95%CI: 0.83-0.85) for the Chonyi, Junju, and Ngerenya cohorts, respectively (Figure 3:8).

Table 3-4 Univariate and multivariable analysis of predictors of malaria infections

	Univariate analysis		Multivariable analysis	
	RR(95% CI	P value	RR (95% CI	P value
<i>Chonyi cohort</i>				
Weighted local prevalence (1km radius) [§]	2.19(1.77-2.70)	<0.001	1.78(1.38-2.29)	<0.001
Proximity to nearest infected case [§]	0.18 (0.03-0.84)	0.03	0.33(0.07-1.40)	0.135
Proximity to the second nearest case [§]	1.07(0.65-1.76)	0.763	NA#	
Proximity to the nearest uninfected case [§]	1.88(1.30-2.72)	0.001	1.13(0.78-1.65)	0.499
Proximity to the second nearest uninfected case [§]	0.93(0.72-1.21)	0.619	NA#	-
Residence in malaria hotspot	1.23(1.15-1.32)	<0.001	1.14(1.06-1.22)	<0.001
Age (years)*	NA	<0.001	NA	<0.001
<i>Junju cohort</i>				
Weighted local prevalence (1km radius) [§]	1.99(1.75-2.26)	<0.001	1.51(1.21-1.87)	<0.001
Proximity to nearest infected case [§]	0.37(0.28-0.50)	<0.001	0.57(0.40-0.81)	0.002
Proximity to the second nearest case [§]	0.74(0.62-0.87)	<0.001	NA#	-
Proximity to the nearest uninfected case [§]	1.72(1.48-2.0)	<0.001	1.16(0.93-1.43)	0.172
Proximity to the second nearest uninfected case [§]	1.63(1.40-1.90)	<0.001	NA#	-
Residence in malaria hotspot	1.29(1.19-1.41)	<0.001	1.19(1.10-1.30)	<0.001
Age (years)*	NA	<0.001	NA	<0.001
<i>Ngerenya cohort</i>				
Weighted local prevalence (1km radius) [§]	2.25 (1.90-2.67)	<0.001	1.49(1.24- 1.81)	<0.001
Proximity to nearest infected case [§]	0.52(0.42- 0.66)	<0.001	0.52(0.38- 0.71)	<0.001
Proximity to the second nearest case [§]	0.77(0.56- 1.05)	0.101	NA#	
Proximity to the nearest uninfected case [§]	1.49(1.35- 1.65)	<0.001	1.07(0.93- 1.24)	0.286
Proximity to the second nearest uninfected case [§]	1.42(1.31- 1.54)	<0.001	1.17(1.05- 1.30)	0.003
Residence in malaria hotspot	1.45(1.35-1.55)	<0.001	1.26(1.16- 1.36)	<0.001
Age (years)*	NA	<0.001	NA	<0.001
* Multivariable polynomial fraction showed age has a non-linear effect in all the cohorts (see Figure 3:6, #: The best fit model was obtained with only first nearest distances in the model, §: Risk ratios are for each step increase in 0.35 and 0.45 power function of distance to the infected and uninfected child respectively				

3.3.5 Local malaria prevalence and Merozoite antibodies based models in predicting malaria risk

Merozoite antibody levels were assessed in 291 children in the Junju cohort (median age 20.5 months, IQR: 11.6-28.1) at a cross sectional bleed. Merozoite antibodies levels were associated with increase in prospective risk of malaria infection in the index child (

Table 3-5). Univariate predictive models for AMA-1 and MSP-1₄₂ antibodies produced areas under the ROC curve of 0.75 and 0.76 respectively.

In the same group of children weighted local malaria prevalence within 1km radius was associated with the risk of malaria in the index child in the univariate model providing area under ROC curve of 0.69 (95%CI: 0.64-0.73). A multivariable model incorporating weighted local malaria prevalence, distance to the next nearest infected child, distance to the next nearest uninfected child and residence in a malaria hotspot had an area under the ROC curve of 0.72 (95%CI: 0.67- 0.76) which was not markedly different from either weighted local malaria prevalence or antibody level specific univariate models. The area under the ROC curve for the multivariable model incorporating weighted local malaria prevalence and antibodies to AMA1 and MSP₁₄₂ was 0.83 (95%CI: 0.79-0.88) (Table 3-5).

Table 3-5 Merozoite antibody versus weighted local prevalence models in predicting malaria infection in Junju sub-cohort

	RR	[95% CI]	P value	AUC (95%CI)
<i>Univariate specific antibody-based model</i>				
AMA1	2.27	1.80-2.86	<0.001	0.75 (0.70-0.80)
MSP1 ₁₄₂	2.03	1.70-2.42	<0.001	0.76 (0.72-0.82)
<i>Multivariable weighted local prevalence-based model</i>				
Weighted local prevalence	2.29	1.22-4.30	0.009	0.72 (0.67- 0.76)
Malaria hotspot	1.16	0.89-1.51	0.245	
Proximity to the nearest infected case	0.76	0.43-1.32	0.337	
Proximity to the nearest uninfected case	1.08	0.62-1.87	0.768	
<i>Univariate weighted local prevalence-based model</i>				
Weighted local prevalence	3.00	2.28- 3.94	<0.001	0.69 (0.64-0.73)
<i>Combined weighted local prevalence and anti-merozoite antibody</i>				
Weighted local prevalence (1 km)	2.14	(1.60-2.87)	<0.001	0.83(0.79-0.88)
AMA1	1.36	(1.06-1.74)	0.015	
MSP1 ₁₄₂	1.56	(1.28-1.89)	<0.001	
AUC: Area under the curve, RR: Risk Ratio				

Figure 3:6 Mi Multivariable fractional polynomial plots of effect of age on the risk of malaria infection

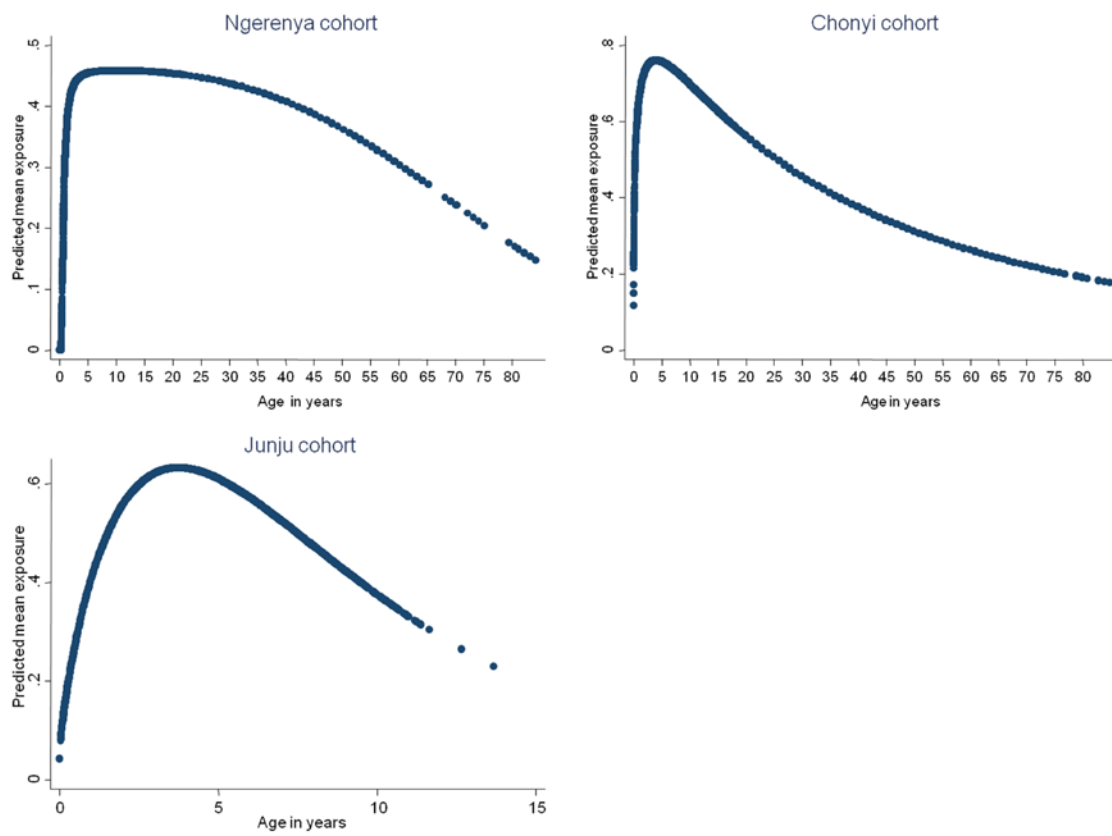
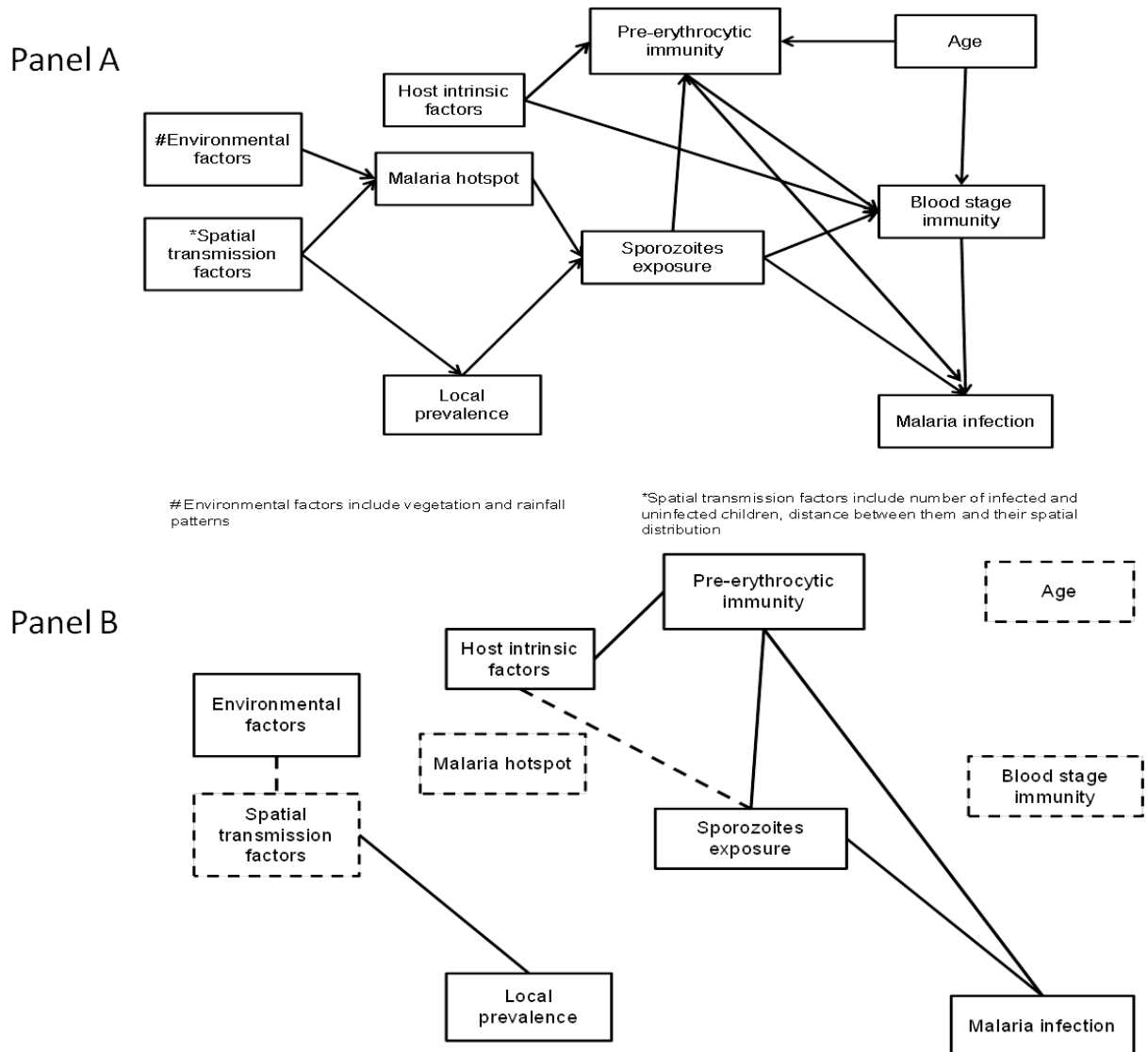
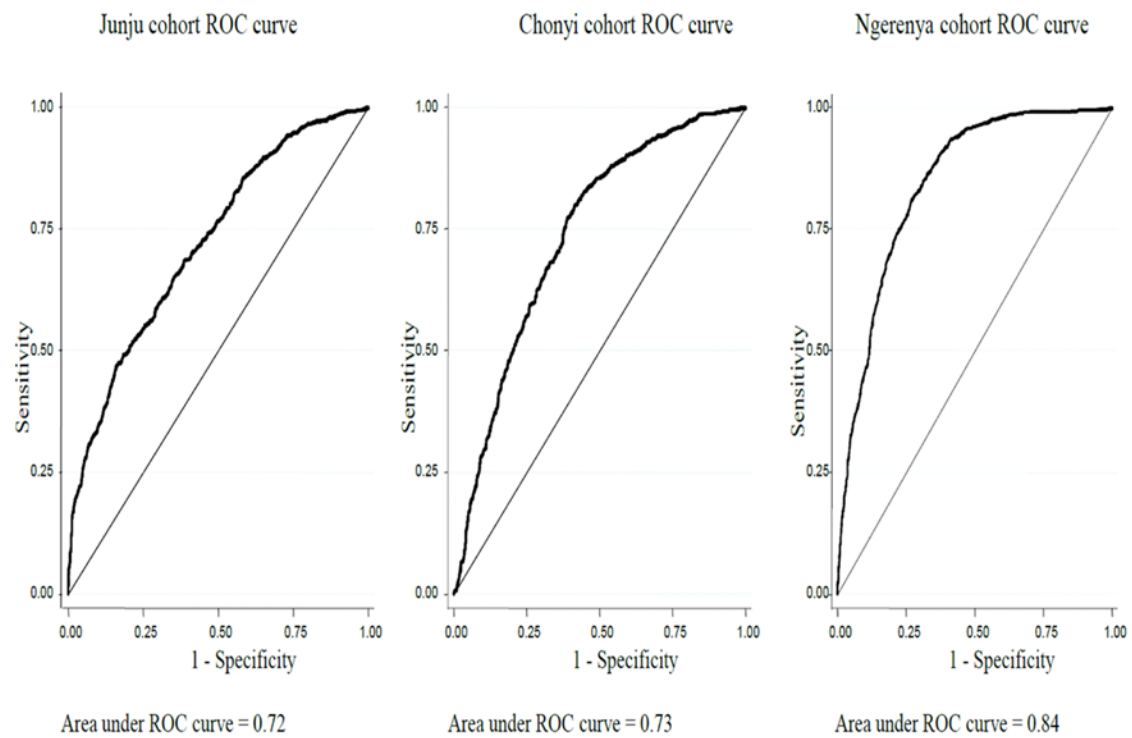


Figure 3:7 Causal directed acyclic graphs (DAG)



Panel A represents the causal diagram for the data; Panel B represent causal diagram after 6 step DAG approach and if one conditions on Age, malaria hotspot, spatial transmission factors (distance from infected and uninfected children) and blood stage antibodies (dashed boxes). Dotted lines represent conditional associations.

Figure 3:8 Area under the ROC curves for the multivariable weighted local prevalence based models



3.4 Discussion

Based on this study I proposed a measure of individual malaria exposure that uses the distance-weighted local prevalence of malaria infection (composite endpoint including asymptomatic infection or febrile malaria) within a 1 km radius. The measure is empirical, being derived from active malaria surveillance and location data, and not based on any assumed distribution of exposure. The weighted local malaria prevalence demonstrated moderate to good discriminatory ability for malaria infection in the index child (ROC of 0.71, 0.72 and 0.82 in Junju, Chonyi and Ngerenya respectively). The discriminatory ability of a multivariable model incorporating the distance-weighted local malaria prevalence (within a 1 km radius), age, distance to the next nearest infected, distance to the next nearest uninfected children and the presence or absence of a malaria hotspot was not statistically different from that of distance-weighted local prevalence within a 1 km radius alone (Table 3-5).

In 291 children in Junju who had antibody levels measured, merozoite surface protein-1 (MSP-1₁₄₂) and apical membrane antigen-1 (AMA-1) antibody levels were also good predictors of the individual prospective risk of malaria infection as described before [256,265] and their discriminatory ability for malaria infection was comparable to that of weighted local malaria prevalence. The combined model incorporating both of the antibodies data as well as and the weighted local malaria prevalence had slightly higher discriminatory ability than either alone (ROC of 0.83). Weighted local malaria prevalence captures exposure related to the spatial distribution of local infections. However antibody responses likely reflects both geographical variations in exposure and

individual variations resulting from factors such as bed net use, individual attractiveness to mosquitoes [208] or genetic variation in susceptibility[186]. This could explain the improved predictive power of the model incorporating the two measures. However, using antibody levels as marker of exposure could be circular in observational studies of natural immunity, particularly when one intends to assess the potential protective value of the same antibody response or a closely correlated antibody response. Under such circumstances adjusting for weighted local malaria prevalence as a marker of exposure may improve the estimates of antibody effect. Furthermore, antibody levels to blood stage antigens may be misleading if half the cohort has been randomized to a pre-erythrocytic vaccine that prevents exposure to blood stage parasites. On the other hand, provided a standardized assay is used, antibody levels will be more readily generalized between cohorts, and give an indication of the average transmission intensity of the cohort that can be compared with other cohorts.

Heterogeneous exposure to malaria complicates the analysis of efficacy of candidate malaria vaccines [134]. Calculating the weighted local prevalence of malaria infection for each child will allow for more sophisticated analyses, such as dividing the cohort into “high exposure” and “low exposure” groups, and examining interactions between intensity of malaria exposure and vaccination. Other indirect measures of exposure such as entomological inoculation rate and parasite prevalence may also be used at a larger scale in large multi-centre studies involving sites with known transmission intensities. However for a single site such measures will provide only the average exposure for the population and not reflect the underlying variability of exposure at homestead or individual level.

To avoid circular reasoning I avoided using the index child's own malaria infection status to calculate the individual weighted local malaria prevalence. A causal diagram suggests that geographical heterogeneity in malaria risk is caused by unmeasured environmental factors. Therefore, although both local malaria prevalence and malaria hotspot shared spatial transmission factors as common ancestor, they represented two different causal pathways to exposure to sporozoites. This could explain why both the effect of malaria hotspot and local malaria prevalence remained significant in the multivariable model.

The risk of malaria infection (i.e. the composite endpoint of asymptomatic and symptomatic parasitaemia) increased with age early on in life and decreased with age later in life consistent with findings from previous studies [266]. Lower exposure to mosquito bites due to small body surface area in children could explain the early trend [267], and the apparent observed decline in the risk of malaria infection later in life could be attributed to the development of effective pre-erythrocytic immunity or of blood stage immunity which suppresses asymptomatic parasitaemia below the level of detection [268].

My study has limitations. The surveillance approach identifies acute clinical malaria by weekly surveillance and asymptomatic parasitaemia on yearly cross-sectional blood films. I would therefore miss brief asymptomatic infections, asymptomatic infections below the level of detection by microscopy, and exposure that does not result in a blood stage infection because of pre-erythrocytic immunity. I have assumed that individuals remained in the same location. Although most infections are likely to be acquired in the evening or night when individuals are at the homestead, it is possible that some few infections were acquired during travel and this cannot be captured in the calculation of

the weighted local malaria prevalence. Nevertheless I have identified empiric evidence that weighted local malaria prevalence predicts the risk of malaria infection in the index child with reasonable accuracy. I conclude that any bias resulting from the limitations described do not preclude the utility of the approach. Furthermore, these limitations may result in an under-estimate of the local prevalence of infection, but in the absence of a geographical bias, the local prevalence will still reflect the intensity of exposure relative to the rest of the cohort.

My findings may not be directly applicable to other settings where the transmitting vectors and human behaviour patterns vary. The optimal radius for calculating local prevalence may be different, and the relative predictive power of malaria hotspots, weighted local malaria prevalence and antibody levels may reflect the local setting. However, heterogeneity on a fine-scale is observed in many different settings [204,269,270] and it is likely that this approach to determining weighted local malaria prevalence could be adapted to these settings given adequate data.

3.5 Conclusion

In conclusion I have used a conceptually straightforward approach to generate weighted local malaria prevalence as an estimate of individual's intensity of exposure to malaria. I have demonstrated that the weighted local malaria prevalence has satisfactory discriminatory ability, particularly when combined with anti-merozoite antibody levels. I propose that it could be used as general marker of exposure to malaria and used as a

covariate in models assessing the efficacy of potential malaria vaccines or immune correlates of protection to adjust for the heterogeneity in malaria exposure.

4 Long term efficacy of RTS,S/AS01E

Four-year efficacy of RTS,S/AS01E against clinical malaria and its interaction with malaria exposure in children aged 5-17 months in Kilifi, Kenya.

4.1 Introduction

RTS,S/AS01E is the candidate pre-erythrocytic malaria vaccine developed by GSK Biologicals, currently under clinical development. The introduction on RTS,S/AS01E antigen and its clinical development history has been described in section 1.6 . The results from early phase II studies in malaria endemic countries have shown promising results.

The objective of this work was to determine the duration of protection of RTS,S/AS01E against clinical malaria in children aged 5-17 months in Kilifi, Kenya during 4 years of follow-up.

The duration of vaccine efficacy will be critical to public health policy decisions on vaccine introduction. Reductions in estimated vaccine efficacy over time may reflect; a) waning in the vaccine-induced protective immune responses to sporozoites b) delayed acquisition of natural immunity to blood-stage parasites in the RTS,S/AS01E group because of reduced exposure to blood-stage parasites in the presence of vaccine-induced immunity to sporozoites c) an artefact in survival analysis produced by micro-heterogeneity in malaria exposure within the cohort [210]. In order to adjust for

variations in malaria exposure within our cohort, I used the distance-weighted local malaria prevalence as a marker of individual malaria exposure, which I will subsequently refer to as the “malaria exposure index”[271]. The methodology of developing this measure of individual malaria exposure has been described in chapter 3. Malaria exposure index is a relative measure of the intensity of malaria exposure, as distinct from absolute measures such as the prevalence of asymptomatic parasitaemia or the entomological inoculation rate (EIR). These absolute measures are frequently used to compare different populations, but would be very labour intensive if used to accurately measure the individual variation within a population.

4.2 Methodology

4.2.1 Study design

The original study was a double blind randomized controlled trial at two sites; Korogwe, Tanzania and Kilifi, Kenya to evaluate the efficacy and safety of RTS,S/AS01E between March 2007 and November 2008[132]. The double blind phase lasted for an average of 8 months (range: 4.5 to 10.5 months). Follow-up in Korogwe stopped at 12 months but was extended in Kilifi, Kenya. Therefore only children from Kilifi, Kenya who were recruited in the original double blind control trial were recruited in the extended follow-up to assess the longevity of the vaccine efficacy against clinical malaria. As part of this work for this thesis I analysed data from Kilifi, Kenya from randomization to 48 months post dose 3. During the single blind phase, principal investigators were unblinded to

allow analysis of the data at 8 months, but parents/guardians of participants, project manager, laboratory staff and healthcare workers in the community who performed active and passive surveillance remained blinded.

The original study was sponsored by GlaxoSmithKline Biologicals. Extended follow-up beyond 12 months was investigator-led, and sponsored by the KEMRI-Wellcome Trust Research Programme and which I supervised. Both original and extended follow-up studies were registered with ClinicalTrials.gov, number NCT00380393 and NCT00872963 respectively.

4.2.2 Participants

The study population comprised healthy children between the ages of 5 and 17 months from Junju and Pingilikani sub-locations in Kilifi, Kenya. The local residents are Mijikenda and their main activity is subsistence farming. The long rainy season is between March and May while the short rainy season is during November-December. Malaria transmission follows the rainy seasons. The main mosquito vector is *Anopheles gambiae s.l.* and the entomological inoculation rate (EIR) is reported between 4 and 60 per year [272]. The areas are served by two dispensaries namely Junju and Pingilikani. The roads are rough and difficult to pass during rainy season. Kilifi District Hospital is the main referral hospital and is located approximately 35 kilometres to the North.

The original study and its extensions were approved by the Kenya Medical Research Institute National Ethics Committee, Western Institution Review Board in USA and Oxford Tropical Research Ethics Committee. Written informed consent for the extension was obtained from all children's parents/guardians using approved Swahili or Giriama

consent forms. Illiterate parents thumb printed the forms with independent literate witness countersigning. The study was overseen by an Independent data monitoring committee and local safety monitors, and conducted in accordance with the Helsinki Declaration of 1964 (revised 1996)[273] and Good Clinical Practice guidelines[274].

4.2.3 Study procedure

At screening, medical history and physical examination were done and blood samples were taken for haematological and biochemical tests. Participants were excluded from the trial if they had acute diseases with or without fever at the time of enrolment, serious acute or chronic disease at enrolment, a history of severe allergic reactions, a history of a previous blood transfusion, or a medical disorder not permitted by the protocol (eg, a weight-for-age Z score of less than -3 or other clinical signs of malnutrition at screening, major congenital defects, or a confirmed or suspected immunosuppressive or immunodeficient disorder). However no HIV test was conducted at the time of enrolment to exclude children with HIV infection.

Children with haemoglobin level below 8g/dL, total white cell count $< 4.0 \times 10^3/\mu\text{L}$ or $> 17 \times 10^3/\mu\text{L}$, platelets of $< 100 \times 10^3/\mu\text{L}$, alanine aminotransferase (ALT) $> 60\text{IU/L}$ and creatinine $> 60\mu\text{mol/L}$ were also excluded at enrolment.

Other exclusion criteria included the following; a) Planned administration/administration of a vaccine not foreseen by the study protocol within 30 days of the first dose of vaccine(s) with the exception of tetanus toxoid or scheduled diphtheria, pertussis or measles vaccine; b) Use of any investigational or non-registered drug or vaccine within 30 days preceding the first dose of study vaccine, or planned use during the study period;

c) Administration of immunoglobulins, blood transfusions or other blood products within the three months preceding the first dose of study vaccine or planned administration during the study period; d) Chronic administration (defined as more than 14 days) of immune-suppressants or other immune-modifying drugs within six months prior to the first vaccine dose (for corticosteroids, this will mean prednisone, or equivalent, 0.5 mg/kg/day. Inhaled and topical steroids are allowed); e) Previous participation in any other malaria vaccine trial; f) Simultaneous participation in any other clinical trial; g) Same sex twin; h) Any other findings that the investigator feels would increase the risk of having an adverse outcome from participation in the trial.

All recruited children who presented with the fever or severe illness on the day of vaccination were deferred to another day within the time window for vaccination. However children with minor illnesses such as diarrhoea or mild upper respiratory infection without fever (axillary temperature < 37.5°C) were vaccinated. The absolute contraindication for vaccination was appearance of acute allergic reaction, significant IgE-mediated event or anaphylactic shock following the administration of vaccine investigational product and any confirmed or suspected immunosuppressive or immunodeficient condition, including human immunodeficiency virus (HIV) infection.

4.2.4 Randomization and masking

RTS,S/AS01E and Rabies vaccines were packed in the identical boxes labelled with the treatment numbers. Treatment numbers were supplied in a separate randomization list generated by GSK Biologicals. Participants were assigned treatment numbers on attendance to the dispensary

4.2.5 Vaccination

447 children aged 5-17 months old from Kilifi, Kenya were randomized and received either RTS,S/AS01E or human diploid-cell rabies vaccine (Sanofi Pasteur, Swiftwater, PA, USA) intramuscularly at the left deltoid in a 1:1 ratio according to 0, 1, 2 month schedule.

Participants were vaccinated between March, 2007, and August, 2007. Participants received three doses of vaccine: dose one at enrolment, dose two 1 month after enrolment, and dose three 2 months after enrolment. Children were observed for one hour after each vaccination for acute adverse events.

All vaccinations took place at Junju dispensary and were administered by a study nurse who did not take part in any other study activity. Resuscitation facilities and qualified personnel were available in case of anaphylactic reaction following administration of vaccines.

4.2.6 Surveillance of clinical malaria cases

Weekly active surveillances for clinical malaria were conducted by trained fieldworkers and clinicians provided passive surveillance at local dispensaries [132].

Under active surveillance, study participants were visited weekly by trained field workers and had their temperature measured. Only if the child had documented fever (axillary temperature ≥ 37.5 C) was a blood slide prepared. A rapid malaria test was also taken and formed the basis of prompt management of malaria cases. Blood slides were not read in real time. If the parent complained that the child had fever but axillary temperature was

<37.5 °C, the child was revisited between 6-12 hours later to recheck the temperature. If fever was confirmed at the revisit the child was managed as above. If on the other hand fever was not present at the revisit, the study clinician was informed and appropriate to the clinical features, the child was either seen at the field health facility, transferred to Kilifi District Hospital or the parents were reassured that no further action was required. If no fever was present then the child was seen the following week. Parents/guardians were always encouraged to bring their children to the dispensary if they were concerned about their health. In case of positive rapid malaria test children were treated with Artemisinin combined treatment (AL) as recommended by Kenya national malaria guideline[275].

Under passive surveillance all children who presented at the dispensary had their temperature measured and only if they were febrile (axillary temperature $\geq 37.5^{\circ}\text{C}$) was a malaria blood slide prepared. If the child's temperature was less than 37.5 °C they were revisited 6-12 hours later by trained field worker and re-examined. All children with severe illness which required admission to hospital were referred to Kilifi District Hospital.

4.2.7 Cross sectional bleeds

In addition blood samples were obtained from all study participants regardless of their axillary temperature to study antibodies to *Plasmodium falciparum* CS protein repeat region (anti-CS protein antibodies) and/or asymptomatic parasitaemia from every child; before vaccination at 1 month, ~8 months (range 4–10 months), 12 months, ~15 months

(range 12-18 months), ~25 months (range 21-27 months), ~38 months (range 34-40 months) and ~49 months (range 45-51 months).

4.2.8 Malaria Exposure

The malaria exposure index was calculated as the distance-weighted proportion of asymptomatic or symptomatic malaria infected cases within a 1km radius of each individual over a 6-month interval, using data from 870 children under active surveillance in the same study area as the vaccinated cohort as described in chapter 3. Children were categorized into lower and higher exposure groups if they were below or above the cohort mean during the four years of follow-up, respectively.

4.2.9 Statistical analysis

The primary study endpoint was clinical malaria defined as axillary temperature $\geq 37.5^{\circ}\text{C}$ and *P. falciparum* parasitaemia of more than 2500/uL. The secondary study endpoint was clinical malaria defined as axillary temperature $\geq 37.5^{\circ}\text{C}$ and *P. falciparum* parasitaemia of any level. The according to protocol (ATP) cohort included all children who received three doses of vaccine according to protocol and for whom surveillance data from 2 weeks post dose three were available. Each participant's data were censored at four years of follow-up. The intention to treat (ITT) cohort included all randomized children.

The sample size for extended follow-up was limited by the number of children randomized in Kilifi at the start of the trial. Based on the observed clinical malaria

cumulative incidence of 60% over 4 years, this study had 85% power to detect a vaccine efficacy of 30% at the 5% significance level.

4.2.9.1 Analysis of efficacy against first episode

First malaria episodes were evaluated by Cox proportional hazards models. Vaccine efficacy was defined as 1 minus the hazard (HR).

4.2.9.2 Analysis of efficacy against all episodes

Multiple episodes were analyzed a) by ordinary negative binomial regression with clustering to adjust for repeated measures (NB) and b) by Andersen and Gill Cox regression (AG) for multiple event analysis[276].

Multiple episodes were also analyzed by negative binomial regression (NB) through generalized estimating equations (GEE) [211] with specified working correlation structure in the model and robust SE estimates taking into account multiple outcomes per participants. To select the best working correlation structure I determined “quasi-likelihood under the independence model criterion” or QIC. Initially I estimated the dispersion parameter α by running an ordinary negative binomial regression without considering the repeated nature of data [277,278]. Then using STATA package the full model with all covariates was run and QIC values obtained for several correlation structures. The correlation structure with the lowest QIC was selected. The results of the three models estimating vaccine efficacy against all clinical malaria episodes (Ordinary NB clustering to adjust for repeated measures, NB using GEE and AG models) were then compared.

Vaccine efficacy was defined as 1 minus the hazard (HR) or incidence rate ratio (IRR). Vaccine efficacy was adjusted for age at first vaccination, village, exposure index (as a time-varying covariate), use of insecticide treated bed net (as a time varying covariate) and distance to the health facility.

4.2.9.3 Assessment of waning in vaccine efficacy

Waning of vaccine efficacy was assessed by time-dependent interactions between logarithm of failure time (or year of follow-up) and RTS,S/AS01E vaccination. Plots of adjusted vaccine efficacy over time were produced from the regression coefficients from Cox and AG regression models. I also conducted yearly stratified analysis of efficacy against all episodes by NB and NB (GEE) in order to inspect for evidence of waning in efficacy over time.

4.2.9.4 Assessment of cases of malaria averted

Vaccine-attributable reduction was calculated as a difference between malaria incidence in control and RTS,S/AS01E groups in the ITT cohort and expressed as number of cases averted per 100 children per year of follow-up. The cumulative averted cases were calculated by summing the yearly averted cases.

Differences in prevalence of asymptomatic *P. falciparum* parasitaemia were assessed by use of Fishers exact test. Data were analyzed using STATA Version 12.0 (StataCorp LP, College Station, TX, USA).

4.3 Results

4.3.1 Trial profile

Of the 447 children eligible for randomization in Kilifi, 223 were randomized to RTS,S/AS01E and 224 to the Rabies vaccine. 320 (72%) children completed 4 years of follow-up (Figure 4:1).

415 children (209 RTS,S/AS01E vaccinees and 206 rabies vaccinees) received all 3 planned doses of vaccines according to the study protocol (ATP) and were included in the ATP analysis. 169 and 151 children from RTS,S/AS01E and Rabies group respectively completed four years of follow-up. Baseline characteristics were similar in the two groups. The median duration of follow-up was 47.5 months (47.7 in RTS,S/AS01E group and 47.1 in rabies group), with no significant difference between the groups (Table 4-1).

Figure 4:1 Consort diagram

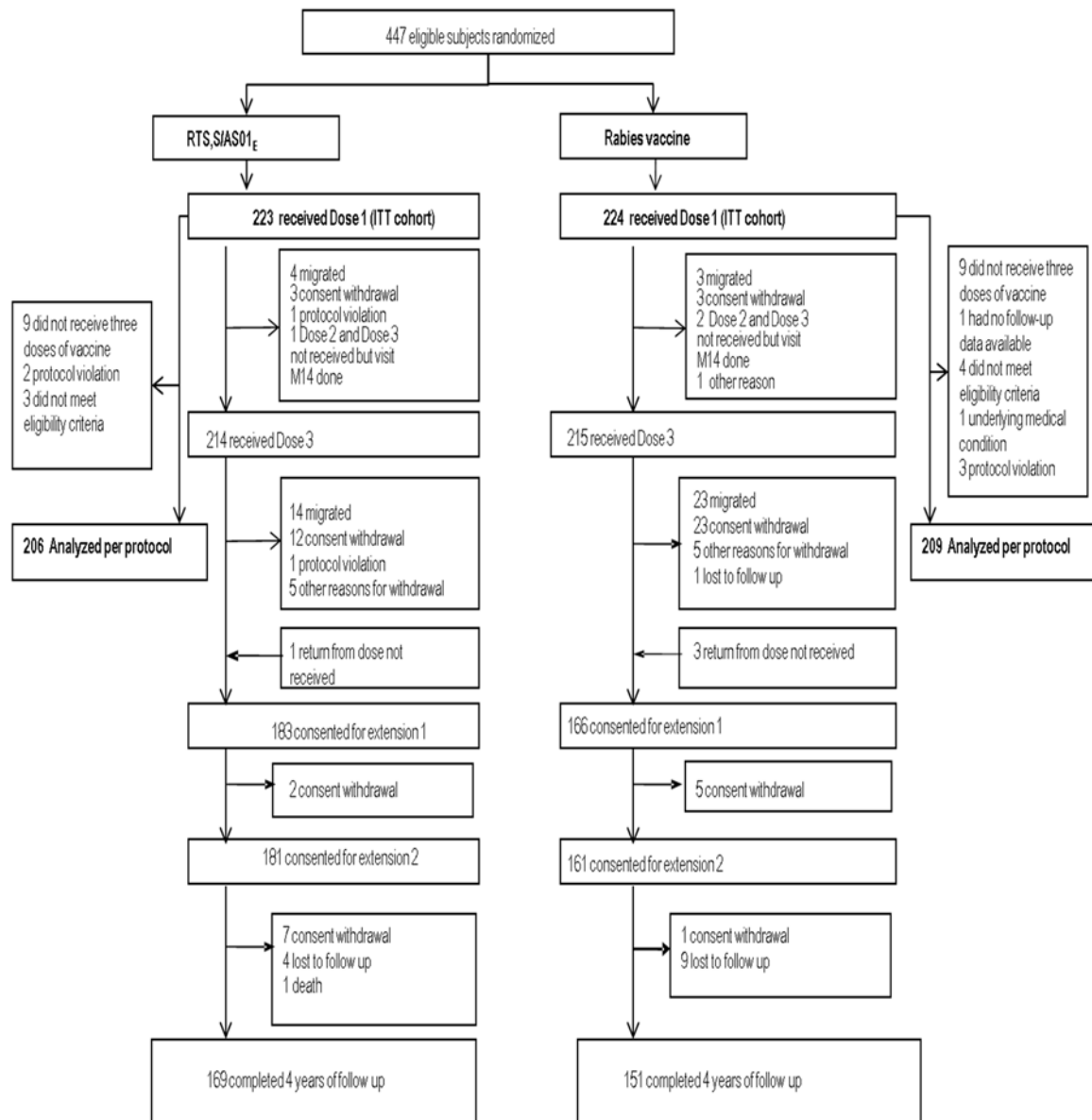


Table 4-1 Baseline characteristics and follow-up time summary (per protocol cohort)

Characteristics	Parameters or Categories	RTSS/AS01 _E N = 209		Rabies N = 206		Total N = 415	
		Value or n	%	Value or n	%	Value or n	%
Follow-up time (Months)	Median (5 th -95 th Centile)	47.7(12.5-48.6)	-	47.1(11.9-48.5)	-	47.5(12.1-48.6)	-
Age (months)at vaccination	Median (SD#)	10(3.6)	-	11(3.4)	-	11(3.5)	-
	Minimum/Maximum	5/17	-	5/17	-	5/17	-
Gender	Female/Male	106/103	50.7/49.3	102/104	49.5/50.5	208/207	50.1/49.9
Geographical Area	Area 1	58	28.2	44	21.4	103	24.8
	Area 2	48	22.9	54	26.2	102	24.6
	Area 3	50	23.9	56	27.2	106	25.5
	Area 4	52	24.9	52	25.2	104	25.1
Distance from Heath Centre (Km)	0-5	154	73.7	144	69.9	298	71.8
	5-10	55	26.3	62	30.1	117	28.2
Exposure index\$	Median (SD#)	0.37 (0.21)	-	0.37(0.24)	-	0.37(0.23)	-
	Unknown	7	1.6	3	0.7	10	2.3
Bed net use year 1*	Yes	154/204	75.5	148/199	74.4	302/403	74.9
Bed net use year 2	Yes	94/203	46.4	89/189	47.0	183/392	46.7
Bed net use year 3	Yes	47/174	27.0	34/145	23.5	81/319	35.4
Bed net use year 4	Yes	46/156	29.5	37/139	26.7	83/295	28.1

* Bed net was used as time varying covariate in the statistical models (the denominator was the number of children with available data on bed net use), # SD: standard deviation, \$: mean exposure over the entire follow up period. Area 1=Bodoi, Bomani and Junju villages, Area 2= Gongoni, Kolewa, Mapawa and Mwembe-tsungu villages, Area 3= Chodari, Kadzinuni, Kapecha and Pingilikani villages, Area 4=Bokini, Dindiri, Makata and Ng'ombeni villages.

4.3.2 Efficacy against the first or only malaria episode

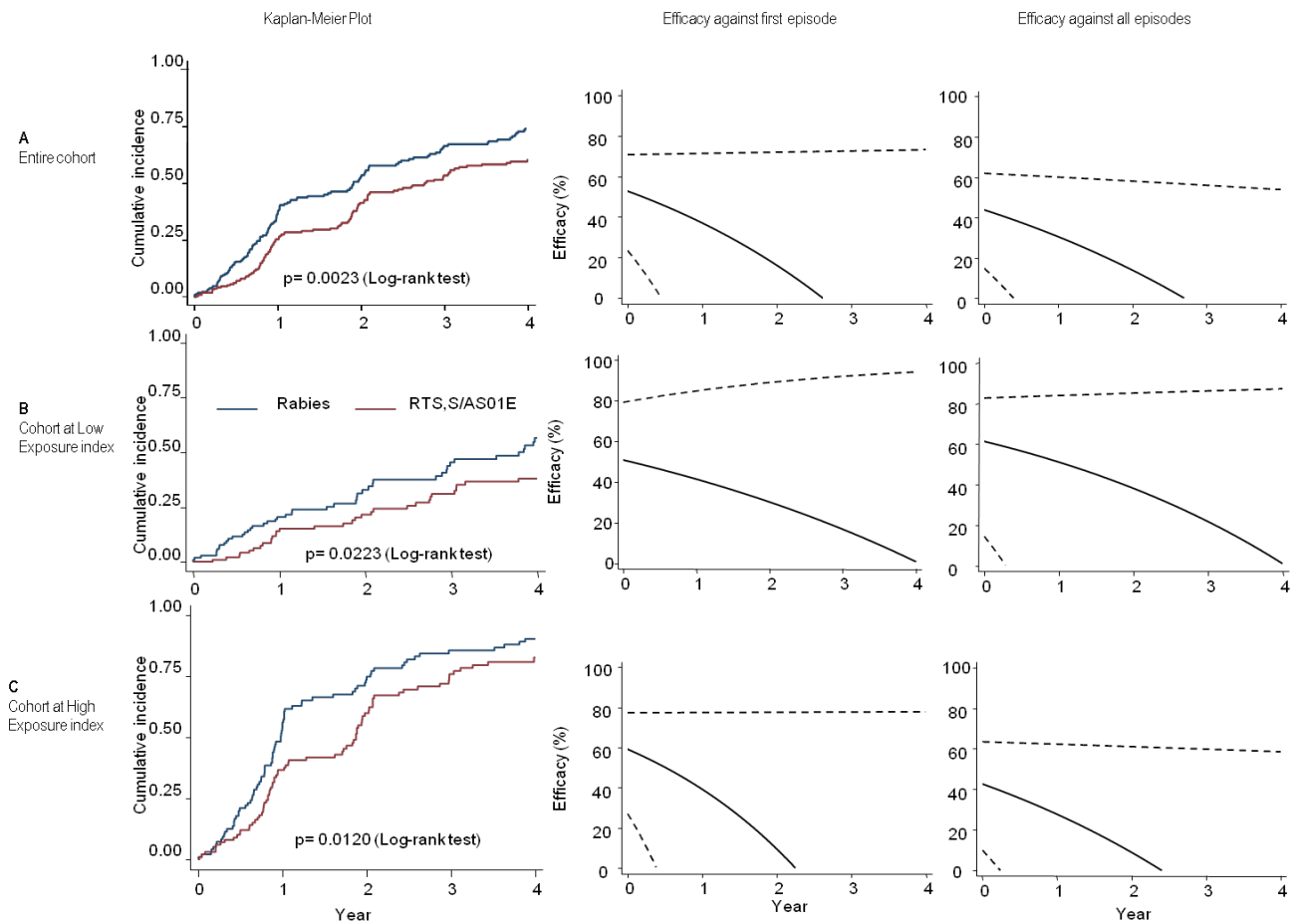
In the ITT cohort, 118 first or only clinical malaria episodes meeting the primary case definition were documented in the RTS,S/AS01E group compared with 138 episodes in the control group, giving an unadjusted efficacy of 29.9% (95%CI: 10.3%-45.3%, p=0.005) by Cox regression.

In the ATP cohort (Table 4-2 and Figure 4:2), 111 and 130 first or only clinical malaria episodes were documented in the RTS,S/AS01E and control group, respectively, giving an adjusted vaccine efficacy of 32.1% (95% CI: 11.6% to 47.8%, $p=0.004$) by Cox regression. There was weak evidence of non-proportionality of the hazard associated with vaccination by time-dependent Cox regression model; HR=1.33 (95%CI: 0.98 to 1.81, $p=0.07$). A plot of adjusted vaccine efficacy over time showed statistically non-significant waning in efficacy over time (Figure 4:2).

Table 4-2 Efficacy of RTS,S/AS01E against Plasmodium falciparum clinical malaria and asymptomatic parasitaemia

RTS,S/AS01E					Rabies		Vaccine efficacy*					P value
Subjects (N)	No. of events€	PYAR	Rate§		Subjects (N)	No. of events€	PYAR	Rate§	(95%CI)			
Intension to Treat analysis												
First episodes (Cox regression model)												
≥2500 parasites per µL	223	118	501.9	0.24	224	138	404.9	0.34	29.9 (10.3 to 45.3)		0.005	
>0 parasite per µL	223	128	481.6	0.27	224	141	387.2	0.36	26.7 (6.8 to 42.3)		0.01	
All episodes (Andersen and Gill Cox regression)												
≥2500 parasites per µL	223	551	836.9	0.65	224	618	791	0.78	16.8 (-8.6 to 36.3)		0.18	
>0 parasite per µL	223	632	836.9	0.75	224	705	791	0.89	16.7 (-8.8 to 36.2)		0.18	
All episodes (Negative binomial regression)												
≥2500 parasites per µL	223	551	836.9	0.65	224	618	791	0.78	18.6 (-7.2 to 38.3)		0.14	
>0 parasite per µL	223	632	836.9	0.75	224	705	791	0.89	17.9 (-6.8 to 36.9)		0.14	
All episodes (Negative binomial regression through GEE)												
≥2500 parasites per µL	223	551	836.9	0.65	224	618	791	0.78	14.9 (-11.9 to 35.4)		0.25	
>0 parasites per µL	223	632	836.9	0.75	224	705	791	0.89	13.9 (-11.7 to 33.6)		0.26	
According to protocol cohort												
First episodes (Cox regression model)												
≥2500 parasites per µL	209	111	466.9	0.24	206	130	360.3	0.36	32.1 (11.6 to 47.8)		0.004	
>0 parasites per µL	209	118	450.4	0.26	206	136	342.2	0.39	34.0 (15 to 48.7)		0.001	
All episodes (Andersen and Gill Cox regression)												
≥2500 parasites per µL	209	475	730.8	0.65	206	518	668.9	0.77	24.3 (1.9 to 41.6)		0.04	
>0 parasites per µL	209	542	730.8	0.74	206	592	668.9	0.88	24.1 (1.6 to 41.5)		0.04	
All episodes (Negative binomial regression)												
≥2500 parasites per µL	209	475	730.8	0.65	206	518	668.9	0.77	23.5 (-0.7 to 41.9)		0.06	
>0 parasites per µL	209	542	730.8	0.74	206	592	668.9	0.88	23.8 (0.9 to 41.3)		0.04	
All episodes (Negative binomial regression through GEE)												
≥2500 parasites per µL	209	475	730.8	0.65	206	518	668.9	0.77	23.4(-1 to 41.8)		0.06	
>0 parasites per µL	209	542	730.8	0.74	206	592	668.9	0.88	24.2 (1.5 to 41.6)		0.04	
PYAR: Episodes/Person Years at Risk; VE: Vaccine Efficacy (1-HR or 1-IRR); CI: Confidence Interval; *: Efficacy is adjusted for age, bed net use, area, malaria exposure index and distance from health center in classical Cox model. In negative binomial and Andersen and Gill Cox models malaria exposure index was excluded from adjusting covariates because of its interaction effect with vaccine efficacy. For Intention to treat analysis efficacy estimates are unadjusted.												

Figure 4:2 Kaplan-Meier curves and vaccine efficacy over time (According to protocol cohort)



Kaplan–Meier plots of the cumulative incidence of malaria and corresponding vaccine efficacy over time are shown for the entire cohort (Panel A) and for the cohorts with low and high malaria exposure indexes (Panels B and C, respectively). Clinical falciparum malaria was defined as the presence of fever (temperature $\geq 37.5^{\circ}\text{C}$) and a *Plasmodium falciparum* density of more than 2500 parasites per cubic millimeter. A log (time) interaction model was used to produce the fit for the vaccine-efficacy plots. In these plots, the solid line indicates the point estimates of efficacy and the dotted lines indicate 95% confidence intervals. Vaccine efficacy was truncated at 0% as the lower limit; hence, the lower limit of the confidence interval is visible only at the start of monitoring. The lower limit of the 95% confidence interval for vaccine efficacy against the first or only episode in the cohort with a low exposure index is not visible because it is below 0 and has been truncated

4.3.3 Efficacy against all episodes

The histogram showing the distribution of malaria episodes in the RTS,S/AS01E and Rabies group is shown in Figure 4:5. I used two different analyses to examine efficacy against all episodes, both allowing for possible variations in efficacy and the malaria exposure index over time; a) a modified Cox-regression model, namely the Anderson-Gill model (AG) and b) a negative binomial regression model (NB), which fitted the data significantly better than the Poisson model (Likelihood ratio test of over-dispersion, Chi-square=504.21, df=1, p value<0.001, Figure 4:3).

Independent working structure was the best correlation structure for the events within subject as indicated by the lowest QIC (Table 4-3). Furthermore a model with vaccine, distance to dispensary, area and bed net use covariate was the model with lowest QIC (Table 4-4). However I included age of participant at the time of vaccination in the final model. This is because age is known to be an important predictor of malaria risk. The efficacy estimate was similar whether or not I adjusted by age.

In the ITT cohort, there were 551 and 618 clinical malaria episodes among 223 RTS,S/AS01E and 224 control vaccinees respectively, giving an unadjusted efficacy against multiple episodes of 16.8% (95%CI: -8.6% to 36.3% p=0.18), 18.6% (95%CI: -7.2 to 38.3, p=0.14) and 14.9% (95%CI:-11.9% to 35.4%, p=0.25) by AG, NB and NB(GEE), respectively.

In the ATP cohort, there were 475 and 518 episodes of clinical malaria among 209 RTS,S/AS01E and 206 control vaccinees, giving an adjusted vaccine efficacy against all

episodes of 24.3% (95%CI: 1.9% to 41.6%, p value=0.04) by AG, 23.5% (95%CI: -0.7% to 41.9%, p=0.06) by NB and 23.4% (96%CI: -1% to 41.8%, p value=0.06 by NB (GEE) as shown in Table 4-2.

Figure 4:3 Comparison of Negative binomial and Poisson distribution fit of data (According to protocol cohort)

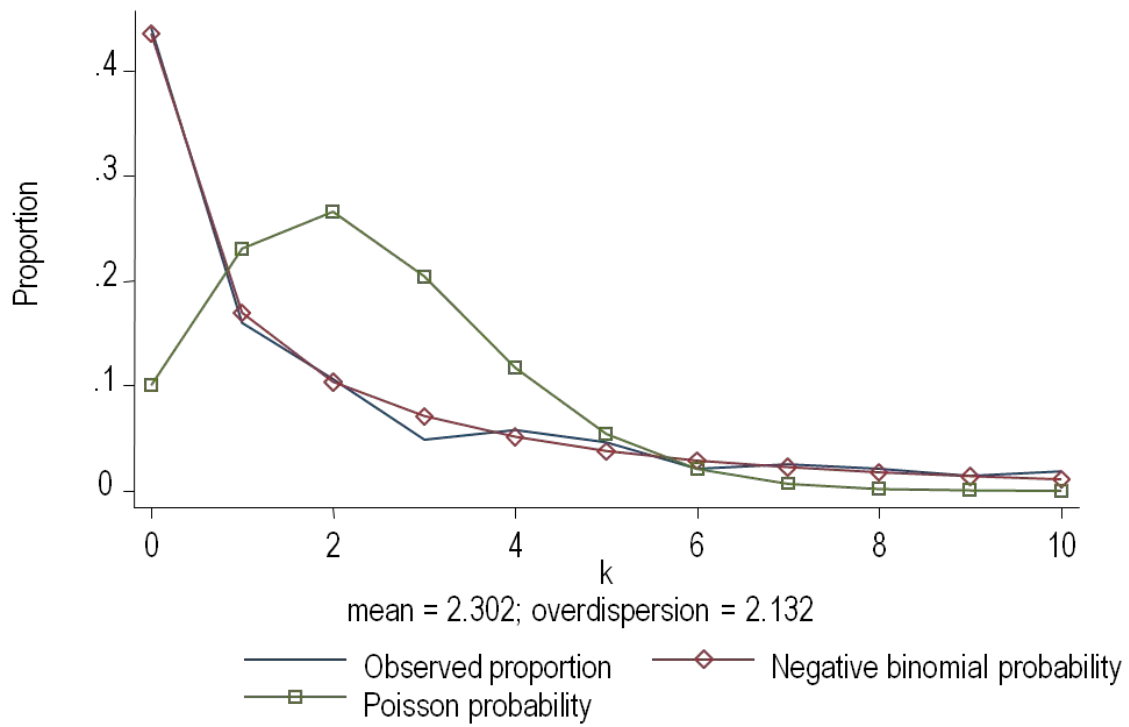


Table 4-3 Selection of working correlation structure

Correlation	P	QIC
Unstructured	8	1444.325
Exchangeable	8	1442.921
Independent	8	1435.016
Autoregressive	8	1435.420
Static	8	1435.696

Table 4-4 Model selection for analysis of efficacy against all clinical malaria using negative binomial model through GEE

Correlation	Covariates	p	QIC
Independent	Vaccine, Bed net, Area, distance, age	6	1625.6
Independent	Vaccine, bed net, area, distance	5	1622.2
Independent	Vaccine, area, distance, age	4	1681.7
Independent	Vaccine, bed net, area	4	1724
Independent	Vaccine, distance, age	4	1743.7
Independent	Vaccine, bed net	3	1777.9
Independent	Distance, age	3	1745.0
Independent	Vaccine	2	1829.2
Independent	Distance	2	1741.9
Independent	Age	2	1833.2
Independent	Bed net	2	1777.7
Independent	Area	2	1777.8

4.3.4 Efficacy and interactions with time and malaria exposure

As shown in Table 4-5 , vaccine efficacy was lower at later time points as indicated by significant interaction between vaccine efficacy and follow-up time (HR=1.28 versus 1, $p=0.004$) by AG and (IRR=1.65 versus 1, 95%CI: 1.03 to 2.63, $p=0.04$ in 4th year) by NB. Efficacy was lower in children with high than low malaria exposure index as shown by the interaction between vaccination and malaria exposure index (HR=5.17 versus 1, $p=0.001$ by AG) and (IRR=2.48 versus 1, $p=0.02$ by NB). Over time, the effect of malaria exposure on clinical malaria risk decline as indicated by interaction between malaria exposure index and follow-up time (HR=0.71 versus 1, $p=0.04$) by AG and (IRR=0.35 versus 1, $p=0.02$ and IRR=0.35 versus 1, $p=0.01$ in the 3rd and 4th year, respectively) by NB. There was no interaction between bed net use and vaccination (HR=0.95, 95%CI 0.64 to 1.4, $p=0.78$).

Based on the NB model, stratified efficacy estimates by year of follow-up were 46 (95%CI: 21 to 63, $p=0.001$), 25 (95%CI: -19 to 52, $p=0.23$), 22 (95%CI: -17 to 48, $p=0.23$) and -1 (95%CI: -47 to 31, $p=0.95$) during the four years of follow-up as shown in Table 4-7.

Based on the AG model, vaccine efficacy was 43.6% (95%CI 15.5% to 62.3 %) in the first year and -0.4% (95%CI -32.1 to 45.3%) in the fourth year after vaccination (Figure 4.2). The decline in estimated vaccine efficacy was more rapid among children with a higher malaria exposure index (Figure 4:2) although terms for 3-way interactions between vaccination, malaria exposure index and time (to examine if vaccine efficacy declined more rapidly at high exposure than low exposure) were not statistically significant (HR=

1.56 versus 1, 95%CI 0.81 to 2.99, p value=0.18 and IRR=1.28 95%CI: 0.76 to 2.14, p value=0.35 for AG and NB respectively).

Interactions between vaccination and malaria exposure index, vaccination and time of follow-up and malaria exposure index and time of follow-up were also demonstrated by the negative binomial regression model through GEE as shown in Table 4-6.

Table 4-5 Interaction analyses between vaccination, malaria exposure index and year of follow-up (According to protocol cohort)

Covariate	Negative binomial regression			Andersen and Gill Cox regression		
	RTS,S/AS01E N=209	Rabies N=206	P value	RTS,S/AS01E N=209	Rabies N=206	P value
	IRR	95% Confidence interval		HR	95% Confidence interval	
RTS,S/AS01E	0.37	0.22-0.61	<0.001	0.23	0.12-0.45	<0.001
EI	4.67	2.18-10.03	<0.001	3.92	2.15-7.12	<0.001
RTS,S/AS01E*EI	2.48	1.18-5.21	0.02	5.17	1.98-13.47	0.001
Area1	1	-	-	1	-	-
Area2	0.78	0.44-1.35	0.37	0.80	0.47-1.37	0.42
Area3	0.49	0.34-0.73	<0.001	0.56	0.38-0.84	0.005
Area4	0.45	0.29-0.69	<0.001	0.53	0.35-0.82	0.004
Bed net	0.82	0.66-1.02	0.08	0.77	0.62-0.95	0.02
Distance to dispensary	0.84	0.75-0.95	0.005	0.88	0.78-1.00	0.05
Age (months)	0.99	0.96-1.03	0.67	1.01	0.97-1.04	0.73
RTS,S/AS01E* year 1	1	-	-			
RTS,S/AS01E*year 2	1.37	0.83-2.27	0.22			
RTS,S/AS01E* year 3	1.39	0.86-2.26	0.17	1.28 [§]	1.08-1.51	0.004
RTS,S/AS01E* year 4	1.65	1.03-2.63	0.04			
EI*year 1	1	-	-			
EI*year 2	0.62	0.28-1.34	0.22			
EI*year 3	0.35	0.15-0.82	0.02	0.71 [€]	0.51-0.98	0.04
EI*year 4	0.35	0.16-0.79	0.01			
Year 1	1	-	-	NA	-	-
Year 2	0.96	0.62-1.49	0.85	NA	-	-
Year 3	1.41	0.87-2.27	0.16	NA	-	-
Year 4	1.56	1.02-2.37	0.04	NA	-	-

§: interaction between vaccination and time as continuous variable, €: interaction between exposure index and time as continuous variable, EI: Malaria exposure index,; IRR: Incidence rate ratio; The models with three-way interactions (vaccination, year and exposure) didn't fit the data well; N=Number of children

Table 4-6 Interactions analysis between vaccination, malaria exposure index and year of follow-up by binomial regression through GEE (According to protocol cohort)

Covariate	IRR	95% Confidence Interval	P value
RTS,S/AS01E	0.37	0.22 to 0.61	<0.001
EI	7.86	3.74 to 16.54	<0.001
RTS,S/AS01E*EI	3.17	1.36 to 7.43	0.008
Year 1	1	-	
Year 2	0.97	0.61 to 1.54	0.903
Year 3	1.33	0.77 to 2.30	0.304
Year 4	1.67	1.04 to 2.67	0.033
RTS,S/AS01E*Year 1	1	-	
RTS,S/AS01E*Year 2	1.31	0.82 to 2.11	0.259
RTS,S/AS01E*Year 3	1.29	0.81 to 2.06	0.277
RTS,S/AS01E*Year 4	1.58	1.01 to 2.45	0.043
EI*Year 1	1	-	
EI*Year 2	0.54	0.25 to 1.21	0.136
EI*Year 3	0.36	0.15 to 0.84	0.019
EI*Year 4	0.29	0.13 to 0.62	0.002
Age	1.00	0.96 to 1.04	0.964
Area 1	1	-	
Area 2	0.84	0.47 to 1.50	0.551
Area 3	0.53	0.35 to 0.81	0.003
Area 4	0.48	0.30 to 0.77	0.002
Distance to dispensary	0.88	0.77 to 1.00	0.042
Bed net	0.82	0.63 to 1.07	0.142

Table 4-7 Stratified adjusted vaccine efficacy against all clinical malaria by malaria exposure index and year of follow-up by negative binomial regression (According to protocol cohort)

	Total cohort			Low exposure group			High exposure group		
	RTS,S/AS01E (N)=209			RTS,S/AS01E (N)=98			RTS,S/AS01E (N)=99		
	Rabies (N)=206			Rabies (N)=104			Rabies (N)=96		
	VE (%)	95% CI	P value	VE (%)	95% CI	P value	VE (%)	95% CI	P
All years	23.5	-0.7 to 41.9	0.06	45.1	11.3 to 66.0	0.01	15.9	-11.0 to 36.4	0.22
Year 1	46	21 to 63	0.001	58	7 to 81	0.03	40	10 to 60	0.01
Year 2	25	-19 to 52	0.23	39	-38 to 73	0.24	31	-1 to 53	0.06
Year 3	22	-17 to 48	0.23	62	24 to 82	0.006	-4	-61 to 33	0.86
Year 4	-1	-47 to 31	0.95	42	-8 to 69	0.09	-29	-98 to 15	0.22

VE: Adjusted vaccine efficacy estimates, CI: 95% Confidence Interval, NB: Negative Binomial, (N) Number of children

4.3.5 Clinical malaria episodes averted

Incidence of clinical malaria episodes increased in both groups during follow-up (Figure 4.4A). On analysis of the ITT cohort, it was estimated that the number of malaria cases per 100 children averted in each year of the four-year follow-up period were 26, 22, 18 and -1, respectively giving a total of 65 cases averted over 4 years, out of a total of 618 cases per 100 children in the control group (Figure 4:4 D). The number of cases averted over the 4 years of follow-up in low and high exposed children was 62 and 78 per 100 children respectively (Figure 4:4 B and Figure 4:4 C).

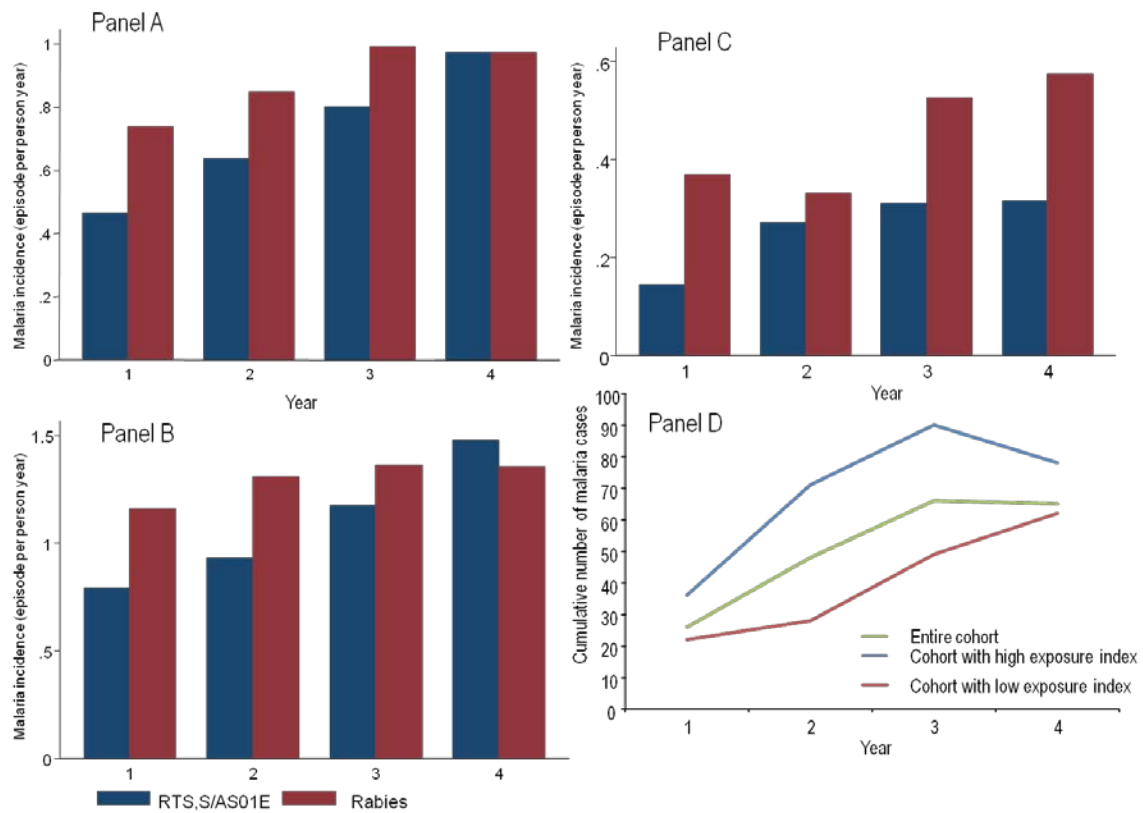
4.3.6 Cross sectional survey analysis

Prevalences of asymptomatic *P. falciparum* parasitaemia among RTS,S/AS01E vaccinees were lower compared to the control group at all cross sectional surveys except at 49 months (range: 45-51 months) post-vaccination (Table 4-8). There were no significant differences in mean haemoglobin concentration between the groups.

Table 4-8 Asymptomatic parasitaemia at cross-sectional bleed in the per protocol cohort

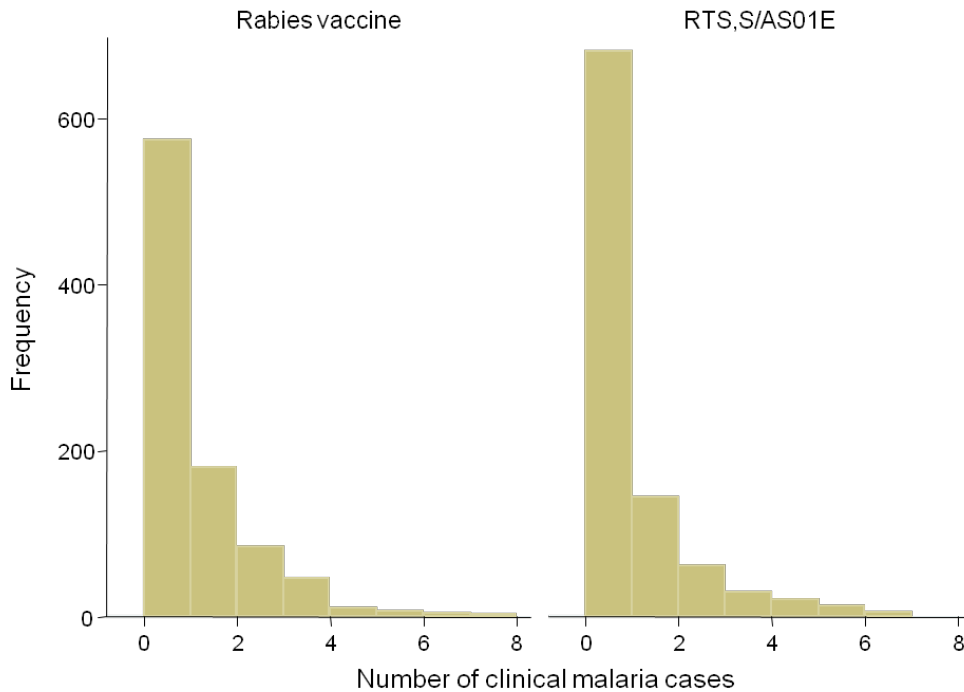
Mean month (range)	RTS,S/AS01E Vaccine		Rabies Vaccine		Efficacy (95% CI)	P Value
	Participants Tested (N)	Participants with Positive Slides (N (%))	Participants Tested (N)	Participants with Positive Slides (N (%))		
8 (4-10)	193	2 (1)	184	8 (4)	76.9 (-17.9 to 97.6)	0.06
12	185	9 (5)	175	24 (14)	67.8 (25.4 to 87.2)	0.005
15(12-18)	167	2 (1)	146	9 (6)	81.5 (8.3 to 98.1)	0.03
25 (21-27)	161	6 (4)	139	10 (7)	50.1 (-56.8 to 85.5)	0.21
38 (34-40)	154	14 (9)	139	28 (20)	60.4 (17.5 to 81.6)	0.008
49 (45-51)	148	11 (7)	136	7 (5)	-47.9 (49.5 to -27.5)	0.47
Efficacy against asymptomatic parasitaemia calculated from Odds Ratios and p value based on Fisher’s exact test.						

Figure 4:4 Malaria incidences by exposure group and Vaccine-attributable reduction (Intention to treat cohort)



Panel A shows the incidence of malaria according to year of follow-up in the entire cohort, Panel B shows the incidence in the cohort with a high exposure index, and Panel C shows the incidence in the cohort with a low exposure index. Panel D shows the cumulative number of malaria cases averted in the entire cohort and in the high-exposure and low-exposure cohorts.

Figure 4:5 Histogram showing frequency of clinical malaria episodes (primary case definition) by vaccine group



4.4 Discussion

During four years of follow-up, RTS,S/AS01E was associated with 29.9% and 16.8% efficacy against first and all *P. falciparum* clinical malaria episodes, respectively, for children vaccinated at 5-17 months of age in a malaria endemic country. All three models of efficacy against all malaria episodes gave similar results for the ATP cohort analysis. NB using GEE gave slightly lower point estimates in the ITT cohort compared to AG and NB.

All models (NB, NB using GEE, and AG) demonstrated significant variations in vaccine efficacy over time and by intensity of malaria exposure (as measured by our malaria

exposure index). The efficacy estimate during follow-up is surrounded by considerable uncertainty implying possible sustained efficacy at later time points. However, significant interaction between time and vaccination confidently rejected the null hypothesis that efficacy is constant over time. Furthermore, the upper confidence limits for efficacy in the 4th year exclude greater than 31% efficacy overall, and exclude greater than 15% efficacy in the high exposure group.

Waning in vaccine efficacy observed in our data is unlikely to be the consequence of heterogeneity in malaria exposure because reductions in efficacy over time were similar whether first or all episodes were analyzed, and also because they persisted even after including the exposure index in the model.

I identified significant interactions between time since vaccination and vaccine efficacy, and between exposure to malaria and vaccine efficacy. These interactions indicate how vaccine efficacy varies in the presence of other covariates. For instance, in the NB model the theoretical efficacy of vaccination at an exposure index of 0 and in the first year is given by $IRR=0.37$ (i.e. 63% efficacy). As exposure index rises to 1, vaccine efficacy can be calculated by multiplying the $IRR=0.37$ by the interaction term of $IRR=2.48$ (i.e. 0.91 or 9% efficacy). These variations in efficacy may be more intuitively appreciated on the graphs shown in Figure 4:2 and Figure 4:4.

The efficacy estimates of RTS,S/AS01E were similar to previously described estimates in older Mozambican children (1-4 years) who received RTS,S/AS02 and followed for 4 years using passive surveillance at health facilities. The efficacy against first or only clinical malaria episode in my study was 29.9% (95% confidence interval [CI]: 10.3% to

45.3%, $p=0.005$) compared to 30.5% (95% CI 18.9% to 40.4%; $p < 0.001$) in Mozambican children. The efficacy against all malaria episodes in my study was 24.3% (95%CI 1.9% to 41.6%, $p=0.035$) compared to 25.6% (95%CI, 11.9% to 37.1%; $p < 0.001$) in Mozambican children.

In contrast, a long term follow-up study evaluating efficacy of RTS,S/AS02 in Mozambican children found no evidence of waning efficacy against first episode over time[134]. The difference in our respective findings may be because a) in our study waning in efficacy was more readily apparent on analysis of all episodes in Anderson-Gill survival models rather than first or only episodes in Cox regression, and the former analysis was not examined in Mozambique; b) whereas the incidence of clinical malaria was sustained (and in fact increased) during follow-up of our cohort, it fell over time in Mozambique, reducing the power to identify waning efficacy. AG regression includes all malaria episodes ($n=243$ “first episodes” versus $n=1169$ “all episodes” in our trial), and in particular includes all the 2nd, 3rd and 4th episodes (Figure 4:5) that occur later during follow up but would be censored in Cox regression. In addition, AG estimates total effect, a sum of direct and indirect effect as a result of event dependency. Such estimates are more relevant from public health perspective than time to first event only. Other differences between the two cohorts were; higher transmission in Mozambique compared with Kilifi (EIR of 38 versus 22 and parasite prevalence of 20% versus 15%), higher median age at vaccination in Mozambique compared with Kilifi (35.9 months versus 10.8 months) and different adjuvants (AS02 in Mozambique versus AS01 in Kilifi, Kenya). However in an exploratory analysis of time to first event resetting the clock after each year of follow-up, the Mozambican study showed a trend towards reduced efficacy

against first episode over time but the authors could not rule out the effect of heterogeneity of malaria exposure.

Increases in malaria incidence over the follow-up period reflect an increase in transmission in our study area (i.e. Junju/Pingilikani). The mean exposure index increased from 0.27 (95%CI: 0.26 to 0.28) in first year to 0.43 (95%CI: 0.42 to 0.44) in the fourth year. This increase in Junju/Pingilikani sub-locations contrasts overall declines in malaria transmission in Kilifi [45], suggesting marked local heterogeneity in transmission within the district [199,204].

I found significantly lower vaccine efficacy estimates in children with a higher malaria exposure index, suggesting that vaccine efficacy may appear to wane because of slower acquisition of natural immunity to blood-stage parasites among RTS,S/AS01E vaccinees, due to reduced exposure to blood-stage parasites. Alternatively, vaccine efficacy may wane because anti-CS protein antibody levels fell with time. Anti-CS protein antibodies may mediate protection, and were associated with reduced risk of clinical malaria in this study. The details of the anti-CS protein antibody analysis are given in chapter 5. Lower efficacy estimates immediately following vaccination at high malaria exposure could be due to a heavy sporozoite challenge that overcomes the vaccine-induced immunity [279,280].

Despite waning efficacy over time and with increasing transmission intensity, vaccination with RTS,S/AS01E resulted in an overall reduction of clinical malaria episodes over four years of follow-up (65 cases averted in total per 100 vaccinated children, from the estimated 618 cases per 100 children in the control group). Whereas vaccine efficacy

determined from survival models provides a useful estimate of the biologic effect of vaccination[197], there is concern that such estimates may be misleading in public health terms[281]. The vaccine-attributable reductions provide a further metric in evaluating possible public health impacts[282].

Although the confidence intervals are wide, efficacy against asymptomatic parasitaemia may persist longer than efficacy against clinical malaria. A similar pattern has been noted by others [134]. A pre-erythrocytic vaccine might, in theory, achieve such an effect if blood-stage immunity was lower among vaccinees, thus resulting in a greater likelihood of clinical disease resulting from any given infection.

4.5 Conclusion

In conclusion, RTS,S/AS01E was associated with reduction in first and in all *P. falciparum* clinical episodes, but vaccine efficacy waned during four years of follow-up. Vaccine efficacy against all episodes was 46% (95%CI 21% to 63%) in the first year but -1% (95%CI -47 to 31%) in ATP cohort. Waning in vaccine-induced immunity and more rapid acquisition of blood stage immunity in the controls may both contribute to waning efficacy over time, and the latter may explain variations in efficacy according to malaria exposure.

5 Correlates of protection: Anti-CS protein antibodies

5.1 Introduction

RTS,S immunization induces high levels of anti-CS protein antibodies. Although the peak anti-CS protein antibody response correlates with protection from malaria infection in challenge studies [131], no correlation with clinical malaria has been described in the field [129,157]. Identification of such a marker would aid selection of the best candidate malaria vaccines and facilitate determination of the duration of protection and timing for booster dose. Using data from a randomized controlled trial in Kilifi, Kenya I conducted an analysis to describe the dynamics of anti-CS protein antibodies in RTS,S/AS01E immunized children and determine their association with protection from clinical malaria.

5.2 Methodology

5.2.1 Study design

The samples came from children who received RTS,S/AS01E in Kilifi, Kenya and Korogwe, Tanzania in a randomized trial as described in chapter 4. Children were vaccinated between March and August 2007. Blood was taken for serological studies before vaccination at 1 month, ~8 months (range 4–10 months) and 12 months from

Korogwe, Tanzania and Kilifi, Kenya. In addition serum samples at ~15 months (range 12-18 months), ~25 months (range 21-27 months), ~38 months (range 34-40 months) and ~49 months (range 45-51 months) were obtained from Kilifi, Kenya only.

5.2.2 CS antibody measurement

Measurement of vaccine-induced CS repeat region antibodies was conducted at CEVAC laboratory in University of Ghent, Belgium and results reported in EU/mL. The method is described in detail in Appendix B. I did not conduct the laboratory work to measure antibody levels to CS protein.

5.2.3 Statistical analysis

I conducted two analyses; a) the first analysis was on data from Korogwe, Tanzania and Kilifi, Kenya from 2 weeks to 12 months post dose 3 (combined data). In this data set I used first or only clinical malaria episodes as my endpoint to assess the relationship between anti-CS protein titres and protection from clinical malaria and b) the second analysis was on data from Kilifi, Kenya only from 2 weeks to 38 months post dose 3 where my endpoint was all clinical malaria episodes

5.2.3.1 Analysis of combined data

I conducted exploratory post hoc analyses of log-transformed anti-CS protein antibody titres on the According-To-Protocol RTS,S/AS01E vaccinees. I first calculated the predicted anti-CS protein antibody titres per week per child by fitting an exponential decay model using linear regression of log-transformed anti-CS protein titre as a function of time of follow-up.

I then used the Cox regression model to evaluate the relationship between anti-CS protein antibody titres and protection from first clinical malaria episode with anti-CS protein antibody titres as a time-varying covariate. The optimal two-group model was selected by varying the dichotomization point to maximize the log-likelihood, and bootstrapping was used to calculate confidence intervals. A step function was then approximated using a cumulative normal distribution. I also explored other non-linear fits with power functions and fractional polynomials using the multiple fractional polynomial function on STATA version 12, with $p < 0.1$ to retain additional power functions.

5.2.3.2 Analysis of Kilifi data

I made two changes in the analysis of these data; a) First I did not assume exponential decay in anti-CS protein antibodies and b) secondly I included all clinical malaria episodes in assessment of the relationship between anti-CS protein antibody titres and protection. I calculated imputed weekly anti-CS protein titers by fitting fractional polynomial of time and log transformed anti-CS protein titers to allow for non exponential decay of anti-CS protein. The Cox regression model with spline functions was used to assess the relationship between imputed anti-CS protein antibodies as time-

varying covariate and protection. Linear and spline model fit were compared by log likelihood ratio test whereas Akaike information criterion (AIC) was used to compare step function and linear model given the non-nested nature of models in the latter comparison. Optimal threshold anti-CS protein titer was determined in the Cox regression model by dichotomizing imputed anti-CS protein titers at different points to maximize log likelihood ratio and using bootstrapping with 1000 iterations for 95 CI's. Data were analyzed using STATA Version 12.0 (StataCorp LP, College Station, TX, USA).

5.3 Results

5.3.1 Combined data

A total of 447 children, who had received RTS,S/AS01E had their anti-CS protein titres available for analysis.

5.3.1.1 Anti-CS protein decay overtime

Box plots showing the distribution of anti-CS protein (CS) antibody titres after vaccination for RTS,S/AS01E group are shown (Figure 5:1). There was a significant increase in anti-CS protein titres one month post dose three in the RTS,S/AS01E group to a peak geometric mean titre of 540 EU/mL (95% CI 501-582). Over time, the titres declined to a geometric mean of 41.6 EU/mL (95% CI 38-46) at 12 months and then to 31.3 EU/mL (95% CI 26-38) at 15 months.

Baseline anti-hepatitis B surface antigen (HBsAg) seroprotection rate $\geq$ (10 mIU/mL) were similar in RTS,S/AS01E and rabies group (approximately 95% of the participants). At 12 months, the seroprotection rates were higher in the RTS,S/AS01E group; 99.7% (95%CI: 98.4 % to 100%) compared to the rabies vaccine group; 91.6% (95%CI 88.3% to 94.2%). Mean geometric titres of HBsAg antibodies were 12358.5 mIU/mL (95%CI 10707.0 to 14264.8) in RTS,S/AS01E vaccinees compared to 108.0 mIU/mL (95% CI 91.8-127.1) in rabies vaccinees.

Figure 5:1 Anti-CS protein antibody titres in RTS,S/AS01E vaccinees (Kilifi and Korogwe combined data)

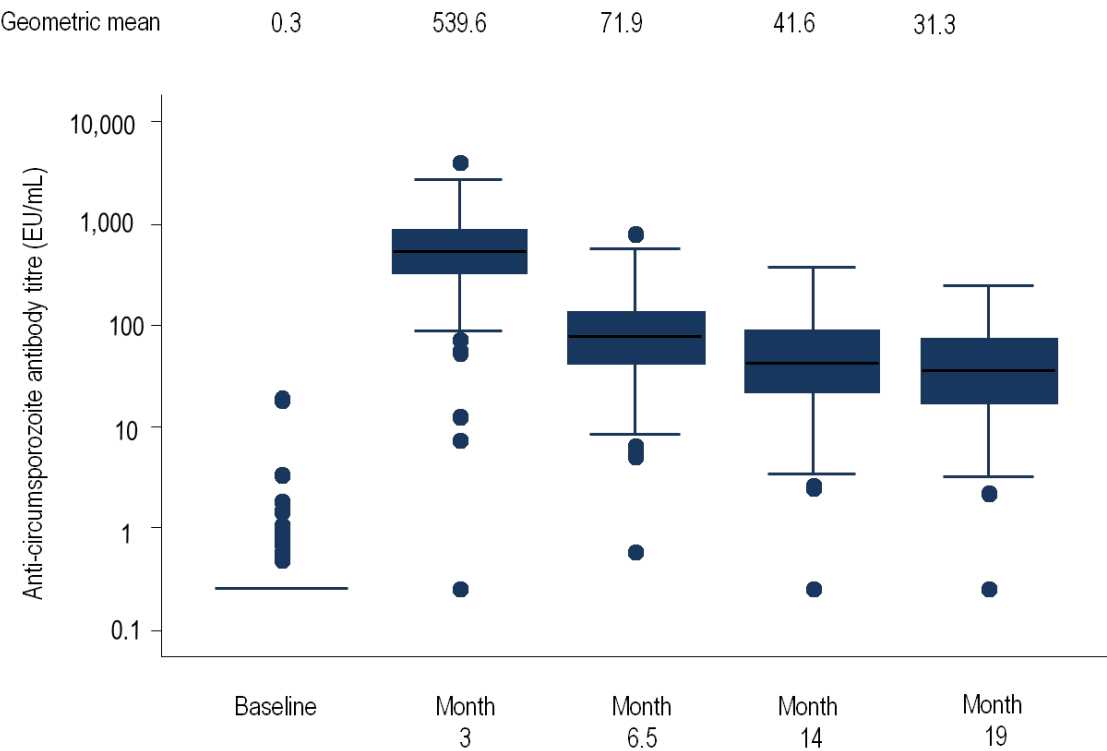


Figure shows anti-circumsporozoite antibody titres during follow-up in children who received the RTS,S/AS01E vaccine. Anti-circumsporozoite antibody titres in the control group were consistently low or undetectable throughout follow-up and are not shown. The horizontal line within each box represents the median, the top and bottom of each box represent the 25th and 75th percentiles, respectively, and the I bars represent the highest and lowest values within 1.5 times the Interquartile range. The circles denote outliers. The horizontal line under the baseline values indicates that the values for the median and interquartile range were identical

5.3.1.2 Association between anti-CS protein titres and protection

Among RTS,S/AS01E vaccinees, there was no correlation between anti-CS protein titres one month post dose three and protection from clinical malaria over 12 months monitoring. (Hazard Ratio for log increase in anti-CS protein titre=0.87 [95% CI 0.7-1.7], $p=0.7$). However titres at month 6.5 showed a correlation with protection over 12 months (HR for log increase in anti-CS protein titre=0.43 [95% CI 0.2-0.8], $p=0.006$). On examining the survival function by tertile of anti-CS protein titre, it appeared that the lower tertile of responders were at increased risk compared with the upper two tertiles 6.5 months after vaccination. Figure 5:2 shows the KM plots of anti-CS antibody tertiles measured at one month post dose three and Figure 5:3 shows the KM plots for the anti-CS antibody tertiles measured ~8 months post dose 3). The mean response in the lower tertile at 8 month post dose 3 was 26 EU/mL, compared with 225 EU/mL in the lower tertile one month after dose three.

Figure 5:2 KM plots for the risk of clinical malaria based on the anti-CS protein titres at 1 month post dose 3

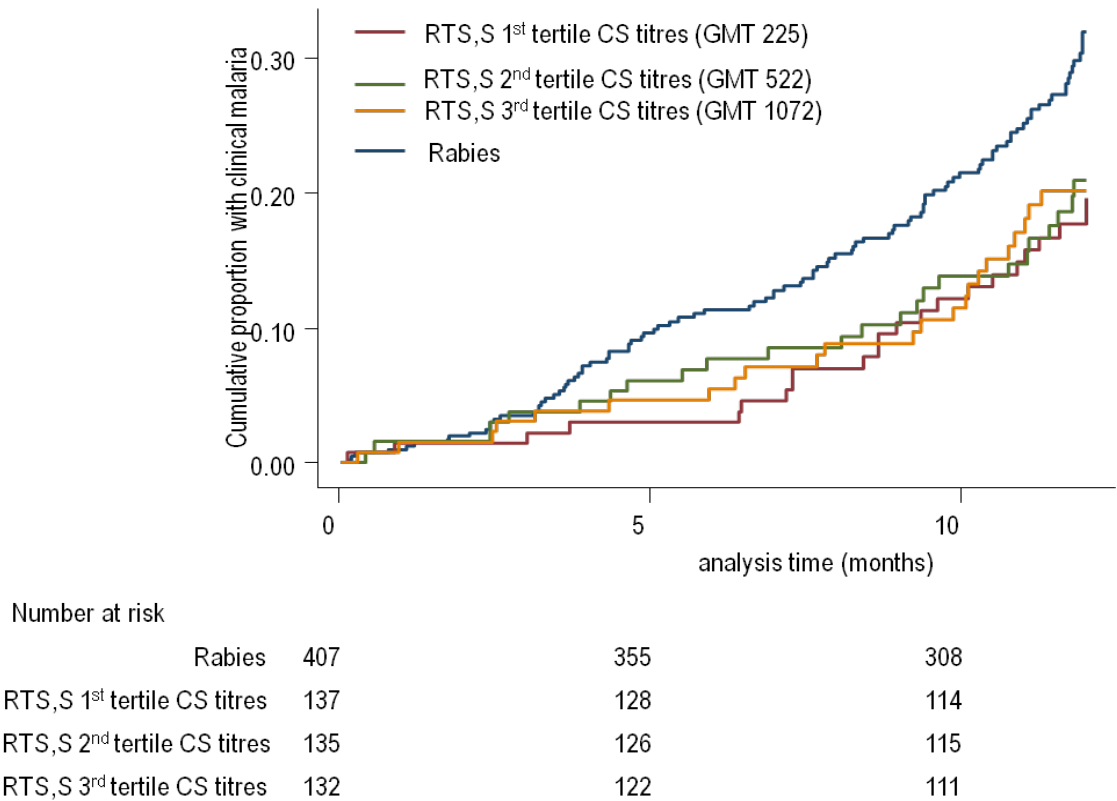
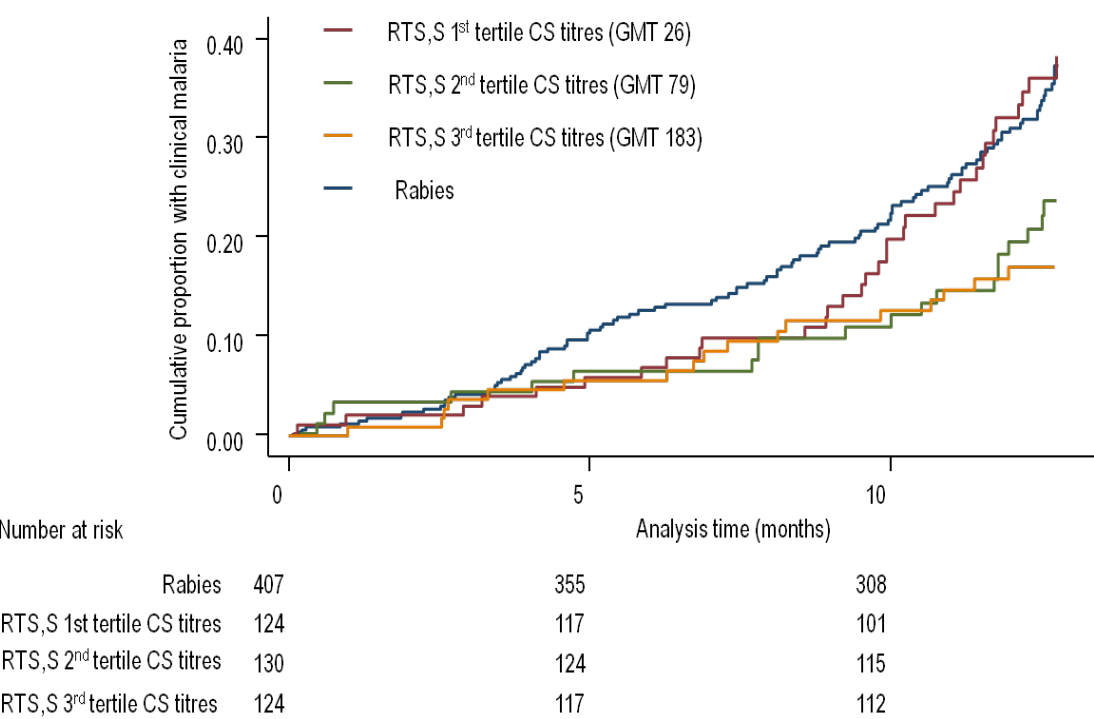


Figure 5:3 KM plots for the risk of clinical malaria based on anti-CS protein titres at 6.5 month



In order to analyze the outcomes related to concurrent anti-CS protein titres throughout the study (rather than at a single time-point), I used an exponential model of antibody decay, which fitted the anti-CS protein titres data well (Figure 5:4 for examples of selected individual fits). I then used the calculated weekly anti-CS protein titre as a time-varying covariate in a survival model. A linear fit between antibody titres and risk of clinical malaria was of marginal significance (HR=0.68, 95% CI 0.45-1.03, $p=0.07$). As described above, the survival function plots by anti-CS protein tertiles showed that the increase in risk of clinical malaria was specific to the lower tertile of anti-CS protein titres at month 6.5. I therefore reasoned that models dividing anti-CS protein titres into groups might fit the data better than a linear model. I examined two-level models, dichotomizing at a range of titres from 10 to 350 EU/mL (Figure 5:5). An optimal fit (as measured by the log likelihood) was produced when the calculated anti-CS protein titres were dichotomized at 40 EU/mL (95% CI 32-59 by bootstrap), with a hazard ratio of 0.52 (95%CI 0.35-0.78, $p=0.001$) for the high titre group.

Figure 5:4 Linear decay of anti-CS protein titres in selected RTS,S vaccinees (combined data)

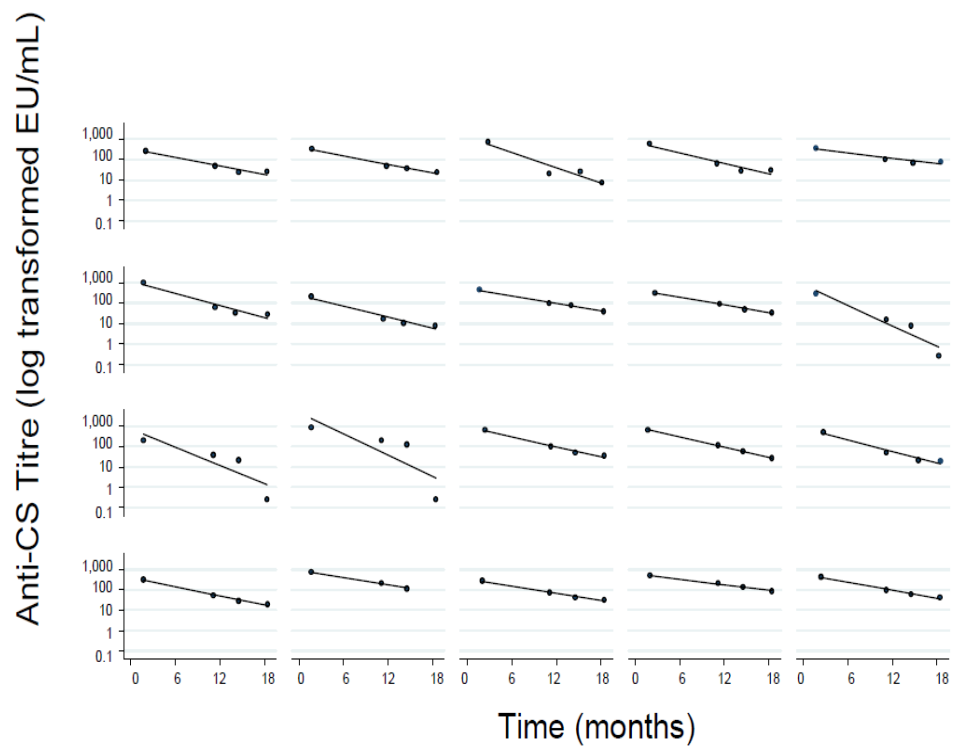
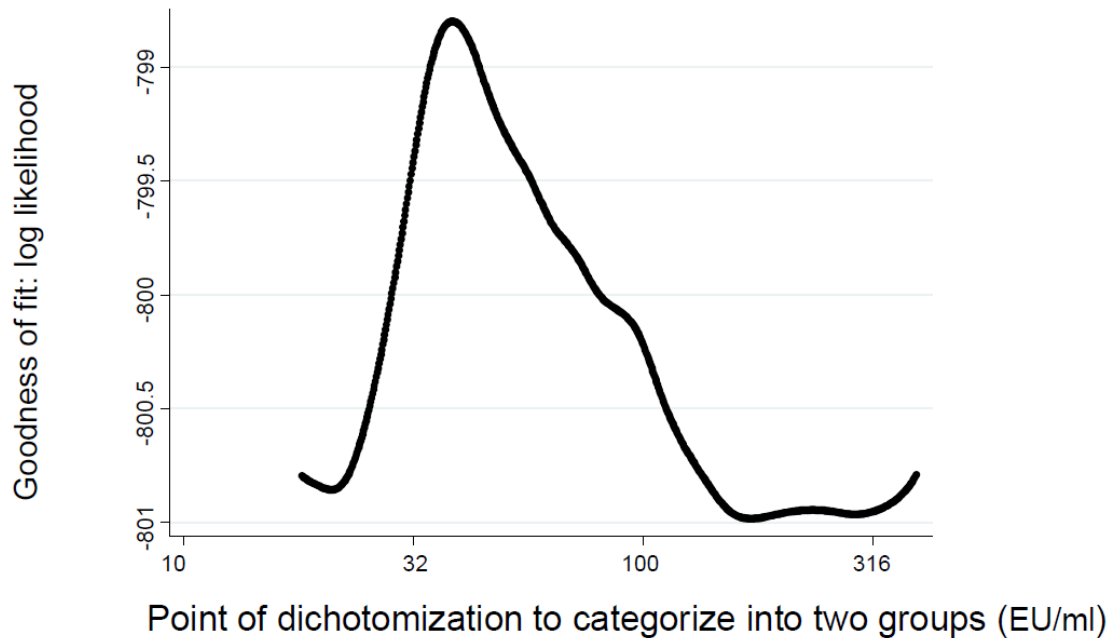


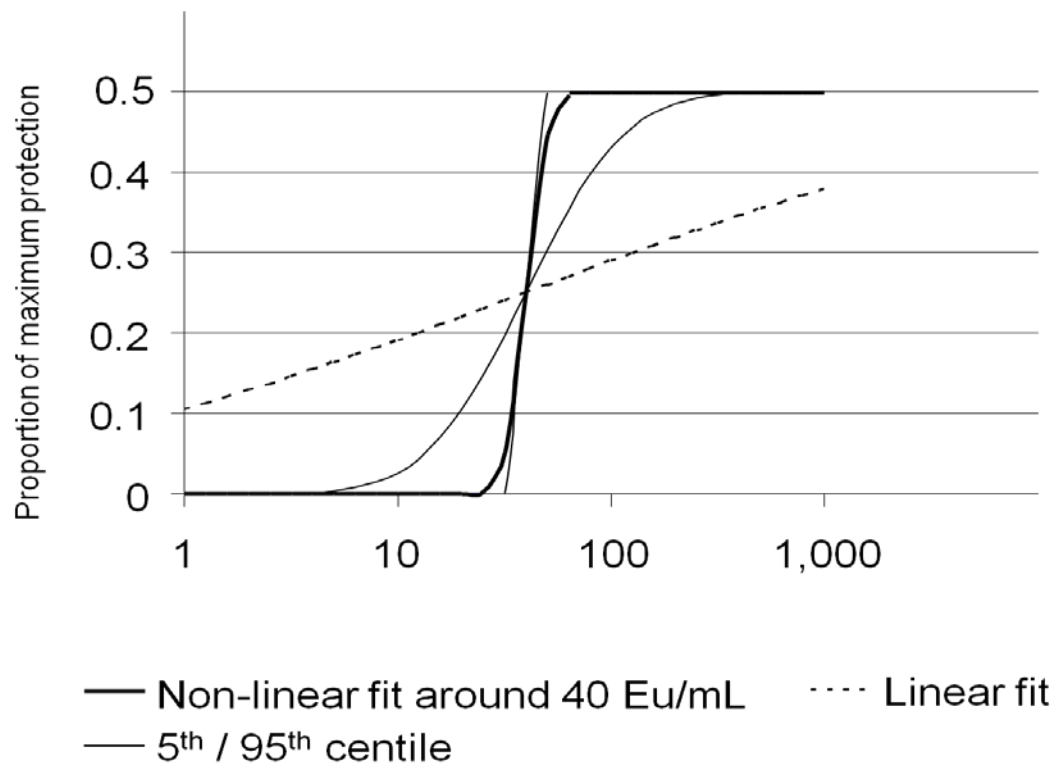
Figure 5:5 Log likelihood ratio graph for the optimal dichotomization level of anti-CS protein titres (combined data)



A two-level model implies an immediate change in risk of clinical malaria at a particular titre, and may be an over-simplification. To estimate how abrupt the change in risk was around 40 EU/mL, I fitted a cumulative normal distribution of anti-CS protein titre and protection. The cumulative normal distribution model may approximate a two level model with an abrupt change, but can also model more gradual rates of change in protection between the two groups. The cumulative normal distribution that fit the data had a standard deviation of 0.1 on a log scale (95% CI 0.05-0.3). This meant that 70% of the change in level of protection occurred between 30 and 50 EU/mL (i.e. the transition between groups is quite sudden, as plotted in Figure 5:6). I calculated the uncertainty around this estimate by bootstrap. The 5th centile indicates the most abrupt transition with 70% of the change in level of protection occurring between 35 and 45 EU/mL, and the

95th centile indicates a more gradual transition with 70% of the change occurring between 16 and 100 EU/mL (Figure 5:6).

Figure 5:6 Cumulative distribution curve for association between anti-CS protein titres and protection (combined data)



5.3.2 Kilifi data

5.3.2.1 *Anti-CS protein titres during four years of follow-up*

The Kilifi dataset showed similar trends with peak anti-CS protein mean of 543 EU/mL (95% CI: 479 to 614) and declining to 77EU/mL (95%CI: 68 to 87), 44 (95% CI: 38 to 50), 32(95%CI: 26 to 38), 26(95%CI: 22 to 30) and 17(95%CI: 15 to 20) at 6, 14, 18, 25 and 36 months post vaccination. However, titres remained higher in RTS,S/AS01E vaccinees compared to control vaccinees who had undetectable anti-CS protein antibodies titres throughout the study (Figure 5:7).

Imputed anti-CS protein antibody titres dynamic is shown in Figure 5:8. The decay of anti-CS protein titre demonstrated biphasic kinetics with earlier faster decay followed by a more subtle decay beyond about one year since the last dose of vaccination.

There was no difference in the geometric mean peak titres or decay kinetics of anti-CS protein by malaria exposure level (p value=0.66) as shown in Figure 5:9 and Figure 5:10.

Figure 5:7: Anti-CS protein antibody titres over time (Kilifi data)

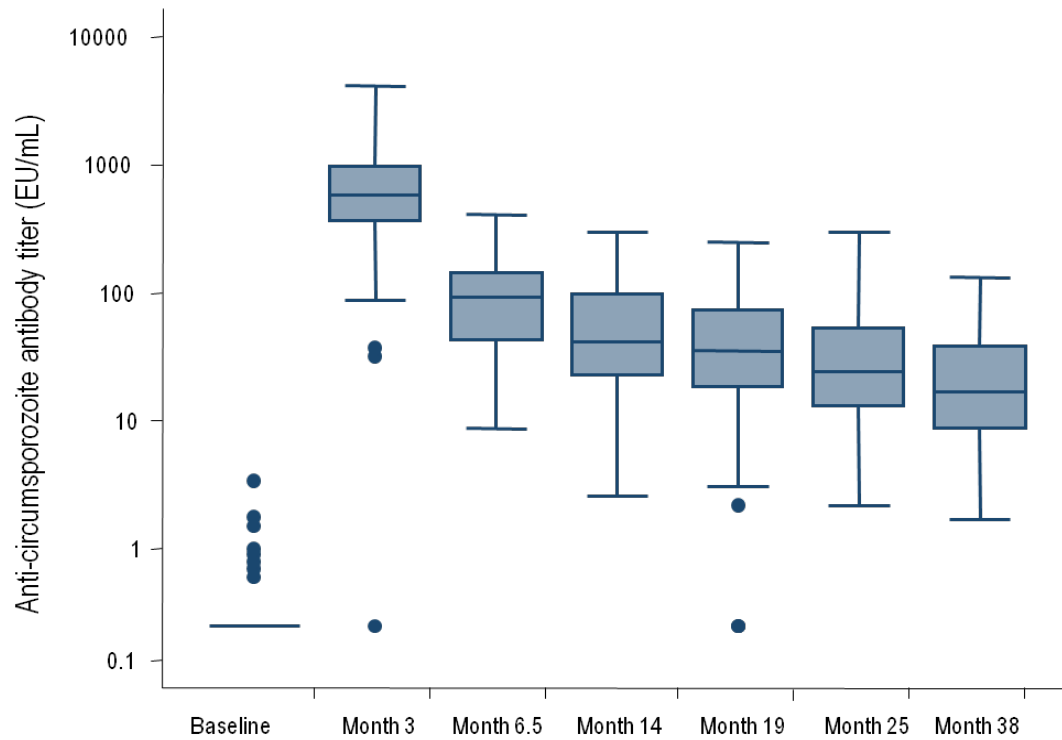


Figure shows anti-circumsporozoite antibody titres during follow-up in children who received the RTS,S/AS01_E vaccine. Anti-circumsporozoite antibody titres in the control group were consistently low or undetectable throughout follow-up and are not shown. The horizontal line within each box represents the median, the top and bottom of each box represent the 25th and 75th percentiles, respectively, and the I bars represent the highest and lowest values within 1.5 times the Interquartile range. The circles denote outliers. The horizontal line under the baseline values indicates that the values for the median and interquartile range were identical

Figure 5:8: Imputed individual anti-CS protein antibody titres (Kilifi data)

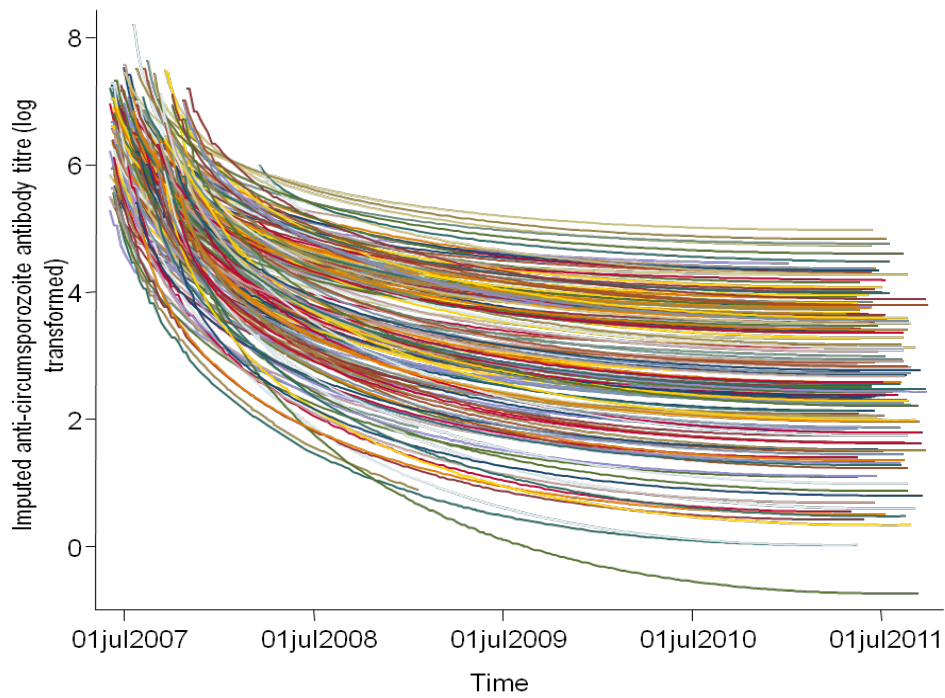


Figure 5:9 Imputed individual anti-CS protein antibody titres by malaria exposure

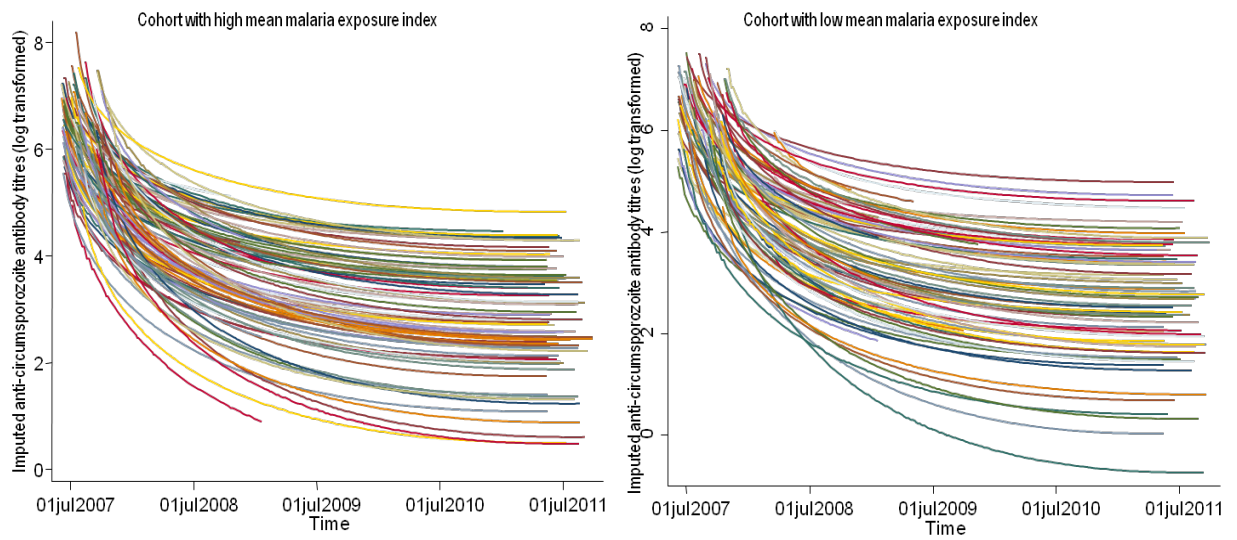
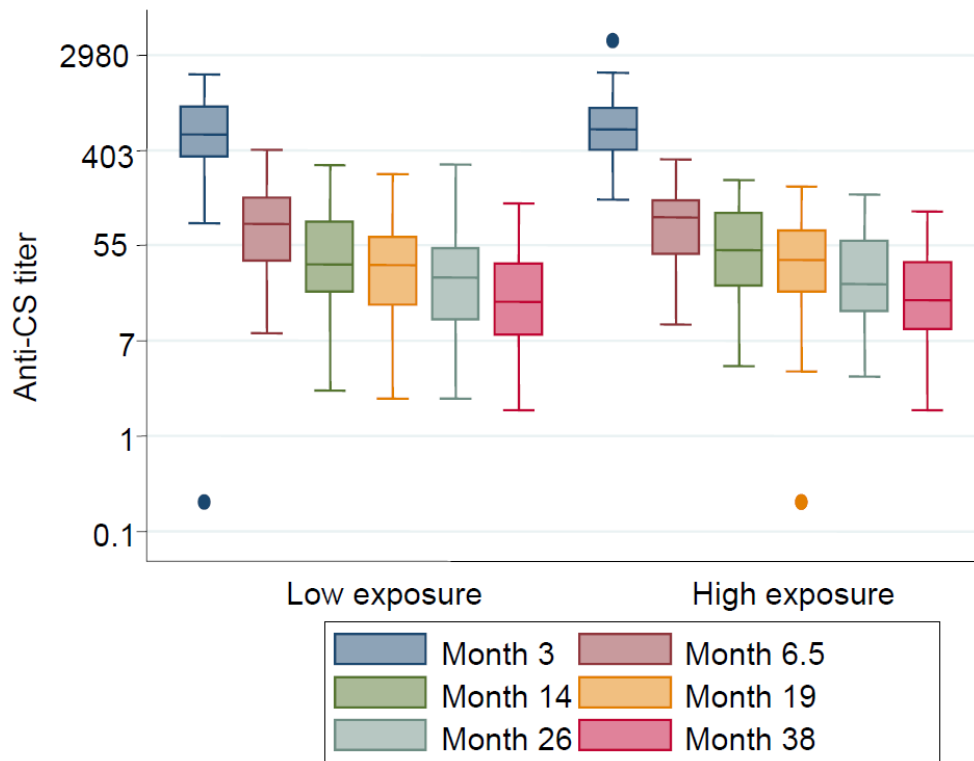


Figure 5:10 Anti-CS protein titres by malaria exposure group and follow-up (Kilifi data)



5.3.2.2 Association between anti-CS protein titres and protection

As in the combined dataset I found no linear association between imputed anti-CS protein titre and protection from clinical malaria (HR 0.91; 95%CI: 0.76 to 1.08, p value=0.286). I demonstrated a non linear relationship between anti-CS protein titres and protection from clinical malaria by cubic spline Cox regression model and step function model. The cubic spline model with 3 knots was selected for the best fit (Table 5-1). Graphical representation of hazard rate ratio from cubic spline Cox regression model showed no significant variation in risk between 1 and 100 EU/ml (i.e. the confidence intervals include HR=1), but above 100 EU/ml there was a reduced risk of clinical malaria with increasing antibody levels (Figure 5:11).

The best categorization level which given maximum log likelihood ratio was 85 EU/mL (Figure 5:12). Hence using step function model levels above 85EU/mL (95%CI: 30-105) were associated with protection from all clinical malaria (HR=0.55 (95%CI: 0.36 to 0.85; p=0.007).

Both cubic spline Cox regression and step function models were a better fit for the data than the linear model (Likelihood-ratio test p value=0.0021 for comparison with the linear model and AIC= 3507.3 versus 3515.7 for the step model and linear model respectively).

Table 5-1 Comparison of cubic spline models

Model	Observation	Log likelihood for null	Log likelihood for model	df	BIC
Model with 3 knobs	30504	-1916.905	-1866.795	8	3816.194
Model with 4 knobs	30504	-1916.905	-1865.322	9	3823.575
Model with 5 knobs	30504	-1916.905	-1865.028	10	3833.313

Figure 5:11 Non-linear association between protection and anti-CS protein titres based on cubic spline model (Kilifi data)

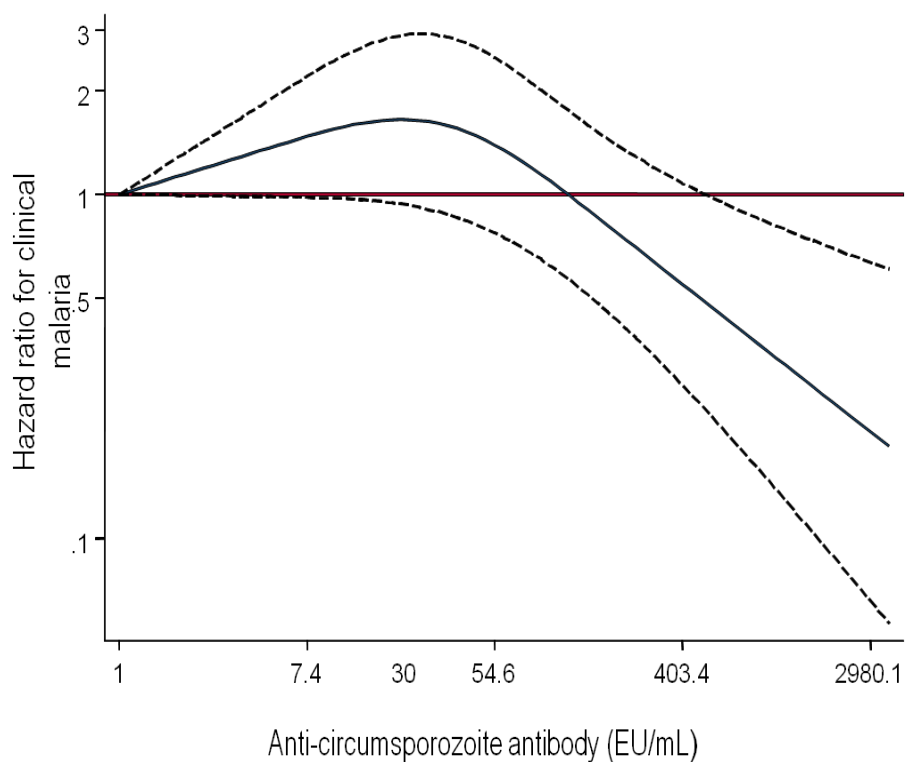
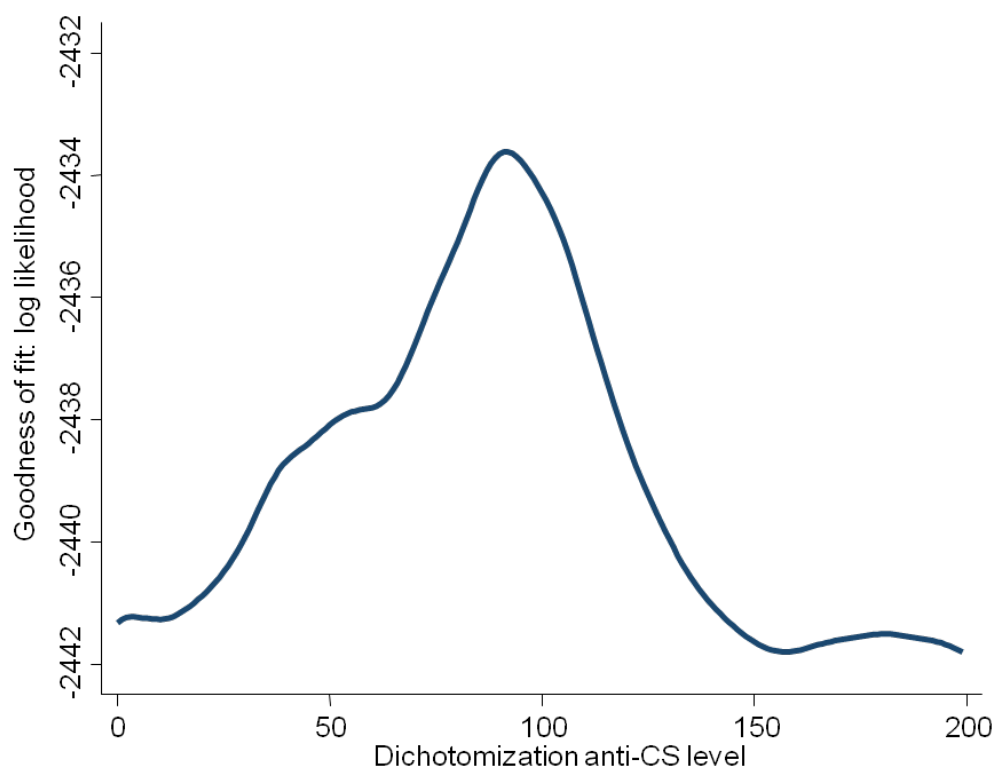


Figure shows the association between imputed anti-circumsporozoite antibody titres and the hazard ratio for clinical malaria episodes among children who received the RTS,S/AS01E vaccine, according to a Cox regression model with cubic splines and with a baseline titre of 1.0 enzyme-linked immunosorbent assay unit (EU) per milliliter as the reference. The dotted lines indicate the 95% confidence interval. There was no significant variation in risk between 1 EU per milliliter and 100 EU per milliliter (i.e., the confidence intervals include a hazard ratio of 1.0); at values above 100 EU per milliliter, however, there was a reduced risk of clinical malaria with increasing antibody titres.

Figure 5:12 Log likelihood ratio for the optimal dichotomization level of anti-CS protein titres (Kilifi data)



5.3.3 Discussion

I demonstrated a non linear association between anti-CS protein titres and protection from clinical malaria in children aged 5-17 month at first dose of vaccination residing in malaria endemic countries who were vaccinated with RTS,S/A01E.

The relationship was apparent when current anti-CS protein rather than peak titres were used in the model. This is probably because there is insufficient time for an anamnestic response during the brief time that sporozoites spend in the peripheral tissues and circulation before invading hepatocytes.

Better fit models were obtained by dichotomizing at 40 EU/mL (95%CI: 32-59) and 85EU/mL (95%CI: 30-105) in short-term combined data and long-term Kilifi data respectively. The linear model fitted the data more poorly than step-function model. The difference in time of follow-up, number of participants included in the analysis as well as the difference in endpoints (first or only clinical malaria versus all clinical malaria episodes) may have been responsible for the difference in dichotomizing point in the two analyses.

A linear association between anti-CS protein titre and protection from malaria infection was found in Mozambican young infants [156]. In my study, the linear association albeit was of marginal significance between anti-CS protein titre and clinical malaria. However, the mean peak levels were much lower in Mozambican infants (peak mean 199EU/mL) compared to level in my study (543EU/mL versus 274EU/mL).

In contrast to my findings, Mozambican children were protected from clinical malaria at a mean level of 8.8EU/mL [134] and experimental malaria challenge studies reported linear association between protection and anti-CS protein well above the dichotomization points observed in my analyses [131]. My step-function would have predicted no protection with levels in Mozambican children at 4 years and very little or no protection in experimental malaria challenge studies. However protection from malaria is the function of many factors such as age, acquisition of natural immunity to malaria and exposure, and these factors differ from one study to another.

However in the Kilifi-only dataset, the best fit model supported a more progressive increase in protection with increasing anti-CS protein titres above a certain cut-off (i.e. 105EU/mL), without a plateau as described in the combined data set.

How biologically plausible is the step function model compared to a curve relationship?

A curve relationship argues for the situation where rising antibody titres would steadily inhibit more sporozoites until near to 100% efficacy is reached, rather than reach a plateau at 50% efficacy (Figure 5:6). However, there may be sub-populations of sporozoite that reach the liver through different routes and after spending different periods of time in the skin [283,284]. A given anti-CS protein titre might inhibit only a sub-population of sporozoites that take a particular route [285]. In order to resolve this question and given the large 95% intervals surrounding the dichotomizing points there is a need to combine large numbers of studies to obtain an accurate representation of the relationship between anti-CS protein titres and protection from clinical malaria.

In theory, a partially effective pre-erythrocytic vaccine might either positively [286] or negatively [287] impact naturally acquired immunity to blood-stage parasites. Until further data are available, using a single correlate to predict clinical outcomes may not be appropriate. Furthermore, antibody may be a non-causal correlate. For instance, sustained antibody titres might closely correlate with an additional mechanism such as a cellular response [131,178]. Alternatively, other properties of the antibody response may be more accurate correlates, such as isotype, avidity, functional properties or binding to intact sporozoites.

5.4 Conclusion

In conclusion I showed the relationship between RTS,S/AS01E-induced anti-CS protein antibodies and protection from clinical malaria is non linear. The model is based on a single dataset and needs to be validated using other data sets. The current vaccine development process is empiric, and requires costly and time-consuming clinical studies. Models that predict efficacy from immunological studies will greatly accelerate malaria vaccine development.

6 Correlates of protection: T cell immune responses in adults

(A re- analysis of correlates of protection from a phase 2a trial of the falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults.)

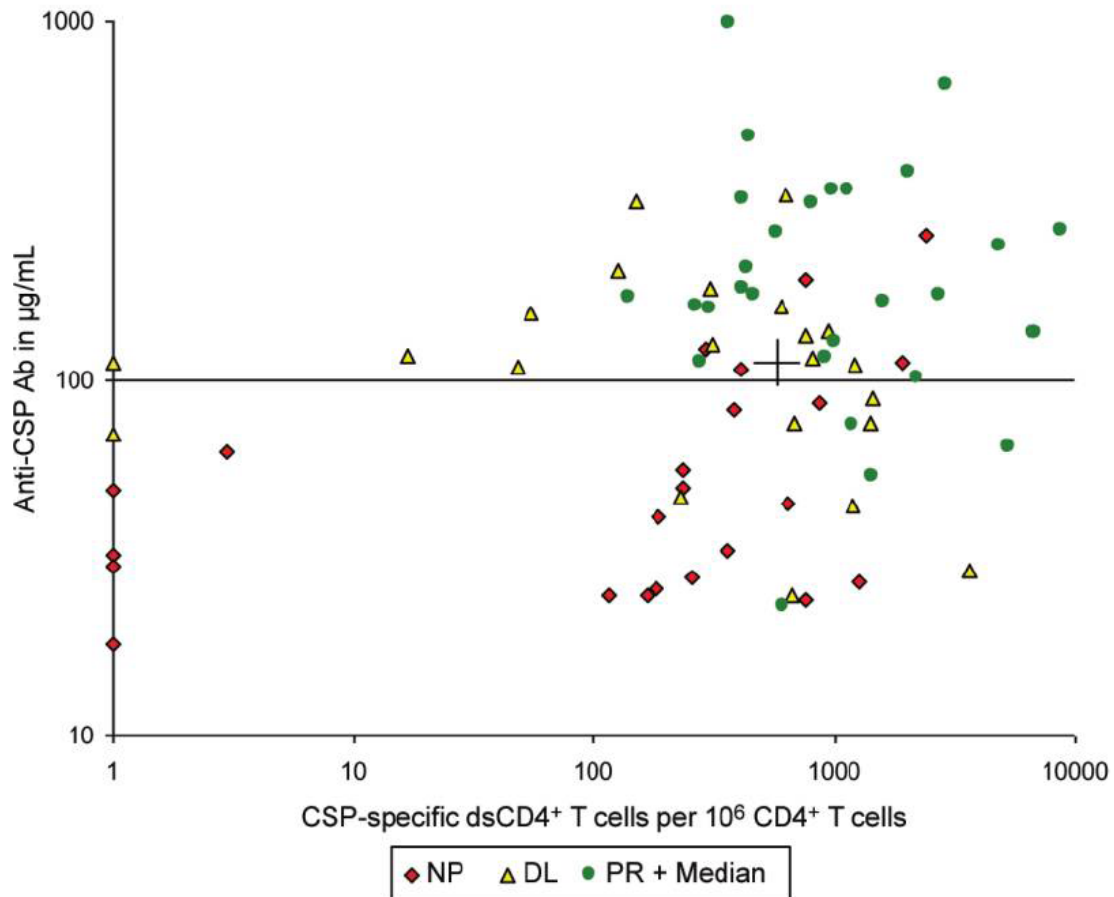
6.1 Introduction

Cell mediated immune responses are important vaccine effectors. RTS,S/AS01E candidate vaccine is believed to work through both anti-CS protein antibodies and cell mediated immune responses in preventing infection of the hepatocyte (chapter 1).

In the publication by Kester et al, both anti-CS protein antibody titres and CD4 cell responses were suggested to be important correlates of protection against experimental challenge in malaria naive adults [131]. In figure 7 of their article, the authors presented a scatter plot of antibody titres and CD4 cell responses for 73 individuals, using symbols to show outcome (Figure 6:1). The authors suggested that both antibody and CD4 cell responses may be independently protective against malaria challenge, based on the observation that, on the scatter plot, individuals with both high CD4 cell responses and high antibody titres were more likely to be protected than individuals with only high CD4 cells or only high antibody titres. In this chapter, I conducted multiple regression analysis

using the presented data to test this hypothesis, quantify the effect size, and test the significance of the associations.

Figure 6:1 CS protein specific CD4⁺ T cell and antibody responses and their relation to protection against malaria-infected mosquito challenge (Modified from Kester et al JID 2009[131])



Red diamond=Not protected; Yellow triangle=delayed time to infection; Green circle=protected from challenge

6.2 Methods

I used the coordinates from the scatter plot to deduce the values of the antibody titres and CD4 cell responses by individual. In the plot individuals were classified as either fully protected, partially protected (i.e. a delay to appearance of parasites in the blood) or not protected. For our analysis, we used two definitions of protection; the first definition included only fully protected individuals, the second included both fully protected and partially protected individuals. I reasoned that time to event models did not match the biology of the situation, and logistic models to calculate odds ratios as the measure of effect were not appropriate given the high frequency of the outcome. I therefore used a modified Poisson regression model [262] with a robust error variance to determine the relative risk of infection after malaria challenge as a function of log transformed antibody titres and CD4 cell responses using Stata version 9, StataCorp, Texas. I investigated possible interactions between CD4 cell responses and antibody titres by comparing the fit of models with and without interaction terms.

6.3 Results

Antibody titres and CD4 cell responses were both independently associated with protection from malaria challenge (Table 6-1). Increasing antibody titre was associated with reductions in relative risk (RR) of infection following malaria challenge corresponding to an efficacy of 65% (95%CI: 41-80%) for each 10-fold rise in antibody titre for the first definition of protection, and an efficacy of 91% (95%CI 71-97%) for the

second definition. Increasing CD4 cell responses were associated with more modest reductions in risk, corresponding to efficacies of 13% (95%CI 3-21%) and 11% (95%CI -10 - +26%) for each 10-fold rise in cellular responses, but were of marginal statistical significance.

There was no significant difference between the models with and without an interaction term; LR χ^2 test= 2.09; P value=0.15 and LR χ^2 test= 0.34; P value=0.56 for the first and second definition, respectively. Terms for the interaction between antibody titre and CD4 cell count gave relative risk estimates of 0.46 (CI 0.16-1.35) and 1.54 (CI 0.36-6.5) for the first and second definitions of protection, respectively. These relative risks suggest non-significant tendencies towards synergistic and antagonistic interactions, respectively; assessments that are limited by a modest sample size to examine interactions.

Table 6-1 : Multiple regression analysis for the first and second definition of protection

Covariates	First definition of protection*		
	Adjusted RR	95% CI	P value
Antibody titres	0.35	(0.2-0.59)	<0.0001
CD4 cell responses	0.87	(0.79-0.97)	0.011
	Second definition of protection[∞]		
	Adjusted RR	95% CI	P value
Antibody titres	0.09	(0.03-0.29)	<0.0001
CD4 cell responses	0.89	(0.74-1.1)	0.202

* Protection defined as full protection from infection after malaria challenge

[∞] Protection defined as full protection plus delayed time to infection

RR: Relative risk

6.4 Discussion

In adults, the findings from further analyses of data published by Kester et al [131] suggest that anti-CS protein antibody titres are strongly associated with vaccine efficacy, and suggest that CD4 cell responses are less strongly associated with protection. Despite the lack of evidence for interactions in these data, there is evidence for synergy between antibody and cellular responses in mouse challenge models [288], and interactions between antibody and cellular responses in humans need to be examined further with larger sample sizes from field studies.

7 Correlates of protection: T cell immune responses in children

7.1 Introduction

Having quantified the relative importance of cell mediated immune responses in adults vaccinated with RTS,S/AS01E, I now describe the T cell immune responses in the 447 children aged 5-17 month old who were vaccinated with RTS,S/AS01E and assess if these responses predicted protection from clinical malaria.

The blood volumes sampled in children prevented us from using an ICS assay previously reported in adult studies [131], but a whole blood ICS assay requiring smaller blood volumes has been developed and used in two phase II trials in Ghana [289] and Gabon [290]. These studies showed that the vaccine induced CD4⁺ IL2, TNF α or IFN γ producing cells, but CD40L was not detectable using the whole blood assay for children in Sub-Saharan Africa. Therefore CD40L staining was not included in this study. The assays were conducted partly in Kilifi, Kenya and at GSK Biologicals laboratory in Belgium.

The qualification of correlates of immunity and surrogates of protection has been recently reviewed [291,292]. One of the proposed methods of identifying a surrogate marker of protection is application of the Prentice criteria which I used to assess if anti-CS protein titres and T cell immune responses were surrogates of protection[293]. The Prentice criteria require that: a) vaccination predicts protection; b) vaccination predicts the potential surrogate; c) the surrogate predicts protection among vaccinees and d) that the

surrogate accounts for all the effect of vaccination. If vaccination is an independent predictor of outcome after including the potential surrogate in the analysis, this suggests that other mechanisms are involved. On the other hand, if including the potential surrogate in analysis removes vaccination as a predictor, this is consistent with the effect of vaccination being mediated by the surrogate marker.

7.2 Methods

The details of the study design and blood sample collection are described in chapter 4. Briefly this was a randomized controlled trial to evaluate the efficacy and safety of RTS,S/AS01E against clinical malaria episodes due to *P. falciparum* infection in Kilifi, Kenya and Korogwe, Tanzania. The primary end point was clinical malaria, defined as the presence of fever (axillary temperature $\geq 37.5^{\circ}\text{C}$) and *P. falciparum* parasitaemia of more than 2500/ μL . Active and passive surveillance for malaria was conducted by field workers and study personnel at local dispensaries.

There were 894 children between the two sites, of which the 447 children enrolled in Kilifi were assessed for vaccine induced cellular immunity using ICS and ELISPOT. Blood was taken for immunological studies before vaccination, one month post dose 3, then on March 2008 irrespective of the time of recruitment (i.e. between 4 and 10 months post dose 3, mean 8 months), 12 months post dose 3 and in October 2008 irrespective of time of recruitment, (i.e. between 12 and 18 months post dose 3, mean 15 months). Peak malaria transmission was between May and August 2008.

7.2.1.1 CS protein antibody measurement and peptide used

Antibodies to the *P. falciparum* CS protein protein (CS) tandem repeat epitope were assessed by ELISA at the Center for Vaccinology, Ghent University Hospital in Belgium as described in 5.2.2. Results were reported in EU/mL.

Laboratory immunological methods including ELISPOT and whole intracellular staining assays are explained in appendix A.

7.2.2 Statistical analysis

All T cell responses were log transformed before analysis. I calculated geometric mean responses and used Student's T test on log-transformed values to compare between vaccination groups. I used a paired T test on log-transformed values to compare time-courses and used Pearson's product moment calculation on log-transformed values to examine the correlations between assays.

I used Cox regression to assess the association between cellular responses and first or only clinical malaria (axillary temperature $\geq 37.5^{\circ}$ C with *P. falciparum* density of more than 2500/ μ L). The adjustments were made for age at first vaccination, village, distance to the health facility, bed net use and anti-CS protein (CS) antibody levels by dichotomizing concurrent anti-CS protein titres at 40 EU/mL. Cellular responses were analyzed as time-varying covariates, applying the result from the time of the most recent clinic visit. I applied a Bonferroni correction for all the independently significant explanatory variables to adjust for the multiple comparisons. Responses were log

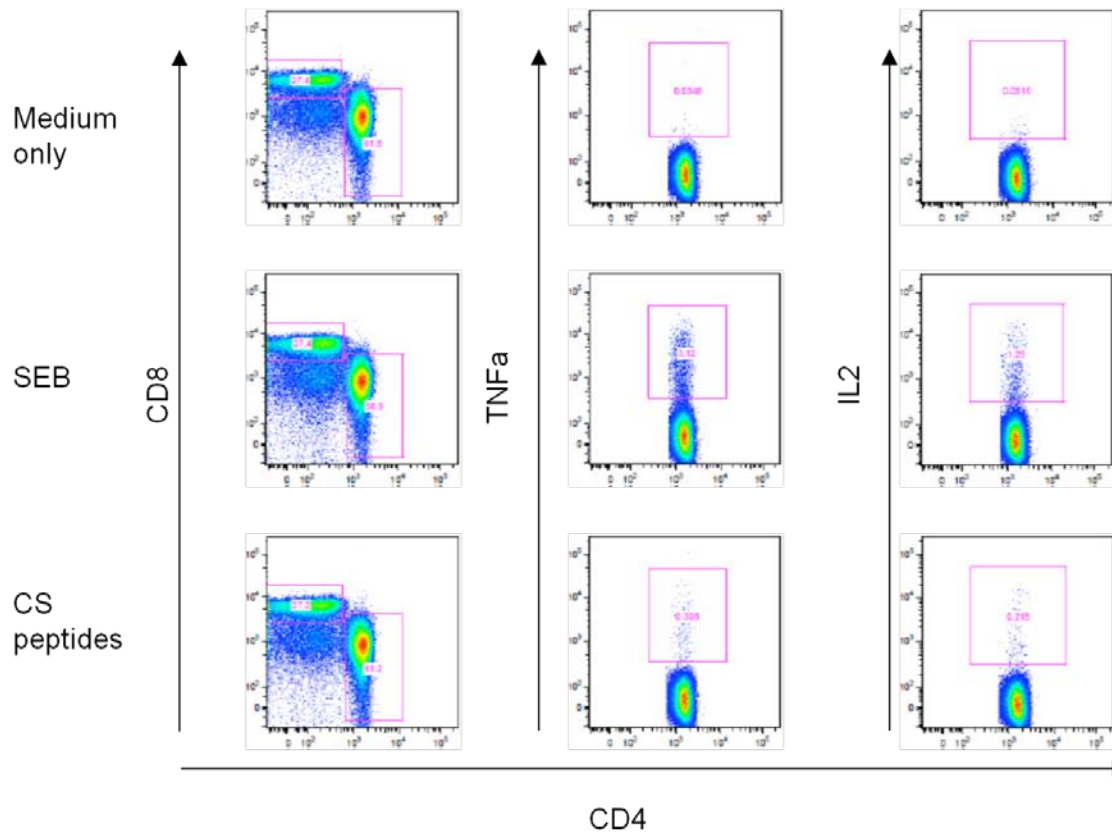
transformed to produce normal distributions before inclusion in the Cox regression models. Analysis was conducted on the According To Protocol vaccinees. STATA version 10 was used.

7.3 Results

Blood samples were processed from 407 children. Data were acquired from 1,066 ICS assays (from three different clinic visits), 660 cultured ELISPOTs (from four different clinic visits), 780 *ex vivo* ELISPOTs for IFN γ (from three clinic visits) and 453 *ex vivo* ELISPOTs for IL2 production (from 3 clinic visits). 56 (8%), 12 (2%) and 21 (5%) assays failed quality control criteria for positive and negative controls for cultured, and *ex vivo* IFN γ and *ex vivo* IL2 ELISPOTs, respectively. For ICS assay, the results from the positive control were at least 100 cells per million above the negative control for 1045 (98.0%), 1057 (99.1%) and 1055 (99.0%) for IFN γ , IL2 and TNF α , respectively.

The geometric mean responses to the negative control were 75, 165 and 159 cells per million for IFN γ , IL2 and TNF α ICS results respectively, and average responses to positive control were 3,768, 18,895 and 3,454 cells per million. The mean responses to CS antigen vary by time point and by vaccination group, but the ranges were 11 to 25, 10 to 681 and 8 to 426 for IFN γ , IL2 and TNF α , respectively. There was no variation in responses to control by time point ($p=0.15$, $p=0.15$, $p=0.6$) or by vaccination group at the first time point post vaccination ($p=0.4$, $p=0.36$ and $p=0.39$) for IFN- γ , IL-2 and TNF- α respectively. An example of the flow cytometry analysis is shown in **Figure 7:1**.

Figure 7:1: An example plot of FACS data acquired following intra-cellular cytokine staining is shown for negative control (medium only), positive control (i.e. staphylococcal enterotoxin B, SEB) and CS peptides.



7.3.1.1 Vaccine induced anti-CS protein T cell responses: ICS assays

CD4⁺ and CD8⁺ anti-CS protein T cell responses were detected in both vaccination groups using ICS. There were no significant differences between the groups pre-vaccination. Vaccination with RTS,S/AS01E induced CD4⁺ but no CD8⁺ anti-CS protein T cell responses. The strongest responses were seen for IL2 producing CD4 T cells at one-month post vaccination (a mean of 681 cells per million, 95%CI 585-792),

followed by TNF- α (426 cells per million, 95%CI 362-502), and weak IFN- γ responses (25 cells per million, 95%CI 18-34) Table 7-1 and Figure 7:2. These levels corresponded to 3.2 fold, 2.3 fold and 1.9 fold increases for IL2, TNF- α and IFN- γ , respectively.

Figure 7:2: The time course of anti-CS protein CD4+ ICS responses and summed ELISPOT responses is shown per time point for RTS,S/AS01E and control vaccination groups.

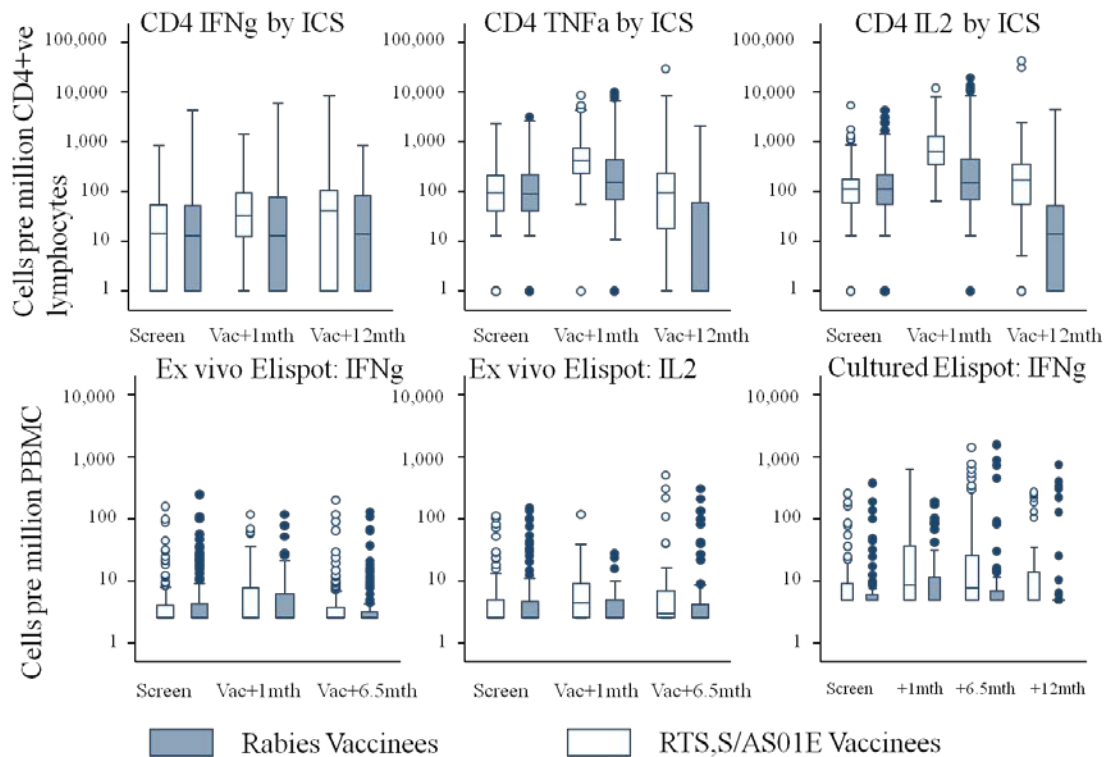


Table 7-1: Geometric means of ICS assays by clinic visit and by vaccination group.

Visit	Rabies		RTS,S/AS01E		
	Mean (95%CI)	N	Mean (95%CI)	N	p
ICS: CD4+ve cells: IFNγ					
Screen	11(8-14)	197	12(9-16)	182	0.6
Vac + 1 mth	13(10-18)	182	25(18-34)	170	0.01*
Vac + 12 mths	11(7-15)	167	20(14-29)	168	0.009**
ICS: CD4+ve cells: IL2					
Screen	103(84-127)	197	94(76-117)	182	0.52
Vac + 1 mth	212(183-245)	182	681(585-792)	170	2x10-13**
Vac + 12 mths	10(7-14)	167	102(73-142)	168	9x10-19**
ICS: CD4+ve cells: TNFα					
Screen	86(69-108)	197	81(64-102)	182	0.69
Vac + 1 mth	182(156-214)	182	426(362-502)	170	1x10-08**
Vac + 12 mths	8(6-11)	167	48(34-68)	168	6x10-12**
ICS: CD8+ve cells: IFNγ					
Screen	12(9-17)	178	7(5-10)	175	0.028#
Vac + 1 mth	19(12-28)	168	19(12-28)	156	0.99
Vac + 12 mths	10(6-15)	137	11(7-17)	127	0.73
ICS: CD8+ve cells: IL2					
Screen	156(128-191)	178	190(155-233)	175	0.24
Vac + 1 mth	200(164-246)	168	215(175-266)	156	0.64
Vac + 12 mths	9(6-13)	137	20(13-32)	127	0.004**
ICS: CD8+ve cells: TNFα					
Screen	49(36-68)	178	59(43-81)	175	0.43
Vac + 1 mth	133(106-167)	168	162(127-205)	156	0.26
Vac + 12 mths	15(10-24)	137	16(10-25)	127	0.91

* for $p < 0.05$ or ** for $p < 0.005$ where Mean for Rabies group < Mean for RTS,S/AS01E group. # for $p < 0.05$ or ## for $p < 0.005$ where Mean for Rabies group > Mean for RTS,S/AS01E group.

7.3.1.2 *ELISPOT assays*

Three different peptide pools namely; a) the conserved region including the NANP repeats, b) the variant TH2R region and c) the variant TH3R region and conserved CS.T3T region were used for the ELISPOT assays, allowing a more detailed analysis of immunogenicity. Cultured ELISPOT results were higher among RTS,S/AS01E vaccinees than among rabies vaccinees at 1 month and 6.5 months post vaccination, but not at 12 months (Table 7-2). IFN γ *ex vivo* ELISPOT results did not vary by vaccination group at any time point. IL2 *ex vivo* ELISPOT responses were significantly higher in RTS,S/AS01E vaccinees at 1 month post vaccination, but not at 6.5 months (Table 7-3) compared with rabies vaccinees.

For both the cultured IFN γ ELISPOT and *ex vivo* IL2 ELISPOT, the vaccine induced cellular responses were limited to two peptide pools (i.e. TH2R and TH3R/CS.T3T pools) Table 7-2. No responses were detected to the third peptide pool (NANP and conserved region peptides; Figure 7:3).

Figure 7:3: ELISPOT responses are shown for the individual stimulating peptide pools at 1 month post vaccination with RTS,S/AS01E.

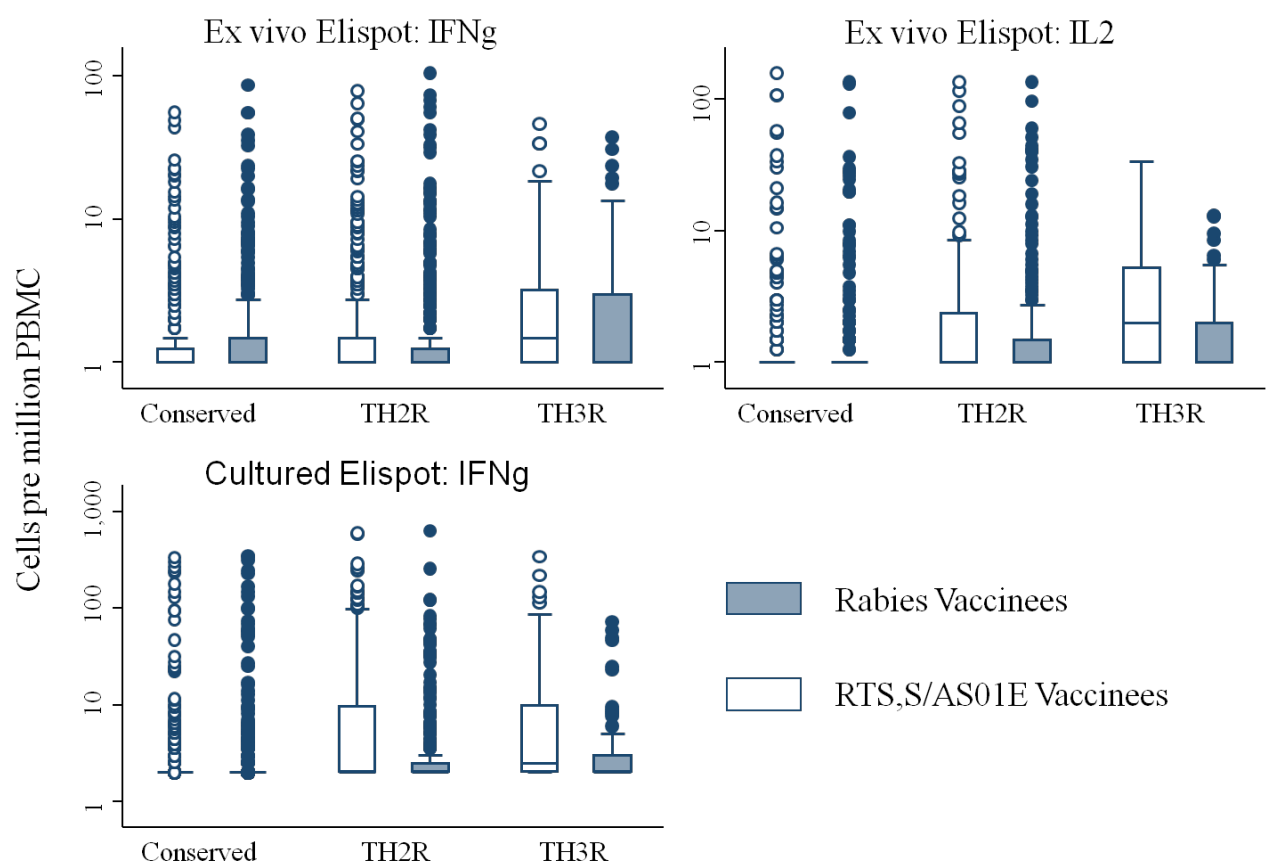


Table 7-2: Geometric means of cultured ELISPOT assays by clinic visit and by vaccination group.

Visit	Rabies		RTS,S/AS01E		p
	Mean (95%CI)	N	Mean (95%CI)	N	
IFNγ cultured ELISPOT: NANP and conserved region					
Screen	27(21-35)	72	33(26-43)	70	0.22
Vac + 1 mth	32(27-38)	86	28(24-32)	109	0.26
Vac + 6.5 mths	29(25-35)	82	26(22-30)	122	0.34
Vac + 12 mths	31(23-42)	55	27(21-36)	64	0.54
IFNγ cultured ELISPOT: TH2R region					
Screen	33 (26-43)	72	36 (28-48)	70	0.66
Vac + 1 mth	34(25-47)	86	66(50-88)	109	3x10-4**
Vac + 6.5 mths	30(22-41)	83	60(47-78)	122	2x10-4**
Vac + 12 mths	26(20-34)	55	39(30-51)	64	0.023*
IFNγ cultured ELISPOT: TH3R/ CS.T3T region					
Screen	31 (23-41)	72	37 (28-48)	70	0.38
Vac + 1 mth	30(23-40)	86	55(43-70)	109	3x10-4**
Vac + 6.5 mths	32(23-44)	83	58(45-76)	122	0.003**
Vac + 12 mths	33(24-46)	55	36(26-48)	64	0.79
IFNγ cultured ELISPOT: All CS peptides summed					
Screen	75 (58-97)	72	90 (69-117)	70	0.7
Vac + 1 mth	88(66-117)	86	151(117-195)	109	0.002**
Vac + 6.5 mths	81(59-110)	82	145(113-187)	122	0.002**
Vac + 12 mths	83(59-115)	55	104(76-141)	64	0.33

* for p<0.05 or ** for p<0.005 where Mean for Rabies group < Mean for RTS,S/AS01E group. # for p<0.05 or ## for p<0.005 where Mean for Rabies group > Mean for RTS,S/AS01E group.

Table 7-3: Geometric means of ex vivo ELISPOT assays by clinic visit and by vaccination group.

Visit	Rabies		RTS,S/AS01E		p
	Mean (95%CI)	N	Mean (95%CI)	N	
IFN γ ex vivo ELISPOT: NANP and conserved region					
Screen	15(13-17)	152	14(12-16)	137	0.59
Vac + 1 mth	15(13-18)	100	16(13-19)	104	0.83
Vac + 6.5 mths	13(12-14)	145	12(11-13)	142	0.21
IFN γ ex vivo ELISPOT: TH2R region					
Screen	15(13-17)	152	14(12-16)	137	0.56
Vac + 1 mth	16(13-19)	100	18(15-22)	104	0.23
Vac + 6.5 mths	14(13-16)	145	14(13-17)	142	0.96
IFN γ ex vivo ELISPOT: TH3R/ CS.T3T region					
Screen	18(15-20)	152	17(15-20)	137	0.79
Vac + 1 mth	19(15-23)	100	21(17-25)	104	0.43
Vac + 6.5 mths	13(11-15)	144	15(13-17)	141	0.23
IFN γ ex vivo ELISPOT: All CS peptides summed					
Screen	40(35-46)	152	39(34-45)	137	0.69
Vac + 1 mth	44(36-54)	100	48(40-59)	104	0.5
Vac + 6.5 mths	35(31-40)	144	35(31-40)	141	0.94
IL2 ex vivo ELISPOT: NANP and conserved region					
Screen	15(13-18)	107	15(12-18)	89	0.8
Vac + 1 mth	12(10-14)	62	14(12-17)	56	0.11
Vac + 6.5 mths	15(12-20)	76	15(11-21)	63	0.98
IL2 ex vivo ELISPOT: TH2R region					
Screen	16(13-19)	107	16(13-20)	89	0.93
Vac + 1 mth	13(10-17)	62	21(16-28)	56	0.003**
Vac + 6.5 mths	18(14-23)	76	17(13-22)	63	0.76
IL2 ex vivo ELISPOT: TH3R/ CS.T3T region					
Screen	17(15-21)	107	17(14-21)	89	0.9
Vac + 1 mth	15(12-19)	62	24(19-31)	56	0.003**
Vac + 6.5 mths	18(13-24)	76	29(20-41)	62	0.022*
IL2 ex vivo ELISPOT: All peptides summed					
Screen	44(37-53)	107	42(34-51)	89	0.71
Vac + 1 mth	35(27-45)	62	56(44-73)	56	0.002**
Vac + 6.5 mths	45(34-60)	76	53(39-72)	62	0.43

* for $p < 0.05$ or ** for $p < 0.005$ where Mean for Rabies group < Mean for RTS,S/AS01E group. # for $p < 0.05$

or ## for $p < 0.005$ where Mean for Rabies group > Mean for RTS,S/AS01E group.

7.3.1.3 Time course of responses (ICS assays)

The frequencies of IL2, TNF α and IFN γ producing CD4 $^{+}$ T cells by ICS was significantly higher at one month after the final vaccination with RTS,S/AS01E compared with pre-vaccination levels ($p < 0.0001$, $p < 0.0001$, $p = 0.0006$, respectively). There was then a fall in responses between 1 month and 12 months post vaccination, falling to pre-vaccination levels for IL2 ($p < 0.0001$) and TNF α ($p < 0.0001$). IFN γ producing CD4 $^{+}$ T cells remained above pre-vaccination levels, albeit at low frequency throughout. However, there was an even more pronounced fall in CD4 $^{+}$ T cell responses among control vaccinees (Table 7-1), and so RTS,S/AS01E vaccinees had substantially higher T cell responses than control vaccinees at 12 months post vaccination ($p < 0.0001$ for TNF α and IL2, $p = 0.009$ for IFN γ).

7.3.1.4 Inter assay correlations

There were strong correlations between the different cytokines detected by ICS, and also between IL2/IFN γ ELISPOT results one month after vaccination with RTS,S/AS01E (Table 7-4). Cultured ELISPOTs were significantly associated with ICS results, but not with *ex vivo* ELISPOT results (IFN γ or IL2). Antibody titres were associated with all the cellular assays except *ex vivo* IFN γ ELISPOTs (Table 7-4).

Table 7-4: Inter-assay Correlation coefficients of CMI assays at 1 month post vaccination with RTS,S/AS01E.

	CD4+ IFN γ	CD4+ IL2	CD4+ TNF α	Antibody (CS)	Cultured IFN γ	IFN γ ELISPOT	IL2 ELISPOT
CD4+ IFN γ	1						
CD4+ IL2	0.38***	1					
CD4+ TNF α	0.32***	0.66***	1				
Antibody (CS)	0.14***	0.35***	0.26***	1			
Cultured IFN γ	0.14**	0.15**	0.18***	0.22***	1		
IFN γ ELISPOT	-0.05	-0.05	0.02	0.03	-0.02	1	
IL2 ELISPOT	0	0.01	0.15*	0.11*	-0.06	0.31***	1

*=p<0.05, **=p<0.001, ***=p<0.0001

7.3.1.5 Correlates of immunity

After vaccination with RTS,S/AS01E, an increasing frequency of TNF α producing, CS-specific CD4+ cells detected using ICS was associated with a reduced risk of clinical malaria (HR=0.64 for each 10 fold increase in the frequency of CD4+ TNF α + T cells, 95%CI 0.49-0.86, p=0.002). On ICS, IFN γ production by CS-specific CD4+ T cells was associated with a weak evidence for reduced risk of clinical malaria (p=0.07) (

Table 7-5). TNF α and IFN γ producing CS-specific CD4+ T cells were at much lower frequencies among control vaccinees, but nevertheless were associated with reduced risks of clinical malaria of borderline significance. When data from both RTS,S/AS01E vaccinees and control vaccinees were combined (with adjusting for vaccination group), the overall hazard ratios were 0.74 (95%CI 0.62-0.89, p=0.001) and 0.79 (95%CI 0.67-0.94, p=0.007) for TNF α and IFN γ , respectively. On Bonferroni adjustment, these p

values were 0.018 and 0.13, respectively. Similar results were observed when adjusting for anti-CS protein antibody titres as a continuous variable (HR=0.75, 95%CI 0.62-0.91, $p=0.003$ with $p=0.054$ after Bonferroni adjustment, and HR=0.81, 95%CI 0.68-0.95, $p=0.01$ with $p=0.13$ after Bonferroni adjustment for TNF α CD4 $^{+}$ T cells and IFN γ CD4 $^{+}$ T cells, respectively).

In order to display the effect graphically, the cellular responses were split by tertile (Figure 7:4). The middle tertiles for TNF α are at intermediate risk, suggesting a continuous change in risk as the frequency of TNF α cells increases rather than a threshold effect.

When the frequencies of TNF α producing CD4 $^{+}$ T cells and IFN γ producing CD4 $^{+}$ T cells were combined in the same model, TNF α CD4 $^{+}$ T cell frequency was an independent factor (i.e. HR=0.76, 95%CI 0.64-0.89, $p=0.001$ compared with HR=0.84, 95%CI 0.71-1.00, $p=0.050$ for the frequency of IFN γ producing CD4 $^{+}$ T cells). An interaction term generated by multiplying the frequency of TNF α CD4 $^{+}$ T cells by antibody concentrations was not significant in determining risk (HR=0.79, 95%CI 0.51-1.2, $p=0.29$).

On applying the fourth of the Prentice criteria, we found that vaccination group was still an independent predictor of clinical malaria risk in a multivariable model including CD4 $^{+}$ TNF α^{+} cells (HR=0.69, 95%CI 0.48-0.97, $p=0.036$). In other words, only 42% of the effect of vaccination could be accounted for by CD4 $^{+}$ TNF α^{+} cells. However, when anti-CS protein titres were added to the model the effect of vaccine became non-significant

(HR=0.93, 95%CI 0.62-1.42, p=0.76, i.e. 87% of the effect of vaccination was accounted for).

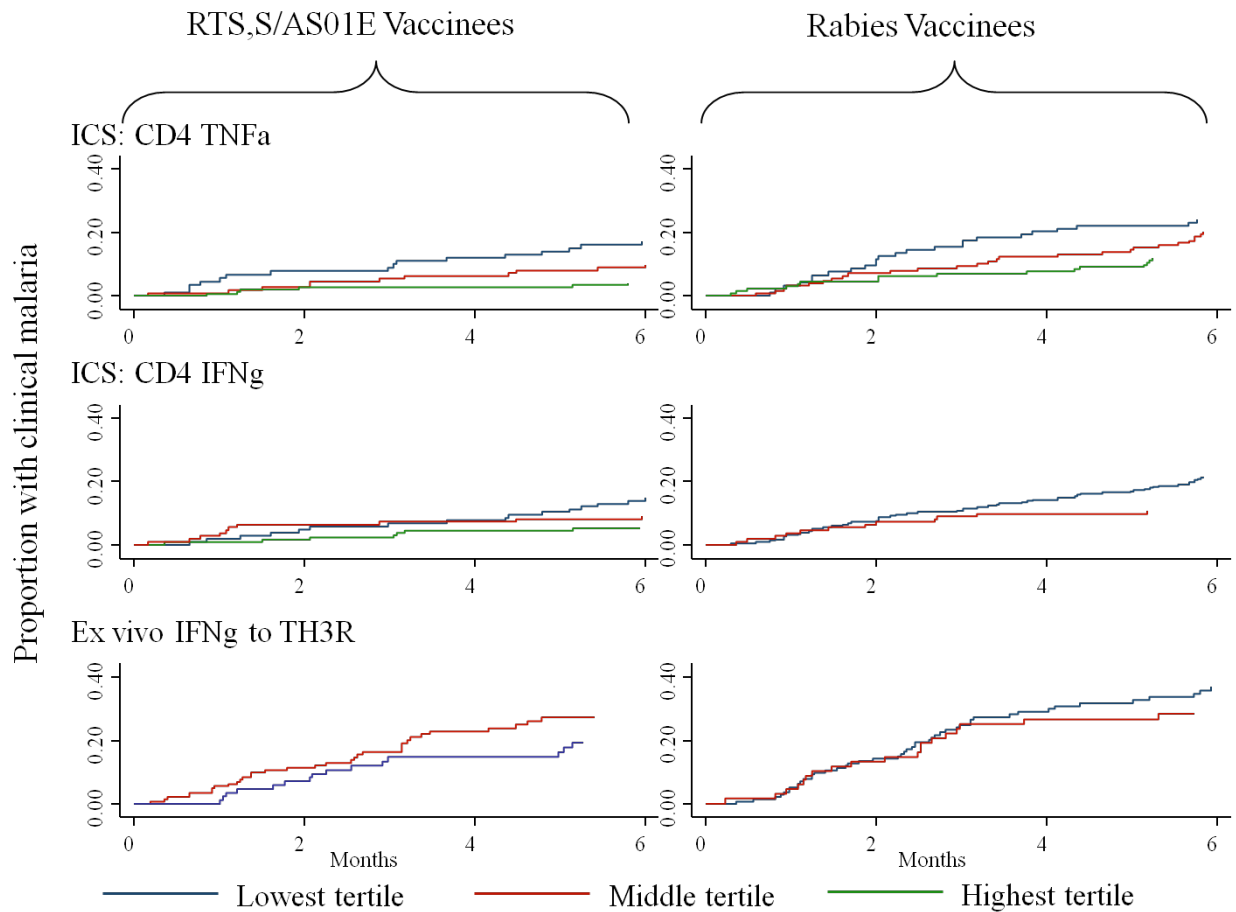
Hence, while neither CD4⁺ TNF α ⁺ cells nor anti- CS protein antibodies alone accounted for all of the effect of vaccination with RTS,S/AS01E on clinical malaria risk, the combination of CD4⁺ TNF α ⁺ T cells and anti-CS protein antibodies together could account for all of the statistical effect of vaccination.

Table 7-5: The hazard ratio from Cox regression models (with 95% CI) for the outcome clinical malaria by CMI assays.

Assay	Both datasets HR (95%CI)	p	Rabies Vaccinees HR (95%CI)	p	RTS,S/AS01E Vaccinees HR (95%CI)	p
ICS: CD4 cells						
IFN-γ	0.79(0.67-0.94)	0.007	0.81(0.66-1.01)	0.058	0.77(0.59-1.02)	0.07
IL2	0.9(0.76-1.07)	0.23	0.97(0.78-1.22)	0.81	0.77(0.55-1.08)	0.13
TNF-α	0.74(0.62-0.89)	0.001	0.8(0.62-1.03)	0.084	0.64(0.49-0.86)	0.002
ICS: CD8 cells						
IFN- γ	1.07(0.91-1.25)	0.43	1.13(0.93-1.37)	0.21	0.93(0.68-1.27)	0.65
IL2	0.85(0.69-1.05)	0.13	0.92(0.7-1.21)	0.56	0.79(0.56-1.12)	0.19
TNF- α	0.87(0.72-1.04)	0.12	0.83(0.66-1.05)	0.11	0.93(0.68-1.28)	0.66
IFN γ cultured ELISPOT						
NANP	1.01(0.65-1.57)	0.95	0.9(0.54-1.52)	0.7	1.08(0.44-2.68)	0.87
TH2R	0.76(0.51-1.14)	0.18	0.67(0.3-1.52)	0.34	0.79(0.49-1.27)	0.33
TH3R	0.94(0.67-1.32)	0.72	0.99(0.61-1.61)	0.97	0.86(0.53-1.39)	0.54
Sum	0.95(0.67-1.34)	0.77	0.92(0.54-1.57)	0.77	0.92(0.58-1.47)	0.73
IFN- γ ex vivo ELISPOT						
NANP	1.61(1.01-2.55)	0.044	1.54(0.87-2.72)	0.14	1.38(0.62-3.08)	0.44
TH2R	1(0.59-1.69)	1	0.9(0.44-1.85)	0.78	1.1(0.49-2.45)	0.81
TH3R	1.62(1.04-2.52)	0.032	1.57(0.82-2.99)	0.17	1.4(0.75-2.64)	0.29
Sum	1.35(0.86-2.12)	0.2	1.32(0.73-2.4)	0.35	1.23(0.61-2.47)	0.57
IL2 ex vivo ELISPOT						
NANP	1.18(0.56-2.51)	0.67	1(0.29-3.39)	0.99	1.38(0.46-4.17)	0.57
TH2R	0.57(0.23-1.45)	0.24	0.49(0.13-1.79)	0.28	0.81(0.2-3.2)	0.76
TH3R	0.94(0.45-1.97)	0.87	0.73(0.21-2.49)	0.61	0.97(0.39-2.42)	0.94
Sum	0.83(0.38-1.83)	0.65	0.81(0.25-2.56)	0.72	0.78(0.23-2.62)	0.69

HR= Hazard Ratio for each log (i.e. ten-fold) increase in frequency of T cells. Confidence intervals are 5-95%. HRs are adjusted by anti-CS antibody titre (in 2 groups), age, area of residence, ITN use and distance from the dispensary. NANP=NANP and conserved region peptides pool, TH2R=TH2R region peptides pool, TH3R=TH3R and CS.T3T region peptide pool, Sum= all three peptide pools summed.

Figure 7:4 Survival plots with time to first episode of clinical malaria plotted for RTS,S/AS01E (left columns) and control vaccinees (left and right columns) according to tertile of CD4+, TNF- α responses (top row), CD4+ IFN γ responses (middle row) and IFN- γ ex vivo ELISPOT responses to TH3R/CS.T3T peptides pool (lower row)



Where more than one third of responses were at the lower limit of detection, the lower two tertiles are combined (and hence only 2 tertiles are displayed on some plots). For CD4+ TNF- α responses, the tertiles were 1 to 154 (lower), 155 to 407 (middle) and 408 to 28,840 (upper) cells per million for RTS,S/AS01E vaccinees, and 1 to 26 (lower), 27 to 165 (middle) and 166 to 10,000 (upper) cells per million for control vaccinees. For CD4+ IFN- γ responses the tertiles were 1 to 12 (lower), 13 to 66 (middle) and 67 to 8,320 (upper) cells per million for RTS,S/AS01E vaccinees, and 1 to 40 (lower) and 41 to 5,980 (upper) cells per million for rabies vaccinees. The time point “0 months” refers to the time of a blood draw. Cellular responses were analyzed as time-varying covariates, where the effect of cellular responses from all available blood draws was related to clinical malaria episodes during the period of monitoring after each measurement. Therefore, each RTS,S vaccinee could contribute to 2 periods of monitoring. These three assays were selected for the figure because significant associations on Cox regression were seen

7.4 Discussion

I have shown that RTS,S/AS01E vaccination induced significant CS protein specific T cell responses in 5-17 month-old children living in a malaria endemic area. The frequency of CD4⁺ TNF- α ⁺ T cells on ICS was associated with protection from clinical malaria. The strength of association between TNF- α producing CD4⁺ T cells and protection was lower (HR=0.7) than that between anti-CS protein antibody titres and protection (HR=0.5) confirming the results in adults which demonstrated anti-CS protein antibody titres to be a stronger predictor of protection than T cell responses. Although the use of 15-mer peptides may have been sub-optimal to demonstrate CD8⁺ T cell responses, I identified both CD4⁺ and CD8⁺ responses above negative control conditions for both RTS,S/AS01E and control vaccinees. However, the CD8⁺ responses were apparently not induced by vaccination, and presumably were the result of exposure to malaria parasites [294].

If TNF- α producing CD4⁺ T cells are causally related to protection, they should be associated with protection whether they are acquired by vaccination or by natural exposure to malaria parasites. Indeed, there was weak evidence for a correlation between TNF- α CD4⁺ T cells and a reduced risk of clinical malaria among control vaccinees. However, the overall level of protection afforded by T cells among RTS,S/AS01E vaccinees was greater, since the vaccinated children had 2-3 fold more TNF- α CD4⁺ T cells at 1 month and 8-10 fold more at 12 months post vaccination. The frequency of TNF- α CD4⁺ T cells at 12 months post vaccination was below screening (i.e. pre-vaccination) levels, despite the fact that clinical protection was sustained at 15 months

[295]. Since clinical protection is determined by contemporaneous comparison with control vaccinees, it is therefore theoretically possible that the relative difference between RTS,S/AS01E vaccinees and control vaccines is relevant despite this fall.

T cell responses to CSP rose between screening and 1 month post-vaccination, and then fell to lower levels at 12 months post vaccination. This was seen in both ELISPOT and ICS studies, and could not be explained by greater background responses (which did not change over time and were subtracted from antigen-specific responses), or by non-specific responses detected in changing positive controls over time. The temporary increase in CSP specific T cell responses parallels the increase and decrease in antibodies to blood stage antigens seen in the same children [256]. Antibody responses made by young children to blood stage antigens are often short-lived [296], and may reflect short-term changes in exposure [297]. I therefore concluded that T cell responses rise as a result of increase in malaria exposure during transmission season. However this rise is short-lived and levels decline once the malaria exposure falls.

I did not identify an association between protection and T cell responses detected by the cultured IFN- γ assay, as previously reported [178]. However, we tested pools of peptides rather than individual peptides, and the previously reported association was specific to the CS.T3T peptide. The CS.T3T peptide was contained in a pool of TH3R/CS.T3T peptides. In other studies, the CS.T3T peptide accounted for more than half of the overall response seen in the TH3R/CS.T3T peptide pool [298]. Furthermore, very little IFN- γ production was identified in this study. Previous studies showing marked IFN- γ production were done in adults [155], and IFN- γ production may be suppressed in children in malaria endemic areas [299].

Ex vivo ELISPOT studies did not correlate with ICS studies for the same cytokines, even though both use overnight stimulations, and ICS results were 10-fold higher than ELISPOT results. This may be partially explained by measuring ELISPOT assays per million PBMC, whereas ICS is measured per million CD4⁺ T cells. However, *ex vivo* and cultured ELISPOTs identify different cell populations, the latter more closely reflecting a central memory phenotype [300,301,302]. Hence it is possible that ICS and cultured ELISPOT identify central memory cells, but *ex vivo* ELISPOTs identify effector phenotypes. ELISPOT assays failing internal control standards were excluded, but similar control standards were not pre-defined for ICS data. However, this applied to a minority of assays (2% and 5%, respectively for *ex vivo* IFN- γ and IL2 ELISPOTS) which did not correlate with ICS data, and 8% for cultured ELISPOTS, which did correlate. Hence it seems unlikely that lack of quality control standards for ICS explained the lack of correlation.

The CD4⁺ T cell response associated with protection in my analysis (i.e. TNF- α production) was at a low frequency (mean 426 cells per million CD4 cells at peak). Higher frequency responses have been required to achieve protection in sporozoite challenge studies [131]. However, the antibody concentrations associated with protection are also higher in sporozoite challenge studies [131,295,303]. These differences in outcome may be explained by the greater sporozoite inoculum used in challenge studies compared with exposure in the field [235]. The IFN- γ response that was apparently associated with protection in this study was very low and barely above the limit of detection (25 cells per million), and the apparent association is likely to reflect the association between IFN- γ and TNF- α rather than an independent effect.

Multiple comparisons (i.e. 18) have been undertaken to identify the association between TNF- α producing CD4⁺ T cells and protection from clinical malaria. However, the association was highly significant ($p=0.001$) and remains significant after the statistically conservative Bonferroni correction ($p=0.018$).

7.4.1 Surrogates of protection

The Prentice criteria have been proposed as a way of qualifying surrogate endpoints [304,305] and include four criteria [293]. I found that the combination of anti-CS protein titres and TNF- α producing CD4⁺ T cells met all the criteria (i.e. vaccination was associated with protection; anti-CS protein titres and CD4⁺ TNF- α ⁺ T cells were both independently associated with vaccination; were both independently associated with protection; and the combination of anti-CS protein titres and CD4⁺ TNF- α ⁺ T cells, but not either alone, could account for the effect of vaccination in multi-variable Cox regression analysis). I found no significant interaction between anti-CS protein titres and TNF α ⁺ T cells.

Micro-heterogeneity of malaria exposure has been observed in Kilifi [257], and may confound the association between antibodies to blood stage malaria antigens and the risk of malaria [256]. However, this is unlikely to explain the association between CD4⁺ TNF α ⁺ T cells and protection from clinical malaria for two reasons: The direction of confounding was in the opposite direction in this cohort (i.e. micro-heterogeneity led to a strengthening of the association between increasing antibody levels and increasing risk of

malaria rather than protection), and the association with protection is more marked in RTS,S/AS01E vaccinees rather than control vaccinees.

There is strong evidence that anti-CS protein antibodies inhibit sporozoite invasion [159], supporting a causal relationship and TNF- α may reduce the parasite's intrahepatocytic development [306,307]. However, it is possible that the frequency of CD4⁺ TNF- α producing T cells is associated with another causal mediator of immunity (for instance better quality antibody responses, enhanced T cell memory or polyfunctionality). These further characterizations of the immune response should now be a priority, since establishing an immunological surrogate endpoint will accelerate the development of candidate malaria vaccines, inform monitoring the persistence of immune responses and inform the timing of a booster dose.

7.5 Conclusion

In conclusion RTS,S/A01E vaccination in young children induced significantly higher levels of CD4⁺ T cell responses but no CD8 T cell responses. The TNF- α producing CD4⁺ T cells response were independently associated with protection from clinical malaria. The combination of TNF- α producing CD4⁺ T cells and anti-CS protein antibody levels accounted for the protection conferred by the RTS,S/AS01E vaccination. Anti-CS protein antibody titres are a stronger predictor of protection than TNF- α CD4⁺ T cell response and which is in agreement with the studies in malaria naive adults.

8 Anti-CS protein antibody avidity and protection

8.1 Introduction

RTS,S/AS01E is highly immunogenic [123,131,132,308], inducing both high titres of CS protein specific antibodies and CD4 T cell responses. There is evidence that anti-CS protein antibody titres correlate with protection against infection in experimental malaria challenge studies in malaria naïve adults, [131,181] and natural malaria infection in adults and children in malaria endemic regions [124,156]. In chapter 5, I demonstrated a non-linear association between anti-CS protein titres and protection from clinical malaria in children 5-17 months residing in malaria endemic country [295]. Recent studies in infants 6-10 weeks of age have also shown correlation between anti- CS protein titres and protection from clinical malaria [309]. In contrast no such association was present in older children in Mozambique [129]. In chapter 7, I described the induction of CS protein-specific CD4 T cell responses by RTS,S/AS01E in young children in Kilifi, Kenya and showed that these responses correlate with protection from clinical malaria.

However, substantial variability in protection remains unexplained even after accounting for anti- CS antibodies and CD4 T cell responses (i.e. one frequently observes unprotected children with high titre antibodies and strong CD4 T cell responses).

In addition to antibody levels and cellular immune responses avidity could also play an important role in mediating protection conferred by RTS,S/AS01E. However, no study has reported the role of avidity in RTS,S/AS01E-induced protection in the field. I

therefore undertook the analysis to assess the RTS,S/AS01E-induced anti-CS protein antibody avidity in children 5-17 month residing in Kilifi, Kenya who were immunized with RTS,S/AS01E.

8.2 Materials and Methodology

8.2.1 Vaccine and subjects

Serum samples were obtained as described in chapter 4. Briefly the serum samples were collected at screening, 1 month, March 2008 (range 4–10 months) and at 12 months post dose 3 for the assessment of antibodies to *P. falciparum* CS protein repeat region (anti-CS protein antibodies).

8.2.2 Study design

I conducted a nested case-control study to investigate the association between vaccine-induced anti-CS protein antibody avidity and protection from clinical malaria. Cases were defined as children who had at least one episode of clinical malaria (axillary temperature $\geq 37.5^{\circ}\text{C}$ and *P. falciparum* parasitaemia $>2500/\mu\text{L}$) during the 15 months of follow-up beginning 2 weeks after the 3rd dose of vaccine while controls were children who did not suffer from clinical malaria during this period.

Malaria exposure was measured as the weighted local prevalence of malaria cases within a 1 km radius of each index child, or “exposure index”, as previously described in chapter 3[271]. Malaria exposure was considered “high” if the exposure index was above the cohort mean and “low” if the exposure index was below the cohort mean.

I selected 19 cases and 19 controls among RTS,S/AS01E vaccinees frequency matched for similar peak anti-CS protein levels. I also studied avidity in a further 19 children with low anti-CS protein antibody levels, who appeared to be protected despite having a relatively high malaria exposure, in order to determine whether high avidity was the explanation for protection despite low anti-CS protein levels.

All selected cases and controls had their samples assessed for the anti-CS protein avidity at three time points i.e. 1 month, March 2008 (range 4–10 months) and at 12 months post dose three. Screening samples were not analyzed because anti-CS protein titres were undetectable before vaccination.

8.2.3 IgG antibody concentrations

Anti-CS protein antibody concentrations were determined by standard enzyme-linked immunosorbent assay (ELISA) developed by GSK Biologicals 5.2.2. A cut-off point for positive titres was 0.5 EU/mL.

8.2.4 TNF- α CD4 T cell immune responses

Whole blood intracellular staining assay was used to determine the frequency of CD4 T cells producing TNF- α as previous described in 11.1.4 [310]. Stimulation of blood was done within 3 hours after blood withdrawal and samples were stored 3 to 4 months before staining.

8.2.5 Anti-CS protein avidity assay

I used a single concentration ammonium thiocyanate (NH₄SCN) elution ELISA to determine the avidity of the polyclonal anti-CS protein antibodies (defined as the antigen binding capacity resulting from the addition of all epitope-specific affinities of CS-specific IgG antibodies).

Polystyrene microtiter plates were coated overnight at 4 °C with R32LR (3.2mg/ml) protein diluted to 2.5 μ g/ml in coating buffer. Following washing, the plates were blocked with 200 μ l PBS-5% skim milk per well for 1 hour at 25 °C in a horizontal orbital shaker (Skatron 300).

I conducted 8-fold serial dilutions in duplicate. A pre-dilution at 1:100 was done for all serum samples with anti-CS protein titer above 200 EU/mL before the beginning of serial dilutions. Serum samples with anti-CS protein titer below 200 EU/mL were not pre-diluted. Standard (serum from malaria naïve adult vaccinated with R32LR attributed an arbitrary value of 109EU/mL kindly provided by GSK Biologicals), negative control (i.e. anti-CS protein negative serum from malaria naïve adult kindly provided by GSK

Biologicals) and positive control (i.e. a pool of anti-CS protein human sera approximately at 100 EU/ml that demonstrated an avidity index (AI) of 43.1 ± 3.8 , kindly provided by GSK Biologicals) and were added in each plate and serially diluted like samples. The plates were then incubated for 2 hour at 37 °C.

Each serum sample was processed in two different plates; one treated with NH_4SCN , and one untreated plate. Both plates were washed twice with PBS in 0.05% tween. A 1M solution of NH_4SCN in laboratory grade water was added in the treatment plate while PBS in 0.05% Tween was added in the control plate and both were incubated for 30 min at 25°C. After a third wash, anti-human IgG conjugated to HRP was added and incubated for 30 min at 25 C before washing. After 30 min incubation with chromogen substrate 3, 3',5,5' tetramethylbenzidine (TMB) and Hydrogen peroxide (H_2O_2) at 25°C (yielding a blue colour), the reaction was stopped with 50µl of 1N sulphuric acid changing the colour to yellow. The intensity of the colour was proportional to the titer of the anti-CS protein IgG antibodies contained in the sample. Absorbance at 450 nm was read by use of an automatic microtiter plate reader. Samples OD's were converted into EU/ml using a standard curve at the linear portion of the curve and mean values used to estimate avidity index. Linearity of the assay was demonstrated by the observation that for a high concentration sample, serially diluted sample was able to reproduce the same AI over the entire anti-CS antibodies analytical range. The Avidity Index (AI) is the ratio of the quantity of anti-CS protein antibodies (in EU/ml) that remained bound to the coated antigen after treatment with NH_4SCN divided by the quantity of antibodies (in EU/ml) that remained bound to the coated antigen in the control plate.

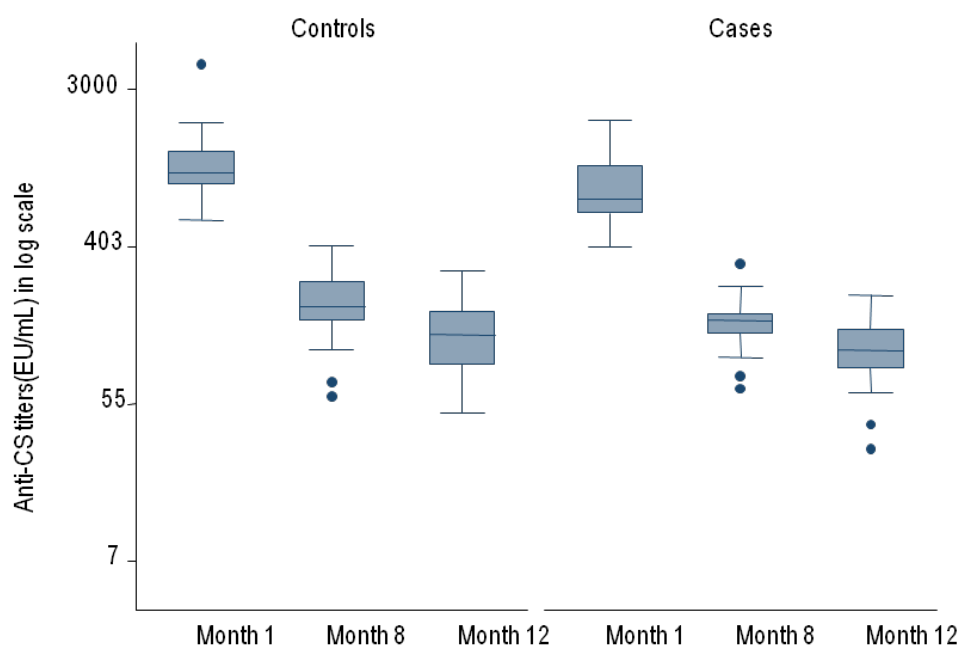
8.2.6 Statistical analysis

Anti-CS protein avidity measures were not transformed in the analysis. The anti-CS protein titres and avidity indices were presented for each group as arithmetic mean $\pm$ 95% confidence interval per group. Student T-test was used to compare the avidity indices and antibody titres between groups. A one-way analysis of variance (ANOVA) was used to test for the difference in antibody avidity during follow up (1 month, ~8 and 12 months post dose three). I used unconditional logistic regression analysis to estimate odds ratios (ORs) and 95% Confidence intervals adjusting for TNF α -producing CD4 T cells, bed net usage and malaria exposure (high or low) clustering on multiple entries per child. All analyses were done in Stata (version 12; Stata Corp).

8.3 Results

A total of 183 sera (61 sera obtained 1 month post dose 3, 61 sera obtained on March 2008 (~8 months post dose 3) and 61 sera obtained 12 month post dose 3) from 61 study participants (age range, 5 to 17months) were available for the analysis. Anti-CS protein titres declined over time in all groups but remained significantly higher compared to the pre-vaccination levels (Figure 8:1).

Figure 8:1: Anti-CS protein titres in cases and controls during 15 months of follow-up



8.3.1 Anti-CS protein avidity

The arithmetic mean avidity index (AI) of polyclonal anti-CS protein antibodies at 1 month, ~8 months and 12 months post dose 3 was 45.2 (95%CI: 42.4 to 48.1), 45.3 (95%CI: 41.4 to 49.1) and 46.2 (95%CI; 43.2 to 49.3), respectively. The AI did not differ between the three time points (F test=0.12 p=0.9 by ANOVA test).

I observed correlations between the AI at 1 month post-dose 3 and at ~8 months post-dose 3 ($r=0.33$, $p=0.0111$), as well as AI at 1 month and 12 months post-dose 3 ($r=0.48$, $p=0.0002$) (Figure 8:2).

There was no correlation between AI and anti-CS protein titres at all three-time points ($r=-0.03$; $p=0.859$, $r=0.05$; $p=0.704$ and $r=0.16$; $p=0.220$ respectively) or between AI and age at vaccination ($r=0.003$, $p=0.656$) (Figure 8:3).

Figure 8:2: Matrix diagram showing correlation between antibody avidity measured at three time points during the follow up

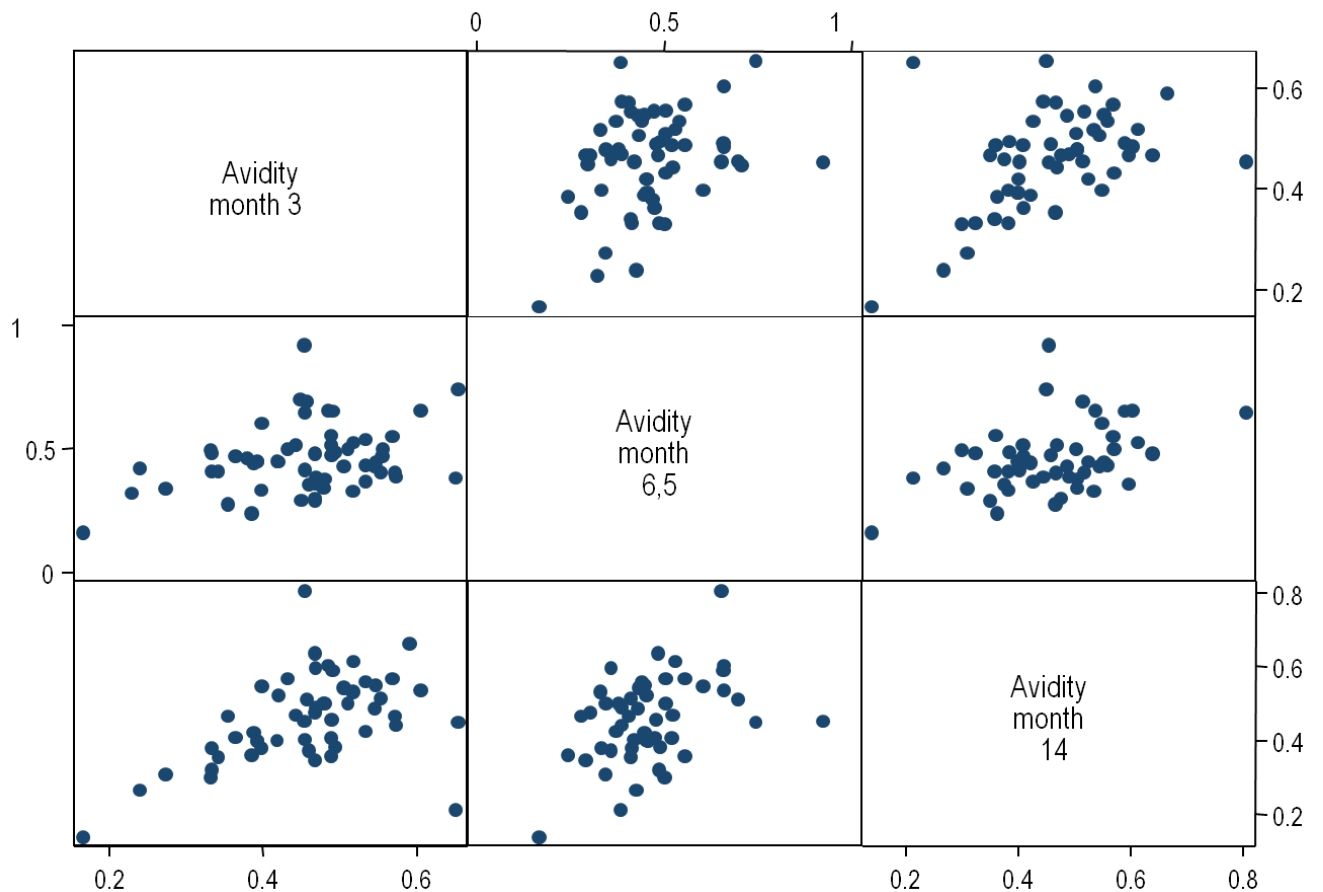
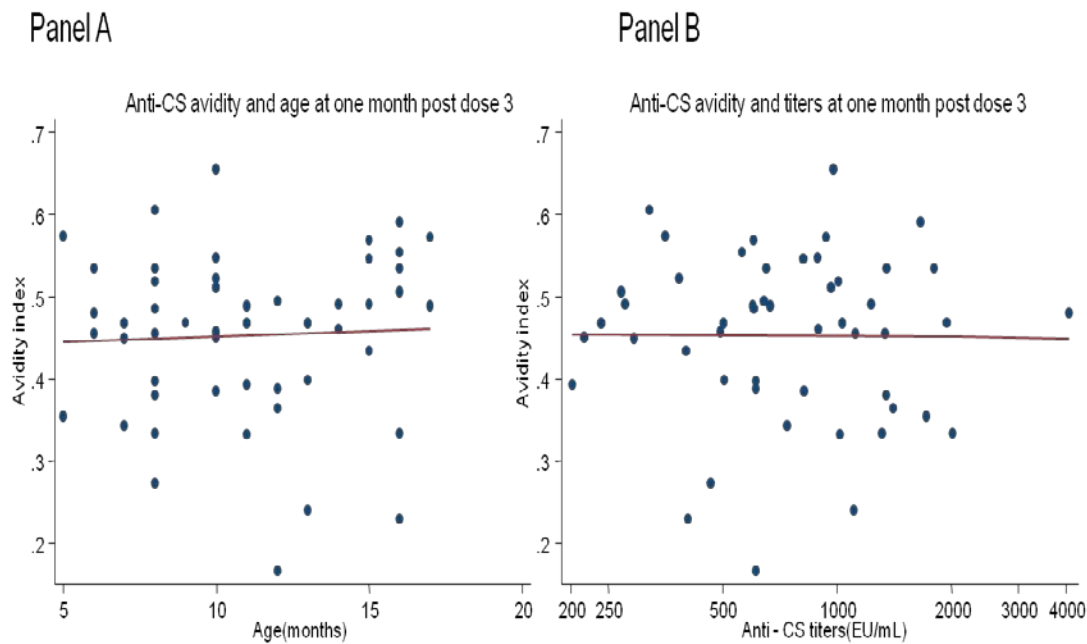


Figure 8:3: Panel A: correlation between anti-CS protein antibody avidity at 1 month post dose 3 and age; Panel B: correlation between anti-CS protein antibody avidity and anti-CS protein antibody titres at 1 month post dose 3



8.3.2 Avidity and protection from clinical malaria

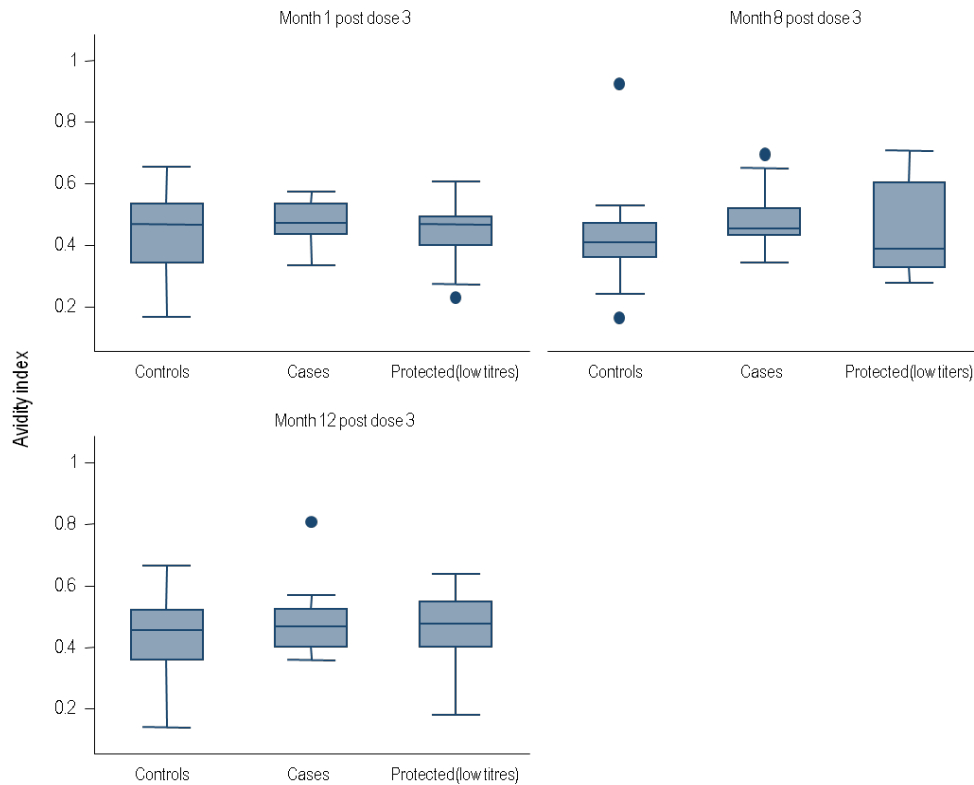
The AI in cases and controls was 47.1 (95%CI: 42.8 to 51.4) versus 44.6 (95%CI: 38.6 to 50.6) $p = 0.503$, 48.4 (95%CI: 43.8 to 52.9) versus 42.2 (95%CI: 33.9 to 50.4) $p = 0.173$ and 48 (95%CI: 43 to 53) versus 44.7 (95%CI: 39.2 to 50.1) $p = 0.354$ at 1, ~8 and 12 months post dose three, respectively. In 19 protected children with relatively high malaria exposure and low anti-CS protein titres, the avidity was 44.4 (95%CI: 39.4 to 49.4), 45.2 (95%CI: 37.5 to 52.9) and 46.3 (95%CI: 40.1 to 52.4) at 1, 8 and 12 months respectively. The avidity at all time points were not different from those recorded in cases and controls (F test=2.69, $p = 0.103$ by ANOVA) (Figure 8:4).

The unadjusted odds ratio for clinical malaria was 1.36 (95%CI: 0.89 to 2.1; $p= 0.165$) for each 10% increase in avidity index. Multivariable logistic regression showed no association between the avidity index and protection from clinical malaria Table 8-1). As previously described TNF- α producing CD4 T cells were independently associated with protection from clinical malaria. Having high malaria exposure was also associated with increased risk of clinical malaria.

Table 8-1: Multivariable logistic regression analysis for the effect avidity on clinical malaria

Variables	Odds Ratio	95% Confidence interval	P value
Avidity (per 10% increase in avidity).	0.90	0.49-1.66	0.744
CD4 ⁺ -TNF- α cells (per 10-fold increase in frequency)	0.63	0.41-0.95	0.027
Bed net	1.39	0.42-4.58	0.588
Malaria exposure	11.24	2.08-60.68	0.005

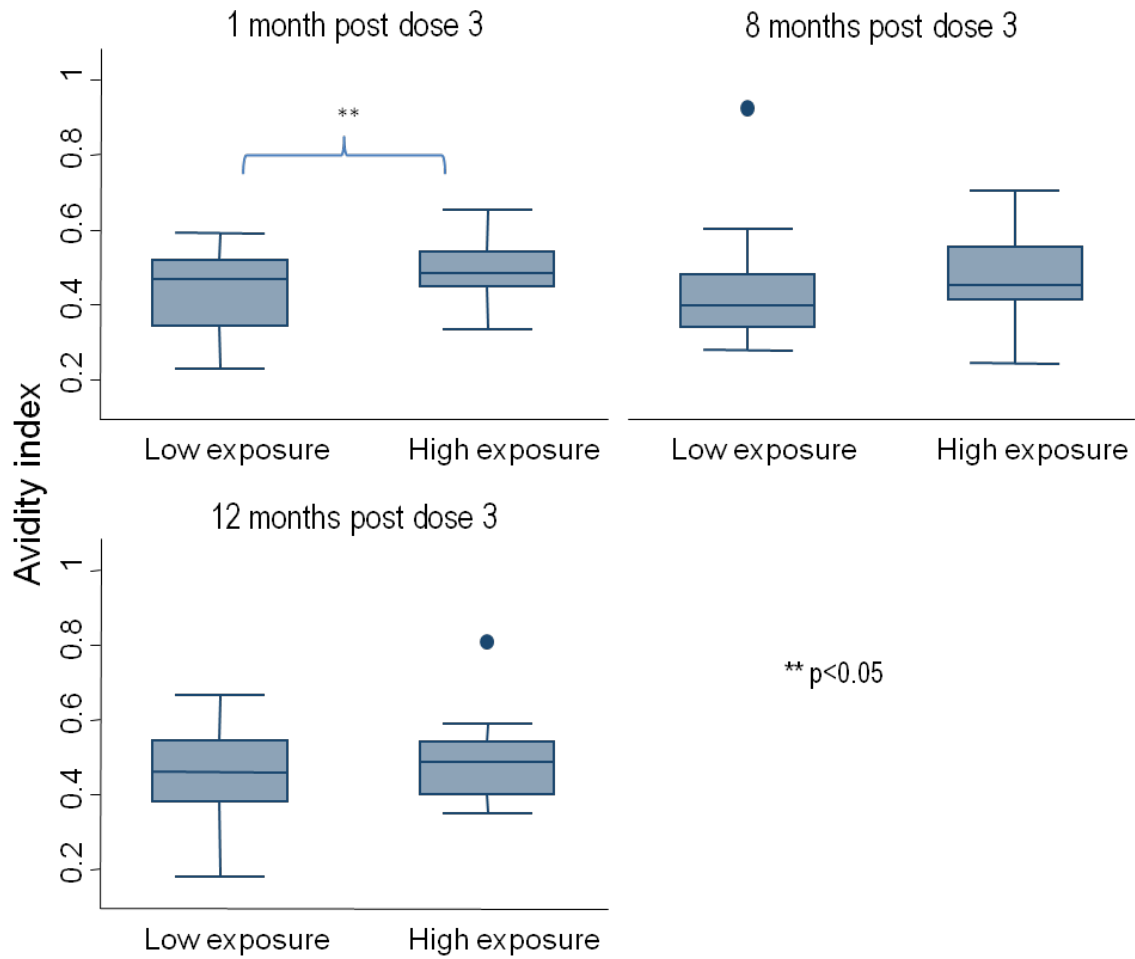
Figure 8:4: Box plot of anti-CS protein antibody avidity in cases, controls and 19 protected children with low antibodies titre and high malaria exposure at 1, ~8 and 12 months post dose 3



8.3.3 Avidity and malaria exposure

AI at 1-month post dose 3 was significantly higher in children with high malaria exposure compared with those with low malaria exposure (48.6%, 95%CI: 45.4 to 51.8, versus 42.8%, 95%CI 38.2 to 47.4, $p = 0.035$ by Students T test) (Figure 8:5). This difference was also observed at ~8 months; 47.9% (95%CI: 42.4 to 53.5) versus 43.2% (37.6 to 48.8) $p = 0.22$ and at 12 months; 48.2% (95%CI: 44.3 to 52.1) versus 45.3% (95%CI: 40.6 to 49.9) $p = 0.34$ post dose 3 although it was not statistically significant.

Figure 8:5: Box plot of anti-CS protein antibody avidity by level of malaria exposure (based on malaria exposure index) at 1, ~8 and 12 months post dose 3



8.4 Discussion

Although RTS,S-induced humoral and cellular immune responses correlated with protection in children in my study (chapter 5 and 7), there is a substantial variation in the rate of protection within individuals of similar level of immune responses. In this analysis, I show that antibody avidity does not predict protection from clinical malaria among RTS,S/AS01E vaccinees with similar levels of anti-CS protein antibodies aged 5-17 month residing in a malaria endemic country.

There was no evidence of anti-CS protein avidity maturation beyond one month post dose 3. Avidity maturation follows B cells activation in a CD4 T cell dependent (protein antigens) manner and is the hallmark of immunologic memory[169]. However there is substantial variability in the capacity of vaccines to evoke avidity maturation[311]. Following Hepatitis B vaccination no avidity maturation was observed beyond the third dose although significant increase in avidity occurred between the first and the third dose [173]. Avidity maturation persisted between the third and fourth dose of meningococcal serogroup C (MCC) conjugated vaccine, but there was no change in antibody avidity beyond the fourth dose[312]. In our situation, it is probable that the avidity maturation process may have been complete by 1 month post dose three. I only examined avidity maturation after the third dose, and not between doses.

I did not observe any correlation between age of the child and antibody avidity. Despite declining anti-CS protein titres, antibody avidity was sustained throughout the 15 months of follow-up. Although the quantity of antibody may reflect the immediate response to a

vaccine, the quality of the antibody response may be more important in determining the immune status months after vaccination. Therefore even in the backdrop of declining antibody titres, sustained antibodies with high avidity could be an important determinant of long-term protection.

The avidity index (AI) was significantly higher in children with high malaria exposure compared to those with low malaria exposure. In theory, this could be due to progressive anti-CS protein avidity maturation as a consequence of natural boosting by repeated malaria exposure. However, there is no evidence of boosting of RTS,S-induced immune responses by natural exposure in previous studies, and the avidity did not increase over the duration of the study. In fact we found no evidence of avidity maturation of anti-merozoite antibodies with increasing natural malaria exposure in individuals residing in the study area [313]. Alternatively, prior exposure to malaria (as measured using our exposure index) may have primed children to respond with higher avidity antibodies on vaccination [155]. Thus, those with higher exposure may have had higher frequencies of naturally acquired anti-CS protein memory B cells than those with low exposure. In keeping with this suggestion is the finding, correlation between AI and malaria exposure was only significant at 1 month post dose 3 and not at subsequent time points.

A similar phenomenon has been described in viral infections where the avidity of specific IgG antibody is lower in primary viral infections than in previously and/or chronically infected individuals[314] allowing for the differential diagnosis between primary and chronic infections.

I found no association between avidity and protection from clinical malaria. Data in one animal model (*P. berghei*) suggests that the immunity induced by CSP-based subunit vaccines may depend on antibody avidity[174] whereas a study in another animal model (*P. yoelii*) found protection against sporozoite challenge to be independent of antibody avidity and/or isotype [315]. One possible explanation for lack of correlation between avidity and protection may be that the majority of the antibodies had exceeded a minimum avidity threshold required for protection and hence I couldn't detect any variation in outcome. For instance, Bachmann et al observed lack of correlation between antibody avidity and protection against vesicular stomatitis virus (VSV) once the antibody avidity had reached a minimum threshold [316]. It should be noted that my analysis focused on the antibodies to the central repeat region of the RTS,S and antibodies to the C- terminal flanking region may behave differently.

There are other qualitative aspects of the antibody responses that I did not examine; such as the IgG isotypes in the children and ability of antibodies to inhibit hepatocyte infection in functional assays. IgG3 and IgG1 are the most effective isotypes at mediating antibody dependent cellular mechanisms like phagocytosis and complement fixation against blood stage malaria antigens, and have been associated with protection from clinical malaria in the field [317,318]. On the other hand IgG2 may compete with IgG1 and IgG3 interfering with their activity[319]. Thus, similarly in the case of RTS,S immunization the isotype distribution of the induced response may determine the mechanism by which sporozoites may be taken up and destroyed by monocytes and macrophages[158]. Although the anti-CS protein antibody response induced by RTS,S in

naïve adults is skewed towards IgG1 and IgG2 [122], the isotype distribution in children is unknown.

8.5 Conclusion

In conclusion, antibody avidity did not predict protection in RTS,S/AS01E vaccinees with similar levels of anti-CS protein antibody titres. Antibody avidity was higher in children with high malaria exposure suggesting a possible priming effect of natural infection on the RTS,S-induced response.

9 Concluding remarks

9.1 Introduction

RTS,S/AS01E is a clinically advanced pre-erythrocytic malaria vaccine currently in phase III studies in seven countries across Africa. The main objective of my thesis was to determine the duration of efficacy of RTS,S/AS01E in children 5-17 month of age residing in a malaria endemic country. I also set to characterise humoral and cellular immune responses to RTS,S/AS01E in vaccinated children and thereby determine the correlates of protection. Before examining the chief scientific questions I dealt with two methodological issues in analysis. The first was related to the comparison of malaria surveillance methods to inform appropriate choices of surveillance methods, and the second involved estimation of individual malaria exposure in the field in order to account for its effect in the analysis of vaccine efficacy.

The main empirical findings are chapter specific and have been described within the respective empirical chapters. I will now briefly summarize these findings, and then synthesise their implications together.

Is there a difference between active and passive only malaria surveillance in terms of specificity of malaria case definitions and capture rate of malaria cases?

- Malaria case definitions from passive and active surveillance have similar specificity and sensitivity contrary to the belief that the former is more likely to misclassify fever of other causes harbouring coincidental parasitaemia as true malaria.

- Active surveillance is more sensitive in capturing clinical malaria cases than passive surveillance.

How do you measure individual malaria exposure in the field in order to account for the effect of heterogeneity in malaria exposure on efficacy estimates?

- Using data from three cohorts under different transmission settings I demonstrated that, the risk of clinical malaria is indirectly proportional to the distance from the nearest infected individual
- Distance weighted malaria prevalence within 1km radius (malaria exposure index) has good predictive power to identify children with malaria infection (asymptomatic and/or symptomatic) and therefore can be used as individual marker of malaria exposure in the field.

What is the duration of efficacy of RTS,S/AS01E against clinical malaria episodes by P. falciparum in young children residing in malaria endemic country, and how does it vary with time and by intensity of malaria exposure?

- The efficacy of RTS,S/AS01E waned over time from around 45% in the first year to -1% in the fourth year of follow-up. The positive effect of the vaccine lasted for 3 years after which no more benefit was observed.
- Efficacy appears to be lower in children with high rather than low malaria exposure and there was a tendency for faster waning of efficacy in children with high compared to low malaria exposure.
- Despite waning in efficacy, for every 100 children vaccinated with RTS,S/AS01, 65 cases of clinical malaria were averted during the 4 years of follow-up.

What is the association between RTS,S-induced anti-CS protein antibodies and/or RTS,S-induced T cell responses and protection from *P. falciparum* clinical malaria in young children residing in malaria endemic country?

- RTS,S/AS01E induced high levels of CS protein specific antibodies and CD4 T responses but not CD8+ T cell responses and were independently associated with protection from clinical malaria.
- Combined effect of CS protein specific antibodies and CD4 T cell responses accounted for almost all the protection conferred by RTS,S satisfying the Prentice criterion as surrogate markers of clinical protection.

Does the avidity of anti-CS protein antibodies as measured by elution assay predict protection from *P. falciparum* clinical malaria in young children residing in malaria endemic country who have been immunized with RTS,S/AS01E?

- The avidity of RTS,S/AS01E-induced anti-CS protein antibodies did not correlate with clinical protection.

9.2 Theoretical Implications

The duration of efficacy of RTS,S/AS01E is an important public health research question. Malaria vaccine evaluation or clinical trials for that matter have always been conducted over short period of time. This is because vaccine trials are expensive and the focus has

largely been on the proof of concept that a vaccine can provide protection rather than its duration of efficacy.

Apart from my study, the only other long term follow-up study of RTS,S vaccinated cohort is the one involving Mozambican children who were vaccinated with RTS,S/AS02. In that study which followed children for 4 years the conclusion was “These results show evidence that RTS,S/AS02A maintained protection during the 45-month surveillance period”[134]. This is contrary to my finding which shows significant waning in efficacy during the 4 years of follow-up. The vaccine efficacy in my cohort gradually waned to a point where the vaccine candidate was no longer protective during the fourth year of follow-up.

Chapter 4 discusses some of the possible reasons for the difference between the two studies. Three key differences were changes in malaria transmission during follow-up, malaria surveillance method and type of analysis used. Analysing the data using the Andersen and Gill Cox regression (AG) model allowed inclusion of all malaria episodes in the model and improved the power of my analysis to identify waning. In fact waning was more apparent on all episodes than on single episode analysis. Analysis of the Mozambican study faced two fundamental challenges. On one hand episodes of malaria were declining overtime, presumably due to increased natural immunity since older children had been recruited into the study and were presumably becoming immune to malaria, and perhaps also because transmission was declining in the study area.

Furthermore the analysis in Mozambique did not take into account all episodes when assessing for waning. I found that taking into account all malaria episodes was more likely to identify waning, since one avoids censoring later episodes of malaria, which are particularly informative in terms of the duration of efficacy.

Andersen-Gill (AG) Cox regression was employed in the analysis of all malaria episodes in my study. This approach provides robust estimates of total efficacy which is the sum of direct and indirect effect as the result of event dependency [276]. From a public health perspective the total effect is a more relevant measure than an effect as measured by time to first event analysis, since all events will need to be treated at a health care facility, and not only first events. AG Cox regression has been used to study total efficacy estimates of other vaccines such as pneumococcal vaccine [320]. Evaluations of other malaria vaccine candidates should also consider AG Cox regression in their analyses.

Another important contribution that my thesis has is with regard to the choice of surveillance method in malaria vaccine clinical trials. Passive malaria surveillance at health facility is felt to be more practical and representative of the real world situation and has widely been used in malaria vaccine evaluation in phase II and phase III trials. It is however noted from my work that it can lead to an underestimate of malaria incidence (chapter 2). Active malaria surveillance on the other hand captures malaria cases of similar specificity as passive surveillance (contrary to suggestions that it may identify “false positive” malaria cases) and captures more malaria cases than passive surveillance.

My thesis therefore suggests use of active malaria surveillance in phase II clinical trials. Phase III trials to evaluate the efficacy of a candidate malaria vaccine will be too large to accommodate active surveillance of malaria episodes. Furthermore they are designed to simulate “real world” situations where children rely on health care provided at health facilities. However when there are outstanding questions regarding duration of efficacy or correlates of protection that require the assurance of capturing all episodes, there may still be a case for nested cohorts with active surveillance even within phase III and IV trials.

In chapter 3, I demonstrated that distance weighted malaria prevalence within 1km radius can be used as individual marker of malaria exposure in the setup where adequate data exist. This malaria exposure index can be used in models assessing efficacy of vaccine candidates. It also provides an opportunity to assess the interaction between vaccine efficacy and malaria exposure in situations where conducting several clinical trials in areas of different malaria transmission is financially challenging. The variation in protective efficacy of RTS,S/AS01 with malaria exposure described in my thesis is consistent with predictions by simulation models of reduced impact of pre-erythrocytic malaria vaccine on malaria morbidity in high compared to low malaria transmission areas [321]. These findings underscore the need to evaluate malaria vaccine efficacy under different malaria transmission settings to understand its potential impact. The current Phase III trial is being conducted in sites at different malaria transmission conditions, and a site-specific analysis of efficacy where sites are grouped according to transmission intensity is indicated.

Identifying correlates of protection has important implications such as a) facilitating basic research for identification of potential antigens as candidate malaria vaccine b) hastening

the evaluation and licensing of new malaria vaccines and c) monitoring the variation of protection over time and to inform on the need and timing of booster. Evidence from my thesis confirmed results from previous studies on the ability of RTS,S to induce high levels of anti-CS protein antibodies and CD4 but not CD8 T cell responses. Previous studies have also shown that RTS,S-induced anti-CS protein and CD4 T cell responses correlate with protection from malaria infection but not clinical malaria. In my thesis I demonstrated an association between both anti-CS protein antibody titres and TNF- α producing CD4 T cell responses with protection from clinical malaria. The association between anti-CS protein antibody titres and clinical malaria was non linear. My thesis proposes two possible models. The first is a threshold model which suggests that above certain antibody levels RTS,S/AS01 vaccinees will be protected and those below the threshold will have no or little protection. A second model is a curve relationship where above a threshold the probability of protection increases with increasing antibody level. However, validation of these models requires large data sets such as those from phase III trial.

I demonstrated that, a combination of TNF- α producing CD4 T cells and anti-CS protein titres satisfied Prentice criteria for surrogate marker of protection. This is mainly a statistical association and might not imply direct biological significance. The markers may simply be the by-products of other more relevant surrogate markers (mechanistic correlate of protection)[152] or lying on the pathway to a more relevant surrogate marker. Because the definitive target of immune correlate is to establish immunological measurement which is predictive of vaccine efficacy across all potential settings, including those which differ from those under current trial [292], further meta-analyses

will be necessary to establish a level 2 Surrogate of protection for RTS,S/AS01E-induced TNF- α producing CD4 T cells and anti-CS protein antibodies according to the Prentice criteria.

Avidity of RTS,S-induced CS-specific antibody as measured by elution assays has never been described in RTS,S vaccinees residing in countries endemic to malaria. Assessment of antibody avidity after a third dose of vaccine revealed absence of avidity maturation. Contrary to my expectation avidity of anti-CS protein antibodies measured during the 15 months of follow-up did not correlate with protection. It could be that, what is more important is isotype-specific avidity rather than overall avidity. However avidities to malaria blood stage antigens have also been shown to not be predictive of protection from clinical malaria[313] .

Finally, my thesis has shown that, despite waning in efficacy, vaccination with RTS,S/AS01 was beneficial to children. I estimated that for every 100 RTS,S/AS01 vaccinated children, 65 cases of clinical malaria were averted during the four years of follow-up. From a public health point of view, the absolute risk reduction is more useful way of understanding the potential impact of the vaccine than relative risk reduction and readily feeds into cost-effectiveness analyses. However, my demonstration that vaccine efficacy varies depending on the intensity of malaria transmission implies that this figure cannot readily be generalized to other settings.

9.3 Potential policy implications

My analysis suggests that RTS,S/AS01 will have variable magnitude of protection and rate of waning depending on the malaria transmission. Despite lower relative efficacy in a cohort of children with high malaria exposure compared to a cohort at low malaria exposure, the absolute number of clinical malaria cases averted was higher in the former than the latter. It is therefore expected that, in areas of high malaria transmission RTS,S would have bigger impact than in areas of low transmission. This is similar to other vaccines such as rotavirus, which despite moderate efficacy at settings of high transmission (around 49.5% in Malawi) is expected to save millions of lives given the large burden of the disease [322].

Beyond three years, RTS,S/AS01 does not appear to confer any protective benefit. Despite the wide 95% confidence intervals around vaccine point estimates during follow-up, the significant p value for the interaction with time allows me to reject the “no variation over time” null hypothesis. If RTS,S/AS01E is introduced, a booster dose should be considered, preferably in the second year after the primary vaccination. However the appropriate timing of the booster dose should be assessed in the context of malaria transmission since the waning may be faster in high than low transmission areas.

9.4 Recommendations for future research

Despite waning in efficacy there was, as yet, no evidence of rebound. In view of the declining efficacy and protective anti-CS protein antibody titres, it is important to continue with the follow-up for additional two to three years to assess if rebound in malaria cases among RTS,S/AS01 vaccinees will occur.

An extended follow-up of the cohort would also provide an opportunity to assess in details the interaction between RTS,S/AS01 and blood stage immunity in mediating long term protection. During 15 months of follow-up it was shown that RTS,S/AS01 vaccination resulted in lower levels of blood stage antibodies [256]. Although these antibodies were not associated with risk of malaria, children vaccinated with RTS,S/AS01 were at increased risk of clinical malaria if exposed to malaria infection. With declining efficacy it will be important to understand how fast will the blood stage immunity recover in children vaccinated with RTS,S/AS01 compared to controls.

Measuring the quality of anti-CS protein antibodies using other functional assays may shed more light on the mechanism of action of RTS,S. Potential assays to consider include; invasion assays, hepatocyte traversing and cytofluorometric opsonisation assay assays. Furthermore studying anti-CS protein antibody isotypes could also shed more light into the role of the isotypes in mediating the protection.

10 References

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11 Appendices

11.1 Appendix A

This appendix describes the peptides used in the cellular assays, ELISPOT and whole blood Intracellular staining assay (ICS).

11.1.1 Peptides used in cellular assays

A set of 32 15-mer, peptides were used, overlapping by 11 amino acids to cover the full length of the CS antigen used in the vaccine (3D7 strain). All these peptides were used in a single pool for the ICS studies, but they were divided into three pools for ELISPOT studies, namely; a) the conserved region including the NANP repeats, b) the variant TH2R region and c) the variant TH3R region and conserved CS.T3T region.

Table 11-1 Peptide pools used in ELISPOT assays

Peptide	Sequence
NANP and conserved region peptides pool	
Pept 1	MMAP DPNANPNANPN
Pept 2	NANP NANPNANPNAN
Pept 3	DPNA NPNANPNKNNQ
Pept 4	NPNA NPNKNNQGNGQ
Pept 5	NPNK NNQGNGQGHNH
Pept 6	NNQG NGQGHNPNDP
Pept 7	NGQG HNMPNDPNRNV
Pept 8	HNMP NDPNRNVDENA
Pept 9	NDPN RNVDEANANAS
Pept 10	RNVD ENANANSVKN
Pept 11	ENAN ANSAVKNNNNE
TH2R region peptides pool	
Pept 12	ANSA VKNNNNNEE PSD
Pept 13	VKNN NNEE PSDKHIK
Pept 14	NNEE PSDKHIKEYLN
Pept 15	PSDK HIKEYLNKIQN
Pept 16	HIKE YLNKIQNLSLST
Pept 17	YLNK IQNSLSTEWSP
Pept 18	IQNS LSTEWSPCSVT
Pept 19	LSTE WSPCSVTCGNG
TH3R/CS.T3T region peptides pool	
Pept 20	WSPC SVTCGNGIQVR
Pept 21	SVTC GNGIQVRIKPG
Pept 22	GNGI QVRIKPGSANK
Pept 23	QVRI KPGSANKPKDE
Pept 24	KPGS ANKPKDEL DYA
Pept 25	ANKP KDEL DYA NDIE
Pept 26	KDEL DYANDIEKKIC
Pept 27	DYAN DIEKKICKMEK
Pept 28	DIEK KICKMEKCSSV
Pept 29	KICK MEKCSSVFNVV
Pept 30	MEKC SSVFNVVNSSI
Pept 31	KCSS VFNVVNSSIGL
All three peptide pools were combined for the ICS assay. The pools were used separately for the ex vivo and plating out of the cultured ELISPOT assay.	

11.1.2 ELISPOT assays

Peripheral blood mononuclear cells (PBMC) were separated and incubated in RPMI medium (Sigma-Aldrich) with 10% Human AB serum. We used Millipore MAIP S45 plates and MabTech antibodies for ELISPOT assays according to the manufacturer's instructions. For *ex vivo* ELISPOT assays (IFN γ and IL2), 2×10^5 per well of freshly isolated PBMCs were incubated in 100 μ l final volume at 2.5 μ g/ml CS protein antigen peptides (see Table 11-1) for 18-20 hours before developing the plates. The positive and negative controls were 20 μ g/ml Phytohaemagglutinin (Sigma-Aldrich) and media alone, respectively. For cultured ELISPOTs, 1×10^6 PBMC were incubated in 0.5mls of 10 μ g/ml/peptide of pooled peptides in a 24-well plate. On days 3 and 7, 250 μ l of culture supernatant was replaced with 250 μ l culture medium containing 20 IU/ml recombinant IL 2. On day 9, the cells were washed three times and left overnight before an ELISPOT assay (IFN γ only) was done according to the method used for *ex vivo* ELISPOTs. Spot forming cell numbers were counted by ELISPOT plate reader (Autoimmun Diagnostika, version 3.0).

11.1.3 ELISPOT analysis

ELISPOT wells were assayed in duplicate, and the final result was the mean of two wells. The negative control well result was subtracted from each peptide well. ELISPOTs failed quality control if the negative control well had more than 25 spots or the positive control

had less than 50 spots. The results from the three peptide pools were added to calculate total responses. Results are presented as number of spots per million incubated PBMC.

11.1.4 Whole blood ICS assay.

Whole blood was stimulated in Kilifi within 2 hours of being drawn. 350 µl of whole blood plus 100 µl of phosphate buffered saline (PBS) was incubated in three different 15 ml Falcon tube, with 1 µg/ml of anti-CD28 anti-CD49d monoclonal antibodies (supplied by BD). After 2 hours, Brefeldin A was added to a final concentration of 1 µg/ml and incubation was continued overnight at 37°C ± 1- CO₂ 5 to 7%. EDTA was then added to a final concentration at 5 mM, and after 15 minutes 1 ml FACS lysing solution (BD). The positive control was stimulated using staphylococcal enterotoxin B (SEB) and negative control was PBS without peptides. CS protein antigen peptides were added to the third tube to a final concentration of 1 µg/ml (see Table 11-1). The cells were then washed in PBS and re-suspended in PBS with 10% DMSO and stored at -70°C for transport to GSK in Rixensart. In GSK, cells were thawed, washed and stained with alexa-fluor 700 conjugated anti-CD3 (Pharmingen), peridinin-chlorophyll (PerCP)-conjugated anti-CD4 (BD Biosciences) and allophycocyanin (APC)-H7 conjugated anti-CD8 antibodies (BD Biosciences). Cells were fixed and permeabilized using the Cytofix/Cytoperm buffer kit (Pharmingen), and stained with APC conjugated anti-IL-2 (Pharmingen), fluorescein-isothiocyanate (FITC)-conjugated anti-IFN-γ (Pharmingen) and phycoerythrin (PE) cyanin-7 (Cy7)-conjugated anti-TNFα (Pharmingen). Cells were washed, re-suspended in fetal-calf-serum (FCS)-containing phosphate buffered saline

(PBS) and analyzed on a BD™ LSR II flow cytometer (BD Biosciences). Events were counted using the automatic gating on the FACSDiva software (BD Biosciences). Conventional rules were used to gate on single cells, then the lymphocyte subset based on forward and side scatter. CD3 and CD4/CD8 positive cells and then cytokine expression was classified into positive/negative using FACSDiva software. An example of the output with gating shown is given in supplementary file 1. We required at least 10,000 CD4 + events and 5,000 CD8+ events. Acquisition was stopped when 75,000 CD4+ events had been acquired, and we acquired more than 50,000 CD4+ events for the majority of samples (>90%).

Results from antigen-stimulated cultures were not excluded from analysis on the basis of positive/negative control results, in the absence of established criteria. Data are represented as background subtracted CS-specific events per million CD4+ or CD8+ T cells.

Assays were conducted according to sample availability, since blood samples were limited to 5mls. In order of priority, the assays conducted were; ICS, IFN γ *ex vivo* ELISPOT, IL2 *ex vivo* ELISPOT and cultured ELISPOT. Samples were processed within 3 hours of being taken. ICS samples were stored for 3 to 4 months at -70° C before staining. The samples were processed during the double-blind phase of the study.

11.2 Appendix B

This section briefly described the measurement of anti-CS protein titres in the samples from children in my study. Anti-CS protein antibodies were tested at the CEVAC Laboratory, University of Ghent, Belgium.

R32LR protein containing the sequence [NVDP(NANP)₁₅]₂LR was coated onto a 96-well polystyrene plate. Serum samples 8 serial dilutions were added directly to the plates. After washing, polyclonal rabbit anti-IgG HRP was added in order to bind to the anti-CS protein antibodies in the wells. After the final wash, a chromogen substrate solution 3, 3',5,5' tetramethylbenzidine (TMB) and Hydrogen peroxide (H₂O₂) specific for HRP was added in order to detect anti-CS protein bound to the pre-coated antigen by the HRP-conjugated anti-IgG. The HRP catalysed a colour reaction producing a blue colour. The reaction was stopped by addition of sulphuric acid which changed the colour to yellow. The intensity of the yellow colour formed was proportion to the titre of the anti-CS protein IgG antibodies contained in the sample. The titres were calculated from a standard curve with the software SoftmaxPro (using a four parameters equation) and expressed as EU/ml. The cut-off for the anti-CS protein ELISA was 0.5 EU/ml.