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Assessment of humoral and cellular immune responses of the RTS,S/AS02D malaria vaccine candidate administered to infants living in a malaria endemic area in Mozambique

Aide, Pedro Carlos Paulino

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ASSESSMENT OF HUMORAL AND CELLULAR IMMUNE RESPONSES OF THE RTS,S/AS02D MALARIA VACCINE CANDIDATE ADMINISTERED TO INFANTS LIVING IN A MALARIA ENDEMIC AREA IN MOZAMBIQUE

Pedro Carlos Paulino Aide

A research report submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Science.

Johannesburg, 21st of October, 2009

DECLARATION

I declare that this research report is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

(Signature of candidate)

Johannesburg, 21st of October 2009

In memory of my father

António Moisés Aide

1948 – 2008

ABSTRACT

Background:

RTS,S candidate malaria vaccine has been shown to be highly immunogenic in children and infants, but the protective immune mechanisms still remain to be clearly elucidated. It is believed that RTS,S elicits a strong neutralizing humoral immune response directed against surface-exposed sporozoite proteins and cell mediated immune (CMI) responses characterized by predominantly $CD4^+$ Th1 cells. The objective of this study was to investigate humoral and cell-mediated immune responses to the RTS,S/AS02D malaria vaccine and its association with protection against infection and disease by *P. falciparum.*

Methodology and Principal Findings:

This secondary data analysis from data of a phase I/IIb randomized, double-blind, controlled trial, included 154 healthy infants living in rural Mozambique, previously immunized with RTS,S/AS02D candidate malaria vaccine or the control Engerix-B™ vaccine.

Antibodies against circumsporozoite protein (CSP) and hepatitis-B surface antigen (HBsAg) were measured with a standard ELISA. Fresh blood intracellular staining assay was performed to evaluate the expression of IL-2 and IFN-γ by $CD4^+$ and $CD8^+$ cells in response to *in vitro* stimulation of specific peptides. Data was evaluated for association with the risk of malaria detected by both active and passive case detection of infection over a period of 6 months post dose 3.

Anti-HBs antibody geometric mean titers declined from 10,082 mIU/mL one month post Dose 3 to 2,751 mIU/mL at 12 months post Dose 3 in the RTS,S/AS02D group; anti-HBs

geometric mean titers were 392.4 mIU/mL and 263.9 mIU/mL, respectively in the Engerix-B^{TM} group. Anti-CSP antibody geometric mean titers declined from 199.9 EU/mL one month post Dose 3 to 7.3 EU/mL at 12 months post Dose 3 in the RTS,S/AS02D group. Median stimulation indices of HBs-specific IL-2 and IFN-γ producing $CD8^+$ T cells was higher in the RTS,S/AS02D group than in control group (Wilcoxon rank sum p-values for IFN-γ = 0.015, for IL-2 = 0.030) at 10.5 weeks post immunization. Median stimulation indices of anti-CSP specific IFN-γ producing $CD8^+$ T cells at the same time point was 1.13 (IQR: 0.79 - 1.67; p=0.029). For specific IL-2-producing $CD4^+$ T cells, the median SI was 1.14 (IQR: 0.74 – 1.60, p=0.043) at 10.5 weeks post dose three.

The reduction in hazards of malaria infection were 18.3 % (95% CI: -267.9 – 81.8, p=0.793) and -12.0 % (95% CI: -295 – 68.2, p=0.86) for specific IL-2 $CD4^+$ stimulation indices; For specific $CD8^+$ IFN-γ stimulation indices the hazards were -103.6% (95% CI: -690.9 – 47.6; p=0.305) and 48.8% (95% CI: -97.0 – 86.7; p=0.33) at four and 10.5 weeks post immunization respectively.

Conclusion:

The RTS,S/AS02D vaccine was immunogenic and has elicited detectable levels of CSP-specific cell mediated responses. No evidence of association was found between the antibodies anti-CSP and specific cell-mediated responses and the risk of malaria.

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PUBLICATIONS

Some of the data presented in this research report have been included in the following publication:

Aponte, J.J., Aide, P., et al., *Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial*. Lancet, 2007. 370(9598): p. 1543-51.

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NOMENCLATURE

ADI: Active Detection of Infection

anti-CS: antibody to the *P. falciparum* circumsporozoite (CS) repeat protein

anti-HBsAg: antibody to the hepatitis B surface antigen

CISM: Centro de Investigação em Saúde de Manhiça, Moçambique.

CMI: Cell-mediated immunity

CSP: Circumsporozoite protein

DTPw/Hib: Diphtheria, tetanus, whole-cell pertussis and *Haemophilus influenzae* type B vaccine (TETRAacHib)

ELISA: Enzyme Linked Immunosorbent Assay

EPI: Expanded Programme on Immunization

GMT: Geometric mean titer

GSK: GlaxoSmithKline

HBsAg: Hepatitis B surface antigen

Hib: *Haemophilus influenzae* type B

HIV: Human immunodeficiency virus

ICS: Intracellular cytokine staining

ICH/GCP: International Conference of Harmonization/ Good Clinical Practices

IFN-γ: Interferon gamma

IL-2: Interleukin 2

TNF-α: Tumoral necrosis factor alfa

TRAP: Thrombospondin-Related Adhesion Protein

MPL: 3-deacylated monophosphoryl lipid A

MSP: Merozoite Surface Protein

MVI: Malaria Vaccine Initiative

P. falciparum: *Plasmodium falciparum*

PATH: Program for Appropriate Technology in Health

PCD: Passive Detection of Infection

PBMC: Peripheral blood mononuclear cells

QS 21: '*Quillaja saponaria* 21': a triterpene glycoside purified from the bark of *Quillaja saponaria*

RF1: A protective epitope against HBsAg

RESA: Ring-infected Erythrocyte Surface Antigen

RTS: Hybrid protein comprising S (hepatitis B surface antigen) and CSP portions

RTS,S: Particulate antigen, containing both RTS and S (hepatitis B surface antigen) proteins

S: Surface antigen of hepatitis B virus (HBsAg)

SI: Stimulation indice

CHAPTER ONE: INTRODUCTION

1.1. Introduction

Malaria caused by the parasite *Plasmodium falciparum* is responsible for approximately 1 million children deaths every year, mainly in sub-Saharan Africa, and is the most common reason for hospital admission. Every year there are around 250 million malaria clinical episodes in children under five years [1]. Various reasons account for the increase in incidence of malaria in much of Africa during the 1980's and 1990's; amongst others, the emerging resistance to conventional antimalaria drugs such as chloroquine and insecticide resistance of the anopheline vectors.

In recent years, increased efforts of malaria control that include long lasting insecticide treated nets (LLINs) and new first line treatments of malaria with more effective combinations, is leading to a reduction in malaria morbidity which yet has to be fully documented [1]. However in some areas particularly in regions where there is ongoing intensive malaria research it is estimated that the overall numbers of cases of clinical malaria will more than double over the next 20 years without effective control [2].

Hepatitis B is an infection of the liver due to hepatitis B virus (HBV) and is an important public health problem across the developing world. World-wide, approximately 350 million people carry HBV, and about 1 million chronically ill die annually [3]. The likelihood of the infection becoming chronic depends largely on the age at infection: 90% if infected in infancy, 30% to 50% if infected between the ages of 1 to 4 years, and low in adulthood. For those that become chronically infected during childhood, the risk of death from HBV-related liver cancer or cirrhosis in adult life is approximately 25% [4].

The RTS,S/AS02 candidate malaria and hepatitis B vaccine consists of sequences of the circumsporozoite protein (CSP) and hepatitis B surface antigen (HBsAg) with the adjuvant AS02D (proprietary oil-in-water emulsion, MPL® and QS21 immunostimulants).

The RTS,S vaccine with the AS0 family of adjuvant is being developed for the routine immunization of infants living in malaria-endemic areas and would offer protection against both malaria and hepatitis B.

A vaccine that induces partial immunity against pre-erythrocytic stages of malaria disease may provide protection to vulnerable young children from the severe forms of the disease, whilst continuing exposure allows them to build up natural immunity. Acquisition of natural immunity may be important to prevent a shift of severe disease burden to older age groups upon waning of vaccine-induced immunity [5].

Previous studies have demonstrated that RTS,S/AS02 vaccine is a powerful inducer of antigen-specific humoral and cell mediated immunity (CMI) [6-9]. The RTS,S/AS02A vaccine is believed to elicit a strong neutralizing humoral immune response directed against surface-exposed sporozoite proteins and elicit CMI responses characterized by predominantly $CD4^+$ Th1 cells, that are hypothesized to either destroy infected hepatocytes and/or limit intracellular parasite development through appropriate cytokines. Th1 cytokines such as IFN-γ, TNF-α and IL-2 have been described to be produced after stimulation with RTS,S or peptides derived from the circumsporozoite protein [10]. It is unclear whether $CD8^+$ cells play a role in protective immunity, thus this remains to be elucidated.

Antigen-specific humoral and cell mediated immunity induced by a vaccine antigen formulated in AS02 adjuvant has never before been measured in infants. As it is believed to

be a key component in protecting vaccinees agains the *P. falciparum* parasite, it has been measured in a trial with Mozambican infants [11] and was further analyzed (as secondary data) in this study.

1.2. Literature review

The GlaxoSmithKline (GSK) Biologicals candidate vaccine against *P. falciparum*, the RTS,S/AS02A malaria vaccine, has been shown to be safe and immunogenic in malaria-experienced adult populations in The Gambia and Kenya and malaria-naive adult populations in Belgium and the USA [6-8]. According to Kester et al. (2001), the vaccine has been shown to protect between 42% and 86% of healthy non-immune volunteers against infection in homologous sporozoite challenge studies, when given according to a 2 or 3 dose vaccination schedule [12]. In addition, a prolongation of the pre-patent period was observed in the majority of non-protected volunteers [12].

Subsequently RTS,S/AS02A studies were done in children aged 6 to 11 years in The Gambia, and demonstrated the safety of the vaccine in this age group as well as its highly immunogenic profile with regard to the mounting of anti-CSP and anti-HBs antibodies [13].

Overall, the GMT values observed in this population of children aged 6 to 11 years were within the ranges seen in previous studies with the RTS,S/AS02A vaccine in malaria-naïve adult subjects [12].

Subsequent to these studies, three sequential double-blind randomized controlled trial of the RTS,S/AS02A administered according to a 0, 1 and 2 months schedule were conducted in Mozambique [5, 14, 15]. In all these studies the vaccine proved to be safe and well

tolerated, with an acceptable reactogenicity profile, and inducing significant humoral immune responses against both CSP and HBsAg epitopes [5, 14, 15]. The non-inferiority of the HBsAg response of RTS,S/AS02A compared to the licensed vaccine Engerix-B™ (GSK Biologicals, Belgium) was also demonstrated by Macete et al [15]. The vaccine efficacy for the first malaria clinical episode was 29.9% (95% CI 11 – 44.8; p=0.004) and the prevalence of *P. falciparum* infection was 37% lower in the RTS,S/AS02A group compared with the control group at the end of a six-month observation period in Mozambican children aged 1 to 4 years [5].

The first administration of the RTS,S/AS02D to infants was carried out in Mozambique and showed that the vaccine was safe and immunogenic. The study showed a 65.9% (95% CI: 42.6 - 79.8, p <0.0001) delay in time to new infection over a 3.5 month period [11].

A recent trial of the same vaccine carried out in Tanzanian infants showed no interference with the immunologic response to WHO's Expanded Programme on Immunization (EPI) vaccines and a 65.2% reduction in malaria infection incidence [16]. Another trial in children 5 to 17 months old with the adjuvant AS01 in Tanzania and Kenya demonstrated a 53% (95% CI 28 – 69, p<0.001) reduction in clinical malaria [17]. These estimates of vaccine efficacy are remarkably consistent with previous data from Mozambique.

There is evidence and proof of concept efficacy in preventing infection, clinical malaria and severe malaria [5, 11]. However, there is still a lack of understanding of the types of immune responses needed for protection [18].

By now, the RTS,S is the most advanced pre-erythrocytic vaccine candidate in development. Other similar vaccines, based on CSP, thrombospondin-related adhesion

protein (TRAP) and other liver stage antigens, have been evaluated in clinical trials, with initial encouraging results [19, 20].

Several Phase III trials of the SPf66 vaccine candidate, a synthetic multiepitope, multistage peptide vaccine have reported low efficacy results and halted its development [21].

Blood-stage (erythtocytic) vaccines aim to stimulate immune response against surface proteins of merozoites, reducing incidence and severity of clinical disease. One of these vaccine candidates, with the *Plasmodium falciparum* merozoite surface protein 3 (MSP3) antigen has been shown to induce cellular and humoral immune responses [22]. In a phase 2 clinical trial of a combined vaccine containing the antigens MSP1, MSP2 and ring-infected erythrocyte surface antigen (RESA) in Papua New Guinea, the authors found that the vaccine reduced the parasite density by 62 % (95% CI: 13% – 84%) in children not pretreated with antimalarial drugs [23]. In a phase Ib dose-escalating trial among Tanzanian children aged 12 to 24 months, a blood stage malaria vaccine candidate MSP3 adjuvanted by aluminium hydroxide has been shown to elicit strong cytophilic IgG responses (subclasses IgG 1 and 3), both recognized in the parasite-killing mechanisms by monocytes [24].

Other interesting approaches are the transmission-blocking vaccines. These are designed to break the chain of transmission by inducing antibodies in the human host that inhibit parasite development in the salivary glands of mosquitoes preventing the development of infectious sporozoites [25].

Despite promising, further research and development still needed for these candidates, vaccine before proceeding for phase 3 trials.

1.3. Objectives

The main objective of this study was to assess the humoral and cell-mediated immune responses to the RTS,S/AS02D malaria vaccine in infants, and its association with protection against infection and disease by *P. falciparum,* and specifically:

- To describe the levels of antibodies against CSP antigens in infants prior to vaccination, four weeks, 10.5 weeks and 12 months after the third dose of RTS,S/AS02D or Engerix-B™;
- To describe the levels of antibodies against HBs antigens in infants prior to immunization, four weeks and 12 months after the third dose of RTS,S/AS02D or Engerix-B™;
- To compare specific $CD4^+$ and $CD8^+$ cell mediate immune responses in infants immunized with RTS,S/AS02D against the response of infants immunized with Engerix-B™ prior to vaccination, at four and 10.5 weeks after the third dose;
- To investigate the association between CMI (IFN-γ and IL-2) and CSP antibody responses in infants vaccinated with RTS,S/AS02D.
- To investigate the association between CMI (IFN-γ and IL-2) and the risk of infection or disease due to *P. falciparum* in infants vaccinated with RTS,S/AS02D.

CHAPTER TWO: MATERIALS AND METHODS

2.1. Study Population

The study population comprised healthy male and female infants aged 6 to 12 weeks at enrollment living in Ilha Josina Machel and Taninga, two rural villages located in the Manhiça demographic surveillance site, Mozambique, from June 2005 to December 2007.

2.2. Study Design

This study constitutes a secondary data analysis from the Malaria-038 clinical trial carried out in Mozambique between June 2005 and December 2007, using information collected for immunogenicity and efficacy. The original study was a phase I/IIb randomized, double-blind, controlled trial of the safety, immunogenicity and proof-of-concept of RTS,S/AS02D, a candidate malaria vaccine in infants living in a malaria-endemic region. Infants were randomized and immunized with either the RTS,S/AS02D or the control vaccine Engerix-B™ at 10, 14 and 18 weeks of age staggered with the EPI vaccines (TETRActHib) at 8, 12 and 16 weeks of age [11]. The study was registered with ClinicalTrials.gov identifier NCT00197028.

Briefly, infants were screened between 6 and 12 weeks of age after an informed consent was obtained from parents or guardians. At the first EPI vaccination visit, they were randomly distributed in two immunization groups to receive either the RTS,S/AS02D or the hepatitis B Engerix-B™ (GSK Biologicals, Belgium) study vaccines. All immunizations were given intramuscularly.

The study included all participants with available data for immunogenicity endpoints (received the three immunization doses, had available samples processed and complete follow up data). Those children who had received immunoglobulins and/or any blood product, chronic immunosuppressants (more than 14 days) or other immune-modifying therapy were excluded from the study.

Infants at high risk of vertical transmission of hepatitis B infection were excluded from the study because it used an experimental hepatitis B vaccine. A licensed hepatitis B vaccine in a schedule beginning at birth was offered to these infants.

Infants of HIV-positive women were excluded to avoid confounding the safety pattern associated with the investigational vaccine.

2.3. Methodology

2.3.1. Information program

Prior to study start, a community information program informed the local population of the study. Throughout the period of enrollment, study information was presented at antenatal clinics to expectant mothers.

2.3.2. Methods of data collection

Information related to all study visits and procedures was collected using a conventional case report form (CRF).

2.3.3. Laboratory tests

Blood samples for immunogenicity were drawn from children prior to first immunization (screening) and then at 4 and 10.5 weeks after the third immunization with RTS,S/AS02D or Engerix-B™.

Cases of malaria infection by *P. falciparum* were monitored by active detection of infection (ADI) commencing 2 weeks after dose 3 and performed every-other two weeks for 12 weeks. Blood slides for parasitaemia determination were collected and axillary temperature recorded irrespective of symptoms. In all children, parasitaemia was presumptively cleared with amodiaquine plus sulfadoxine and pyrimethamine 2 weeks before the final dose of RTS,S/AS02D or Hepatitis-B vaccine. Only children without parasitaemia started the active detection of infection. Children found positive for *P. falciparum* infection were treated with the first line treatment and excluded from further assessment of active detection of infection. Re-infections were assessed by passive case detection (PCD).

PCD was performed through monitoring of all attendances to health facilities and ascertainment of episodes of clinical malaria including blood smear for infants with documented fever (temperature equal or superior to 37.5°C) or history of fever in the preceding 24 hours. This method was also used to follow-up cases of re-infections by *P. falciparum.*

Antibodies against the CS repeat region were measured by a standard ELISA using plate absorbed with recombinant R32LR with an assay cut-off of 0.5 EU/mL. Anti-HBsAg antibody levels were measured using a commercial radioimmunoassay (AUSAB, Abbott, IL, USA) with an assay cut-off of 10 IU/mL.

Fresh whole blood intracellular staining was done to evaluate the expression of IL-2 and IFN-gamma by $CD4^+$ and $CD8^+$ T cells in response to *in vitro* stimulation with peptide pools of either CSP or HBsAg. After the stimulation, erythrocyte lysis was performed using 1X FACS lysing solution (BD Biosciences) at a room temperature during 10 minutes.

Fluorochrome-conjugated antibodies to the surface markers [(anti-CD8+/APC, anti-CD4+/FITC and anti-CD3/PerCP (BD, PharMingen)] were used to stain the cells. Then the cells were fixed and permeabilized with a solution containing paraformaldehyde and saponin (Cytofix/Cytoperm, BD Biosciences) for 20 min on ice/darkness, split into two tubes and stained with PE-conjugated anti-IFN-γ or anti-IL-2 antibodies (BD, FastImmune). Stimulation indexes were calculated as ratios between the proportions of peptide-specific cells over proportion of control stimulated cells (denominator).

2.4. Data Processing and Statistical Methods

The original dataset was cleaned and verified for completion/missing data using the study number as the identifier key. The variables of interest were extracted and analyzed using STATA® (College Station, Texas, USA) version 10.

The Geometric Mean Titers (GMTs) calculations were calculated by taking the anti-log of the mean of the log10 titer transformations. Antibody titers below the cut-off of the assay were given an arbitrary value of half the cut-off for the purpose of GMT calculation agreed by the investigators previously to unblinding of the primary data.

The seroprotective level for anti-HBs is ≥ 10 mIU/mL. The percentage of subjects with protective levels of anti-HBs (≥ 10 mIU/mL) with 95% confidence interval (95% CI) were determined at each blood sampling time point (screening, one and 12 months after third dose).

The percentage of subjects with sero-positive levels of anti-CSP (proportion of subjects with anti-CSP antibody titers greater than or equal to 0.5 EU/mL) with 95% CI were determined at screening, one, $3^{1/2}$ and 12 months after third dose.

The anti-HBs and anti-CSP antibody titers were summarized by GMTs with 95% CI at all time points at which serological samples were taken. Reverse cumulative distribution plots were used for visual assessment of distributions of the antibody titers and a Wilcoxon rank sum test was used to evaluate if the distribution were similar.

The proportion of responders in each group was compared using a chi squared test. Differences between both vaccine groups in intracellular median stimulation indexes and were assessed using the Wilcoxon rank sum test.

The association between specific anti-CSP immune responses (IFN-γ and IL-2 for both $CD4^+$/$CD8^+$ responses) and the risk of malaria was done using Cox regression models. Data on malaria incidence was derived from the primary efficacy data [11].

2.5. Ethical considerations

The study protocol was approved by the Wits Ethics Committee on Human Research (ethics clearance in appendix A). The primary study was authorized by the Mozambican National Bioethics Committee, the Hospital Clinic of Barcelona Ethics Review Committee and the PATH Human Subjects Protection Committee and implemented according to the International Conference of Harmonization Good Clinical Practices (ICH/GCP) guidelines. GSK Biologicals (Rixensart, Belgium) monitored the study.

CHAPTER THREE: RESULTS

A total of 154 participants were included in the immunogenicity cohort analysis, 75 in the RTS,S/AS02D group and 79 in the Engerix-B™ group. The proportion of missing data was similarly distributed between the two groups. Table 1 summarises the general characteristics of the participants. Mean age at enrolment was 8.3 weeks in both groups. There were relatively more males than females in the RTS,S/AS02D group than in the control group. The opposite figure was observed in terms of female participants (more females in the control groups). The comparison groups had similar distribution in terms of weight and the main haematological indicators.

Table 1: Baseline characteristics of infants prior to vaccination

	RTS,S/AS02D (n=75)		Engerix-B™ (n=79)	
Age(weeks): mean (SD)	8.3	(1.4)	8.3	(1.0)
Gender				
Male	40	(53.3)	34	(43.0)
Female	35	(46.7)	45	(57.0)
Weight (Kg): mean (SD)	5.01	(0.6)	5.04	(0.6)
Haemoglobin (mg/dl): mean (SD)	108.3	(11.1)	108.4	(12.8)
Total WBC: median (IQR)	9.5	(8.0 - 11.2)	9.9	(8.5 - 11.6)

SD= Standard Deviation; IQR=Inter Quartile Range; WBC= Whole Blood Count

Table 2 shows the anti-HBs, anti-CS seroprotection rates and GMTs at screening, one and twelve months after the third dose of RTS,S/AS02D or Engerix-B™ vaccination. Prior to vaccination, the proportion of participants with seroprotective levels of anti-HBs antibodies was similar in both groups (36.1% (95% CI: 25.1% – 48.3%) and 41.4% (95% CI: 29.8% - 53.8%) for RTS,S and Engerix-B™ respectively). Pre-vaccination anti-HBs GMTs were below 17 mIU/ml.

Anti-HBs GMTs had declined from 10082 mIU/ml (95% CI: 7495 – 13744) at one month post dose 3 to 2751 mIU/ml at 12 months post dose 3 in participants receiving RTS,S/AS02D. Anti-HBs GMTs were 392.4 mIU/ml (95% CI: 297 – 519) and 263.9 mIU/mL, respectively, in the Engerix-B™ group.

In terms of anti-CSP response at the screening, similar proportions of participants were seropositive in the comparison groups, with 31.6% (95% CI: 21.4% – 43.3%) in the RTS,S/AS02D and 33.8% (95% CI: 23.4% - 45.4%) in the Engerix-B™ group. The pre-vaccination GMTs were below the assay cut-off (0.5 EU/mL).

At one month post dose 3 of RTS,S/AS02D or Engerix-B, 98.6% of recipients of RTS,S/AS02D and 4.4% of recipients of Engerix-B™ were seropositive for anti-CSP GMTs.

A marked increase in anti-CSP antibody GMTs was observed at one month post dose 3 of RTS,S/AS02D (199.9 EU/mL) while no increase was observed one month post dose 3 of Engerix-B™ (<0.5 EU/mL).

At 10.5 weeks post dose 3 of RTS,S/AS02D, anti-CSP antibody GMTs had decreased to 58.8 EU/mL; however, 98.1% of subjects were still seropositive for anti-CSP antibodies.

At the end of the follow up period (12 months post dose 3), anti-CSP antibodies reached the lowest observed value (7.3 EU/mL) in the RTS,S/AS02D group.

At 10.5 weeks post dose 3 of Engerix-B™ , 19.7% of subjects were seropositive for anti-CSP GMTs; anti-CS antibody GMTs were below the assay cut-off (<0.5 EU/mL).

Table 2: Seroprotective rates and GMTs for anti-CSP and anti-HBs antibodies titers

	RTS,S/AS02D						Engerix-B™					
			Seropositive		GMT (EU/ml)				Seropositive		GMT (EU/ml)	
Antibodies	N	n	%	95% CI	value	95% CI	N	N	%	95% CI	value	95% CI
Anti-CSP												
Baseline	76	24	31.6	21.4 - 43.3	0.4	0.3 - 0.5	77	26	33.8	23.4 - 45.4	0.4	03 -0.4
4 weeks PD3*	71	70	98.6	92.4 -100	199.9	150.9 - 264.7	68	3	4.4	0.9 - 12.4	0.3	0.2 -0.3
10.5 weeks PD3*	53	52	98.1	89.9 -100	58.8	41.8 - 82.8	61	12	19.7	10.6 - 31.8	0.4	0.3 -0.5
12 months PD3	65	64	98.5	91.7 - 100	7.3	5.1 - 10.6	64	4	6.3	1.7 - 15.2	0.3	0.2 - 0.3
Anti-HBs												
Baseline	72	26	36.1	25.1 - 48.3	14.0	9.6 - 20.5	70	29	41.4	29.8 - 53.8	16.6	11.0 - 25.0
4 weeks PD3*	68	68	100.0	94.7 - 100	10081.6	7304.9 - 13744.4	64	63	98.4	91.6 - 100	392	297.0 - 518.5
12 months PD3	65	65	100.0	94.5 - 100	2751.1	2182.0 - 3468.6	64	64	100.0	94.4 - 100	264	213.8 - 325.6

GMT: Geometric Mean Titers, PD3: Post Dose 3; *Aponte et al[11]

Reverse cumulative distribution function for anti-CSP and anti-HBs antibodies GMTs immunization groups at one, 3.5 months (except for anti-HBs) and 12 months after dose 3 are presented in figure 1. The mean concentration of GMTs values for anti-CSP antibodies were high at all time points after dose 3 of RTS,S/AS02D immunization. Although a decrease in GMTs was observed with time, the levels of anti-CSP GMTs remained far above the cut-off point (0.5 U/mL). A small proportion of participants immunized with Engerix-B™ showed positive anti-CSP GMTs, especially at 3.5 months after dose 3.

At all time points, the anti-HBs GMTs were above the seroprotective level (more than 10 mIU/mL), although the response was higher for the recipients of the RTS,S/AS02D vaccine.

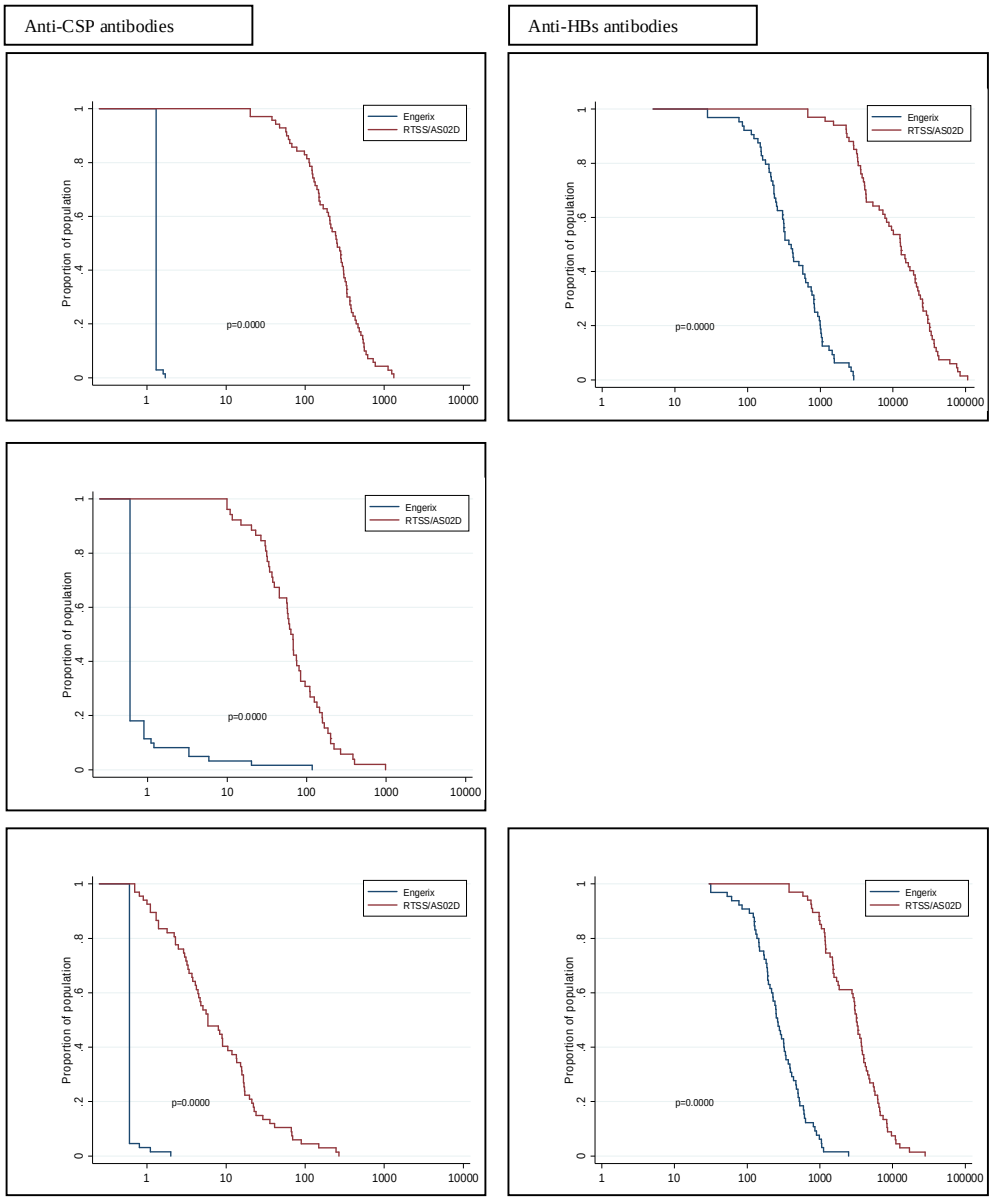


Figure 1: Reverse cumulative distribution of anti-CSP (left side) and anti-HBs (right side) antibodies GMTs after immunization. Plots for four weeks, 10.5 weeks and 12 months post-immunization are shown in top, middle and bottom rows respectively. Immunization groups are represented by red and black lines (for RTS,S/AS02D and Engerix-B™ respectively). Wilcoxon ranksum p-values for comparison of mean GMTs concentrations are shown in each plot.

Tables 3 and 4 compare the anti-CSP and anti-HBs-specific IFN-γ and IL-2 $CD4^+$ and $CD8^+$ responses between RTS,S/AS02D and Engerix-B™ vaccination groups.

The baseline-specific levels of IFN-γ and IL-2 were low and not statistically significant between the groups ($p>0.05$). The median range of stimulation indices for $CD4^+$ and $CD8^+$ T cells varied from 0.81 to 1.34 in both immunization groups.

Participants who received RTS,S/AS02D had a higher median SI of CS-specific IFN-γ producing $CD8^+$ T cells and CSP-specific IL-2 producing $CD4^+$ T cells as compared to the Engerix-B™ group (Wilcoxon rank sum p-values for IFNγ $CD8^+ = 0.029$ and for IL-2 $CD4^+ = 0.043$) at 10.5 weeks post dose three). In terms of HBs-specific responses, the median SI of HBs-specific IL-2 and IFN-γ producing $CD8^+$ T cells was higher in the RTS,S/AS02D group when compared to the Engerix-B™ vaccinated participants (Wilcoxon rank sum p-values for IFNγ = 0.015 and for IL2 = 0.030) at 10.5 weeks post immunization.

The median SI of anti-CS specific IFN-γ producing $CD8^+$ T cells at the same time point was 1.13 (IQR: 0.79 - 1.67; $p=0.029$). For specific IL-2 producing $CD4^+$ T cells, the median SI was 1.14 (IQR: 0.74 – 1.60, $p=0.043$) at 10.5 weeks post dose three.

Table 3: Anti-CS specific IFN-γ and IL-2 $CD4^+$ and $CD8^+$ responses between RTS,S/AS02D and Engerix-B™ vaccination groups.

Table 3

Anti-CSP antibodies	Engerix-B™			RTS,S/AS02D			
	n	Median SI	IQR	n	Median SI	IQR	p*
IFNγ $CD4^+$							
Baseline	62	0.98	0.71 - 1.40	64	1.07	0.72 - 1.56	0.693
4 weeks post D3	75	1.20	0.77 - 1.61	75	1.16	0.85 - 1.75	0.611
10.5 weeks post D3	69	1.09	0.85 - 1.47	63	1.18	0.90 - 1.59	0.213
IFNγ $CD8^+$							
Baseline	62	1.09	0.78 - 1.76	64	1.07	0.70 - 1.73	0.872
4 weeks post D3	75	1.04	0.67 - 1.57	75	1.04	0.66 - 1.57	0.891
10.5 weeks post D3	69	1.05	0.67 - 1.41	63	1.25	0.84 - 1.85	**0.029**
IL2 $CD4^+$							
Baseline	62	1.00	0.55 - 1.58	64	1.17	0.70 - 1.44	0.762
4 weeks post D3	69	1.05	0.71 - 1.65	70	1.32	0.95 - 2.00	0.052
10.5 weeks post D3	68	1.00	0.66 - 1.44	59	1.25	0.92 - 1.74	**0.043**
IL2 $CD8^+$							
Baseline	62	1.14	0.74 - 1.82	64	1.37	0.84 - 1.78	0.617
4 weeks post D3	69	1.04	0.66 - 1.86	70	1.14	0.78 - 1.86	0.626
10.5 weeks post D3	68	0.86	0.54 - 1.38	59	1.04	0.76 - 1.61	0.086

*p value: Wilcoxon Rank Sum test

Table 4: Anti-HBs specific IFN-γ and IL-2 $CD4^+$ and $CD8^+$ responses between RTS,S/AS02D and Engerix-B™ vaccination groups.

Anti-HBs antibodies	Engerix-B™			RTS,S/AS02D			
	n	Median SI	IQR	n	Median SI	IQR	p*
IFNγ $CD4^+$							
Baseline	62	1.04	0.56 - 1.31	64	0.88	0.52 - 1.33	0.503
4 weeks post D3	75	1.17	0.71 - 1.75	75	1.24	0.76 - 1.67	0.533
10.5 weeks post D3	69	1.03	0.74 - 1.47	63	1.08	0.77 - 1.59	0.769
IFNγ $CD8^+$							
Baseline	62	0.86	0.49 - 1.50	64	0.83	0.47 - 1.67	0.970
4 weeks post D3	75	1.08	0.63 - 1.71	75	1.06	0.74 - 1.70	0.423
10.5 weeks post D3	69	0.81	0.58 - 1.21	63	1.08	0.68 - 1.49	**0.015**
IL2 $CD4^+$							
Baseline	62	0.97	0.58 - 1.32	64	1.14	0.84 - 1.17	0.220
4 weeks post D3	69	1.19	0.69 - 1.79	70	1.34	0.95 - 1.79	0.185
10.5 weeks post D3	68	1.04	0.72 - 1.37	59	1.18	0.81 - 1.88	0.065
IL2 $CD8^+$							
Baseline	62	0.91	0.60 - 1.40	64	1.05	0.60 - 1.51	0.830
4 weeks post D3	69	1.14	0.62 - 1.67	70	1.16	0.83 - 1.65	0.451
10.5 weeks post D3	68	0.89	0.52 - 1.26	59	1.28	0.63 - 1.65	**0.030**

p* value: Wilcoxon Rank Sum test

We found no evidence of association between antibodies anti-CSP and specific cell mediated responses ($CD4^+$ SI for IL-2 and $CD8^+$ SI for IFN-γ) (figure 2).

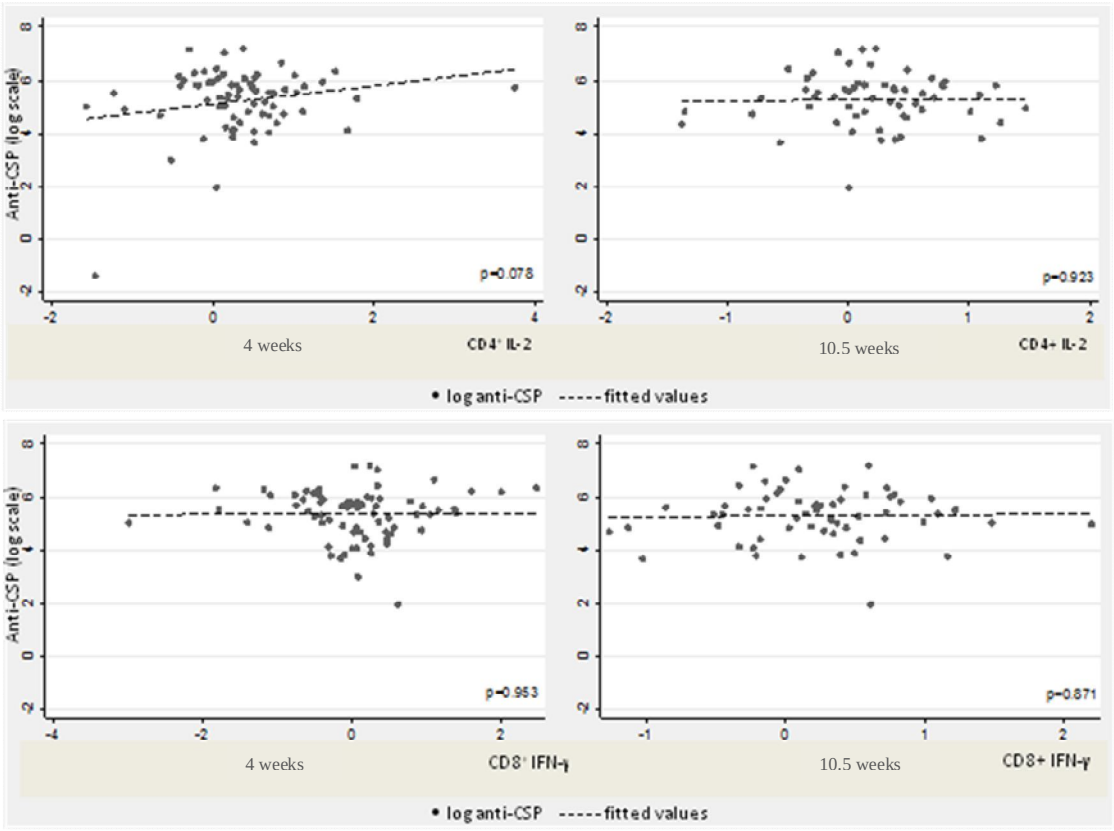


Figure 2: Association between anti-CSP specific cytokine responses ($CD4^+$ IL-2 and $CD8^+$ IFN-γ on top and lower panel respectively) and anti-CSP antibodies. Plots for four and 10.5 weeks post-immunization are shown at left and right side respectively. CSP-specific cytokines and antibodies are expressed in log scale and p-values were obtained from linear regression models.

For the comparison of children in the highest tertile of anticircumsporozoite response versus those in the lowest tertile in terms of anti-CS specific $CD4^+$ SI for IL-2 and IFN-γ the reduction in hazard of malaria infection were not statistically significant as shown in table 5. These responses were only evaluated for specific $CD4^+$ SI for IL-2 and $CD8^+$ SI for IFN-γ that previously was statistically significant (tables 3 and 4). When the analyses were adjusted by gender, no statistical significant differences were noted (data not shown).

Table 5: Association between anti-CSP CMI responses and the risk of malaria infection

Stimulation indices Anti-CS	**Tertiles**	**Episodes**	**Time At Risk** (PYAR)	**Incidence** (Episodes per PYAR)	**Reduction in Hazard** (1-HR)%	**(95% CI)**	**p***
IL-2 $CD4^+$ (4 weeks PD3)	Lower	4	5.59	0.72	18.30%	(-267.9; 81.8)	0.793
	Higher	3	5.57	0.54			
IL-2 $CD4^+$ (10.5 weeks PD3)	Lower	5	4.23	1.18	-12.00%	(-295.3; 68.2)	0.86
	Higher	5	4.36	1.15			
IFNγ $CD8^+$ (4 weeks PD3)	Lower	3	5.23	0.57	-103.60%	(-690.9; 47.6)	0.305
	Higher	7	5.78	1.21			
IFNγ $CD8^+$ (10.5 weeks PD3)	Lower	8	5.15	1.55	48.80%	(-97.0; 86.7)	0.33
	Higher	3	4.54	0.66			

*p-value from Cox regression model using Wald test;
PD3: post Dose 3

Limitations of the study

The cell mediated immune response was measured at four and 10.5 weeks after the third doses of RTS,S/AS02D or Engerix™ immunization, periods that do not coincide necessarily with the times were these responses can reach optimally high titers (before four and 10.5 months). Data on malaria incidence was derived from the primary efficacy study [11].

Cell-mediated immunity data presented here is only related to intracellular cytokine staining of $CD4^+$ and $CD8^+$ lymphocytes. Data pertaining cytokine measures in supernatants were not included in this analysis.

The sample size was estimated to evaluate safety and humoral immunogenicity as well as the proof-of-concept of efficacy. The process to obtain the CMI is complex, mainly due to a high volume of blood required and long and complex procedures, thus leading to a considerable amount of missing data that further reduced the power to detect differences. For the same reason, i.e. incomplete data related to CMI, multivariate analysis on the relationship between humoral and CMI responses as well as its association with the risk of malaria led to inconclusive findings and were excluded from this report.

There is no power calculation to estimate the effect between the immune response and the risk of malaria. We did not adjust the p-values for the multiple comparisons. So the p-value limit of 0.05 has been considered as indicating statistical significance.

CHAPTER FOUR: DISCUSSION

Previous studies with GSK Biologicals candidate malaria vaccine RTS,S/AS02D in Mozambique, Kenya and Tanzania showed that vaccination of infants (less than one year of age) confers protection against infection and clinical malaria endemic areas [11, 16, 17]. Over a period of 12 months, RTS,S/AS02D was shown to be immunogenic in Mozambican infants, inducing in the RTS,S/AS02D group high GMTs anti-CSP antibody levels after three doses, despite indication of waning over time (especially from 10.5 to 12 months after dose three). This indicates that anti-CSP antibodies, probably together with other cellular immune responses, may be involved in the initial protection against malaria in infants vaccinated with the RTS,S vaccine.

The RTS,S vaccine is being developed as a conjugate vaccine to protect children against malaria and hepatitis B. Data from the evaluation of cellular mediated responses to both the *P. falciparum* circumsporozoite protein and hepatitis B surface antigen shows that the RTS,S/AS02D vaccine was immunogenic in infants eliciting levels of detectable cellular immune responses to both CS and HBs antigens following immunization.

Increased secreted levels of IFN–γ and IL-2 to both antigen vaccine components were observed by intracellular cytokine staining up to 10.5 weeks after immunization. HBS-specific IFN-γ and IL-2 responses were more frequently induced by the RTS,S/AS02D vaccine than by the control Hepatitis-B vaccine, possibly due to a stronger Th1 adjuvant effect of AS02D compared with alum present in the Engerix-B™ Hepatitis B vaccine.

Although RTS,S/AS02D immunization has induced CSP-specific cellular immune responses detected by intracellular cytokine staining that are statistically significant when compared to the Hepatitis-B group or pre-immune responses, the percentage of positive cells was very low and the biological significance cannot be assured.

The median stimulation indices for CSP-induced responses were approximately 0.25 higher in the RTS,S/AS02D group than in the control Engerix-B^{TM} vaccine group, when taking in consideration only the observed statistical significant differences between these groups. Making approximations, this could be translated to around 0.08 and 0.09% increment in the CSP-specific $CD4^+$ and $CD8^+$ T cell population in children immunized with RTS,S.

We found no association between CSP-specific T cell immune responses to RTS,S vaccine and the risk of malaria infection. However, a potential limitation of the ICS assay performed was the use of whole blood instead of peripheral blood mononuclear cells (PBMC), due to the small volumes of blood available for the assays. Whole blood assays may give more background in ICS than PBMC thus weakening the signal to noise ratio for the antigen-specific response. The true differences between the immunization groups may thus be greater than detected in this study.

The current analysis is an initial description of CSP-specific T cell immune responses elicited in infants immunized with RTS,S vaccine. The identification of correlates of the RTS,S vaccine protection constitutes a goal still to be achieved, hence further investigation is needed. The role of cytokine-producing T cells has been under intense study in efforts to identify correlates of malaria vaccine efficacy. However, there is a wealth of possible

complications mainly due to the use of different assays to measure antigen-specific T cell activity and the lack of functional assays. The RTS,S vaccine candidate has been shown to elicit cellular immune responses by various techniques in the past [5, 8, 26, 27], but it is still unclear how these cellular responses correlate with vaccine protection.

The next steps in the development of this vaccine candidate are the phase III trials in preparation for the next 3-4 years. It has been suggested that IFN-γ T-cell responses to natural malaria exposure are infrequent in children [5, 11] and it is hypothesized that repeated exposure and a mature immune system may be required. Thus a lot of opportunities to better understand and identify how this vaccine acts to elicit immunity are to be considered and have to be included in upcoming trials to accelerate further development of malaria vaccines.

Despite having found weak CSP-specific T cell responses induced by the RTS,S/AS02D immunization, the results indicate that specific cell-mediated immunity can be elicited in infants less than one year of age.

Hopefully, with a successful completion of these plans, a first generation of malaria vaccine would be available to protect African infants in the near future.

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

In conclusion, we found that the RTS,S/AS02D vaccine has induced seropositivity rates for anti-CSP antibodies and seroprotection rates for anti-HBs antibodies that were detectable after 12 months following vaccination with RTS,S/AS02D in staggered co-administration with TETRActHib. The anti-HBs responses were higher in recipients of RTS,S/AS02D than of Hepatitis B control vaccine.

The analysis also demonstrated that this vaccine elicits detectable levels of cytokines CSP-specific cell mediated responses (namely $CD4^+$ and $CD8^+$ producing IL-2 and IFN-γ). However, no evidence of association was found either between anti-CSP antibodies and specific cell-mediated responses; or between the former one and the risk of malaria in this group of infants immunized with the RTS,S/AS02D vaccine.

In the light of the above-mentioned, our first recommendation is the continuity of further research of the type and specificity of immune responses against CSP during phase III studies of this vaccine candidate.

Secondly, we recommend the optimization of ICS assays using total whole blood to be used in African infants due to the less amount of blood required for this assay and fewer ethical concerns (contrary for example to ICS assays in peripheral blood mononuclear cells, technique already optimized for adults but not infants).

Finally, evaluation of cost-effectiveness of this intervention as well as its expected integration in the EPI schedules are recommended to be included in the next phase III studies.

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APPENDIX

Appendix A

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Aide

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M081044**
PROJECT	Assessment of Humoral and Cellular Immune Responses of the RTS,S/AS02D Malaria accine Candidate Administered to Infants in a Malaria Endemic Area in Mozambique
INVESTIGATORS	Mr PCP Aide
DEPARTMENT	School of Public Health
DATE CONSIDERED	08.10.31
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 08.11.03 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof MP Grobusch

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

02/12/2008