MALVAC Meeting 2004: Evaluation of malaria vaccines

Clinical Evaluation Group: End-points and Trial Design of Phase 2b Clinical Trials of Blood-stage Vaccines

Report from a WHO/IVR Malaria Vaccine Advisory Committee meeting

Montreux, Switzerland,

11-14 October, 2004

IVR would like to acknowledge the contribution of the Malaria Vaccine Initiative of PATH in co-sponsoring and organizing this meeting

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Executive Summary

Specific scientific and technical challenges in the assessment and evaluation of candidate malaria vaccines were discussed at the 2004 meeting of the WHO Malaria Vaccine Advisory Committee (MALVAC), in Montreux, Switzerland on 11-14 October, 2004. In addition to the committee members, and representatives of the major malaria vaccine funding agencies, the meeting also gathered experts in immunology, parasitology, malaria epidemiology, vaccine development and clinical trials from diverse malaria vaccine research laboratories, clinical trial sites and industry. The meeting participants (Annex 2) were divided into two groups, addressing either pre-clinical/non-clinical evaluation or clinical evaluation. This section addresses only the outcome of the meeting of the clinical evaluation group. The outcome of the meeting on preclinical evaluation is published in a separate report.

'Considerations of End-points and Trial Design of Phase 2b Trials of Blood-stage Vaccines'

Guidance for the conduct of malaria vaccine field trials were published in the ***Guidelines for the Evaluation of Plasmodium falciparum vaccines in populations exposed to natural infections, WHO/TDR/1997***. Since the publication of the guidelines, there has been significant progress in the field, including field trials of other candidate vaccines, and the development of multiple other candidates that will likely require field evaluation in the coming years.

In the absence of reliable surrogates of protection, the clinical development of blood-stage vaccine poses several challenges. Earliest proof-of-concept for blood-stage vaccines requires large field efficacy trials, prior to any firm knowledge if the vaccine will be protective or not. Given the potential numbers of vaccine candidates requiring field trials, and the resources consumed in the conduct of trials, early demonstration of a vaccine's biological effect in smaller trials would be useful, before such an investment is undertaken.

Smaller field trials, designed and powered to detect the vaccine's putative biological effect are being considered. Therefore at this meeting, the clinical evaluation group focused on discussing if and how parasitological end-points could be used to design these smaller efficacy trials, that could potentially be useful at screening more candidate vaccines faster and using fewer resources than that required by current field efficacy trials.

End-points considered were parasite density, anemia, breakthrough genotypes and multiplicity of infections.

Parasite density

The correlations between parasite density and risk of disease in different

populations have been observed in multiple studies. It was generally agreed that evaluating a blood-stage vaccine's effect on parasite density levels provided useful information and should be done in malaria vaccine trials. However, age, threshold levels and epidemiological background remain unpredictable confounders of this correlation and an exact, predictive relationship is not known. Therefore parasite density cannot be currently used as a proxy measure or valid surrogate for clinical disease and current evidence does not support using it as a primary end-point, but as a secondary end-point in Phase 2b and 3 trials. However, in specific endemic circumstances, where preliminary efficacy is sought and at a site with well-characterized parameters, initial, small, Phase 2b trials using parasite density as a primary end-point could be conducted. Given the confounders previously mentioned, these trials should be conducted in populations that are as homogeneous as possible with regards to the potential confounding factors. Follow-up larger trials with clinical efficacy end-points could then provide the opportunity to validate this approach.

It was also suggested that given the limitations of current knowledge on the inter-relationship between parasite density and clinical outcomes of malaria attacks, severe disease and death, a systematic review and analysis of data from trials of other malaria interventions such as ITNs and IPTi could help in better defining this inter-relationship, and potentially highlight other potentially valuable end-points for future trials.

Microscopy remains the gold standard method of measurement. In terms of read-outs, parasites per high power field may yield far less variation than parasites per 200 white blood cells. The lack of highly trained field microscopists and the need for training more microscopists were expressed by the participants. In some instances, such as large-scale field trials, parasite antigen load measurement (HRPII, LDH) and PCR may be considered as alternatives.

Another measurement related issue was that of individual fluctuations in parasite density levels, which must be considered when deciding on the end-point measurement. The preferred and simple measure is the geometric mean for parasite density as a continuous variable. An alternative suggestion was the arithmetic mean which has several advantages over the geometric mean in some situations. Other alternative approaches were discussed, and for each, it was emphasized that it was important to consider baseline imbalance caused by exclusion of relevant data from those below the 'set' threshold, as this could impair the analysis.

Anemia

Infection with *P. falciparum* is among the major cause of childhood anemia in malaria-endemic areas. In epidemiological studies, a strong correlation has been reported between the incidence of severe anemia, age-specific rates of anemia, and the intensity of *P. falciparum* transmission. Severe anemia often complicates clinical malaria in young children in areas of intense transmission, increasing the risk of blood transfusion and death.It was generally agreed that anaemia should not be a primary end-point for phase 2b trials and that as an end-point, anaemia would be more useful once more is known about a vaccine and its effect in a community.

However, if Phase 2b trials are designed with the aim of looking for impact on clinical disease, anaemia needs to be measured. It should be a secondary end-point and

should be a measurement of mean Hb rather than only of severe anaemia. Trials designed to explore if the intervention has an impact on anemia must balance statistical considerations which may require frequent sampling with the impact of treatment that will be required with detection of anemia, making assessment of the efficacy of the intervention in preventing anemia difficult. Confounding factors such as age, endemicity, treatment, hookworm infestation, and others must be adjusted for in the design and analysis plan of the trials. Anemia must also be considered in the context of the local endemic background, as the age distribution and features of clinical malaria would be different under different conditions. The measurement of hemoglobin rather than packed cell volume or haematocrit was the preferred method for measuring anemia

It was also generally agreed that small scale trials could use Hb measurement or levels as a primary end-point in certain circumstances, such as when a vaccine has been proven to be safe, and then smaller trials could be designed to look at the impact on this end-point. At some future point, anaemia could be a very useful tool in well-designed trials aimed comparing vaccine performance to eliminate some vaccines and prioritize others.

Parasite genotyping

For parasite genotyping, although evidence from past vaccine trials highlight its importance as an exploratory end-point, data on correlation between breakthrough parasitemia and risk for clinical malaria was not conclusive enough to recommend parasite genotyping as a primary end-point. Parasite genotyping should be considered as an essential secondary end-point in vaccine trials to detect selection of non-vaccine type parasites in the case of vaccines based on polymorphic antigens and explore possible changes in parasite subpopulations due to vaccination.

Evidence was discussed which suggests that for polymorphic vaccines where only one allelic type is utilized, this could induce variant specific immune responses and selection of the non-vaccine type parasite. Therefore, parasite genotyping, which helps to determine and characterize parasite dynamics could be used to assess and clarify the relationship between the parasite dynamics, multiplicity of infection, specificity and selective effect and the clinical end-points of clinical and severe malaria. Practical issues such as lack of resources that would be required to routinely conduct molecular monitoring of malaria vaccine trials in the field and concerns over the issue of frequency of blood sampling in each individual were highlighted as potential limitations to routine molecular monitoring of trials.

Multiplicity of infection

Multiplicity of infection (MOI) refer to infections that contain multiple *Plasmodium. falciparum* clones, or multiple different genotypes in a single individual. These infections are often due to super-infection, which is the acquisition and accumulation of multiple infections, from different mosquitoes, where individuals who have been bitten and are already infected, get bitten again and accumulate multiple infections. It has also been demonstrated that multiple infections are often also due to a single inoculation of multiple genotypes by one mosquito. it should be used as an exploratory tool and not as a primary end-point in vaccine trials. A direct and predictive correlation is lacking between MOI and clinical or parasitological end-points, and these can be simply measured without any requirement of MOI data. Given the concerns of

potential effects of vaccine polymorphism, it could play a role in the detection of selective pressure by the vaccine and impact on parasite dynamics and parasite population, and like breakthrough genotypes, should be explored as a way of better understanding parasite dynamics.

Most participants felt that given the state-of-the-art of genotyping, MOI analysis requires resources and expertise that may stretch current field-trial capacity. It should be approached from a research perspective, and it was generally agreed that the information gathered from monitoring of MOI in vaccine trials had the potential to contribute to improved assessment of vaccine effect that may help improve vaccine and trial design.

Conclusion and Recommendations

The meeting participants concluded that the primary efficacy end-points recommended in the *Guidelines for the evaluation of Plasmodium falciparum vaccines in the population exposed to natural infection.* (Geneva: WHO/TDR, 1997), which are parasitemia, clinical disease, severe disease, direct malaria deaths and total deaths were still valid and appropriate. The recommended primary efficacy end-point for blood-stage vaccines is incidence of disease.

It was recommended that further knowledge on clinical evaluation of malaria vaccines that have emerged since the publication of the guidelines should be incorporated into a revised and updated version of the guidelines. Recommendations were also made to address the parasitological end-points discussed at this meeting, and incorporate the meeting discussions into the update of the guidelines. As more trials are conducted, the opportunity to gather more data on these end-points should be maximized, through efforts to develop a general agreement on a standard panel of secondary end-points such as parasite density, anemia, and genotyping to be assessed during all trials, as well as consensus on general standards for collection, sampling and analysis methods of these end-points.

An international collaborative working group was recommended to be established that could address issues related to clinical evaluation of malaria vaccines such as:

- study design of efficacy trials and analytical methods

- standardization of study methodology (i.e. parameters of microscopy read-outs in terms of numbers of microscopists and limits of the readings) and trial specimen collection methods, including the frequency, timing and duration of sampling.

- sharing of clinical development plans, clinical lab and trial results and assistance in the conduct of trial-related activities.

This could lead to the development and sharing of ' best practices' that are accessible, compatible and harmonized in the clinical evaluation of malaria vaccines, thus improving comparability of field trial analysis.

2. Report from the Clinical Evaluation Group: End-points and Trial Design of Phase 2b Clinical Trials of Blood-stage Vaccines

2.1 Introduction

In the clinical testing of a candidate vaccine, following the demonstration of safety in small numbers of healthy adults in Phase I trials, Phase 2 studies involve larger numbers of subjects and are intended to obtain preliminary information about a vaccine's efficacy in the target population and to gather further safety data. Phase 2b and Phase 3 are field efficacy trials and are distinguished mainly by scale. Phase 2b trials are usually viewed as efficacy trials to gather the preliminary data essential to guide decisions and appropriate design of pivotal phase 3 trials.

For the development of pre-erythrocytic vaccines, early proof-of-concept of vaccine effect may be provided by Phase 2a trials utilizing the sporozoite challenge model. Although similar challenge models are being developed to assess blood-stage vaccines, they remain to be optimized before being available for use for screening and prioritization of candidate vaccines. In the absence of reliable surrogates of protection, the earliest proof-of-concept for blood-stage vaccines will require large field efficacy trials, prior to any firm knowledge if the vaccine will be protective or not. Given the numbers of candidate vaccines requiring field trials, and the investment required by clinical trials, early demonstration of a vaccine's biological effect in smaller trials would be useful. Current guidance for the conduct of malaria vaccine field trials published in the ***Guidelines for the Evaluation of Plasmodium falciparum vaccines in populations exposed to natural infections, WHO/TDR/1997***, do not extensively address the use of biological or parasitological end-points as vaccine trial end-points.

Therefore, the meeting was aimed at gathering scientific advice and expertise on possible end-points and design of these smaller Phase 2b field trials, powered to detect a vaccine's putative biological effect. Clinical trial experts, malaria epidemiologists, academic researchers, malaria vaccine development program managers, representatives from major funding agencies, and industry considered the following parasitological end-points, parasite density, anemia, multiplicity of infection and breakthrough genotypes for their potential as preliminary measures of asexual malaria vaccine activity and efficacy. These end-points were discussed in terms of their predictive value and potential role as marker of clinical disease, methods of detection, trial design and statistical considerations. The meeting participants also discussed strengths and weaknesses of the end-points, and usefulness and limitations for decision-making on vaccine development.

This report details the issues considered and discussed at the meeting. The meeting concluded with recommendations for the WHO to address and incorporate new knowledge on clinical evaluation of malaria vaccines that have emerged since the publication of the guidelines, by revising and updating the guidelines. These recommendations are described in further detail in the recommendation section of the report.

2.2. Phase 2b Trial Design of Blood-stage Vaccines- EMVI Workshop

(Contributors: Pierre Druilhe, Brian Greenwood)

Natural exposure to malaria progressively induces the development of some protective immunity. Complex host-parasite interactions, influenced by age, transmission intensity, and number of infections leads to the development of the various types of immunity to malaria (anti-toxic immunity or anti-disease, resistance to cerebral malaria, strain dependant versus strain independent anti-parasite immunity, anti-Var immunity). This naturally raises concerns about the difficulties of field testing a blood stage vaccine. Therefore importantconsiderations in the clinical evaluation of blood-stage vaccines include the identification of conditions, in terms of target population and endemicity that will convincingly demonstrate vaccine efficacy. The outcome of a recent workshop held to discuss this and other related questions is described below.

2.2.1 Testing the efficacy of blood-stage vaccines - considerations of immunity, age, endemicity and clinical disease in the selection of the 'ideal' population

Given that interaction with pre-existing immune responses may interfere with vaccine efficacy assessments and therefore epidemiological considerations for the testing of a blood stage vaccines were discussed where the optimal characteristics of a population in which to accurately test these vaccines were proposed, as described below:

- Some degree of anti-toxic immunity,
- An absence of anti-parasite immunity,
- A sufficiently high level of infection to allow a vaccine trial of reasonable size to be conducted.

With these considerations, it was suggested that African children are not the best target for early proof-of-concept testing of blood stage vaccines, and that trials in less endemic areas, e.g. parts of Asia would be better.

However, it was pointed out finding a site where there had been no priming of the immune system, but yet where the attack rate was high enough to allow a vaccine trial to be done would be difficult. In later discussions the possibility of using migrant (including military) populations moving to endemic areas or populations at risk of epidemics was

raised, but the practical and ethical difficulties of doing trials in these populations could be a limitation. In addition, these populations do not meet all of the above characteristics and it is uncertain how much more useful than an experimental challenge study such a trial would be.

Data were presented describing the age and clinical disease profile from a number of sites. In some situations only a relatively small proportion of the population has experienced an attack of malaria or an asymptomatic infection by the age of one year – 60% in a population in PNG, and these young children might meet the desired criteria for a vaccine study. In an area of Senegal with seasonal malaria transmission, the effects of season of birth, on age at first attack of malaria, and on parasite density during such attacks was not seen during first, second or third attack.

The issue of the fever threshold and its' role in defining clinical disease was discussed. The fever threshold has been used to define clinical malaria attacks in evaluating malaria interventions in order to increase specificity. However, it was presented that the use of different levels of parasite density to define a clinical attack of malaria had little effect on measurement of protective efficacy when using the protective effect of the genotype AS. Limited data from vaccine trials has shown a similar effect. This suggests that the importance of parasite density in definition of a clinical attack of malaria, which should include a history of fever as well as observed fever, may have been over-emphasized, and needs further evaluation.

A potential problem is the differences of this threshold in different populations at different ages, and that it is unlikely that regulatory authorities would allow different definitions to be used in licensure trials, and that a standard definition will be required.

Data was presented using the Smith and Schellenberg method[1] for malaria attributable fraction estimates for several populations living under different levels of transmission. Reasonably consistent results found that the threshold was low in those under one year, and increased from ages one to ten years and then declined. Data from Dielmo, Senegal presented by Jean-François Trape supported this trend. In this population, nearly all infants who became parasitaemic were symptomatic regardless of the degree of parasitemia.

2.2.2 Learning from the model of natural immunity developed under natural exposure

Comparison of incidence of clinical malaria under different transmission settings, from Dielmo(EIR 200), Northern Ghana, and Papua New Guinea (EIR between 15 and 40) showed that identifying a cohort of young children without considerable exposure to malaria and experience of clinical malaria under high transmission settings would be difficult. However, under low to moderate transmission conditions, it is likely that a significant proportion of children (possibly between 25% and 50%) may reach their first birthday without having experienced an obvious clinical attack of malaria. Comparisons of surveillance conducted by different groups at various sites must take into account differences in data collection methodology. Preparatory site surveillance studies with

[1] Smith T, Schellenberg JA, Hayes R. Attributable fraction estimates and case definitions for malaria in endemic areas. Stat Med. 1994 Nov 30;13(22):2345-58.

standardized methodology, incorporating both active and passive surveillance and immunological measurements, will be necessary to define the exact degree of malaria experience.

In order to further explore how to identify individuals with little malaria experience, but at high risk of clinical attacks, data from Asia was compared with data from other sites in Africa. In Myanmar, it was observed that adolescents and young adults with relatively little evidence of previous exposure to malaria may be exposed to a clinical attack rate of around about one attack per year. Data obtained from surveillance of a cohort of transmigrants in Indonesia reportedly showed that this level of exposure was insufficient to lead to any immunity in the cohort. However, as the measure of immunity was reduction in the incidence of clinical attacks over a period of three years, it was agreed that this could still be within the experience of young African children in endemic conditions, who may not achieve the ability to limit clinical attacks over this period, but still build up substantial immunity to severe disease and death. Data from the Amani mountains in Tanzania indicates that it was possible to define populations with continuous variation of exposure by altitude, and that these populations could allow the possibility of dissecting the relative contribution of malaria exposure and age. It was agreed that the optimum populations with minimal exposure but high attack rates could be found either in migrant populations, epidemic situations, or situations in which non-immunes such as military personnel are deployed in an endemic situation. However, it was considered that the logistical and ethical considerations of conducting clinical trials in such populations would make vaccine studies extremely difficult.

Specific analysis of naturally induced immune responses to antigens of vaccine potential(MSP3, LSA3, R23, GLURP and crude parasite extract) in a cohort of approximately 50 children, longitudinally followed up for 8 years, was presented. IgG and IgM responses to MSP3 were high and continued to rise over time (IgG).This pattern of well-established responses by the age of three, was also repeated for LSA3, R23 and GLURP, albeit to a lesser degree. In the case of R23, responses declined over time. In an analysis of the relationship between antibody responses to individual antigens and experience of clinical malaria, responses to all antigens including the crude extract, were associated with significant protection ranging between 21% and 45%.

A striking feature of the analysis was the considerable individual variation in responses to different antigens. The third analysis indicated that association with clinical protection was limited to IgG1 and IgG3 in the case of MSP3 and GLURP, to IgG1 in the case of R23 and IgG2 in the case of LSA3.

2.2.3 Determining the desired performance - biological and clinical requirements

Malaria therapy was used to treat neurosyphilitic patients in the early half of the 20th century. It was rarely, if ever, necessary to give treatment on clinical grounds to patients with *P. falciparum* infection, other than in the first wave of parasitemia of any induced infection. It also appears, from limited numbers, that treatment on clinical grounds was rarely necessary during any part of subsequent induced infections. Maximum morbidity therefore seems to be associated with the first wave of parasitisation, and analysis was restricted to these data.

Studies of malariotherapy[1], have demonstrated that one infection lowered the likelihood of fever during subsequent infections, as well as the maximum parasite density, indicating that even one infection does influence the outcome of subsequent exposure, a view supported by the fact that only few infections are required to prevent an African child from dying from malaria.

An analysis of data studying the kinetics of rise and fall of the parasitemia in the first wave for a hundred patients was discussed. A striking observation was the variability in the exponential multiplication rates, and these were related to the eventual maximum parasitemia achieved before parasite densities declined. The variation in multiplication rates appeared to be mostly related to individual patient variation, rather than to being a feature of the parasite. The data were modelled to consider the effects of innate parasite clearance mechanisms and subsequent acquired parasite clearance mechanisms. The innate ability to control the initial rise in parasite density is a critical factor limiting the eventual height of parasitisation.

This analysis supports the idea that the aim of delaying/reducing parasite multiplication should be a key goal for blood-stage vaccines. Individuals with the lowest innate response(per parasite) may be at risk for morbidity and further modelling indicates that limiting severe disease could be easier than reducing clinical symptoms per se by vaccination, as this enhances acquired immunity and not innate immunity.

2.2.4 Making the clinical development rationale

Critical criteria driving a candidate vaccine through the various stages of clinical development of blood stage vaccines were discussed. The evaluation of safety of the product was absolutely essential in Phase 1a and 1b trials. These early trials should be designed to gather maximum information and define the vaccine-induced immune response and therefore parameters that help in defining criteria for vaccine performance in future trials.

The difficulty is in that some candidates are developed without clear parameters in humans (as data supporting vaccine is generated in animal models and in vitro assays) and lack of certainty about what are the desired quality and quantity of the immune response. For certain antigens it appears that cytophilic antibodies are essential, but this is not likely to be the case for all antigens. It is probably desirable to have a high titer of antibodies after immunization, more as a measure of vaccine potency, but knowledge regarding the quality and the functionality of these antibodies is also important

Functional *in vitro* assays, such as GIA, and ADCI, should be optimized and technically validated, and even if currently lacking validation of their predictive value, they could still serve as guidance for prioritizing candidates for further clinical development. In particular, with several constructs and formulations of the same antigen molecule being developed, such as the multiple MSP1 vaccine constructs, it is essential to have a means

[1] Molineaux L., Trauble M, Collins WE, Jeffery GM, Dietz K, Malaria therapy reinoculation data suggest individual variation of an innate immune response and independent acquisition of antiparasite and antitoxic immunities. Trans R Soc Trop Med Hyg. 2002 Mar-Apr96(2):205-9

of prioritizing the options. The influence of prior exposure to malaria on the immune response to a vaccine, and the need to differentiate the immune response as a result of prior exposure and the maturity of the immune system in infants was discussed at length. Some participants supported the view that previous exposure, even ten years prior, might influence the immune response. It was noted that if an exposed population in a Phase 1b trial has a different or markedly inferior response compared to the response in a Phase 1a in a naïve population, this should raise concern and a re-assessment of the vaccine formulation.

It was agreed that a fundamental property required of a blood-stage vaccine is that these vaccines should limit parasite multiplication rates. It was therefore considered whether or not induced infection in non-immunes under tightly controlled conditions could offer a critical screening process for potential vaccines. In principle it was agreed that such a screen, essentially a Phase 2a challenge trial, would be desirable, and that a positive result (i.e. limitation of parasite multiplication rates and therefore delay to parasite appearance) would be a strong indication for proceeding with clinical trials of a vaccine candidate. However, it was noted that this system has not yet been validated, and is limited in certain respects (the reliance on a single parasite strain and probably unnatural levels of challenge). In addition, it is likely to be unable to illustrate effect on density-dependent protective immune effector mechanisms. This means that for evaluating vaccines like MSP1, and AMA1 (which are believed to exert their protection via antibodies directly inhibiting parasite invasion of red blood cells), a Phase 2a challenge would be appropriate, provided adequate immune response quantitively and qualitatively in volunteers was elicited, preferably after a Phase 1a trial shows clear-cut evidence of in *vitro* GIA activity by vaccine-induced antibodies. However for antigens thought to exert an indirect effect via an ADCI like mechanism, where it is likely that parasitemia must reach a critical or threshold level before an ADCI like mechanism takes effect *in vivo,* a Phase 2a challenge would not be appropriate. It might very well be that the threshold could be above the gold standard for treatment of volunteers, i.e. thick smear positivity.

A recent publication[1] described the design and conduct of artificial challenge of Dutch non-immunized volunteers, and it was concluded that further experience, refinement and validation of this model in field efficacy trials were required before it can be considered as a GO / NO GO decision point.

The initial population for a Phase 2b trials was discussed in terms of whether efficacy trials should initially be conducted in adults (where possible), or whether after age de-escalation, for primarily safety demonstration, in younger children. Concern over the importance to avoid co-stimulation of the immune system by pre-existing parasitemia was discussed, and it was noted that in the Papua New Guinea trial of combination B vaccine and an earlier Spf66 study, pre-existing parasitemia did not impair immune responses to the vaccine. A solution to this question could be a stratified study with children treated prior to immunization, and another arm with no prior treatment, to

[1] Hermsen CC, de Vlas SJ, van Gemert GJ, Telgt DS, Verhage DF, Sauerwein RW. Testing vaccines in human experimental malaria: statistical analysis of parasitemia measured by a quantitative real-time polymerase chain reaction. Am J Trop Med Hyg. 2004 Aug.71(2):196-201

demonstrate whether or not existing asymptomatic parasitemia is a problem (this will also have implications for the practical application of any vaccine). Likewise, the possible unwanted immunological effect of concomitant worm infection could be solved by stratifying a child population by prior or concomitant de-worming. There was general agreement that presumed interference with the immune response to immunization from concomitant infections or infestations should be studied.

There was general agreement that the primary end point in the assessment of efficacy of blood stage vaccines would be clinical attacks of malaria. A clinical attack of malaria is the consequence of a new infection. Therefore this end-point should measure the reduction in the expected number of clinical malaria episodes, which might be reflected in the delay of first episodes after immunization, or the number of recorded clinical episodes over a pre-defined period, such as over one or two rainy seasons. The sensitivity and specificity at various parasite densities in defining clinical attacks of malaria were debated. The opinion that the actual medium resting parasite density, instead of the number of positive children in a given population, would be more predictive of the risk of severe disease in the following months was presented with supporting data resulting from results from six surveys. It was also emphasized that high specificity is essential to ensure a study with enough statistical power.

An example of a secondary end point could be anaemia, if the children were immunized during the dry season before transmission. It has been recorded in Ghana that 24% of children, age twelve – twenty four months, suffered from severe anaemia after the end of the rainy season, whereas only 2% of the children suffered from anaemia at the start of the rainy season, i.e. anaemia could be a good biological marker. Secondary end points could also be reduced parasite rate, or reduced mean parasite density, and in public health terms, and reduced hospitalization.

Coming back to the issue of studying the impact of prior exposure to malaria, it was suggested that, not only could one assess vaccine immunogenic and subsequent efficacy in a “horizontal manner” by moving from high to low transmission areas, but that same could be studied in a “vertical manner” with genetically homogenous populations living at various levels above sea level. The results of the latter design might be more influenced by migration than the horizontal design.

2.3. Parasite Density as an End-point

(Contributors: Brian Greenwood, Blaise Genton, Tom Smith, Alan Saul, Christophe Rogier; session chaired by Kevin Baird)

The 1997 guidelines recommend five primary efficacy end-points for field evaluation of malaria vaccines. These are parasitemia, clinical disease, severe disease, direct malaria deaths and total deaths. These end-points are the ones of public health interest but are essentially remote from the molecular biological activity targeted by the vaccines. Parasite density and parasite density threshold are recommended as important secondary end-points in trials.

2.3.1 General Considerations

Parasite growth rates and disease severity have been observed to be reflected by changes in parasite density in various studies, and evaluating vaccine impact on this easily measured and fairly accessible end-point could provide useful data on a vaccine's biological effect. Extrapolation from this effect to its impact on clinical disease for trials will depend on clear evidence of correlation to disease end-points which will then demonstrate the relevance and utility of this end-point.

The relationship of parasite density and clinical malaria was first demonstrated by *Field and Niven*, in 1937. These observations showed rising mortality from malaria from approximately 4% to 20% to 60% from low (5,000-25,000 per ml), moderate (250,000-500,000 per ml), to high (>500,000 per ml) parasite density levels. More recent studies have also shown this, such as in a case control study conducted in Ghana(From *Koram et al*. ASTMH 1995;89:151), which observed a doubling of parasite density levels in cases of mild disease(22,000 per µl) compared to cases of severe disease (49,000 per µl).

Another study observing a correlation between parasite density levels and morbidity was at a health centre in Papua New Guinea, where the risk of reporting to the Kuningini Health Centre with a malaria attributable episode increased steadily for all age groups with increasing levels of parasitemia. (From *Smith et al.,* Parasitology 1994; 109:539-549).

The relationship of fever and parasite density was observed in a study where for parasite density of >10,000 per µl, prevalence of fever was 33 %, and an inverse relationship was seen with asymptomatic parasitemia. Supplemental Vitamin A was shown to reduce both incidence density of clinical attacks as well as parasite density by 31% and 32% respectively (*Shankear et al* 1999, Lancet). In Tanzania with the SPf66 vaccine, a 20% reduction in parasite density was observed in conjunction with a 30% reduction in malaria specific morbidity (*Alonso et al* 1994, Lancet).

The relationship between parasite density and anemia in children has also been observed. In a study of Kenyan children from the ages of two to thirty-six months studied by

McElroy et al (Am. J. Trop. Med. Hug 2000; 62:504-512), it was found that across all age groups, anemia worsened with increasing levels of parasite density.

Despite these examples and that from other studies, a reliable and predictable correlation between parasite density levels and malaria morbidity and mortality indices is still lacking. As it stands, current evidence of this correlation is not conclusive enough to support parasite density as a surrogate for clinical disease, that could be used as an efficacy trial end-point. However the measurement of parasite density in clinical trials as a secondary end-point is essential as it still has the potential of providing useful information on a vaccine's biological effect, and as more information is generated from clinical trials, the relationship to clinical disease may become better defined.

In addition, parasite density is also a component of the often used clinical case definition of a 'malaria episode'. A parasite density threshold - the parasite density above which an associated fever is generally attributable to malaria - is used to increase the specificity of case definitions in trials in highly endemic areas.

Given the limitations of current knowledge on the inter-relationship between parasite density and the clinical outcomes of malaria attacks, severe disease and death, a systematic review and analysis that includes results from other trials of other malaria interventions such as insecticide-treated bednets and IPTi could help in better defining this inter-relationship, and highlight other potentially valuable end-points for future trials.

2.3.2 Method for Detection

Due to the life-cycle of asexual parasites, and parasite pathology in the mammalian host, parasite density levels are subject to fluctuations as well as sequestration (contributing to fluctuations in the measurement of the parasites in the peripheral circulation), presenting biological and technical problems when it comes to accurate measurements that reflect the actual parasite burden.

Four methods of measuring parasite density were discussed. The first two methods, involves obtaining an accurate measurement of either a white blood cell (WBC) or red blood cell (RBC) count, and then determining the percentage of infected ones. Although accurate, these methods require a costly automated machine, the Coulter counter, and are not practical or accurate with low density parasitaemia. The third method requires measuring the number of parasites per WBC in thick film, and then assumes a fixed WBC count in the population in order to convert the measurement from parasite per WBC to number per volume of blood. This method does not require a measurement of RBC or WBC, and could detect low levels of parasitaemia. However, adoption of an arbitrary white blood cell count (usually 8,000/ul) may introduce a substantial level of inaccuracy in the measurement of parasite density as the actual white cell count in a random population of African children can vary from 2,000 to 20,000, causing a potential 3-4 fold error in the determination of parasite density if this method is used. The fourth method, counting the number of parasites per high power field, is simple and, as for the third method, does not require a measurement of RBC or WBC. Calculation of parasite density is based on the assumption that detection of an average of one parasite per high power field represents a parasitaemia of approximately 5,000 parasites per ul. This calculation is based on the assumption that the volume of blood taken to produce the thick blood film examined is approximately 10 ul. The range of blood volumes used by

experienced field staff to make thick films rarely varies from the range of 8 – 12 ul so the inherent error in this method is less than that for method three. However, whenever accurate determination of parasite density is required, method 1 or 2 should be used.

Other alternative methods of measuring parasite density are antigen assays and quantitative PCR. These can play an important role in rapid evaluation of high numbers of blood samples in large field trials but there is a need to standardize and validate the PCR method if this to be used as a quantitative end-point for a vaccine trial. . Given the usefulness of measuring parasite density both as a potential end-point and as part of a case definition of clinical malaria, it is essential that the short-comings in measuring parasite density be addressed, particularly regarding efforts to standardize all related procedures.

2.3.3 Trial Design and Statistical Considerations

2.3.3.1 Trial Design

Given that any study designed to obtain early proof-of -concept of potential vaccine efficacy using parasite density as an end-point must be take into account the validity of such an approach (i.e. relationship to clinical disease) as well as the difficulties determining the threshold, the question remains as to whether parasite density data analysis will be conclusive enough for decision-making on further efficacy studies.

These biological 'proof-of-concept' trials should be designed as a randomized, double-blind, placebo controlled study, and it was discussed that phase I trials for safety in children in the field could be powered for detecting differences in parasite density levels between vaccine versus control groups without much of an increase in sample size. This in effect was the design of the Phase 2b trial of the 'Combination B' vaccine - as a larger safety trial, powered to explore the biological effects of the vaccine. Key determinants to consider in the design of such a trial are -:

- ***Level of malaria transmission*** - Areas of reasonably high endemicity with either seasonal or perennial transmission will ensure an adequate challenge that allows for the smaller sample sizes and shorter duration trials. The level of transmission is also greatly affected by seasonality and a different pattern of immunity is likely compared to perennial transmission.
- ***Target age group*** - Age is a major factor affecting the outcome of the host-parasite interaction. In a study in Senegal, which looked at host factors affecting delay of reappearance of parasites after treatment with quinine, the first cases of new patent infections occurred 3 weeks after treatment in age groups 1-2 and 3-6 years old, without any significant differences in incidence between the two groups (*Sokhna et al* Am J Trop Med 2000/2001). The younger children showed higher cumulative incidence in a shorter space of time than the older children. In the older children, the incidence rates rose more steadily between 2-5 weeks after. Those above age 15 years resisted challenges much better; these differences are likely due to high levels of anti-parasitic immunity acquired by individuals continuously exposed to intense perennial transmission.
- ***Case definition of clinical episodes*** -The proportion of asymptomatic

parasitemias can be high in areas of moderate to high transmission. A case definition will require comparison between clinical malaria and controls in terms of parasite density levels in order to achieve high specificity (minimize inclusion of false positive cases- i.e. non-malarial cases with concurrent parasitemia) and high sensitivity (detect true positive cases with minimal omission)

- ***Baseline surveillance*** - The duration of surveillance, will depend on the expected case definition and in general, 2-4 months, is sufficient for collection of parasite density information. The higher the intensity of surveillance, the better the parasite dynamics can be explored. Once the parasite dynamics and characteristics of clinical malaria in the group are known, a threshold could then be set for parasite density. The trial case definition is then determined and sample size calculated. For the trial, a baseline pre-vaccination sample is essential followed by subsequent bi-monthly samples after vaccination regimen is completed.
- ***Potential confounders*** - The complexities of host-parasite interactions and its outcomes make it critical that the design take into consideration potential confounders, such as age, bed-net use, sickle-cell trait, study period, and pre-treatment parasitemia levels. Host genetic factors and individual differences in exposure to transmission must be controlled during the trial.
- ***Pre-treatment for parasite clearance*** - The current guidelines recommend that the investigation of the impact of malaria infection and of treatment on a vaccine's immunogenicity should be conducted as part of Phase I trials. Pre-vaccination parasite clearance is routine for pre-erythrocytic vaccine trials. However, the rationale for doing so in blood-stage vaccine trials is debatable. Two main concerns regarding the effect of pre-trial parasite clearance are:-

1. **Effect on morbidity**

- Does parasite clearance before transmission season result in a loss or attenuation of premunition, and therefore an increased risk for disease episodes?
- Does it result in decrease risk of disease episodes, especially if the drug used has a long half life?

2. **Effect on immunogenicity**

- How does pre-treatment affect the blood-stage vaccines immunogenicity?

In Combination B trials in Papua New Guinea, where geometric mean parasite density was the study end-point, the differences in parasite density between vaccine and placebo groups were only detected and significant in the untreated cohort. In the groups that were pre-treated, it led to under detection of cases and subsequent loss of power to detect any differences between the groups.

In a randomized trial in Mali, that compared the impact of pretreatment on new infections and disease (case definition - predefined signs and symptoms consistent with malaria and any parasitemia), sulfadoxine-pyrimethamine (SP) treatment delayed the median time to first infection by approximately 30 days. After this, the incidence of disease surpassed that in the untreated group. However, there were no signs of overall significant reduction

of clinical malaria incidence as the age-specific cumulative incidence rates were similar in both treated and untreated groups. (*Coulibaly et al,* Am J Trop Med Hyg. 2002).

In a holoendemic area in Navrongo, Ghana, *Owusu-Agyei et al* TM & IH 2002 looked at the effect of pre-treatment on symptomatology. They compared symptomatic (reported/documented fever, chills, headaches, nausea, dizziness, myalgias) subjects with any parasitemia between treated and untreated cohorts (cohorts were enrolled separately, one year apart), and found that the symptoms were overall more frequent in the treated versus untreated cohorts.

Randomized trials of intermittent preventive treatment for malaria in infants, IPTi, have also observed significant reduction in incidence of clinical malaria and fever in treated versus untreated groups.

In unpublished data from a randomized controlled trial of the SPf66 vaccine in Tanzania looking at the effects of pretreatment with SP prior to vaccination in 38 children ages 1-5 years, it was found that the treated group had lower antibody response to the vaccine, and this was significant particularly for IgG3 responses. However in the trial of the Combination B vaccine, there were no observed differences of antibody responses to the three components of the vaccine between treated and untreated groups. A slightly reduced interferon-gamma response was observed to the MSP1 component in the treated group.

In terms of possible effect or interaction with immunogenicity of the vaccine on trial, although there remains limited information on the interaction of anti-malarials with efficacy of EPI vaccines, several studies have observed a lack of difference in the seroconversion rates to EPI vaccines between treated and untreated groups. (e.g. in randomized trials of intermittent preventive treatment for malaria in infants, IPTi).

It should also be noted that the meeting discussed the issue of pre-treatment with SP, and the potential impact of other anti-malarials on immunogenicity was not discussed. In fact this issue is being explored through the on-going trials within the IPTi consortium, with regards to other widely used anti-malarials such as amodiaquine, artesunate, Lapdap and mefloquine.

In conclusion, current research suggests that for pre-treatment with SP, that-:

- SP treatment did not affect antibody responses;
- SP treatment slightly reduced IFN-g responses to MSP1;
- SP treatment reduced dramatically the power of the study to detect effects of vaccine on parasite densities;
- parasitaemia on the day of immunization did not decrease immune responses, [reverse effect];
- parasitaemia on the day of immunization did not affect vaccine efficacy.

Finally, a pragmatic approach should be taken when deciding whether or not to have pre-treatment parasite clearance in vaccine trials, and take into consideration the advantages of assessment of vaccine effect in a situation that closely approximates reality in the context of future implementation. For example, such systematic treatment is unlikely in an EPI program. Furthermore, efficient, short-acting and cheap schizonticides are

unavailable. It appears that for the assessment of blood-stage vaccines, where the epidemiological background can significantly influence vaccine efficacy, pre-treatment unnecessarily confuses this assessment. Therefore, it may be preferable to avoid such treatments in field trials of blood-stage vaccines.

2.3.3.2. Statistical Considerations

In determining the statistical aspects of a trial designed to detect valid differences using parasite density as an end-point, it was pointed out that parasite density is a continuous variable, and should reflect changes over time, which can be rapid. It has the potential to provide more information as opposed to a binary variable like prevalence which could merely reflect presence or absence of parasites at a given time. The sample size needed to demonstrate a given efficacy is much smaller with continuous variables as outcome measures. For a vaccine trial that uses parasite density as the trial end-point, the sample size required would be in the tens of volunteers compared to a trial with the end-point parasite prevalence which would need 50-100 volunteers. (*Annex 3. Efficacy outcome variables for malaria vaccine trials and their statistical properties, Tom Smith*)

Individual parasite density levels are subject to rapid changes, so each trial participant should be sampled several times and the measurements averaged. The simplest method to measure this effect is by taking the geometric mean of the positive samples for each individual. As the levels of positive samples per individuals may vary, the results need to be weighted differently. A more statistically efficient approach would be a random effects model that partitions variation into group effects, individual effects and within-individual effects. Another consideration discussed in Annex 4 is the advantages of using the arithmetic mean instead of the geometric mean.

With respect to slide/PCR negative results, and how these should be considered, these apparent negative samples in effect are either true negatives or sub-patent samples. Sub-patent samples should be included in the analysis, and true negatives samples not. However, a more conservative approach would be to take all the negatives as true negatives, as was done in the Combination B trial.

A problematic situation could arise if the results show that parasite prevalence is higher in one group but the parasite density is higher in the other group. One possible solution to this interpretational dilemma will be to calculate the overall measurement of parasite burden for each group - the arithmetic mean parasite density, and then perform randomization tests to assess the statistical significance of the difference. Annex 4 (*Analysis of parasite density as a continuous variable: use of arithmetic mean over geometric mean, Tom Smith*) provides further explanation on the advantages and limitations of the arithmetic mean compared to the geometric mean. However, some drawbacks to this are that arithmetic means have distribution problems, as low densities have less impact. Furthermore, the number of observations in each case needs to be the same when using arithmetic means.

Underlying these statistical considerations and essential to improving comparability of field trial analysis, standardization of data collection methods should also be addressed, including the frequency, timing and duration of sampling.

2.3.4 Discussion

The meeting participants discussed other approaches that have been taken when considering parasite density as a trial end-point and potential ways to decrease the size and duration of efficacy trials.

In trials conducted in Mali, parasitemia was used as a discreet variable and outcome measure was the proportion of subjects crossing a defined threshold of parasitemia. In this study, this defined threshold was parasitemia in an 8 year-old, which corresponded to 0.7 attacks per person year. It was proposed that this method could be relevant as it assesses vaccine effect on higher levels of parasitemia, which is presumably where blood-stage vaccines should have an impact in order to be truly protective.

However it was pointed out that the exclusion of unused data from subjects below the given threshold gave the study less power. The determination of the threshold could have an impact on the efficacy assessments.

On the idea of exploring ways to conduct smaller efficacy trials, it was suggested that an intense follow-up program of a trial conducted in a homogeneous population would be an ideal set-up for such trials. Highly trained, "live-in staff" could follow-up clinical attacks, defined as fever with parasite density threshold of X per µl. Data based on 10 years of experience of daily follow-up of African populations in Dielmo and Ndiop, two villages in Senegal, was discussed where this has led to detailed characterization of the population in terms of transmission levels, number of episodes, time to first episodes, clinical malaria attack rates, P. falciparum prevalence rate and the parasite density rates. The data and set-up can be used for planning these small trials.

(*Annex 5. Phase 2b efficacy trials for screening candidate asexual stage malaria vaccines- Christophe Rogier*)

However, it was pointed out that, the trial design needed to take into account that the use of a threshold could possibly lead to statistical imbalance and that this baseline imbalance could cause a serious analytical problem in very small trials. Therefore sample sizes might need to be increased to overcome this possibility.

The relationship of parasite density to a clinical outcome measure was discussed in terms of how this end-point can be validly used as a proxy measure of protection against disease. The determination of the relationship between biological effect and clinical disease still required further study and analysis of past trial data. Some suggested that although the relevance of an end-point to clinical disease was critical, in view of the potentially large number of candidates, the lack of correlates of protective immunity, and the diverse considerations in conducting large-scale trials in children, the measure of biological effect could still be important preliminary information, and help in guiding decisions.

2.3.5 Conclusions

- The correlation between parasite density and risk of disease in different populations has been observed in multiple studies. It was generally agreed that evaluating a blood-stage vaccine's effect on parasite density levels provided useful information and should be done in malaria vaccine trials. However, age, threshold levels and

epidemiological background remain unpredictable confounders of this correlation and an exact, predictive relationship is not known. Therefore parasite density cannot be clearly used as a proxy measure or valid surrogate for clinical disease and current evidence does not support using it as a primary end-point, but as a secondary end-point in Phase 2b and 3 trials. However, in specific endemic circumstances, where preliminary efficacy is sought and at a site with well-characterized parameters, initial, small, Phase 2b trials using parasite density as a primary end-point could be conducted. Given the confounders previously mentioned, these trials should be conducted in populations that are as homogeneous as possible with regards to the potential confounding factors. Follow-up larger trials with clinical efficacy end-points could then provide the opportunity to validate this approach.

- Microscopy remains the gold standard method of measurement. In terms of read-outs, parasites per high power field may yield far less variation than parasites per 200 white blood cells. The parameters of microscopy read-outs in terms of numbers of microscopists and limits of the readings should be clearly defines and if possible standardized between trials. The lack of highly trained field microscopists and the need for training more microscopists were expressed by the participants. In some instances, such as large-scale field trials, PCR may be considered an alternative.
- Individual fluctuations in parasite density levels must be considered when deciding on the end-point measurement. The preferred and simple measure is the geometric mean for parasite density as a continuous variable. An alternative suggestion was the arithmetic mean which ahs several advantages over the geometric mean in some situations (Annex 3). Other alternative approaches were discussed, and for each, baseline imbalance caused by exclusion of relevant data from those below the 'set' threshold could impair the analysis, if not considered.

2.4. Anaemia as an End-point.

(Contributors: Kevin Baird, Paul Milligan; session chaired by Marcel Tanner)

Infection with *P. falciparum* is among the major cause of childhood anemia in malaria-endemic areas. In epidemiological studies, a strong correlation has been reported between the incidence of severe anemia, age-specific rates of anemia, and the intensity of *P. falciparum* transmission. Severe anemia often complicates clinical malaria in young children in areas of intense transmission, increasing the risk of blood transfusion and death. Given that anemia is a component of clinical disease with significant impact on morbidity and mortality and that it is easily measured, it should be explored as a marker for clinical disease in malaria vaccine trials.

2.4.1 General Considerations

Several studies looking into the relationship of anemia and malaria in young children six to 24 months old exposed to seasonal, intense transmission conducted by the Navrongo Health Research Center, Naval Medical Research Centre, and the Noguchi Memorial Institute of Medical Research in Accra, Ghana, were discussed.

The study site, Kassena-Nankana district of northern Ghana, had well-documented rainfall records with the peak wet season being July to September period. Child mortality rates increased four-fold from the last month of the dry season to the month with the highest rainfall. Wet and dry season cohorts (corresponding to high and low transmission periods) were enrolled in May and November respectively.

In a study looking at incidence density of the malaria attack rate following radical cure (quinine sulfate, sulfadoxine-pyrimethamine, primaquine) for parasitemia, the cumulative incidence of any parasitaemia by *P. falciparum*, and of parasite density greater than 20,000/µl, in infants and small children started in May and increased steadily until February, when they both levelled out. The incidence density of infection then decreased moderately during the dry season until the end of the season, when there is an abrupt drop of transmission.

The incidence density of parasitemia was significantly higher for the wet season than for the dry season (7.11 per person year versus 4.71 per person year). In another study measuring hemoglobin (Hb), malaria prevalence, and anthropometric indices of 6-24 month-old infants and young children at the end of the high (May–October, n = 347) and low (November–April, n =286) malaria transmission seasons, a very high prevalence of anemia (<Hb 6.0 g/dL) was observed at the end of the wet season, corresponding to the high malaria transmission season in the district.

Among this randomly selected cohort of children, anemia prevalence was 22.1% at the end of the high transmission season compared to 1.4% at the end of the low transmission season (OR 20.1; 95% CI: 7.1–55.3). Prevalence of parasitemia was 71% and 54.3% at these time points (OR 2.1; 95% CI: 1.5–2.9). In addition, the proportion of children with

Hb >6.0g/dl and parasitemia was 67.9% at the end of the high transmission season and 53.6% in the low transmission season. Similarly, differences were observed for proportion of children with fever; where the prevalence was higher in the high transmission season (10.8% vs. 3.3%). Prevalence was also higher for febrile children with severe anaemia; 43.2% versus 0% between the high transmission and the low transmission seasons. Many children seen at the end of the high transmission season had hemoglobin values below 6 g/dl, while all of those with Hb > 12g/dl were seen after the low transmission season.

The study also compared mean hemoglobin levels and average parasite density among children seen at the end of the high transmission season and low transmission season. In all categories of parasite density, mean hemoglobin values were higher at the end of the low transmission season compared to the high transmission season.

In one study, the number of blood transfusions as a marker of severe anemia was explored. The monthly number of transfusions showed 55 to 80 transfusions in October, and 15 to 20 transfusions in November for infants ≤ 24 months and 25-60 months respectively. These levels diminished substantially until August, September and October of the following year, when they rose again, although not to the levels of the previous year. The high rainfall season correlates with the high transfusion periods. The number of transfusions for young children less than 25 months was almost exclusively two to five times higher than for children. The highest number of transfusions was given to infants between the age of 5 and 12 months with little difference between genders.

Nutritional anemia appeared to have little impact upon this seasonal difference since anthropometric indices were comparable between seasons.

It appears from these studies that severe anaemia will develop in 20% of children, aged 6 – 24 months, if they were exposed to infection for 24 weeks or more. Anaemia was not tested after 9 more weeks of dry season, but a 16 week pause in transmission was sufficient for severe anaemia rates to decline to <2% in this age group.

These Kassena-Nankana District studies were summarized as follows-:

- severe anaemia rarely occurred with the first parasitaemia after radical cure in any season;
- the months of September, October and November, (as the rainy season closes) were the highest risk.
- children aged 6 to 14 months appeared most at risk of severe anaemia;
- hospitalization for transfusion followed sharp seasonal peaks linked to rainfall;

Overall, moderate to severe anaemia is induced after chronic exposure to parasitaemia and this may be linked to parasite induced suppression of hematopoesis.

2.4.2 Method for Detection

Many laboratory methods are available for measuring anaemia. For trial purposes, it was suggested that the most practical, objective and quantitative method is the portable hemoglobin spectrophotometer, which uses reagent-loaded microcuvettes. The hand held HemoCueÆ, was considered an affordable (approx. $800) example of this methodology.

The HemoCue@ takes a small sample of blood from the end of a finger and gives a clear hemoglobin level within seconds. The measurement of haemoglobin was preferable to measurement of packed cell volume (PCV).

2.4.3 Trial Design and Statistical Considerations

The advantage of anaemia as an end-point for phase 2 trials of blood stage vaccines is that anaemia represents cumulative effects of malaria exposure over a period of time, and can be measured at a single point at the end of a period. However, there are practical problems related to multiple and frequent sampling that may be required when using anemia as an end-point that needs to be addressed.

A single point measurement avoids the problems of interference caused by active surveillance. The major disadvantage is that a negative result will be difficult to interpret, because if the intervention has no impact on anaemia, it could be either that the intervention does not protect against malaria, or that the anaemia is not caused by malaria. Other confounders include the use of ITNs, which may decrease anaemia related to malaria and there may be little scope to demonstrate additional benefits from vaccination. In addition, the time scale of changes in haemoglobin (Hb) levels is relatively long so this endpoint may be insensitive for detecting short-lived vaccine efficacy.

The sample sizes required will depend on the case definition of anemia. The definition of severe anaemia is somewhat arbitrary. Hb<8g/dl ("severe"), corresponding roughly to the mean minus 2 standard deviations, and Hb<5 ("very severe"), corresponding roughly to the mean minus 4SD, are often used. Moderate effects on mean Hb can be detected with relatively small sample sizes. Much larger sample sizes are required if severe anaemia is the endpoint. Measuring the effect of the intervention on the mean Hb in a trial can give an indication of the likely impact on the prevalence of severe anaemia, assuming Hb follows a normal distribution. However this could be misleading should the intervention has a particular effect of improving Hb of the most anaemic cases, and where there could be a real improvement in severe anaemia but relatively little impact on the mean measured. It was proposed that existing datasets could be examined, to see in practice how well changes in mean Hb predict changes in the prevalence of severe anaemia.

Mean Hb is useful as a phase 2 endpoint particularly in areas of seasonal transmission where the seasonal drop in Hb is clearly associated with malaria. In a trial, subjects can be enrolled and randomized at the end of the dry season, and then the seasonal drop in Hb or PCV compared between vaccine and control groups. Data from the Gambia shows that the greatest drop in Hb is seen in children under 2 yrs of age, but the magnitude of the drop is reduced in children sleeping under ITN's, and in children kept under weekly surveillance for malaria episodes. Frequent contact with trial subjects for measurement of other endpoints can therefore interfere with the measurement of effects on Hb, since trial staff will be obliged to treat malaria cases and anemic cases.

Although assessing the impact of an intervention on anemia is often thought of in terms of comparing the fall in Hb between the start and end of the trial, between the intervention and control group, this is not the most efficient method of comparison. It is preferable to compare the post-intervention measurements, and adjust for the pre-

intervention measurement by including it as a covariate in the regression model. Subtracting the pre-intervention measurement serves to correct for baseline imbalance between the groups, but it overcompensates, because there will inevitably be a tendency for the large values at baseline to be somewhat smaller at the second measurement, simply due to measurement variability.

Repeated longitudinal sampling has statistical advantages which have to be balanced against the impact of multiple visits with the subjects and the benefits of the information gained. If a baseline (pre-intervention) measurement is taken, there are substantial gains in precision but it will be necessary to act on the information obtained and provide treatment for severely anaemic children at the time. Similarly there are gains from taking repeated post-intervention measurements, where the statistical efficiency is improved, and if measurements are spaced, there is an opportunity to detect transient effects of the intervention. However, anaemic children will have to be treated at each visit and this may reduce the apparent impact of the intervention. Therefore, consideration should be given to a design where observations are restricted to one or two carefully timed cross-sectional surveys in the study period in order to gain the potentially important observation of impact by the intervention. on this end-point.

The gain in terms of sample size from a baseline measurement depends on the correlation between the pre- and post- measurements; a single baseline measurement reduces sample size proportionately to a fraction $(1-\rho^2)$ of the sample size that would be needed without the baseline measurement. For example, in a study in Farafenni in The Gambia, the correlation was $\rho = 0.55$, so that including a baseline measurement reduces sample size to 70%. This will be greater if the correlation is greater.

The statistical gain in efficiency from repeated post-vaccination measurements depends on the correlation between these repeated measurements. If the measurements are highly correlated there is relatively little gain in information. If the correlation is low the gain is greater, the marginal gain is greater when a baseline measurement is also taken, but there are diminishing returns and it is not worth taking more than 3-4 post-vaccination measurements. These conclusions are demonstrated in Table 1 which shows the sample size required in the different configurations, as a percentage of the sample size for the case when a single post-intervention measurement is taken.

Covariates such as age, bednet use and hemoglobin genotype may all impact study results, and need to be factored in the trial design and statistical analysis plan. For analysis purposes, there is a need to define how missing data would be handled, and how various end-points would be weighted.

Table 1. Sample size required with repeated sampling, expressed as a percentage of the sample size for the worst case (a single post-intervention measurement)

Number of post-treatment measurements	Without a baseline measurement	A single baseline measurement
1	100%	70%
2	85%	55%

3	80%	50%
4	78%	47%
5	76%	46%

Table 2. Example of size calculation

Mean Hb g/dl	SD	Difference (g/dl) that is considered important	Power (significance level)	Number per group with one post-intervention measurement	Sample size per group with one baseline and one post-intervention measurement	Sample size per group with one baseline and three post-intervention measurements
10.5	1.3	0.5	90% (0.05)	144	100	72
		0.75	90% (0.05)	65	45	32

2.4.4 Discussion

It was discussed that treatment given for anemia during multiple measurements after randomization would lower detection of anaemia to where all power to detect differences in the vaccine and control groups would be lost. Another factor that could influence the end-point was self treatment. In considering that self-treatment would bias effect towards zero, it was recommended that to overcome this, it would be preferable to have a larger sample size and less surveillance.

The issue of how to best adjust the data for the influence of treatment was discussed. It was noted that anaemia and clinical malaria are competing end-points in a trial. Usually children reaching an anaemic end-point would have been withdrawn from trial analysis for clinical malaria. This is an issue if anaemia is a secondary outcome versus having severe anaemia as a primary outcome. Another potential confounder was hookworm infestation, although it affects older school children and not infants. It was also confirmed that even with treatment for parasitemia, effects on haemoglobin levels can still be measured.

It was discussed that the impact on anemia could only be studied in young children as it is strongly age dependant. Children below the age of two years old are most at risk. In trials in Mali, the baseline Hb measurement of older children started higher although magnitude of the drop of their Hb levels increased with age. Overall, older children rarely presented with severe drops, but they were still apparent.

Anemia must also be considered in the context of the local endemic background, as the age distribution and features of clinical malaria would be different in different situations.

For the most part, it was generally agreed that anaemia should not be a primary end-point for phase 2b trials and that as an end-point, anaemia would be more useful once more is known about a vaccine and its effect in a community. Anemia can be a useful indication, (not a licensing, nor an obligatory guideline), but should not be a Go/No Go tool for decision-making as a primary end-point. It should be a necessary secondary end-point and should be a measurement of mean Hb rather than severe anaemia. It was pointed that

if Phase 2b trials are designed with the aim of looking for impact on clinical disease, anaemia needs to be measured.

It was also generally agreed that small scale trials could use Hb measurement or levels as a primary end-point in certain circumstances. In particular, if a vaccine has been proven to be safe, trials could be designed to look at the impact on this end-point. It was also agreed that to further refine and understand the usefulness of the end-point, the concept "clinically significant Hb levels" needs to be investigated further and clearly defined for trials.

2.4.5 Conclusion

- The measurement of haemoglobin was the preferred method for measuring anemia (i.e. compared to PCV) in trials. For trial purposes, it was recommended that such a device like The HemoCue@ which takes a small sample of blood from the end of a finger and gives a clear hemoglobin level within seconds, be used.
- It is also agreed that anaemia should be a secondary, and not a primary end-point in phase 2b trials. During discussions it was also agreed that there may be certain circumstances, like the one previously seen in Mali, when a series of shorter smaller trials could be conducted with Hb levels as a primary end-point. However, this should not be a general rule and should only be used in special circumstances, and only when it is known that a vaccine itself does not cause anaemia.
- Trials designed to explore if the intervention has an impact on anemia must balance statistical considerations which may require frequent sampling with the impact of treatment that will be required with detection of anemia, making assessment of the efficacy of the intervention in preventing anemia difficult.

It was recognized that anaemia could be a very useful tool at a certain point in the future in eliminating some vaccines and prioritizing others. In this instance, it was agreed that trials could be designed with anaemia as a primary end-point. However, the optimal design and conduct of these trials still need to be explored.

2.5. Breakthrough Genotyping as an End-point

(Contributors: Ingrid Felger, Blaise Genton, Tom Smith, ; session chaired by Robert Sauerwein)

The field of malaria molecular epidemiology has greatly expanded due to advances in PCR amplification of polymorphic ***Plasmodium falciparum*** genes.

In particular, antigenic diversity related to polymorphism is thought to be part of an immune evasion mechanism and there are unresolved questions regarding the potential impact of vaccination with vaccines based on highly polymorphic antigens on parasite sub-populations over the long- term. There is evidence suggesting that for polymorphic vaccines where only one allelic type is utilized, this may induce variant specific immune responses and selection of the non-vaccine type parasite.

Subsequently, parasite genotyping and analysis of parasite population dynamics are seen as potential outcome measures to further explore effect of malaria control interventions in trials, including vaccines.

2.5.1 General Considerations

Four main parameters can be used as molecular outcome measurements. These are listed below, along with the trials which have used them -:

1. ***Detection of selective effects*** - Anti-malarial drug trials have demonstrated selective effects of drug treatment on the frequency of resistant clones. Given the malaria parasite's antigenic diversity and genetic polymorphism, it is anticipated that selective effects of a vaccine will emerge. The Combination B trials in PNG demonstrated a reduction in the prevalence of infections caused by the alleles of the vaccine component (3D7-MSP2) and a reduction in the time taken to first clinical episode with the alternative allelic family (FC27). Other trials (RTS, S/AS02) have not detected selective effects.

   (Genton et al. 2002) Malaria vaccine trials Combination B, Papua New Guinea

2. ***Multiplicity of infection*** - This refers to the number of concurrent infections of P falciparum clones in one individual, usually detected by PCR. MOI appears to be dependant on age and endemicity and some evidence suggests a correlation with host immune status and infection status, although interpretation of this relationship can be difficult.

   (Smith et al. 1999) Trial of insecticide-treated bed nets

(Genton et al. 2002) Phase 2b trial of Combination B malaria vaccine, Papua New Guinea

(Beck et al 1997) Phase 2b trial of SPf66 malaria vaccine, Tanzania.

3. ***Prevalence by PCR***- The sensitivity and specificity of PCR-based methods are higher than microscopy based methods. This is especially true at low parasite density levels. This highly sensitive method will detect breakthrough parasitemia in a vaccine trial.

(Smith et al. 1999) Trial of insecticide-treated bed nets

(Genton et al. 2002)Phase 2b trial of Combination B malaria vaccine, Papua New Guinea

4. ***Infection dynamics*** - This refers to the rates of acquisition, and rates of loss of infectious clones. The ability to do this allows the estimation of average duration of infection.

(Smith et al. 1999) Trial of insecticide-treated bed nets

(Genton et al. 2002) Phase 2b trial of Combination B malaria vaccine, Papua New Guinea

The merozoite surface protein, msp-2, can be used as a marker gene. This highly polymorphic antigen has been used as a molecular marker to examine the relationship of complexity of infections with age and risk of clinical disease in various studies.

In studies where genotyping for infection and parasite dynamics is done, it is important to consider both age and endemicity of the host as this will affect the genetic polymorphism of the parasite. Thus any interpretation regarding parasite dynamics and in trials, selective effects of an intervention, must be done in this context.

Some of the suggested uses of molecular monitoring in vaccine trials were:

1. To assess effects on parasite population
2. To improve the design and components of a polymorphic vaccine
3. To improve the sensitivity of outcome measures by PCR on parasite prevalence rates and real time PCR for parasite density levels
4. To generate additional outcome measures that could provide specific information on a vaccine's biological effect, particularly regarding the rates of new infection
5. To study potential mechanisms of protection caused by the vaccine
6. To improve trial design and decrease the sample size (more frequent sampling from individual subject)

2.5.2 Method for Detection

In a comparison between data obtained from microscopy versus polymerase chain reaction, (PCR) for analysing parasite genotypes, PCR was observed to be more sensitive

and able to identify lost, new or changing genotypes. However, it also demonstrated imperfect sensitivity.

The level of MOI will also be a factor when considering the method of detection. In the Combination B vaccine trial, msp-2 genotypes were determined by PCR restriction fragment length polymorphism (RFLP). In an analysis of four individuals (2 participants each from the vaccine and placebo groups), a baseline reading and bi-monthly readings following vaccination resulted in a mean multiplicity of infection of 1.4, which is relatively low and therefore easy to calculate. The expected MOI in Africa is 5, making genotyping a more challenging and difficult option.

The trial also compared parasite prevalence rates by PCR with parasite prevalence rates by microscopy for efficacy estimates. In the non pre-treated group, the overall proportion of positive parasitaemia was significantly higher than in the pre-treated group, giving an efficacy of 47% and 18% respectively. Results by microscopy showed a 21.5% efficacy for non pre-treated subjects.

2.5.3 Trial Design and Statistical Considerations

2.5.3.1 Trial Design

In a Phase 2a trial, real-time PCR analysis would be able to study the kinetics of pre-patent parasitaemia (pre-patent period is the time period prior to microscopy-detected parasitaemia). In Phase 2b trials, parasite prevalence, multiplicity of infection and parasite dynamics could contribute to assessing mechanism of action or secondary effects of the vaccine. Genotyping could also help to understand the specificity of response of a vaccine. Larger efficacy trials (Phase 3 and Phase 4) could assess selective effects of polymorphic vaccines on parasite population.

The preferred design of a genotyping study is that of a randomized, double-blind, placebo-controlled study. The following outcome measures could be considered:-

7. Prevalence rate of all infections and new infections
8. Incidence rate of all infections and new infections
9. Rate of acquisition and loss of infecting clones
10. Multiplicity of infections
11. Incidence rate of clinical episodes with new infections

Ideally, a trial requiring frequent sampling will result in decreasing the sample size estimates. In children aged over one year there should be three bleeds to establish and characterize the baseline profile, before vaccine or placebo injections followed by bi-monthly bleeds. This will have to be balanced with the implications of frequent blood sampling in young children.

Some determinants that should be considered when designing trials with molecular monitoring are-:

- ***Genotyping profile of the parasite population -*** This is the background epidemiological data;
- ***Duration -*** Sufficient time points prior to vaccination must be considered to circumvent problem of sensitivity.

- ***Surveillance*** - Four months of post vaccination surveillance, (depending on the season), should be sufficient to estimate prevalence/incidence rates and dynamics of new infections. For clinical episodes, this should be extended to between six months to one year of surveillance.
- ***Intensity of sampling -*** Due to the rapid turnover of infectious clones in an infection, the intensity of sampling needs to be relatively high to pick up more events.

In the Combination B malaria vaccine trial, the primary end-point for pilot proof-of-concept was parasite density. Pilot-efficacy by genotyping was explored through molecular monitoring and determining the incidence rate of clinical episodes with new infections in vaccine versus control groups. Genotyping results showed a specific activity against the 3D7 parasite type but not against FC27 parasite type. The trial detected a reduction in the prevalence of infections caused by the alleles of the vaccine component (3D7-MSP2) and a reduction in the time taken to first clinical episode with the alternative allelic family (FC27).

The genotyping in the trial resulted in the following observations:

- Reduction of 3D7 parasite prevalence
  ⇔ Proof of concept of the effect of the MSP2 vaccine component
- Demonstration of strain specific immunity
- Demonstration of selective pressure of an efficacious vaccine on circulating parasites
  ⇔strain specific clinical episodes
- Improvement of the design of the next vaccine in that the next generation vaccine will include 2 components of MSP2 representing the 2 allelic families.

2.5.3.2 Statistical Considerations

Molecular monitoring of trials must ensure that appropriate statistical approaches are used for trial design and analysis of parasite dynamics. Any approach must allow for the 'imperfect detection' of current methods and some examples discussed were the immigration-death models and the hidden markov models.

Current methodology, whether microscopy or PCR, present the challenge of uncertainty due to imperfect sensitivity. Therefore a newly detected strain could be either new or lost. More baseline sampling may help to clarify this issue. Moreover, if smaller sample sizes are desired, regular bleeds of high-enough frequency per individual would be required to power the trial to detect effect on rates of acquisition, loss and duration of infection or assessment of duration of protection.

In order to conduct longitudinal parasite typing in vaccine trials, the following considerations were identified:-

- The need for a high- resolution typing system;
- The need for high-throughput typing methods due to the large sample sizes needed, hence;
- The need to measure and assess multiple baseline samples from each individual;
- The contribution of longitudinal parasite typing towards assessment of longer-term efficacy;
- The importance of further development of statistical methods for estimating efficacy, allowing optimally for sub-patent infections;

- The assessment of this monitoring as the method of choice for evaluation of incidence in trials of blood stage vaccines; and that it should also be applied in trials of pre-erythrocytic vaccines.

2.5.4 Discussion

The meeting participants discussed the potential usefulness of molecular monitoring to gather information on vaccine effects. It was generally agreed that although evidence from past vaccine trials highlight its importance as an exploratory end-point, data on correlation between breakthrough parasitemia and risk for clinical malaria was not conclusive enough to recommend parasite genotyping as a primary end-point.

Some meeting participants raised the concern over the resources that would be required to routinely conduct molecular monitoring of malaria vaccine trials in the field. Others raised concerns over the issue of frequency of blood sampling in each individual, and that this would be of particular concern in young children and infants. It was discussed and generally agreed that frequency of once every two weeks would be accepted but more frequent or daily sampling would raise difficulties regarding acceptability and complexity of analysis. The issues around lab techniques and methods of analysis are critical as it has an impact on how to properly assess this end-point. For example, the handling of multiple infections and minor populations of parasites require further discussion.

Participants also discussed the effect of pre-treatment with anti-malarials, where evidence implies that this will interfere with the interpretation of genotyping particularly if drugs used have a long half-life. In addition, comparison of genotypes at baseline and those collected during follow-up allows for the estimation of vaccine efficacy without pre-treatment.

2.5.5 Conclusions

- Parasite genotyping should be considered as an important secondary end-point in vaccine trials to detect selection of non-vaccine type parasites in the case of vaccines based on polymorphic antigens and explore possible changes in parasite subpopulations due to vaccination
- Parasite genotyping helps to determine and characterize parasite dynamics. The monitoring of acquisition and loss of clones and the complexity of allelic forms can provide additional information on mechanism of vaccine effect. It also can provide useful information on newly acquired infections.
- Laboratory techniques, sample collection and methods of analysis for molecular monitoring of trials need optimization.
- Parasite genotyping could eventually clarify the relationship between the parasite dynamics, multiplicity of infection, specificity and selective effect and the clinical end-points of clinical and severe malaria.
- Allelic complexity of a vaccine molecule differs and the functional consequences of this is yet unknown. This complexity and variability of the target gene impacts on trial measurement sensitivity and sample size. Sample size will increase with increasing allelic complexity.

2.6. Multiplicity of Infection as an End-point

(Contributors: David Conway, Pedro Alonso, John Aponte; session chaired by Ogobara Doumbo)

Multiplicity of infection (MOI) is the term used to describe infections that contain multiple *Plasmodium. falciparum* clones, or multiple different genotypes in a single individual. These infections are often due to super-infection, which is the acquisition and accumulation of multiple infections, from different mosquitoes, where individuals who have been bitten and are already infected, get bitten again and accumulate multiple infections. It has also been demonstrated that multiple infections are often also due to a single inoculation of multiple genotypes by one mosquito. This has been established due to the fact that some of these genotypes have been shown to be clearly sibling parasites. Examination of the multi-locus genotype profiles shows that they are identical. (*Conway et al.* 1991. *Druilhe et al.* 1997, *Paul et al* 1999, *Anderson et al.* 2004).

2.6.1 General Considerations

In order to explore the predictive value of MOI in determining the risk of malaria morbidity, various studies have looked at different ways to investigate the correlation between multiplicity of infection and a clinical end-point. Three case-control studies looking at MOI and correlation with mild versus severe malaria were discussed. In each study, multiple genotype infections were counted, and expressed as a mean number for different groups. No difference was detected between MOI and the groups with severe and mild malaria. (The Gambia *Conway et al* 1991, Senegal *Robert et al* 1996 and Gabon *Kun et al* 1998).

Community-based association studies have also compared MOI between asymptomatic malaria cases with symptomatic infections. In a cross-sectional study in Senegal, (*Zwetyenga et al* 1998), there was higher MOI in the symptomatic infections than in the asymptomatic carriers. However, other similarly designed studies conducted in different locations have found that a higher MOI was associated with a reduction in symptomatic infections (*Beck et al* 1997).

In a longitudinal study in Papua New Guinea (*Al-Yaman et al.* 1997), a higher MOI at a baseline measurement was associated with a decreased risk of symptomatic infection during follow-up. A recent longitudinal study conducted in Tanzania found that the prevalence of parasitemia and MOI increased with age. For children aged more than three years old, MOI tended to appear protective against symptomatic infections compared to

the inverse relationship observed in children aged less than three years old (*Henning et al.* 2004). Although the correlation between MOI and clinical malaria was not statistically significant in either group, the trend does suggest support for the idea that carriage of higher numbers of genotypes is associated with a lower risk of clinical malaria episode. Therefore, although current evidence does not broadly reflect a strongly consistent and predictive relationship between MOI and risk of symptomatic or severe malaria, it would appear that the marginal differences of risk seem to be age and transmission dependent.

The following vaccine trials did consider MOI as a secondary or tertiary end-point.

- ***Phase III trial of SPf66 in Tanzania***: There was no difference in MOI seen between vaccine and control groups in terms of symptomatic infections. However, for subjects with asymptomatic infections, MOI was significantly lower in the vaccine group than in the control group. In the control group, however, there was a significant negative correlation MOI (numbers of infecting genotypes, as identified by MSA2) and morbidity. Asymptomatic children had an average of 5 concurrent infections, whereas symptomatic children had an average of 3.4 infections. No such effect of multiple infection was found in the vaccine group. (Beck *et al.* JID1997;175:921-927)
- ***Phase IIb SPf66 trial in The Gambia***: There was no efficacy demonstrated against the primary end-point of P falciparum disease episodes. However, it was noted during follow-up that the MOI was lower in vaccinated group compared to the control group (Haywood *et al.* 1999);
- ***Phase IIb Combination B trial in Papua New Guinea***: No effect was detected in MOI (Genton et al. 2002);
- ***Phase IIb trial of RTS, S/AS02 trial in The Gambia***: Short-lived efficacy (14 weeks) was seen against the primary end-point of first positive blood film for asexual parasites. Surprisingly, overall MOI was significantly higher in the RTS, S/AS02 group than in the controls, 4.90 and 4.23, respectively (P= 0.05) (Alloueche *et al.* 2003). The MOI increased during the transmission season and was higher at the end of the season than at the beginning.

2.6.2 Methods for detection

Methods for detection and measuring MOI were discussed under non-PCR and PCR methods. Two specific non-PCR methods were discussed:-

- The Starch gel electrophoresis of isoenzymes method which has a low allelic resolution and low detection sensitivity. (Carter & McGregor 1973).
- The monoclonal antibody immunofluorescent serotyping of polymorphic parasite antigens (Conway et al. 1991). It has high allelic and haplotypic resolution but low detection sensitivity and requires parasite culture to the schizont stage.

For PCR based methods, various protocols for looking at the numbers of different alleles that hold polymorphic antigen genes that may be present within an infection are currently used. For the assessment of MOI, two PCR methods were discussed-:

- The typing of antigen genes with repeat sequence polymorphisms (e.g. *msp1,*

msp2 and *glurp*), by allelic size discrimination, restriction fragment length polymorphism (RFLP) patterns, and/or allele-specific probing (different protocols compared by Farnert et al. 2001, each simple and quite robust).

- The typing of micro satellites (shorter, simple sequence repeats occurring every 2-3 kb in the genome), by allelic size discrimination. (Standard protocols e.g. Anderson et al. 1999, universally applicable but some loci give minor 'stutter' bands).

2.6.3 Trial Design and Statistical Considerations

2.6.3.1 Trial Design

An example of an appropriate trial design was illustrated by the Phase IIb trial of RTS, S/AS02 in semi-immune Gambian men, where breakthrough genotypes and MOI were exploratory variables. Breakthrough *P. falciparum* parasites sampled from vaccinated subjects and controls were genotyped at two polymorphic regions of the *csp* gene encoding T cell epitopes (csp-th2r and csp-th3r), to determine if the vaccine conferred a strain-specific effect. Poisson regression was used to produce estimates of the vaccine effect on MOI, adjusted for the effect of time, village, and parasite density. The MOI values are presented as means with SD values.

The overall distribution of *csp* allelic variants was similar in infections occurring in vaccine and control groups. The mean number of genotypes was not reduced compared with the controls. Overall MOI was significantly higher in the RTS, S/AS02 group than in the controls, 4.90 and 4.23, respectively (P= 0.05). The ratio of control to RTS,S/AS02, which was estimated using Poisson regression and adjusting for effects of time period and village by including these variables as factors in the model, was 0.96 (95% CI = 0.81-1.13) for mspl, 0.84 (95% CI 0.71-0.99) for msp2, 0.80 (95% CI = 0.65-0.99) for glurp, and 0.88 (95% CI = 0.76-1.03) overall. The MOI increased during the transmission season and was higher at the end of the season than at the beginning.

2.6.3.2 Statistical Considerations

Statistically, MOI was described as a numeric variable. Therefore, non-parametric tests are used to test difference between groups. However, it is difficult to adjust for other possible confounding variables, such as illustrated by the RTS, S example, namely, time, parasite density, transmission intensity etc. Poisson regression can be used to estimate the effect of the treatment as well as adjust for multiple covariates, keeping in mind the need to still evaluate the assumption of the Poisson distribution.

Longitudinal analysis with multiple samples taken from the same individual requires a more complex analysis. However it could be approached using Poisson regression taking into account the non-dependency of the counts within each individual. For example, should participants require anti-malarial treatment because of symptomatic disease, the longitudinal analysis becomes difficult to interpret, as in addition to measuring the effect of the vaccine, it would have measured the effect of the treatment as well. It is possible to calculate a sample size with a given power to detect a significant difference between groups, using the Poisson distribution of the counts. However, this would require

knowing the relevant difference to use to calculate the sample size as well the effect of the negative samples on the interpretation of the MOI of those that are positive.

2.6.4 Discussion

The participants discussed the dependence of MOI on age of the host and endemicity. Protection from disease episodes appears to be conferred to older children, but not in younger children. For vaccine trials that aim to further analyse the area parasite population and measure the impact of the vaccine on parasite dynamics and parasite population through parasite genotyping, site baseline data must be established.

The relationship of parasite density and multiplicity of infection was also discussed. It was generally agreed that there was a positive correlation between parasite density and multiplicity which appears to decrease with the age of the host. It was noted that some studies have observed a correlation between parasite density and the proportion of infections of from the FC27 allelic family. (Henning et al, 2004; Beck et al, 1999)

Most participants felt that given the state-of-the-art of genotyping, MOI analysis requires resources and expertise that may stretch current field-trial capacity. It should be approached from a research perspective, and it was generally agreed that the information gathered from monitoring of MOI in vaccine trials had the potential to contribute to improved assessment of vaccine effect that may help improve vaccine and trial design.

2.6.5 Conclusion

- MOI should be used as an exploratory tool and not as a primary end-point in vaccine trials.
- MOI is not a surrogate end-point in Phase 2b trials. A direct and predictive correlation is lacking between MOI and clinical or parasitological end-points, and these can be simply measured without any requirement of MOI data. However, it is worth measuring as an exploratory variable, in order to detect subtle or unexpected effects of a vaccine, as with resources and expertise, the techniques have become routine and straightforward for small scale analysis.
- MOI should be explored as a way of better understanding parasite dynamics. As for breakthrough genotyping, given the concerns of potential effects of vaccine polymorphism, it could play a role in the detection of selective pressure by the vaccine and impact on parasite dynamics and parasite population. To this end, with regard to the proposed revision of the 1997 Guidelines, MOI should be addressed as a possible secondary end-point or exploratory outcome measure to be considered for future trials.

2.7. Clinical Evaluation Group: Conclusions

(Contributors: Vasee Moorthy, Kevin Baird, Robert Sauerwein, Ogobara Doumbo; session chaired by Marcel Tanner)

The meeting concluded with a presentation by the Malaria Vaccine Initiative of PATH on what was referred to as the Malaria Vaccinology Hypothesis in which key assumptions used when designing, developing and testing malaria vaccines are identified and the process for confirming or refuting the hypothesi in a stepwise algorithm is described below:

1. Set-up key assumptions/ Malaria Vaccinology hypothesis

   Identify & confirm or refute key assumptions used when developing and evaluating malaria vaccines.

2. Vaccine Component Evaluation

   Identify and characterize antigens and platforms for inclusion in malaria vaccines.

3. Product Development

   Determine the clinical efficacy of promising malaria vaccine concepts

For pre-erythrocytic vaccines - the antigen selection, vaccine design, and preclinical testing lead to evaluation in a Phase 2a challenge trial that is able to demonstrate some measure of efficacy in humans, before proceeding to larger clinical efficacy trials. For blood stage vaccine- the lack of a blood-stage challenge model requires the consideration if measuring biological/parasitological effects in small trials with these parasitological end-points can be used as some indicator of vaccine efficacy that will be confirmed in larger clinical efficacy trials.

Key assumptions underlying this algorithm are that:-

- Signals or trends observed in preclinical testing tools - humoral responses by ELISA and functional activity by GIA in mice, rabbit and monkey; and protective efficacy demonstrated in *Aotus* challenge trials - are 'predictive' of impact on parasitological end-points (effect then measured in Phase 2b 'parasitological' efficacy trial).
- An impact on “parasitological end-points” correlates to efficacy against clinical disease (effect measured in Phase 2b clinical efficacy trial).

Ideally, biological efficacy end-points around which to design a trial will be:

- A robust, reproducible method with acceptable variability
- Feasible to perform on small sample sizes(n <100)
- Measurable in the field
- Specific in order to detect true differences
- Known to correlate with end-points of public health significance

Biological effects as end-points can have other potential uses in the design of trials to optimize dosing, schedule or routes of vaccination or early exploration of efficacy of combination or multistage vaccines.

As mentioned during the meeting the issue of possible imbalance in baseline characteristics for small phase 2b trials when measuring biological end/points must be accounted for in trial design and analysis plan.

As more trials are conducted, the opportunity to gather more data on these end-points should be maximized, through efforts to develop a general agreement on a standard panel of secondary end-points such as parasite density, anemia, and genotyping to be assessed during all trials, as well as consensus on general standards for collection, sampling and analysis methods of these end-points.

The conclusions of the discussions on the parasitological end-points considered at this meeting are summarized below.

- For **parasite density**, although the correlation between parasite density and risk of disease in different populations have been observed in multiple studies, age, threshold levels and epidemiological background remain unpredictable confounders of this correlation and an exact, predictive relationship is not known. Useful information on a blood-stage vaccine's effect can be obtained by measuring parasite density levels and this should be in all malaria vaccine trials. Current evidence does not support using it as a primary end-point, but as a secondary end-point in Phase 2b and 3 trials. However, in specific endemic circumstances, where preliminary efficacy is sought and at a site with well-characterized parameters, initial, small, Phase 2b trials using parasite density as a primary end-point could be conducted. Follow-up larger trials with clinical efficacy end-points could then provide the opportunity to validate this approach. The measurement method of choice was microscopy and the importance of defining and standardizing the parameters of microscopy read-outs in terms of numbers of readers and limits of the readings was strongly emphasized. Statistically, the geometric mean for parasite density appears to be the simpler measurement, but the arithmetic mean should also be considered. For all, consideration must be given to the potential of baseline imbalance affecting the final analysis.
- For **anemia**, it was generally agreed that anaemia should not be a primary end-point for phase 2b trials and that as an end-point, anaemia would be more useful once more is known about a vaccine and its effect in a community. If Phase 2b trials are designed with the aim of looking for impact on clinical disease, anaemia needs to be measured. It should be a secondary end-point and should be a measurement of mean Hb rather than only of severe anaemia. It was also generally agreed that small scale trials could use Hb measurement or levels as a primary end-point in certain circumstances, such as when a vaccine has been proven to be safe, and then trials

could be designed to look at the impact on this end-point. At some future point, anaemia could be a very useful tool in well-designed trials aimed comparing vaccine performance to eliminate some vaccines and prioritize others. Confounding factors such as age, endemicity, treatment, hookworm infestation, and others must be adjusted for in the design and analysis plan of the trials. Anemia must also be considered in the context of the local endemic background, as the age distribution and features of clinical malaria would be different in different situations. The measurement of haemoglobin rather than packed cell volume or haematocrit was the preferred method for measuring anemia

- For **parasite genotyping**, although evidence from past vaccine trials highlight its importance as an exploratory end-point, data on correlation between breakthrough parasitemia and risk for clinical malaria was not conclusive enough to recommend parasite genotyping as a primary end-point. It should be considered as a secondary end-point in vaccine trials as the information gathered could help in assessment of vaccine effects and improve vaccine design. Parasite genotyping helps to determine and characterize parasite dynamics and could clarify the relationship between the parasite dynamics, multiplicity of infection, specificity and selective effect and the clinical end-points of clinical and severe malaria. Practical issues such as lack of resources that would be required to routinely conduct molecular monitoring of malaria vaccine trials in the field and concerns over the issue of frequency of blood sampling in each individual, were highlighted as potential limitations to routine molecular monitoring of trials.
- For **multiplicity of infection/ MOI**, it should be used as an exploratory tool and not as a primary end-point in vaccine trials. A direct and predictive correlation is lacking between MOI and clinical or parasitological end-points, and these can be simply measured without any requirement of MOI data. Given the concerns of potential effects of vaccine polymorphism, it could play a role in the detection of selective pressure by the vaccine and impact on parasite dynamics and parasite population, and should be explored as a way of better understanding parasite dynamics.

2.8. Clinical Evaluation Group: Recommendations

The meeting participants concluded that the primary efficacy end-points recommended in the *Guidelines for the evaluation of Plasmodium falciparum vaccines in the population exposed to natural infection.* (Geneva: WHO/TDR, 1997), which are parasitemia, clinical disease, severe disease, direct malaria deaths and total deaths were still valid and appropriate. The recommended primary efficacy end-point for blood-stage vaccines is incidence of disease.

It was recommended to WHO that further knowledge on clinical evaluation of malaria vaccines that have emerged since the publication of the guidelines should be incorporated into a revised and updated version of the guidelines. This update should address the parasitological end-points discussed at this meeting, and incorporate the meeting discussions.

The meeting participants recommended that an international collaborative working group be established that could address issues related to clinical evaluation of malaria vaccines such as:

- study design of efficacy trials and analytical methods

- Standardization of study methodology (i.e. parameters of microscopy read-outs in terms of numbers of microscopists and limits of the readings) and trial specimen collection methods, including the frequency, timing and duration of sampling. Standardization will improve comparability of trial results.

- sharing of clinical development plans, clinical lab and trial results and assistance in the conduct of trial-related activities.

This could lead to the development and sharing of ' best practices' that are accessible, compatible and harmonized in the clinical evaluation of malaria vaccines, thus improving comparability of field trial analysis,.

Annex 1. WHO MALVAC Committee members, 2004/2005

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List of Participants: Meeting II- Considerations in End-points and Trial Design of Phase 2b Clinical Trials of Blood-stage Vaccines

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Annex 3. Efficacy outcome variables for malaria vaccine trials and their statistical properties This table is intended as an aid to discussion at the WHO Technical Meeting on the Evaluation of Malaria Vaccines only. Not to be quoted (T. Smith, October 2004)					
Outcome	Type of variable	Sample size required	Surveillance required	Statistical methods	Statistical issues
Parasite density	Continuous variable, changes rapidly over time	Few tens of volunteers	Repeated parasitological sampling	t-tests, linear regression, ANOVA etc. (random effects models?)	Sub-patent infections must be treated as equivalent to no infection
Parasite prevalence	Proportion	50-100	Repeated parasitological sampling	Logistic regression (random effects models?)	Optimal analyses require models for longitudinal binary data
Haemoglobin	Continuous variable, assessed cross-sectionally, changes slowly over time	Few tens	Cross-sectional blood samples	See presentation of P. Milligan	Effects may take a long time to become evident (long follow-up)
Time to new infection (after clearance of parasites)	Time to event	50-100	Repeated parasitology after radical cure	Survival analysis (Kaplan-Meier, Cox-regression)	Informative only during the initial post-treatment period
Incidence of new infections (by molecular typing)	Incidence density	Several hundred samples; few tens volunteers	Repeated parasitological sampling	See presentation of T. Smith	Problem of imperfect detection
Duration of infections (by molecular typing)	Transition rates	?	Repeated parasitological sampling	In development	Problem of imperfect detection
Multiplicity of infection	Count	Few tens	Cross-sectional blood samples	See presentation of J. Aponte	
Incidence of clinical episodes	Time to event or incidence density	Small number of hundreds	ACD or PCD	Survival analysis	Case definitions are critical and are

					problematical
Transmission to the vector	Proportion (of vectors infected)	A few tens	Cross-sectional blood samples	Comparison of proportions	-
Incidence of severe malaria	Time to event	Several Thousands	PCD/and or CHW systems etc.	Survival analysis	Case definitions are critical
Malaria specific mortality	Time to event	District level	DSS systems/ Verbal Autopsy	Survival analysis	Verbal autopsy has poor specificity
All cause mortality	Time to event	District level	DSS systems	Survival analysis	-

Annex 4

Analysis of parasite density as a continuous variable: use of arithmetic mean over geometric mean

In trials aiming to demonstrate a biological effect of a malaria vaccine, it is tempting to use the parasite density as an outcome measure. It has the advantage of being a continuous variable, and a continuous outcome generally requires a smaller sample size to demonstrate a given percentage effect than does a dichotomous variable, such as e.g. presence or absence of parasites or densities above a given cutoff value. Parasite densities vary considerably over time in the same individuals, but a single value for each individual can be obtained by averaging.

In principle, if the intention is to assess the effect of vaccination on parasite growth, then it is desirable to exclude from the analysis those individuals who were never infected. These individuals have parasite densities of zero. However, individuals whose parasite density is positive but below the limit of detection, and also those whose parasites were controlled and eliminated, also have parasite densities of zero, and it would be desirable to include both these classes of samples in analysis of the effects of vaccination on parasite density. In practice it is impossible to separate the two classes of samples.

A conservative approach is to strictly separate the analysis of positivity from that of parasite densities- this lends itself to the use of the geometric mean as the measure of average parasite density for a set of samples. However, by separating out the two analysis, there is likely to be a significant loss of power. Alternatively, it is possible that such an analysis might indicate effects in different directions on the positivity and on the geometric mean density: in this case there will be an interpretational problem.

The use of the geometric mean has the advantage that the distributions of parasite densities are generally highly skewed, but the log transformed densities are often not so far from normally distributed, so that the log densities can be compared using conventional t-tests and analysis of variance. However geometric means have a number of unfortunate properties; (i) where multiple determinations from the same individual are included, it is not obvious how to combine the information for the whole sample, because the geometric mean of a set of geometric means is not the same as the geometric mean of all the observations. (i) the data for samples with sub-patent parasitaemia cannot be included with value 0, since zero values cannot be included in the calculation of the geometric mean.

The arithmetic mean is not generally used as the measure of location for skewed distribution because statistics based on normal approximations to the distribution are then invalid. However the arithmetic mean has some advantages as a measure of the average parasite densities (i) consistency: the arithmetic mean of the arithmetic means across all individuals is the same as the crude arithmetic mean of the data, providing each

individual is weighted equally.(ii) samples with zero densities can be included. Hence the measure efficiently combines the information from all the samples. (iii) the skewness of parasite density distributions ensures that the highest densities have the greatest influence on the measure of effect- and since these are likely to be the infections with the greatest epidemiological importance, this is a desirable property.

The problem with the use of the arithmetic mean is that, since this quantity will not be normally distributed, the usual statistical tests (t-tests, ANOVA) are not applicable. It would be worthwhile exploring the power of randomization tests for evaluating the effects of vaccines on the arithmetic mean.

Annex 5

Phase IIb efficacy trials for screening of candidate malaria vaccines

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This paper presents the rationale for a double-blind, randomised, placebo controlled trial to assess the preliminary efficacy of malaria vaccines on the incidence of morbid episode detected by active case detection in naturally exposed homogeneous populations. The design is aimed at reducing the sample size and shortening the duration of follow-up, so that numerous trials with sufficient power to detect a 30-50% impact on the incidence of malaria attacks can be conducted. The scheme is based on previous experience of daily follow-up of African populations over a period of more than 10 years to study the natural history of malaria (Trape J.F., et *al*. Am J. Trop. Med. Hyg., 1994, 51: 123-137; Trape J.F.& Rogier C. Parasitol. Today, 1996, 12: 236-240; Rogier C. *et al*. Parasitologia 1999;41:255-259). The proposed plan is aimed at screening vaccine candidates before embarking on large-scale efficacy trials aimed at vaccine registration and is meant primarily for the evaluation of blood-stage vaccines.

Rationale : The objective is to measure efficacy of malaria vaccines under the best possible natural conditions of malaria transmission to detect an effect.

<u>"Small" population = "homogeneous" population :</u>

Assuming that the effect of the vaccine is similar irrespective of individual variation in the population, the representation of the sample is less important than its homogeneity. The more homogeneous is the sample population in terms of malaria exposure (naturally acquired immunity and risk of uncomplicated malaria), the higher is the power of the study. For this reason, it may be more appropriate to study "small" populations.

<u>Advantages of active case detection :</u>

- The number of clinical episodes detected by active case detection (community-based detection) is generally higher than the one detected by passive case detection (health facility-based detection) (Trape J.F. & Rogier C. Lancet, 1995, 345: 134-135). This increases the power of the trial.
- Active case detection reduces recruitment bias.

- From the first malaria infection, a certain degree of immunity is acquired. This can translates into morbid episodes that are shorter (Rogier C. *et al*. Am. J. Trop. Med. Hyg. 1999;60:410-420), which may resolve without anti-malaria treatment (self-cured). These short clinical episodes are less likely to be detected in health facilities by passive case detection than longer episodes. Although causing less burden (in terms of healthy days lost), these clinical episodes still affect the overall health of the populations and may trigger self-treatment. The trials that do not take into account these clinical episodes may underestimate the efficacy of the vaccine on incidence of morbid episodes
- Active case detection ensures higher degree of safety for the recruited subjects (against the occurrence of severe morbid episode and for the assessment of serious adverse events). It also allows to detect more accurately short adverse events following vaccination.

Study site :

The ideal characteristics of a study site are the following :

- Meso- to holoendemic malaria (WHO definition) with a transmission level (EIR) equal or higher than 20 infected bites per person per year [to ensure a reasonable incidence of malaria episodes in the control group]
- Small catchment area (diameter less than 2 kilometres, less than 30 minutes to cross the study site by foot)[to reduce exposure heterogeneity and to facilitate surveillance work]
- Ill-structured ecosystem with homogeneous distribution of potential larval sites for anopheles vectors [to reduce heterogeneity of exposure]
- Highest possible homogeneity of habitat [to reduce heterogeneity of exposure]
- Target population (e.g. children aged 0 to 4 years) larger than the sample size required for the trial.
- Highest possible homogeneity of the population in terms of sociological and ethnic characteristic as well as behaviour [to ease acceptability of the study and to reduce heterogeneity of exposure, of susceptibility to malaria and of behaviour towards the study and towards recourse to health systems]
- Absence of health facilities in this surrounding 5 to 10 kilometres [to reduce the recourse to health care not related to the trial]

Detection of malaria episode and collection of clinical and parasitological data :

Daily visit of each trial participant must be carried out by the field study team:

- Ensure on-site presence
- Measure body temperature
- Record answers to the questions on health status since the day before
- To perform thick film with immediate parasitological examination for appropriate case management in case of fever or clinical signs associated with fever.
- Perform systematic thick film(whatever the clinical status) once per week with parasitological examination

Medical presence on site 24/24 hours 7 days a week for consultation :

- Of ill participants detected during the daily visits
- Of ill participants attending spontaneously

The treatment of morbid episodes should include short-acting anti-malarial drugs such as quinine or artemisinine derivatives to ensure quick efficacy and to reduce the duration of the exclusion of the population at risk (denominator).

Case definition :

A morbid episode is attributable to malaria if there are clinical signs of fever (hyperthermia, feeling hot or cold, sweating, chills, headache) associated with a parasite density above a defined threshold, estimated from the attributable fraction method (T. Smith et al. Stat Med 1994; 13: 2345-58), or by the demonstration of a pyrogenic threshold (C. Rogier et al. Am.J.Trop. Med. Hyg., 1996, 54: 613-619). This definition requires the estimation of parasite density during an asymptomatic infection and during a morbid episodes. The systematic weekly collection of thick films allows to estimate the density of asymptomatic parasitemia and to assess the effect of the vaccine on parasite density that may be independent of its effect on clinical malaria attacks.

Study scheme and sample size required :

In areas where the ecological and climatic conditions lead to seasonal transmission of malaria, the recruitment and immunization should be performed during the low transmission season and the morbidity surveillance during the high transmission season. The dependent variable is the number of malaria morbid episodes per individual during the follow-up period ; the exposure variable is the vaccine status (vaccinated or placebo). Potential variables to adjust for are age and haemoglobin genotype status (AA; AS; AC). Vaccine efficacy is estimated using a Poisson regression model that provides the relative risk of malaria episodes in the vaccinated group to the control group. A generalised estimating equation approach could be used if several periods of follow-up must be considered for each subject or if the distribution of the number of malaria attacks per individual is over-dispersed in comparison to a Poisson distribution.

For example, in several areas of West Africa, most of the rains fall from June to November and a high transmission period runs from July to December. The mean of malaria episodes detected by active case detection during this semester (July to December) in children aged less than 5 years is of 2.7 morbid episodes in Dielmo (hyper-, holoendemic area, approximately 200 infected bites/person/year) and of 2.8 morbid episodes in Ndiop (meso-endemic, approximately 20 infected bites/person/year). Over such a short period, and in a sample size of about 100 children, the distribution of the number of morbid episodes per person is not significantly different from the one of Poisson (C. Rogier & J. F. Trape. Trans. Roy. Soc. Trop. Med. Hyg., 1993, 87 : 245-246).

Assuming a mean of 2.7 malaria episodes per child during the period of high transmission in the control group, an alpha risk of 0.05 and a ratio of 1 vaccine to 1 control, the total sample size (vaccine group + control group) and lowest

confidence internal limit (CI 95%) according to the expected efficacy are the following :

Efficacy	Power	Total sample size	Lowest limit of efficacy (IC 95%)
50 %	**95 %**	57	26 %
40 %	**95 %**	96	20 %
30 %	**95 %**	181	14.5 %
20 %	**95 %**	433	9.5 %
50 %	**90 %**	46	22 %
40 %	**90 %**	78	17 %
30 %	**90 %**	147	12 %
20 %	**90 %**	350	8 %

Factors that affect level of acceptability of such studies in the community:

- Ongoing presence of the research team on site that allows a better understanding of the work by the population (transparency) and the building of personal relationships between the population and the scientists.
- Access to health care provided to the whole on-site population (not only to the recruited subjects)
- Recruitment of the morbidity reporters within the studied population
- Building of the set-up by the population and local provision of food
- Good preparation of study end and withdrawal of the research team (training of health workers, organisation or assistance to the community for health-related problems)

Collaboration between the local health facilities and the research team at the local and national level (i.e support for control program or technical training).