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PROJECT FINAL REPORT

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4.1 Final publishable summary report

Executive summary

Placental malaria (PM) is caused by the parasite *Plasmodium falciparum.* Approximately 25 million pregnancies are at risk of placental malaria infections in sub-Saharan Africa each year. A vaccine preventing placental malaria will reduce mortality and morbidity for the new-born and can co-exist with a general malaria vaccine, and act in synergy with other malaria control measures such as insecticide-treated nets (ITNs) and intermittent preventive treatment during pregnancy (IPTp), reducing the disease burden originating from malaria.

The pathology of falciparum malaria is exacerbated by the adherence of infected erythrocytes to the receptors of the vascular lining. The adherence is mediated by a family of proteins called *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). A specific PfEMP1, named VAR2CSA, has been identified as the parasite protein responsible for the binding of infected erythrocytes in the placenta. Only women who have had placental malaria have substantial levels of protective antibodies against the VAR2CSA protein, the levels of antibodies are acquired as a function of parity and inhibit the binding of infected erythrocytes in the placenta. As the interaction between the parasite protein VAR2CSA and chondroitin sulphate A (CSA) in the human placenta is a key element in the pathogenesis of placental malaria, a vaccine should aim at eliciting antibodies that block this interaction.

The objectives of the PlacMalVac FP7 project is the clinical development of a VAR2CSA based vaccine against placental malaria. This includes the production and pre-clinical testing of the vaccine, a first in man phase Ia and Ib clinical trial, as well as preparation for a phase II trial.

The VAR2CSA antigen is a complex 350 kDa protein consisting of seven domains. One smaller sub-unit of the antigen (ID1-ID2a) has been demonstrated to bind specifically to CSA and induce antibodies that block interaction with placental tissue. Thus, this antigen was selected as the PAMVAC vaccine. The pre-clinical studies demonstrate remarkable high in vitro efficacy against the homologous parasite strain, and are also cross-inhibitory, although the inhibition of adhesion of heterologous parasites strains was lower.

The primary endpoint of the human clinical trial is safety, hence the first choice of adjuvant was aluminium hydroxide due to the safety profile. As the vaccine efficacy eventually will rely on the ability to mount a high titre of antibodies and preferably a sustainable immunological memory response, a more potent second choice of adjuvants comprises two formulations of monophosphoryl lipid A (MPL) analogue glucopyranosyl lipid adjuvant (GLA); a stable oil emulsion (SE) and a liposome saponin (LSQ), in collaboration with the Infectious Disease Research Institute (IDRI), Seattle, US. The toxicological safety study was performed as a repeat dose study in rabbits concluding that all regiments were well tolerated. The phase 1 clinical trial is completed in Tubingen with the last visit of the last subject. The PAMVAC vaccine is safe and well tolerated in healthy malaria naive volunteers. During the PlacMalVac project a clinical trial site was constructed and audited in Cotonou, Benin. The phase I clinical trial in Benin is almost completed and the vaccine is safe and well tolerated in healthy malaria exposed volunteers.

During PlacMalVac we have as a contingency plan developed a proprietary virus-like particle (VLP) platform. It has become increasingly clear that many protein-in-adjuvant sub-unit vaccine candidates suffer from low immunogenicity. Our strategy combines conventional recombinant vaccine production, which enables stringent quality control of the vaccine antigen, with VLP display of antigens, which enhances both the primary antibody responses as well as the memory immune responses.

Project context

The most deadly form of malaria is caused by the parasite *Plasmodium falciparum*, which is transmitted to humans by infected mosquitoes. Infection leads to different disease syndromes, at least partially as a result of the expression of parasite-derived antigens on the surface of infected erythrocytes and the subsequent adherence to the vascular lining. One such syndrome is placental malaria. Approximately 25 million pregnancies are at risk of placental malaria infections in sub-Saharan Africa each year. In endemic areas the prevalence of infected placentas is high; one out of four pregnant women living in high-transmission areas is estimated to have placental infection at delivery. A vaccine preventing placental malaria will reduce mortality and morbidity for the new-born and can co-exist with a general malaria vaccine, and act in synergy with other malaria control measures such as insecticide-treated nets (ITNs) and intermittent preventive treatment during pregnancy (IPTp), reducing the disease burden originating from malaria.
The adherence to the vascular lining is mediated by a family of proteins called *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by *var* genes, of which there are about 60 in each genome. Naturally acquired protective antibodies block the binding between infected red blood cells and receptors on vascular endothelium. A specific PfEMP1, VAR2CSA, has been identified as the parasite protein responsible for the binding of infected erythrocytes in placenta. Studies have since shown that parasites isolated from infected placentas express VAR2CSA in high quantities, and that this gene family is relatively conserved. Only women who have had placental malaria have substantial levels of protective antibodies against the VAR2CSA protein and the levels of antibodies are acquired as a function of their parity. Furthermore, women who have acquired VAR2CSA-specific antibodies give birth to healthier babies that are on average 450 g heavier than those born from women without these protective antibodies. As the interaction between the parasite protein VAR2CSA and chondroitin sulphate A (CSA) in the human placenta is a key element in the pathogenesis of placental malaria, a vaccine should elicit immunoglobulin that block this interaction. Therefore the aim of the PAMVAC is to protect foetus and mother against the adverse effects of placental malaria during pregnancy by interfering with infected erythrocyte binding to CSA in placental tissue.

Overall Project Objectives

The objectives of the PlacMalVac FP7 project were the clinical development of a VAR2CSA based vaccine against placental malaria, including the production and pre-clinical testing of the vaccine, a first in man phase Ia and Ib clinical trial, as well as preparation for a phase II trial.

Specific objectives with regard to selection of adjuvant, formulation - and analytical development

- To perform down-selection of VAR2CSA antigens using Freund's incomplete adjuvant and Alhydrogel in rats and rabbits.
    - Selection criteria were based on a) cross inhibition titre b) compatibility with Alum c) manufacturability.
- To re-clone and re-express the antigen without polyHIS tags.
- To define adjuvants to be used in the clinical trials for comparison with alum.
    - The main criteria for selection of appropriate adjuvants were 1) induction of high-titered cross-inhibitory IgG 2) accessibility and compatibility with human use of the adjuvant 3) physiological compatibility between VAR2CSA antigen and adjuvant and 4) induction of a long-term immunity.

- To perform analytical development including reagents and methods to monitor and optimize upstream, downstream and stability of the produced antigen.
- To develop conformational mono-specific antibodies for accurate estimative of the amounts of functional protein during production.
- To further develop the potency of the PAMVAC vaccine

Specific objectives with regard to upstream and downstream process development

- To develop an upstream and downstream process for cGMP production of the phase I clinical material
- To perform process documentation in the form of Standard Operating Procedures (SOPs)
- To perform consistency runs entailing running three identical production and purification runs in parallel according to the created SOPs.
- To transfer the upstream and downstream process to the CMO and support the initial transfer runs at the CMO to ensure a smooth transfer of know-how and methods.

Specific objectives regarding process up-scaling, manufacturing, test of DS material, process optimization

- To manage the outsourcing of the cGMP activities to the CMO as well as provide technical and on-site support as needed for the upstream and downstream production process.
- To support the CMO for activities relating to analysis, drug substance (DS) formulation and filling, as well as acquisition of the vialed adjuvant.
- To establish and release a master cell bank
- To perform analytical method validation for all the required methods for production, analysis.
- To set specifications for the drug substance and drug product.
- To perform formulation and vialing of the DP
- To perform pre-clinical repeat-dose toxicity study in rabbits
- To perform GLP and GMP compliant stability studies to provide stability data to cover all Phase I clinical trial periods.
- To will perform further process optimization in preparation for later clinical stages and the market.

Specific objectives regarding phase 1clinical trial

- To write the clinical trial protocol for testing the PAMVAC vaccine
- To implement a combined staggered phase 1 clinical trial in healthy adult volunteers with no exposure to malaria living in Europe and in volunteers living in a malaria endemic region of sub-Saharan Africa.
    - The clinical trial assessed the safety and the immunogenicity of different formulations of the VAR2CSA vaccine candidate, regarding antigen dosage and selected adjuvant. The safety assessment of the vaccine candidate in the non-exposed volunteers living in Europe allowed the transition to the phase Ib clinical trial in semi-immune young women in Africa.
- To establish SOPs and perform independent clinical audits that will assess Good Clinical Practice and Good Clinical Laboratory Practice.
- To standardize clinical trial design and assessment to allow comparison of data in order to harmonize the study with protocols for the testing of alternative placental malaria vaccine candidates.

Specific objectives relating to preparation of a phase 2 clinical trial

- To prepare the future phase 2 clinical trial of PAMVAC, including the provision of data necessary for the drafting of the protocol with regard to target population, end-points and number of subjects required.
- To prepare the strategy of interaction with the eligible population, including identification of the target population according to the opportunities of immunization, national immunization programs, and acceptability and feasibility of malaria vaccination in the socio-economic context of endemic countries.
- To perform follow-up studies of a cohort of nulligravid women in whom the first pregnancy will be systematically screened in order to measure the acquisition of natural immunity against placental stages of the malaria parasite in women to assess time of appearance of protective antibodies, and their response to VAR2CSA.
- To confirm the reliability of methods for selecting candidates for inclusion in a phase II clinical trial.
- To acquire information regarding anthropological and demographic studies in order to evaluate the average ages of puberty, first pregnancy, and the acceptability and feasibility of a malaria vaccine, including identifying elements of membership and resistance to malaria control and immunization.
- To assess opportunities for vaccination from birth to late adolescence using anthropological investigations to be conducted amongst young women, households and opinion leaders.

Main results and achievements

Selection of antigen

The VAR2CSA antigen is a complex 350 kDa protein consisting of seven domains. One smaller sub-unit of the antigen (ID1-ID2a) has been demonstrated to bind specifically to CSA and induce inhibitory antibodies (Clausen et al J Biol Chem. 2012 & Nielsen et al PLoS One. 2015) Thus this was selected as the PAMVAC vaccine antigen to be expressed in Drosophila sneider 2 cells. The pre-clinical studies demonstrated remarkable high inhibition against the homologous parasite strain and antibodies were also cross-inhibitory, although inhibiting adhesion of heterologous parasites strains to a lower extend (Bordbar et al Vaccine. 2012 & Nielsen et al PLoS One. 2015).

Selection of adjuvant formulation: An investigation of freedom to operate was performed prior to commencement of formulation studies, which resulted in a collaboration with Infectious Disease Research Institute, Seattle (IDRI), US as this allowed for analysis of immunogenicity of the antigen in the stable emulsion and liposome delivery platforms, as well as in combination with the potent monophosphoryl lipid A (MPL) analogue glucopyranosyl lipid adjuvant (GLA) that enhance immunological memory responses.

A total of seven groups of mice were immunized three times with ID1-ID2a in different combinations of GLA, stable emulsion (SE), liposome (LS) and alhydrogel. After one year the animals were boosted to evaluate induction of immunological responses. The functional antibody response was tested on malaria parasites by ELISA, flow-cytometry and in binding inhibition assays. The functional antibody response was highest against the antigen in GLA-LS immunizations, closely followed by GLA-SE.

Initially the results indicated that the clinical development plan should include Alhydrogel, due to its excellent safety profile, and GLA-LS. However, GLA-LS had at that point not been tested in humans. Therefore, we chose to go forward with both GLA-SE and GLA-LS.

Formulation

An empirical phase diagram was constructed to characterize the antigen regarding physical stability, facing pH and temperature changes. The protein is most stable in the pH range 6-8 and formulation buffers within this pH range are preferred.

A fluorescent probe (sypro orange) assay was used to screen different generally regarded as safe (GRAS) excipients and stabilisers to increase the physical stability of the protein. Excipients such as sorbitol (10-20%), trehalose (10-20%), sucrose (10-20%) and glycine (0.2-0.4 M) all stabilized the tertiary structure of ID1-ID2a. However, as a shelf life of 1.5-2 years is needed to complete phase Ia and Ib it was decided to continue with a frozen formulation. The stabilisers do not have the same ability to stabilize the protein in the frozen state and they may even crystallize out of solution upon freezing. Since UCPH has years of experience with ID1-ID2a stored in phosphate buffered saline (PBS) buffer this was the final formulation of choice for the clinical batch.

The interaction between antigen and adjuvant was characterized. For the IDRI stable emulsion the antigen adsorption, pH, integrity of the emulsion (dynamic light scattering) as well as the antigen (SDS-PAGE) has been tested. The mixture of antigen and the emulsion adjuvant was stable for the tested conditions 72 h at 5, 25 and 37°C. For alhydrogel studies have been performed to determine the degree of adsorption, kinetics of adsorption, degree of desorp/effect of pH micro-environment on the physical structure of the antigen. The final studies on antigen-alhydrogel interactions will be performed on the same alhydrogel batch that is also used in the clinical trial.

The different batches of development runs of protein batches have continuously been tested in mice and the resulting IgG has been tested on laboratory malaria parasites as previously described, regarding antibody titre, and parasite CSA binding inhibition.

Analytical methods

To monitor protein quality and stability, relevant assays were identified in collaboration with Expres2ion and the CMO - CMC Biologics. The assays and the SOPs were optimized and written at UCPH. Initially five SOPs for HPLC, western blot, capture ELISA, decorin ELISA direct ELISA were transferred from UCPH to the CMO. After further development the size exclusion HPLC, identity western blot and host cell protein method was developed further. In addition the applicability of SDS-PAGE reduced, SDS-PAGE non-reduced, OD280, free SH groups, glycan profile, endotoxin and bio burden was tested and verified at the CMO. Based on the assay validation and transfer, the specifications were set. The acceptance criteria of the release specification for the cGMP batch were formed on the basis of the results for the engineering batch. In addition to the items included in the specification for engineering batch a potency assay was developed for the release of the cGMP batch. The potency assay development was initiated at UCPH. After a tender process the clinical research organisation (CRO) that was chosen to perform good laboratory practice (GLP) potency assay was Huntingdon life sciences, United Kingdom, to ensure GLP standard.

Pre-clinical toxicological studies

After a tender process the CRO to perform the safety toxicological study was decided to be Huntingdon Life Sciences, United Kingdom. The objective of this study was to evaluate the potential local and/or systemic toxic effects of the ID1-ID2a vaccine candidate alone and in combination with Alhydrogel and GLA-SE after four intramuscular administrations to rabbits, and to evaluate the recovery or potential delayed systemic toxic or local effects at 28 days after the fourth administration. The monitoring of animals included: clinical observation: erythema and eschar formation, oedema formation, haematoma formation; mortality; body weight; food consumption; body temperature; ophthalmic examination; haematology: peripheral blood and bone marrow; blood chemistry; biomarker analysis (acute phase protein measurements); antibody analysis; necropsy and histology: macroscopic pathology, microscopic pathology and organ weight.

Repeat dose administration of ID1-ID2a or ID1-ID2a combined with aluminum hydroxide, GLA-LSQ adjuvant or GLA-SE adjuvant elicited no signs that were attributable to treatment, no deaths occurred during either the treatment or recovery period and there was no effect of treatment upon body weight and body weight gain or food consumption. There was no effect of treatment on body temperature and there were no treatment-related ophthalmoscopic changes. The haematological and blood plasma biochemical examinations did not indicate any treatment-related differences from pre-treatment values. Organ weights were unaffected and there were no treatment-related macroscopic changes.

Test article-related histopathological findings were confined to the injection sites and were consistent with a normal immune response following intramuscular vaccine administration. The slight increase in endomysial/perimysial lymphocytes and heterophils at the injection site at which the final (Day 43) administration was given three days before necropsy in animals given ID1-ID2a vaccine, ID1-ID2a with aluminium hydroxide or ID1-ID2a with GLA-SE was attributed to the ID1-ID2a vaccine, but the reason for the absence of these findings in animals given ID1-ID2a vaccine with the GLA-LSQ adjuvant was unknown. In the affected groups there was no clear increase in the incidence and/or severity of these findings at the earlier injection sites on Day 1, 15 and 22 demonstrating that some recovery had occurred since these administrations were given.

An accumulation of cell debris (containing mixtures of degenerate inflammatory cells and myofibres and expanded endomysial and perimysial areas) at the most recently used injection site was confined to some animals given ID1-ID2a vaccine with aluminium hydroxide adjuvant and this was attributed to the aluminium hydroxide adjuvant, rather than to the ID1-ID2a vaccine. Aggregations of foamy macrophages in some animals given ID1-ID2a with aluminium hydroxide occurred at all injection sites and therefore tended to be a persistent finding. This was the only finding that showed no evidence of recovery and was similarly attributed to the aluminium hydroxide adjuvant, which is a commonly used adjuvant with a well-established toxicity profile.

Potency

The objective of these assays is to verify the immunogenicity of the product the PAMVAC vaccine. ELISA assays were developed, optimized and qualified to be transferred to CMOs by UCPH. These were used to test the potency of product from pre-clinical products to cGMP. The assays were performed according to SOPs developed at UCPH (non-GLP) or transferred to CMOs in order to work under current good laboratory or current good manufacturing practices (GLP/GMP). The CMO did further validation of the assays and performed the end point titre analysis as part of the toxicological study and stability studies. Three different assays were used: 1) *In vitro* measurements of antibody induction against the PAMVAC vaccine. Performed by Huntingdon Life Sciences, developed by UCPH. Quality: GLP qualified. Purpose: to measure the potency of the engineering run product in rabbits for preclinical safety evaluation. 2) *In vitro* potency measurements against the PAMVAC vaccine antigen ID1-ID2a used for release of the vaccine and stability testing. Performed by NDA analytics, developed by UCPH, Quality: performed under GMP. Proposed analytical principle: IgG seroconversion in mice following a single administration of ID1-ID2a in alum as determined by cGMP qualified ELISA. 3) *In vitro* measurement of parasite binding inhibitory antibodies in a biological functional assay. Performed by UCPH. Quality: Non GLP qualified. Purpose: to determine the biological potency of vaccine.

cGMP manufacturing of the ID1-ID2a antigen

Selection of CMO

In 2011 we received a grant from the Danish Advanced Technology Foundation (HTF) to prepare for GMP manufacturing of VAR2CSA, the grant was given to a consortium consisting of UCPH, Expres2ion Bio and CMC Biologics (CMO). In this consortium we developed analytical tools and transferred these to CMC as well as developed and transferred the research cell bank. Hence the work at the CMO had already begun at the time of the PlacMalVac initiation. We still performed a tender process (taking into account that the work had to be re-initiated at a given other CMO) and contacted relevant CMOs in Europe, who would have the capacity to manufacture a malaria vaccine in S2 cells. CMC biologics were proven to be competitive on the price, in addition to the obvious advantages of proximity to UCPH and Expres2ion, and was chosen as the CMO to manufacture VAR2CSA for PlacMalVac, co-financed by funds from Denmark through HTF and Germany through Bundesministerium für Bildung und Forschung (BMBF).

Upstream process development

The lead monoclonal cell line candidate (established at ExpreS2ion), formed the basis for evaluating the effects on ID1-ID2a protein production of several process parameters, such as media, temperature, harvest time and production additives. Final refinements to the production process as well as robustness testing lead to a final process that was tested for consistency and robustness through triplicate consistency runs.

Downstream process development

The aim of this sub-task was to develop a high-yielding downstream purification process of consistent product quality. The up- and downstream processes were developed in close collaboration to ensure that the final process yields optimal results. The process was developed starting from the UCPH process, which consisted of a Capto S first column, followed by Size Exclusion Column (SEC). The Capto S column was replaced by a SP Sepharose column after it was found to perform better. SEC cannot be used effectively in large-scale, and was therefore replaced by a Capto Adhere mixed mode column after screening of several alternatives, including Hydrophobic Interaction Columns (HIC). Optimization of each step was performed, as well as robustness studies to determine the effect of changes in upstream material as well as minor changes in process intermediates. The original process used gradient elution profiles, but after consultation with the CMO, this was changed to step elution to simplify the process transfer and cGMP manufacture. Two virus inactivation methods have been developed and optimized for the process: the first is a low-pH hold step, while the second is a nanofiltration step. Two batches of reference material were produced during the process development to support analytical method development and transfer to the CMO.

In conclusion, a robust process for the purification of the VAR2CSA fragment ID1-ID2a was developed at Expres2ion Biotechnologies. This process will be used to produce ID1-ID2a material under clinical good manufacturing practice for a planned clinical phase I trial. The final downstream process isolates homogeneous ID1-ID2a at more than 90% purity in a monomeric state. Several batches of protein purified using this process have been tested in animal studies and has been shown to raise inhibitory antibodies. Further development work at Expres2ion technologies refined the process to suit potential further clinical trials.

Stability of the vaccine

The current stability studies covers

- Up to 6 months stability data for API batch CMC-I-M003 at minus 80°C
- Up to 24 months stability data for API batch CMC-I-M003 at minus 20°C
- Up to 12 months stability data for DP batch CMC-I-0053 at minus 20°C and 2-8°C. API batch CMC-I-M003
- Up to 24 months stability data for DP batch CMC-I-0054 at minus 20°C. API batch CMC-I-0045

All results comply with the specification stated in the IMPD and show a trend well within the limits at both minus 80°C and minus 20°C.

The clinical trial

The phase I clinical trial is a staggered two-centre, dose-escalation trial. The first stage was performed in Germany and the second stage in Benin. The overall clinical trial design as well as the immunoassays have been adapted to allow comparison of placental malaria vaccines across independent clinical centres following recommendation emanating from the workshops on "Clinical Development of Placental Malaria Vaccines" and on "Immunoassays for Placental Malaria Vaccines" organised by EVI in 2014.

The protocol

The first version of the protocol was finalized and signed the 30th June 2015. Additional preclinical results and outcome from the above mentioned workshops lead to several minor and two major changes in the protocol design as compared to the one provided in the proposal.

1) Inclusion of a third adjuvant in the first stage of the trial. Glucopyranosyl Lipid A Liposome-QS-21 formulation (GLA-LSQ) was included in the clinical trial in view of its excellent

performance and dose-sparing effect in pre-clinical models. GLA-LSQ safety and tolerability profile is similar to GLA-SE.
2) The first dosage level of PAMVAC was changed to 20µg. The decision was made following communications with regulators and to harmonize dose escalation with a parallel clinical trial by an independent group testing another placental malaria vaccine candidate.

Clinical trial implementation
Once the protocol was finalized, the University Clinics of Tübingen was officially asked to sponsor the phase I clinical trial (for both stages) in compliance with the principles of § 4(24) German Drug Law and ICH-GCP. The sponsorship was agreed on the 2nd July 2015. The sponsorship was delegated to the University Clinics UTB (Eberhard-Karls Universität Tübingen) clinical team.

For the phase Ia of the clinical trial in Germany, UTB team submitted the relevant documents to the University of Tübingen ethics committee on the 7th July 2015. After clarification of outstanding issues and changes to the clinical trial dossier, the ethics committee provided a favourable opinion on the clinical trial on 22nd October 2015. In parallel, the relevant documents were prepared and submitted to the responsible German regulatory authority, the Paul Ehrlich Institut (PEI) in Langen, on the 13th July 2015. Once all formal and scientific queries solved, PEI provided official approval of the clinical trial on the 5th November 2015.
During this period also:
- All relevant Standard Operating Proceudres (SOPs) were written and approved.
- Insurances were selected and contracted.
- Monitoring of the clinical trial was contracted.
- Case Report Forms (CRFs) were written, approved and electronic CRF preparation was commissioned.
- Subject documents were prepared according to German requirements/regulation.

For the phase Ib of the clinical trial in Benin, the finalized protocol was translated into French prior to submission to the National Ethics Committee of Benin on 15th December 2015. Following modifications resulting from an independent scientific evaluation of the protocol, a formal presentation to the Committee was given by the phase Ib principal investigator Dr Saadou Issifou on 20th January 2016. Oral and written of the protocol was given.

During this period, for the phase Ib stage:
- All relevant SOPs were written.
- Insurances were selected and contracted.
- Monitoring of the clinical trial was contracted.
- CRFs were written, approved and eCRF preparation was commissioned.
- Subject documents were prepared according to the Benin requirements.

As stage 1 of the clinical trial is in Germany, the PAMVAC vaccine clinical trial phase I has been registered at EudraCT, the number of the trial is 2015-001827-21.
The clinical trial has also been posted on a public database that complies with the requirements of the International Committee of Medical Journal Editors, the ClinicalTrials.gov Identifier: NCT02647489.
For the review of the safety data prior to the dosage escalation for each stage of the clinical trial, an independent safety monitoring board (ISMB) has been set-up by EVI team. The experts invited to sit on the ISMB have been selected based on the recommendation of the consortium PSC. An ISMB charter has been prepared according to the operational guidelines for the

establishment and functioning of TDR/WHO. ISMB meetings at specific stage of the clinical trial stage have been pre-defined to provide recommendation to the sponsor on the continuation of the clinical trial and on the dosage escalation. A first meeting prior to the start on the trial conduct has been organised in November 2015 for the appointment of the chair of the ISMB, review of the charter and the protocol. The charter has been approved by all members.
Following assessment visits of the clinical trial sponsor, the phase Ia investigational site (UTB), and the phase Ib investigational site, in 2013 EVI has appointed an independent auditor to conduct ICH-GCP audit of the sponsor and investigational sites. The selection of the auditor was performed following the evaluation of three contract research organisations in accordance with EVI procurement process.
The audit of clinical trial sponsor and phase Ia investigational sites was conducted by the selected independent auditor and EVI (Nathalie Imbault) on 24 November 2014. The outcome of the audit was positive, with suggestions for archiving the clinical trial documents.
For the phase Ib site, as it is a new clinical trial site, it was agreed by the sponsor and the site team that an additional assessment visit would be held prior to the audit. The visit was conducted from 18 to 22 May 2015 by EVI (Nathalie Imbault) accompanied by UTB sponsor representative (Diane Egger). The new building ("Institut de Recherche Clinique du Bénin") was found well organised for its purpose. It was noted that a lot of efforts were made by the site team on the quality management. Additionally, the assessment team had the opportunity to meet the representative of the national ethics committee in Benin.
An audit of the phase Ib site was conducted on 9-10 November 2015 by the selected auditor and EVI (Nathalie Imbault). The audit outcome was acceptable with suggested improvement in a corrective and preventive action plan that is being completed by the team with the support from EVI.
The construction of the "Institut de Recherche Clinique du Bénin" (IRCB) was finished in the summer of 2015. The UAC and IRD team are busy finalising the equipment of the building for the clinical trial, and the development of the necessary SOPs.
The legal status of the IRCB was validated and signed by all concerned parties -Inserm, IRD, Faculté des Sciences de la Santé de l'Université d'Abomey-Calavi and Centre de Lutte Intégrée contre le Paludisme du Ministère de la Santé du Bénin- in February 2016.
For the clinical trial stage in Benin, prior to the effective start of the trial, several visits were conducted by the sponsor and by EVI to assess the capacity of the Benin site and the readiness of the team to conduct the phase I clinical trial. An audit of the clinical trial site was also conducted by an external auditor and by EVI.
The National Ethic Committee of Benin provided a favourable ethical opinion on the protocol, the subject information form/informed consent sheet, the case report form and the investigator brochure. The opinion was provided on 31 March 2016. Insurance were contracted for the volunteers and for the study staff.
The clinical trial monitoring was performed by Clinapharm, a Contract Research Organisation. The Site Initiation Visit was conducted on May 12-13, 2016 by the sponsor representative of the trial, the principal investigator and the local trial monitor. The trial team at IRCB is organized as follows:

- a clinical team of five physicians (including 3 permanent physicians)
- two study nurses and four nurses from Referral Hospital for pre and post-vaccine clinical monitoring;
- a vaccine preparation team: 6 pharmacists (3 per vaccine preparation);
- the laboratory team of two technicians and two senior supervisors
- one data manager
- one driver.

The staff in charge of the clinical trial in Benin has received several training sessions.
A training on Good Clinical Practices and on adverse events post-vaccination was held on 6 May 2016.
A training on Advanced Life Support was provided on 15 June 2016.
A training of the vaccine preparation team was provided by the pharmacists of the University of Tübingen on 1st-5 November 2016.
The requested drug import license was obtained from the Direction chargée des Pharmacies of Benin and PAMVAC vaccine and GLA-SE and Alhydrogel adjuvants were shipped from the University of Tübingen on 9 August 2016 and arrived at IRCB on 16 August 2016. The vaccine and adjuvants are stored in a freezer and refrigerator, respectively at temperatures specified by the study protocol.

3. Conduct of the clinical trial

Stage 1 University of Tubingen, Germany
It was planned to enroll a total of 36 healthy male and female 18 to 45 years old malaria-naïve adults living in Tubingen. A session of information for the participants was organized on 3 May 2016. Subjects were provided with an information sheet and all subjects willing to participate have signed written information consent.
A total of 54 subjects were assessed for eligibility, 15 subjects did not meet the inclusion criteria, 3 declined participation to the trial and 36 subjects were enrolled in the trial.
For the first group 1A-3A (n=9) receiving the lowest dose of PAMVAC, the screening was performed between 4 and 9 May 2016. Nine subjects were enrolled and randomized to receive 20µg PAMVAC adjuvanted either with Alhydrogel (0.17mg), GLA-SE (1mg) or GLA-LSQ (1mg), with 3 participants per group. One person per group (sentinel) was injected 1 day (9 May 2016) before the main group (10 May 2016) as specified in the protocol.
A safety report was prepared by clinical team on the blinded safety data set of group A until 14 days following first injection. The safety report was provided to the Independent Safety Monitoring Board (ISMB) and a meeting was organized by EVI on 3 June 2016. The ISMB provided recommendation to continue the trial as planned as there was no serious adverse event related to the vaccination and no safety concern identified.
For the second group 4A-6A (n=27) receiving the middle dose of PAMVAC the screening was performed between 12 May and 27 June 2016, 27 subjects were enrolled and randomized to receive 50µg PAMVAC adjuvanted either with Alhydrogel (0.43mg), GLA-SE (2.5mg) or GLA-LSQ (2.5mg), with 9 participants per group. The sentinel group was injected on 7 June 2016, at least one day ahead of the main group that was vaccinated between 9-30 June.
A safety report was prepared on the 1st July by the clinical team with the cumulative data set from cohort 1A-3A (14 days post second injection) and cohort 4A-6A (14 days post first injection). The safety report was provided to the ISMB and a meeting was organized by EVI on 14 July 2016. The ISMB provided recommendation to continue the trial as planned as there was no serious adverse event related to the vaccination and no safety concern identified.
The last visit of the last subject was on 20 January 2017 for the group 1A-3A and on 9 March 2017 for the group 4A-6A. Two subjects out of 36 did not attend the last visit, however the site has contacted and asked them to come back at a later time to complete the study.
The conclusion from the clinical team was that the PAMVAC is safe and well tolerated in healthy malaria- naïve volunteers. The tolerability is similar at 20 and 50 µg PAMVAC and also similar when formulated with Alhydrogel, GLA-SE and GLA-LSQ.
A Development Update Safety Report (DUSR) for the reporting period 5 November 2015 to 5 November 2016 was prepared and submitted to the German national competent authority and the ethics committee of University of Tübingen on 29 December 2016.

Stage 2, Institut de Recherche Clinique du Bénin, Benin
It was planned to enroll 30 healthy nulligravid female adults, living in endemic area, 18 to 30 years old. The information process included preparation of flyer and meetings with the Abomey Calavi University, meetings with students of several faculties, meeting with smaller groups of students and personal interviews.
All volunteers enrolled in the trial have signed the informed consent and have agreed to receive injectable contraceptives until the completion of the trial follow-up.
From around 500 subjects informed about the trial, forty-nine volunteers were screened; 9 were ineligible, based on clinical and biological data. Forty participants were eligible, 5 withdrew consent and 21 were enrolled and randomized to receive 50 µg PAMVAC adjuvanted with either Alhydrogel (0.43mg), or GLA-SE (2.5mg), or placebo. It is planned that investigators will remain blinded until the subject follow-up is completed. The 21 participants were divided into sentinel group (3 participants) and 3 main groups (6 participants per group). One person per group (sentinel) was injected 1 day before the main groups and observed for 4 hours after the vaccination.
The first vaccination was on 3 November 2016. All the 21 participants of 50 µg group 1B-3B received 3 doses of PAMVAC vaccine, and 19 completed 7 visits post-vaccination (D84).
A safety report was prepared by the clinical team with blinded data set from cohort 1B-3B (14 days post first injection) on 22 November 2016. The safety report was provided to the ISMB and a meeting was organized by EVI on 9 December 2016. The ISMB provided recommendation to continue the trial as planned as there was no serious adverse event related to the vaccination and no grade 3 adverse event lasting for more than 48hours.
The next step will be the 100µg PAMVAC dose administration adjuvanted with either Alhydrogel (0.85 mg) or GLA-SE (5 mg), or placebo (Group 3B-5B). At this date (18/03/2017), 14 eligible candidates are currently available for enrolment of the 9 remaining participants who will receive 100µg of PAMVAC vaccine candidate. The vaccination is planned to take place during the second quarter of 2017 and participants will be followed until the fourth quarter of 2017.
During the conduct of the trial, monitoring visits by the outsourced local monitor Clinapharm were conducted after the first vaccination of the subjects on 11 November 2016, 30 November 2016 and on 1st December 2016.
Monitoring visits were also conducted after the last vaccination of the group 3B-6BA on
13, 14 and 15 March 2017.

Preparations for further development: phase 2

Parasitology: At the beginning of this project we conducted a detailed longitudinal parasitological study in pregnant women recruited in a previous project (STOPPAM project) between the end of the first trimester and the beginning of the second trimester of pregnancy and documented the deleterious impact of submicroscopic *P. falciparum* parasitaemia on multiple pregnancy outcomes, including maternal anaemia, premature birth and birthweight (Cottrell et al., 2015). We have since optimized and used robust and ultra-sensitive detection methods to track those hidden infections in the PlacMalVac cohort. Submicroscopic infections are very frequent in this cohort, the prevalence of which is 2.5 to 5 times that of infections detectable by microscopy.
Furthermore we have also generated sequence data and analysed the genetic diversity of the ID1-DBL2x-ID2 of var2csa from placental isolates to inform vaccine development efforts. The placental blood samples of women who were followed in the STOPPAM project and in which *P. falciparum* was detected by PCR were selected. We were able to successfully amplify the 1.6kb ID1-DBL2x-ID2 region of var2csa in 45 samples. PCR products were then pooled together for deep sequencing on PacBio circular consensus sequencing (CCS) platform using the P5-C4

chemistry. Overall, var2csa diversity in Benin was high and ID1-ID2 haplotypes subdivided into five clades (Doritchamou et al., Plos One 2015). In this study the largest one was defined by homology to the FCR3 parasite strain. This is rather reassuring to the current vaccine trial where the vaccine antigen was defined on the FCR3 variant. However, compared to women infected with only FCR3-like variants, we observed a trend among women infected with only 3D7-like variants to deliver infants with lower birthweight. Although this observation was made on a relatively small sample size, it supports differential effects of infection with specific parasite strains. The immense diversity coupled with differential clinical effects of this diversity suggests that an effective VAR2CSA-based vaccine may require multivalent activity. A publication based on these data is being prepared for submission.

Humoral immunity: To study the antibody response to the ID1-ID2a vaccine antigen, 470 women were selected from the STOPPAM cohort ('Strategies To Prevent Pregnancy Associated Malaria' supported by the European Union Framework 7 under contract number: 200889) based on clinical outcomes of pregnancy and availability of samples and clinical data. The group included 45 placental infections at delivery, 24 premature babies, 53 intrauterine growth retardations, 12 low birth weights, 64 maternal anaemia. A control group of 272 women having none of the mentioned complications was also constituted. Measurement of plasma levels of IgG subclasses specific to ID1-ID2a antigen by ELISA was optimized and applied to the cohort of 470 women. The results clearly show a preponderant role of IgG3 in the in the protective mechanism. In the PlacMalVac cohort, the analysis of the plasma reactivity shows a high reactivity with the vaccine antigen whereby nearly 80% of the women show a seropositive response throughout the follow-up. We also noticed that nearly 40% of the women had IgG antibodies with specificity for ID1-ID2a before pregnancy. This unexpected observation deserves to be confirmed on another population less exposed to infections compared to this cohort.
In addition to these analyses, we performed the automated microtiter plate-based method for reading cytoadherence of infected erythrocytes and measuring plasma anti-adhesion property. We showed a strong correlation between the level of anti-ID1-ID2a IgG in the plasma of women and the ability to inhibit the cytoadherence of FCR3-infected red blood cells to CSA. These results are encouraging because they strongly suggest that the antibodies induced against this antigen interfere with the adherence of *P. falciparum* infected erythrocytes to CSA, which represents the rationale of the mechanism of action of the vaccine that is being developed in this project.

Cellular immunity: During this reporting period the analyses of the full dataset (B & T cell memory responses) was completed and a publication prepared for submission. The analyses combined the immunological findings with infection histories during pregnancy. In summary, the results revealed that B cell memory (IgG-secreting) responses to the vaccine antigen (PAMVAC/ID1-ID2a) are readily detectable during first pregnancies, with their frequency increasing to a peak at delivery then declining to levels that are sustained for at least 6 months and that are equivalent to those detectable in multigravid, non-pregnant Beninese. B cell memory responses to a parasite antigen (CIDR1a) that has no specific expression pattern associated with infection during pregnancy showed no such variation.
Primigravidae who had microscopically detectable infections during pregnancy had higher frequencies of such cells, most markedly at delivery. Of note, although the group
designated as 'uninfected' comprised some women in whom submicroscopic infections were detected (by qPCR), B cell responses remained at comparatively low levels throughout pregnancy. Although the differences are not statistically significant, it is also noticeable that responses to CIDR1a as well as overall B cell activity (Total IgG) were higher at the beginning of

pregnancy in infected women. Recall immunizations for tetanus, routinely given during pregnancy, boosted tetanus toxoid (TT)-specific B cell memory responses, but these were not modified by *P. falciparum* infections. The latter finding clearly validates the use of TT in this context as a non-parasite control antigen.
Quantification of cytokine production as a measure of T cell memory responses following 72h culture in vitro in the presence of antigen revealed, in summary, pro-inflammatory (IFN-γ, TNF-α) responses to PAMVAC that showed tendencies to decline during pregnancy, but with a rebound post-delivery, whilst the profile of the anti-inflammatory IL-10 response was the opposite, increasing to a peak at delivery and then declining post-delivery.
The modulation of cytokine responses measured at delivery as a function of infections with *P. falciparum* arising during pregnancy showed that primigravidae who had been infected during pregnancy displayed trends towards higher PAMVAC-specific pro-inflammatory responses, whilst also having significantly higher mitogen-specific IL-5 and IL-13 responses.
These findings demonstrate that both B and T cell memory responses to the PAMVAC vaccine antigen are readily detectable during first pregnancies in Beninese women. PAMVAC-specific B cell responses, in particular, were most prominent in women who had infections detectable by microscopy during pregnancy, although the levels of such responses measured 6 months post-delivery were very similar regardless of the women's infection histories. It will be important to determine whether and how PAMVAC-specific antibody profiles in these same women, as well as the functionality of such antibodies, reflect the observations documented here.
In conclusion, these studies have generated data that provide a platform for assessments of cellular immunological responses during future PAMVAC vaccine studies, including Phase II-based efficacy trials.

Further optimization of the vaccine performed after initial selection of antigen and adjuvant.

Previous failures of clinical trials to identify viable candidate vaccines have resulted in part from the inherent low immunogenicity of recombinant sub-unit vaccines. One way to shift this risk curve is to enhance immunogenicity by focusing the response on protective epitopes and improving the longevity of the immune responses. An effective, versatile vaccine delivery system would be important in the development of an efficacious recombinant sub-unit vaccine against malaria. Importantly, the induction of immunological memory responses must be addressed to ensure that protection is long-lasting. The development of a vaccine delivery system that elicits long-lasting, robust protective immune responses is thus pivotal in the development of vaccines for malaria and neglected diseases.

During PlacMalVac we have as a contingency strategy combined conventional recombinant vaccine production, which enables stringent quality control of the vaccine antigen, with a virus-like particle (VLP) vaccine-platform. The advantage is that a VLP display of antigens enhances both the primary antibody responses as well as the memory immune responses. The inherent immunogenicity and longevity of the response potentially enables reductions of dose and number of immunizations, substantially reducing the cost of implementing the vaccine. Due to a versatile VLP-antigen coupling strategy, quality control of the antigen can be performed prior to coupling of the antigen to the VLP (Thrane et al. 2016, Journal of Nanobiotechnology) .
Traditional design of VLP-based vaccines has relied on either genetic fusion of small peptides into viral structural proteins, chemical conjugation using a modular approach for coupling antigens onto pre-assembled VLPs or genetic modification of viral vectors. These strategies have considerable limitations. The new technologies we have developed overcome traditional limitations of generating VLP vaccines. Many vaccine trials in the field of malaria illustrate the fact that induction of strong adjuvant-assisted immune responses incurs safety issues and that long-term memory is difficult to obtain. Some of the most successful sub-unit vaccines on the

market are vaccines, where the sub-unit forms a VLP, exemplified by the Hepatitis B and the human papilloma virus vaccine. Such VLP-based vaccines are able to induce remarkably potent and long-lasting immune responses, setting new standards for vaccine design. Virus-like particles (VLP) are an efficient, safe and cost effective strategy to deliver antigens to the immune system.

During PlacMalVac we have therefore actively pursued the possibility of developing a VLP based placental malaria vaccine as a way to define the optimal formulation of the antigen. The technologies have been benchmarked using antigens involved in diseases other than placental malaria, which increases the possible impact of the research performed during PlacMalVac. We have employed three different technologies described below.

Technology 1: *Generation of biotinylated Human Papilloma virus VLPs:* The L1 sub unit of HPV constitutes an attractive VLP backbone for a placental malaria vaccine as the target population is identical. We used the biotin acceptor site (AviTag™) sequence (GLNDIFEAQKIEWHE), which was genetically inserted at four different positions in the HPV16 L1 sequence based on the crystal structure of HPV type 16 L1 capsid protein. These four different positions correspond to surface exposed loops in the assembled VLP structure. The rationale for choosing these positions was to allow VLP assembly while at the same time exposing the AviTag™ sequence for subsequent biotinylation and coupling of monovalent streptavidin-VAR2CSA.

The antigen tested in the PlacMalVac human clinical trial is the shortest truncated VAR2CSA polypeptide sequence that is able to bind CSA. This construct was genetically fused at the amino terminus to monomeric streptavidin (mSA) which can bind biotin with high affinity. To avoid VLP aggregation we used a monomeric form of streptavidin which is advantageous to native streptavidin as the latter contains four biotin binding sites.

Antigen-coupled VLPs were examined by Transmission electron microscopy, showing particles of a comparably larger size (~70 nm) than the non-coupled VLPs (~30–60 nm). This observation was further examined by dynamic light scattering (DLS) analysis, which confirmed that mixing of mSA-VAR2CSA with biotinylated Avi-L1 VLPs resulted in measurably larger particles with an average diameter of ~70 nm (12.9% Pd) compared to naked Avi-L1 VLPs (≤60 nm, 23.9% Pd). Importantly, this analysis also confirmed that such large complexes were not formed after mixing unbiotinylated VLPs with mSA-VAR2CSA, demonstrating that mSA-VAR2CSA is, in fact, bound to the surface of Avi-L1 VLPs via the specific affinity interaction between mSA and biotin.

Together, these results indicate that the produced mSA-VAR2CSA VLP vaccine consists of non-aggregated HPV16 Avi-L1 VLPs displaying dense, repetitive arrays of the mSA-VAR2CSA antigen presented in a consistent orientation.

The immunogenicity of the mSA-VAR2CSA VLP vaccine was tested in C57BL/6 mice vaccinated three times with three week intervals. ELISA was used to measure total immunoglobulin (Ig) levels against VAR2CSA in sera obtained from mice immunized with mSA-VAR2CSA VLP, soluble mSA-VAR2CSA or soluble naked VAR2CSA. After three immunizations the VAR2CSA specific Ig levels were higher in sera from mice immunized with the mSA-VAR2CSA Avi-L1 VLP vaccine than in sera from mice immunized with soluble naked VAR2CSA. After 1st and 2nd immunization sera from mSA-VAR2CSA Avi-L1 VLP immunized mice had statistically significantly higher Ig endpoint titers compared with sera from mice immunized with the soluble mSA-VAR2CSA vaccine (P = 0.014 and P = 0.018, respectively). This difference was, however, not statistically significant after the 3rd immunization (P = 0.058) as both vaccines seem to have reached a similar plateau.

Antisera were examined for their ability to block the binding between native VAR2CSA expressed on the surface of parasite-infected erythrocytes and immobilized CSA. After first immunization, none of the three vaccines had induced efficient levels of functional binding-inhibitory antibodies, leading to full binding of parasites. However, after the second round of immunizations 1:50 diluted serum from mSA-VAR2CSA VLP immunized mice inhibited the

binding between IE and CSA by approximately 70%. In comparison, the soluble mSA-VAR2CSA vaccine only inhibited approximately 20%, while no inhibition was seen for the soluble naked VAR2CSA vaccine. After three immunizations, 1:200 diluted serum from mice immunized with the mSA-VAR2CSA VLP vaccine showed roughly 90% binding-inhibition, while the sera from mice immunized with the soluble mSA-VAR2CSA vaccine inhibited parasite binding by approximately 60%. By contrast, the soluble naked VAR2CSA vaccine failed to induce any binding-inhibitory antibodies.

Technology 2: *Development, expression and characterization of spy-VLPs vaccines* (Thrane et al. 2016, Journal of Nanobiotechnology). A panel of SpyTag or SpyCatcher presenting VLPs was designed based on the *Acinetobacter* phage AP205 coat protein which spontaneously forms a VLP. Expression of this protein in *Escherichia coli* results in the assembly of 29 nanometer wide icosahedral VLPs consisting of 180 subunits. The 116 amino acid SpyCatcher sequence was fused to the N-terminus (SpyCatcher-VLP) of the AP205 coat protein. In addition, the 13 amino acid SpyTag peptide was fused to N-terminus (SpyTag-VLP) or to both N- and C-terminus (2xSpyTag-VLP) of the AP205 coat protein. Recombinant *E. coli* expression of spy-AP205 coat proteins was confirmed by SDS-PAGE analysis of fractions collected following density gradient ultracentrifugation. Reduced SDS-PAGE showed pure protein bands of expected sizes. VLP-assembly of each spy-AP205 coat proteins was evaluated by transmission electron microscopy (TEM) and dynamic light-scattering (DLS) analysis. For all recombinant particles the DLS analysis revealed a homogenous population of non-aggregated particles with an average estimated size of 36 nm (SpyTag-VLP), 42 nm (2xSpyTag-VLP) and 43 nm (SpyCatcher-VLP). In comparison, unmodified AP205 VLPs were determined by DLS to have an average size of 35 nm.

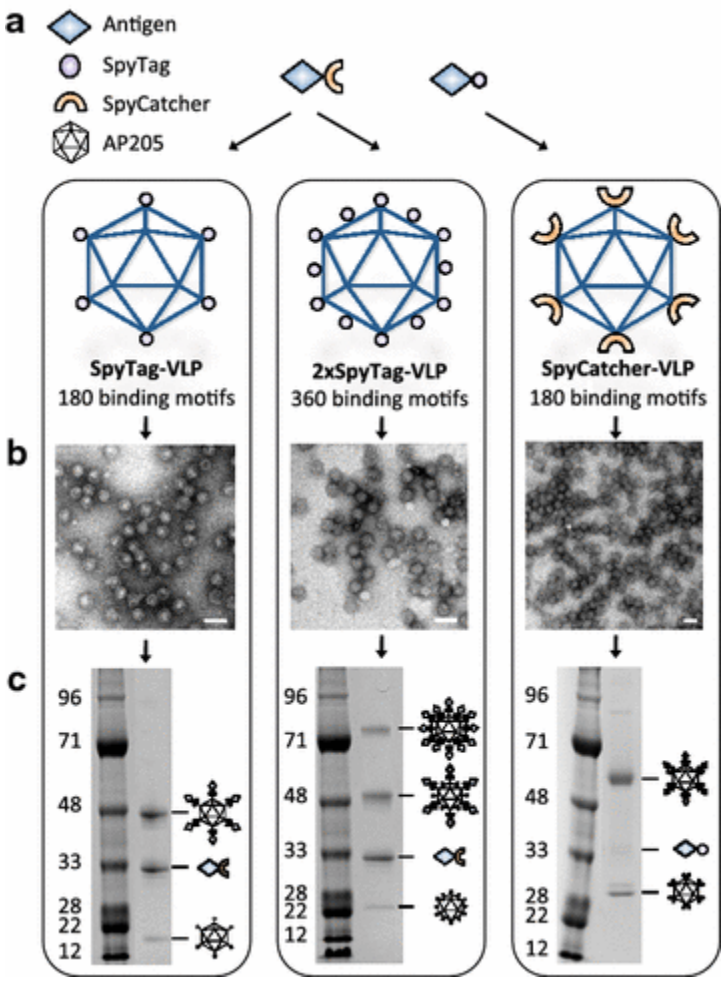

Figure 1 The spy-VLP antigen display platform.
a Three types of spy expressing VLPs were constructed by genetic fusion of SpyTag or SpyCatcher to the virus-like particle (VLP)-forming AP205 capsid protein. (1) "SpyTag-VLP" had the SpyTag fused to the N-terminus of the AP205 capsid protein and present 180 potential SpyCatcher-antigen binding motifs (2) "2xSpyTag-VLP" had SpyTag fused to both the N- and C-terminus of the AP205 capsid protein and present 360 potential SpyCatcher-antigen binding motifs; (3) "SpyCatcher-VLP" had SpyCatcher fused to the N-terminus of the AP205 capsid protein and present 180 potential Spytag-antigen binding motifs. b Transmission electron microscopy (TEM) images showing the SpyTag-VLP, 2xSpyTag-VLP and SpyCatcher-VLP. c Reduced SDS-PAGE gels loaded with VLP vaccines demonstrating that vaccine proteins had formed covalent bonds to the AP205 capsid protein. Left panel shows that mixing of SpyTag-VLPs with SpyCatcher-IL-5 resulted in three protein bands corresponding to the size of an antigen-VLP capsid protein conjugate (48 kDa) (top), uncoupled vaccine antigen (33 kDa) (middle) and unconjugated SpyTag-VLP capsid protein (16.5 kDa) (bottom). The middle panel shows that mixing of 2xSpyTag-VLP with SpyCatcher-IL-5 resulted in four protein bands representing; a conjugate of two vaccine antigens bound to each end of a 2xSpyTag-VLP capsid protein (83 kDa), a conjugate of the 2xSpyTag-VLP capsid protein and a single vaccine antigen (48 kDa), uncoupled vaccine antigen (33 kDa) and unconjugated 2xSpyTag-VLP capsid protein (18.5 kDa). The left panel shows that mixing of SpyCatcher-VLP with PD-L1-SpyTag resulted in three protein bands representing; an antigen-VLP capsid protein conjugate (50 kDa), uncoupled vaccine antigen (33 kDa) and unconjugated SpyCatcher-VLP capsid protein (27 kDa)

To assess the immunogenicity of spy-VLP delivered antigens, we tested, Pfs25 and DBL1-ID2a-VAR2CSA (a longer version of the PAMVAC antigen), and evaluated humoral responses in mice after intramuscular immunizations. Pfs25 is expressed on the *P. falciparum* ookinete surface within the mosquito. The Pfs25 antigen is poorly immunogenic by itself and development of an effective transmission-blocking Pfs25 vaccine has been hampered by the requirement of a

strong adjuvant. SpyCatcher was fused to the C-terminus of Pfs25 and Pfs25-SpyCatcher was expressed and purified from *E. coli* SHuffle® cells. This enabled high level expression of soluble correctly folded Pfs25-SpyCatcher.

SpyTag was genetically fused to the N-terminus of the CSA binding domain of VAR2CSA (domains DBL1-ID2a) and expressed and purified from *E. coli* SHuffle® cells. The protein expressed well and was folded correctly as measured by its binding to decorin. The antigen display capacities for Pfs25 and VAR2CSA were 109 and 61 proteins per VLP, respectively. The antigen-specific IgG titer was measured by enzyme-linked immune-sorbent assay (ELISA) 2 weeks after each immunization (on days 14, 35 and 56) as well as at day 212 (Pfs25) and 137 (VAR2CSA). The Pfs25 spy-VLP vaccine induced higher antigen-specific IgG titers than the control vaccine at all the tested time-points ($P < 0.01$ (day 14, 35 and 56); $P = 0.03$ (day 212), Mann–Whitney Rank Sum Test). At day 212, there was a 37-fold increase in the geometric mean titer (GMT) of IgG in sera from spy-VLP vaccinated mice compared to mice vaccinated with the same amount of soluble Pfs25 plus untagged AP205 VLPs.

The GMT of VAR2CSA-specific IgG was consistently higher in the group immunized with the VAR2CSA spy-VLP vaccine compared to the control group vaccinated with uncoupled VAR2CSA. However, the difference in GMTs between the two groups was not as profound as seen in the Pfs25 study and only reached statistical significance at days 14 ($P = 0.03$, Mann–Whitney Rank Sum Test) and 137 ($P = 0.03$, Mann–Whitney Rank Sum Test).

For most vaccines only a fraction of the induced IgG is biologically active in inhibiting the development of the targeted microorganism and the level and overall avidity of vaccine induced IgG responses does not necessarily reflect the anti-microbial functional activity. The goal of the Pfs-25 vaccine is to block parasite development inside the mosquito. We therefore used the standard membrane feeding assay (SMFA) to measure transmission-blocking (TB) activity of the antibodies induced by the two vaccines. The Pfs25 spy-VLP vaccine showed more than 99 % transmission-reducing activity (TRA) (one oocyst found in the 20 investigated mosquitos) compared to pre-immune serum (82 oocysts detected) or serum from mice immunized with the control vaccine (85 oocysts detected) (Fig. 2a). In a second study, BALB/c mice ($n = 7$) were immunized with the Pfs25 spy-VLP vaccine at days 0 and 14, and different concentrations of purified IgG from pooled anti-Pfs25 serum samples were subsequently tested in the SMFA assay. At the highest IgG concentration (750 μg/ml) serum from the Pfs25 spy-VLP vaccinated mice completely blocked oocyst formation. We compared the vaccines ability to elicit IgG inhibiting the binding between VAR2CSA expressed on infected erythrocytes and chondroitin sulfate in the 96 well in vitro assay (Fig. 2b). The mean EC50 calculated from the dose–response curve of serum from VAR2CSA spy-VLP immunized mice was eightfold higher, [CI 95 % 3.213–14.41], than the mean EC50 in serum from mice vaccinated with VAR2CSA that was not bound to VLPs (Fig. 2b).

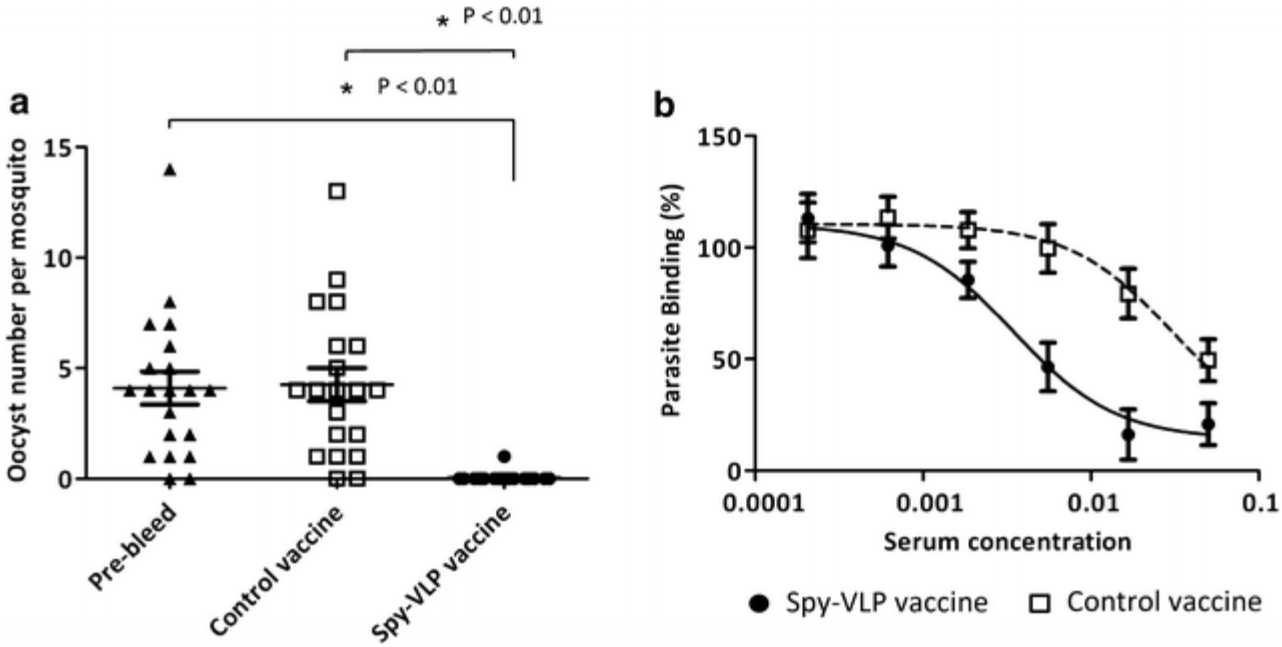

Figure 2 Functional activity of spy-VLP vaccine-induced humoral responses.

a Transmission reducing activity (TRA) of anti-Pfs25 (day 56) sera following immunization of mice with the Pfs25 spy-VLP vaccine or with the control vaccine (soluble Pfs25 + untagged AP205 VLP) as described in Fig. 2a. *Y*-axis shows the number of oocysts identified in the midgut of each of 20 *A. stephensi* mosquitoes. Pre-immunization mouse serum was used as additional negative control. Mann–Whitney rank sum test was used for statistical comparisons. **b** Binding between VAR2CSA expressing infected erythrocytes and CSA in the presence of different concentrations of serum from immunized mice. Binding in the presence of serum from non-immunized mice was set to 100 %. Serum pools were from groups of 5 mice immunized with the VAR2CSA spy-VLP vaccine (*black circle*) or with control vaccine (*empty square*). The EC50 value for the spy-VLP vaccinated mice was 8.8 fold higher [3.213–14.41] than the value from mice receiving the control vaccine

Technology 3: Novel adenovirus encoded virus-like particles displaying VAR2CSA antigen (From Anderson et al Vaccine. 2017 Feb 22;35(8):1140-1147. doi: 10.1016/j.vaccine.2017.01.016.)©

We designed adenovirus encoded virus-like particles (VLP) by co-encoding Simian Immunodeficiency Virus (SIV) gag and VAR2CSA. The VAR2CSA antigen was fused to the transmembrane (TM) and cytoplasmic tail (CT) domains of either the envelope protein of mouse mammary tumour virus (MMTV) or the hemagglutinin (HA) of influenza A. For a non-VLP incorporation control, a third design was made where VAR2CSA was expressed without TM-CT domains. The virus encoded VLP design used in these studies has the advantage that it can include three ID1-ID2a sequences in a single vaccine depending on the viral vector backbone. Such a vaccine might not only induce higher responses, but could potentially stimulate a B cell response against overlapping epitopes. The observation that humans acquire such responses after one pregnancy suggests the presence of B cell precursors with specificities allowing generation of pan-reactive antibodies

The immunogenicity of the huAd5 vectors was investigated in Balb/c mice. The mice were primed with either huAd5 vaccine at d.0 and then boosted at week 17 with the same vaccine. The huAd5 vaccine expressing ID1-ID2a fused to HA TM-CT anchored to Gag VLPs induced significantly higher ID1-ID2a specific antibodies at 7 and 11 weeks after the first immunization compared to the MMTV TM-CT fused ID1-ID2a vaccinated mice (p-values = 0.0159; 0.0317) and compared to ID1-ID2a-S at week 11 (p-value = 0.0079). The immune responses was found to be at a stable level for the VLP encoding immunization groups at week 16 post the first

immunization. Only the mice immunized with the non-VLP vaccine (ID1-ID2a S) were found to display an increase in ID1-ID2a specific responses after the boost at week 17.
The functionality of the induced antibody responses were analysed in a binding inhibition assay, in which the serum samples were investigated for their ability to inhibit parasite IE to bind to CSA. Only the ID1-ID2a HA group exhibited inhibition of IE binding to CSA 4 weeks after the first immunization, albeit low. At 7 weeks after the first immunization, both the ID1-ID2a HA group and ID1-ID2a MMTV group showed inhibition of IE binding to CSA, again most prominently for the ID1-ID2a HA group.

To investigate if the immune responses induced by the huAd5 vaccines could be improved, the huAd5 vaccines were combined with ID1-ID2a protein immunization as outlined in Fig 3A (left schematic). This immunization regimen was compared to prime-boost with huAd5 and prime-double boost with the ID1-ID2a protein (Fig. 3A, right schematic). Boosting either of the huAd5 vaccines with the ID1-ID2a protein significantly increased the ID1-ID2a specific antibody responses compared to the prime-boost regimens utilizing huAd5 only (p-values 2 weeks after 2nd immunization: ID1-ID2a S: 0.0079; ID1-ID2a MMTV: 0.0079; ID1-ID2a HA: 0.0159, Mann-Whitney). The highest responses were observed in the group primed with either huAd5 ID1-ID2a HA or ID1-ID2a MMTV and double boosted with protein. Both of these groups had significantly higher responses at both 2 and 6 weeks after the final immunization compared to the triple protein immunized group (p-values: ID1-ID2a MMTV: 0.0079 (2 and 6 weeks); ID1-ID2a HA: 0.0079 (2 weeks), 0.0159 (6 weeks). The higher levels of antibody responses in these two groups were also mirrored in superior efficiency in inhibiting binding of IE to CSA as compared to protein only in the inhibition assay (Fig. 3C) (p-values: ID1-ID2a MMTV: 0.0008; ID1-ID2a HA: 0.0018; nonlinear regression analysis of $LogEC_{50}$ values, F-test). The group primed with ID1-ID2a S and double boosted with ID1-ID2a protein was also found to be superior in inhibiting binding of IE compared to the triple protein immunization regimen (p-value: 0.0368, nonlinear regression analysis of LogEC50 values, F-test), albeit the superiority was less pronounced.

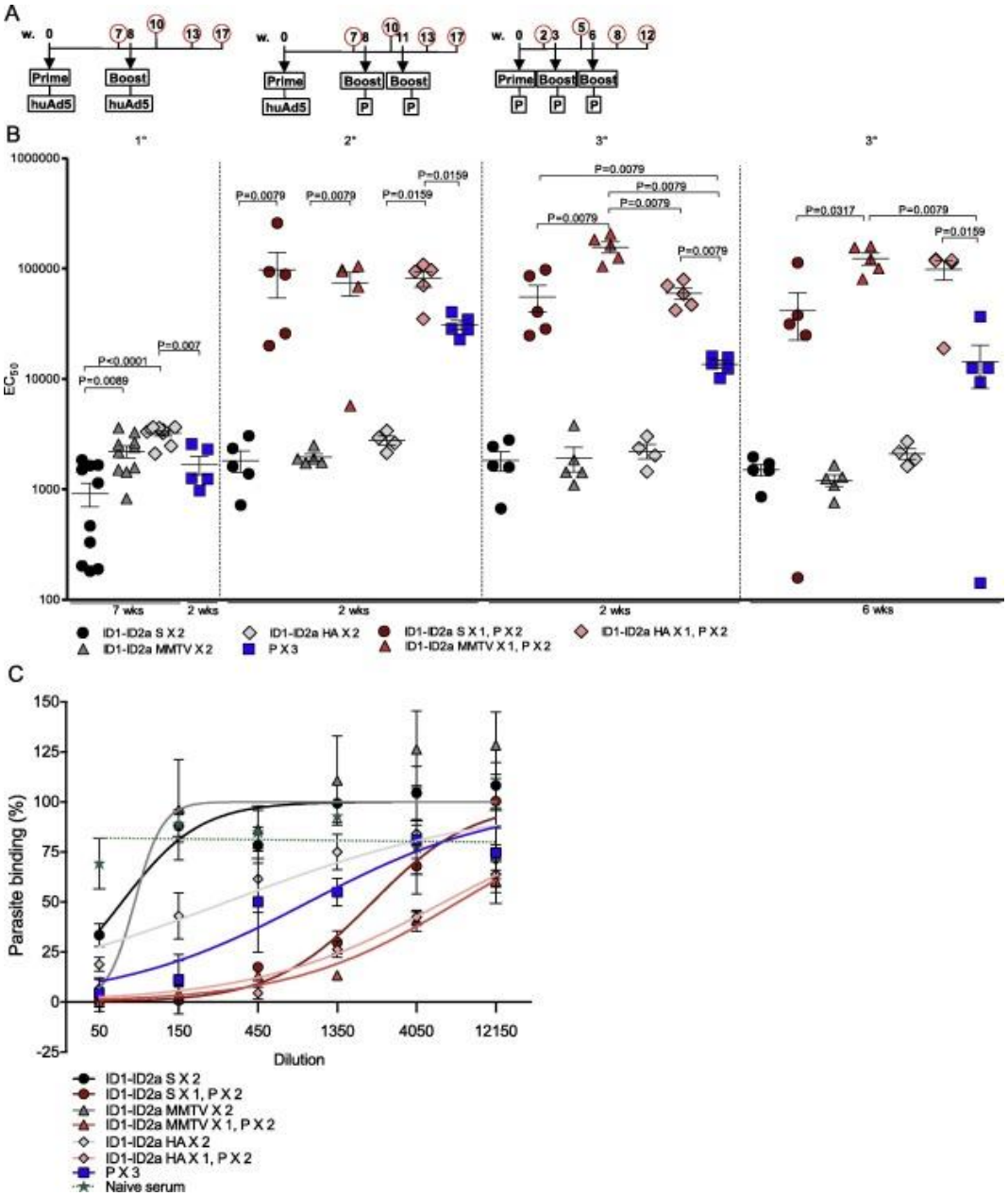

Figure 3 Analysis of responses induced when combining huAd5 priming and protein boost immunizations. (A) Schematic of immunization schedules. The left schematic shows immunization schedule of Balb/c mice primed and boosted with huAd5 vectors encoding ID1-ID2a. The middle schematic shows immunization schedule of mice primed with huAd5 vectors encoding ID1-ID2a and boosted with ID1-ID2a protein. The right schematic shows immunization schedule of mice primed and boosted with ID1-ID2a protein. Encircled numbers indicate time points when serum was harvested. (B) EC50 of antibody titers as determined of individual Balb/c mice immunized as indicated in (A). (C) % parasite binding as determined by inhibition of indicated pooled serum sample dilution of infected red blood cells to bind to CSA coated Falcon plates. Serum samples from 6 weeks post final immunization and a sample of pooled naïve serum were analysed for their functionality. P is protein.

Vaccine potency assays to be used during the clinical development (from Pehrson et al Vaccine. 2017 Jan 23;35(4):610-618. doi: 10.1016/j.vaccine.2016.12.028)©

In the clinical protocol of PlacMalVac/PAMVAC two assays are described, the petri-dish assay and the 96 well assay. The petri-dish assay is relatively low throughput but easy to implement whereas the 96-well assay is high throughput but difficult to implement due to the need for expensive equipment. The inhibitory capacity of antibodies may not be easily translatable

between assays. Therefore, we compared the sensitivity of different assays comparing serial dilutions of serum.
The inhibitory capacity of sera was tested in four binding assays. Three in vitro assays test IE binding to decorin coated on plastic surfaces, while the ex vivo perfusion assay test binding to placental tissue. The 96-well assay is a standardized high throughput assay where several plates can be run simultaneously. Quantification of bound IE relies on the incorporation of tritium labelled hypoxanthine into the parasite nucleic acids, followed by liquid scintillation counting. This requires equipment and facilities for radioactive material, which may limit the transferability of the assay. Alternatively, colorimetric methods may be used. The petri-dish assay requires a minimum of equipment and is easily transferred between laboratories. Dilution series of two sera can be accommodated in one dish, and several dishes can be run simultaneously. Quantification relies on imaging in a microscope, with either manual or automatic counting with appropriate software. The flow assay requires a pump system and associated chips with channels. One sample can be tested in each channel, and dilution series of 2-3 sera can be tested in one day. The perfusion system requires a perfusion chamber, pumps, access to placental tissue and relatively large amounts of reagents. One condition can be tested in each one-hour phase and 4-7 phases conducted in one day.
The binding inhibition results highlighted the importance of running dilution series of immune-serum and assuring comparable parasite binding between experiments. Choice of assay may depend on study objective. It is evident that much higher concentrations of immune-sera are needed to inhibit parasite binding to the placenta as compared to CSA immobilized on plastic. This is likely due to the fact that in the placenta the overall density of CSA surrounding the IE will be much higher as compared to a one dimensional petri dish or a tube. To differentiate constructs that can elicit inhibitory antibodies in pre-clinical development, assays with higher sensitivity for detecting binding inhibition, such as the 96-well and petri dish assays, may be an advantage. These assays, in particular the 96-well assay, also have a higher capacity. The perfusion model more closely represents in vivo conditions and may be more informative regarding the efficacy in humans. However, the model is resource demanding, which limits the number of samples that can be tested and the assay is not sensitive to detect small effects. The overall concordance in determining the inhibitory capacity of individual sera across *the in vitro* and *ex vivo* assays, demonstrates the possibility of using one of these assays in a surrogate endpoint after initial smaller vaccine efficacy trials.
The vaccine formulation containing the ID1-ID2a construct with a potent liposomal adjuvant, part of the PlacMalVac phase I clinical trial, was inhibitory in all assays. Sera from mice immunized with this formulation effectively inhibited parasite adhesion in perfused placental tissue at a 100-fold dilution. This is an encouraging result, conferring hope that antibodies will also inhibit parasite accumulation in vivo.

Clinical development plan
Development and further clinical testing of the PAMVAC vaccine requires funding which is not secured at the moment. Nevertheless, we have developed a plan for how to continue clinical development.
The vaccine against placental malaria should be administered before the first pregnancy, and it would be obvious to vaccinate pre-puberty girls in malaria endemic areas against placental malaria and human papilloma virus at the same time. Thus, the vaccine should elicit sustained high titered IgG responses and should, like the HPV vaccines, be optimized to generate long-lasting plasma cells. For HPV vaccines, the induction of such cells ensures that protective IgG levels are sustained for several decades even in the absence of exposure to the immunogen.
It is not known whether the current PAMVAC-S vaccine based on soluble protein induces long-lived plasma cells, which maintain high vaccine induced antibody levels over long periods. This

question will be answered by following the antibody levels in volunteers participating in the phase 1a & 1b study over the next months. However, since we initiated the development program based on a soluble protein, it has become increasingly clear that induction of sustained IgG responses require that vaccine antigens are presented on particles.
PAMVAC-VLP is based our novel vaccine technology described above enabling binding of soluble proteins antigens to virus like particles (VLP). The technology is the first platform achieving true VLP display of large and complex antigens. To forward the clinical development of the PM vaccine and obtain clinical proof of principle for a vaccine protecting women against placental malaria we present two mutual exclusive development plans.
Plan A: This plan will be activated if prior to project start, the plasma level of anti-VAR2CSA IgG exceeds pre-set thresholds for endpoint titre and binding inhibition in blood samples collected in December 2017 (17 month after the last immunisation) from volunteers participating in the PlacMalVac phase 1 trial testing the safety of PAMVAC-Soluble (PAMVAC-S). Plan A will test PAMVAC-S in phase 2 efficacy trials conducted in three clinical trial sites. Plan B: if the IgG induced by the soluble protein vaccine does not reach the set threshold, the clinical development of the soluble vaccine will be terminated and efforts concentrated on the PAMVAC-VLP vaccine. The work-plan will bring PAMVAC-VLP through phase 2a.

PAMVAC-S: The vaccine encompasses as described the minimal binding region, the ID1-DBL2x-ID2a domains, of VAR2CSA. Formulated in the most promising adjuvant GLA-SE (a synthetic toll-like receptor 4 agonist stable emulsion from IDRI, Seattle, the median (minimal and maximal) IgG Elisa endpoint titre was 51.200 (6.400 – 102.400). Thus, the vaccine is an effective inducer of specific IgG. However, the maximal IgG level at follow up still needs to be assessed, to measure the kinetics of IgG decay.
PAMVAC-VLP: The proposed vaccine has two main constituents. 1) A VLP based on a genetically modified version of the phage AP205 and 2) a recombinant soluble VAR2CSA. The basic constituent of the VLP, is a recombinant protein corresponding to the AP205 structural protein. genetically fused at the N-terminus to a truncated relative conserved L2-HPV protein and at the C-terminus to a Spytag. This recombinant protein spontaneously forms a VLP with protruding N and C ends. This way we incorporate both the element of designing a dual HPV and placental malaria vaccine as described in technology 1 and the stable covalent binding of antigens eluted in technology 2. The recombinant VAR2CSA is essentially identical to the vaccine antigen in PAMVAC-S with the addition of Spycatcher domain that spontaneously forms a covalent bond to Spytag. Simple mixing of the two main vaccine constituents results in a VLP that irreversibly binds VAR2CSA, thereby presenting the vaccine antigens, VAR2CSA and L2-HPV, to the immune system in a densely and highly repetitive fashion. Our pre-clinical testing of the PAMVAC-VLP in mice is as described highly encouraging since the PAMVAC-VLP vaccine produced eight fold higher titers of antibodies than the soluble PAMVAC-S vaccine as well as high titres of functional anti-L2-HPV IgG. The adeno virus particle platform described as technology 3 may become useful should data be acquired indicating that antibodies generated against one variant of the minimal binding epitope will not be sufficient for protection, as this technology allow the expression of up to three variants of VAR2CSA.
The sample size calculations below are based on data acquired during PlacMalVac as this provided data on infections rates, on the number of women that can be expected to become pregnant during a set period and data on the risk of suffering unfavourable pregnancy outcomes. The clinical work plan below will be supplemented by important continued monitoring of malaria prevalence, biological characterisation of vaccine elicited IgG and post-vaccination of break through infections. We will also include social science work on how the vaccine is perceived and accepted among African women. All work will be done according to GCP. We are very aware that the vaccine concept in itself necessitates special ethical considerations and we

will during the whole process seek regulatory and ethical advice from African and European authorities as well as WHO.

Outline of clinical development path A

WP 1: GMP production and reprotox studies for PAMVAC-S

WP2: Phase 2 efficacy trial in three different sites in sub-Saharan Africa

Purpose: to assess safety and immunogenicity during vaccination of non-pregnant women as well as safety in the same women during pregnancy and in children born by vaccinated mothers.

Design: double blinded, randomised 1:1 to receive PAMVAC-S or HPV vaccine.

Description: we will recruit 300 non-pregnant nulli - or primipara women with a wish to become pregnant (100 per site). After completion of vaccination (3 doses 1 month apart), birth control will be lifted and pregnancy tests will be performed every second month. Pregnant and un-pregnant women will be followed. For the pregnant this may entail ultrasound scanning at week 12-14 to assess gestational age and a close follow up including all services offered during the national antenatal programmes. Women will be expected to give birth at hospitals and all relevant health information will be collected at birth and from the children up to 1-month post-partum. We expect 18% of the vaccinated women to get pregnant, with a 10% loss to follow up we expect follow 48 pregnancies in vaccinated women.

Primary end points: safety in pregnant women and offspring and immunogenicity.

Go criteria: no critical safety signals in women or offspring. Plasma collected month after 3rd vaccination: Median end point titre > 100.000. Median inhibition of FCR3CSA binding in plasma concentration 1:20<50%

WP3 Phase 2b in three different sites in sub-Saharan Africa

Purpose: to assess vaccine efficacy

Design: double blinded, randomised 1:1 to receive PAMVAC-S or HPV vaccine.

Description: we will recruit 4614 non-pregnant, HIV negative, nulli - or primipara women with a wish to become pregnant (1538 per site). After completion of vaccination (3 doses 1 month apart), birth control will be lifted and pregnancy tests will be performed every third month. An ultrasound investigation will be performed at week 12-14 of pregnancy to assess gestational age and a close follow up including all services offered during the national antenatal programmes. Women will be expected to give birth at hospitals and all relevant health information will be collected at birth and from the children up to 24 hours post-partum. We expect 28% of the vaccinated women to get pregnant, with a 10% loss to follow up and 97% singleton pregnancies. Thus the pregnancy outcome endpoints available for statistical analyses are expected to be 1292.

Primary endpoint: a composite endpoint for unfavourable pregnancy outcome will be defined as follows: Pregnancy loss at any time after a positive pregnancy test, or birth of a small for gestational weight baby (SGA), or pre-term delivery, or birth of a low birth weight baby, or inter-uterine growth retardation, or peri/neonatal death.

Sample size calculation: Unfavourable pregnancy outcome in HPV vaccinated comparator group: 40%. Unfavourable pregnancy outcome in VAR2CSA group: 32%. A power of 0.8, and a statistical significance of >0.05 requires 564 endpoints measurements in each group or an effective sample size of 1128. Adjusted for singleton pregnancies (97%), loss to follow up (10%) and the expected number of pregnancies among those vaccinated (28%), the adjusted sample size is 4.614.

Secondary endpoints: Malaria positive at any time during pregnancy (slide and/or rdt: yes/no), slide positive at any time during pregnancy (yes/no), highest parasite burden during pregnancy (max. parasite count in peripheral blood at any time through pregnancy), parasite burden in

maternal cord blood, parasite burden in placenta, evidence/burden of acute and/or chronic P. falciparum infections in placenta, birth weight, Z-score, mother haemoglobin at birth, lowest haemoglobin level during pregnancy

Outline of clinical development path B:
WP 1: GMP production and reproductive-toxicological studies for PAMVAC-VLP
WP 2: Phase 1a and phase 1b studies
Purpose: to assess safety, immunogenicity, dose and adjuvant selection
Design: staggered, open labelled non-randomised study employing PAMVAC-VLP
Description: the trial design will be identical to the 1a and 1b trials we have conducted with PAMVAC-S
Go/no-go criteria: Plasma from volunteers from phase 1A, 1 month after 3rd vaccination. Median end point titre > 100.000. Median inhibition of FCR3CSA binding in plasma concentration 1:20<50%. No critical safety signals in phase 1b.
WP2: Phase 2a trial in in three different sites in sub-Saharan Africa
Purpose: to assess safety and immunogenicity during vaccination of non-pregnant women as well as safety in the same women during pregnancy and in children born by vaccinated mothers.
Design: double blinded, randomised 1:1 to receive PAMVAC-S or HPV vaccine.
Description: Essentially as described the phase 2A trial described above for PAMVAC-S, we will however recruit 450 non-pregnant nulli - or primipara women with a wish to become pregnant (150 per site). We expect 24% of the vaccinated women to get pregnant, with a 10% loss to follow up we expect 47 children to be born in each study arm.
Primary end points: safety in pregnant women and offspring and immunogenicity.

Anthropological survey

The individual and social expectations (desire of pregnancy, free treatment, blood in a pregnant, volume of blood collected, social representation of primigravidity) and interpretations of the purpose of the study have greatly hindered the progress of the project. The inadequacy of the information given to the participants in connection with the aims of the study could be involved, including raising suspicions and rumours leading to clustered refusals within the community. The causes of the sharp reduction in the number of women who remained in the study after enrolment were sought in the light of the first results of the anthropological investigation. The choice of the population might have been inappropriate. The population had been chosen because of the high incidence of malaria, including during pregnancy. This population who found refuge on Lake Nokoué in previous centuries is known for its distrust. Our outreach efforts probably were not sufficient to face the special character of the population. In addition, the logistical constraints to achieve the study seemed disproportionate to the findings.

Reluctance of the people from Sô-Ava during PlacMalVac
When implementing PlacMalVac, it appeared 3 months after the beginning of the recruitment, a dramatic fall in inclusions. The causes of the population mistrust have been established:
- Blood sampling was rejected for several reasons: doubt about the use of the blood, sampling in high number of tubes (a factor more taken in consideration than the total volume of blood), blood sampling more frequent than during routine antenatal visits, reimbursement of women's transport from home to health centre assimilated to purchase of the blood;
- Denial of free care of pregnancy (as planned in the protocol) that suggests the husband is "unable" to finance his wife's pregnancy;
- Misunderstanding of placental blood smear, interpreted as an attack of the newborn soul, which can lead to malformation or compromise the destiny of the newborn;

- Rumors disseminated by religious leaders suggesting that the study promoted contraception, and recommending that the faithful not to participate in this type of study
- PlacMalVac competed with private health centres that officiate in the city, which strengthened the circulation of rumours.

Actions taken in chronological order, and according to the identification of the reasons for rejection were as follows:
- We contacted elected local officials and the mayor of the municipality to inform them on problems encountered and review with them the progress of the study;
- We organized sensitization meetings with district chiefs, religious leaders, and the population for information on study objectives, legislative procedures for population-based studies, required authorizations, safety of blood volumes taken with respect to the total blood volume in the body (demonstrations were organized by pouring a coloured liquid representing blood into different containers with volume ranging from 5 mL to 5 L), placental smear, and purpose blood samples taken.
- We met for information and discussion the Board of patients from the Saint Joseph Health Center (about 150 people in addition to the staff of St. Joseph);
- We met each family that included a nulligravid or primigravid woman expressing a rejection or having left the study (parents, aunts, sisters, neighbourhood heads, husband, etc.) to clarify the obscure points, and to remove barriers in cases of concealment;
- We met with religious leaders individually;
- We set up collaboration with main public and private health centres of the municipality (and, when required, we compensated their loss caused by the decline of their customers);
- We offered to all participants and their families to visit the CERPAGE laboratory to show them how the blood samples were processed;
- We gave the opportunity to a family member of each woman to be present during labor and childbirth in order to assess the conditions of placental blood smear realization, and to allow it to recover the placenta thereafter.

The impact – and limits – of these actions was evaluated later in the recruitment.
- We observed a decrease in tension and rumours within the community;
- Recruitment renewal, though slow, was seen among nulligravid women, allowing us recruiting primigravidae in sufficient number during the following year;
- We were able to recover most of the previous refusals;
- Newly recruited women appear to better understand the study and better adhere to it;
- The laboratory was visited by about twenty families of pregnant women that appreciated the opportunity to a better understanding. However, we some resistance remained, causing escape from women at the sight of the dugout of the project, as well as the persistence of sampling refusal by some included women.

The potential impact

Global health impact of a vaccine against malaria in pregnancy: This project addressed an urgent global health challenge that has proven very difficult to roll back. The treatment and prevention of placental malaria is currently pursued through the use of anti-malarial drugs and bed nets. The malaria drugs available to the market are compromised due to increasing drug resistance. In addition, in areas with stable transmission, placental malaria is predominantly sub-clinical. Hence, many pregnant women are not aware of being infected and do not seek treatment. In many instances these silent infections have serious clinical consequences for the foetus. Ideally placental malaria is best controlled through a vaccine. At the moment there are no placental malaria vaccines on the market or in clinical development. Several non-placental malaria vaccine candidates are in development but none of these are likely to be effective against placental malaria.

The highest coverage for a vaccine would be achieved by targeting girls below the age of five years who are already part of the World Health Organization (WHO) Expanded Programme on Immunization (EPI) currently reaching >70% of the target population. This would however, require that life-long protection can be achieved by vaccination during early childhood. If this duration of protection cannot be achieved, the target group will necessarily be adolescent girls, who are currently the targeted population for a Human Papilloma Virus (HPV) vaccine through the Program for Cervical Cancer Prevention. Targeting infants via the EPI gives a target population in sub-Saharan Africa of 15 million infant girls annually. The target population in South-East (SE) Asia would be 22.5 million infant girls. Vaccinating 11-12 year old girls, as for the HPV vaccine target population gives 10 and 20 million girls annually in Africa and SE Asia. In addition there would be a catch-up population, consisting of those women of child-bearing age, who have not received the vaccine. This group would consist of some 316 million girls/women aged up to 25 in Africa, and some 600-800 million in endemic areas of SE Asia. A pregnancy malaria vaccine can co-exist with a general malaria vaccine and will act in synergy with other malaria control measures such as ITNs and IPTp in reducing the total health and disease burden originating from malaria. A successful placental malaria vaccine will have enormous impact on women and child health in endemic countries.

Indirect vaccine development impact: The VAR2CSA vaccine is the first vaccine targeting the pathogenic cyto-adhesion of *P. falciparum* malaria parasites. The work proposed in this application will have a broad impact on the general development of PfEMP1 based cyto-adhesion blocking vaccines, which is an area that has been and is being extensively supported by the EU. It will potentially lead the way for development of compounds that can be used against cyto-adherent malaria parasites in children causing severe syndromes like for example cerebral malaria.

Impact on capacity building: The project was a close collaboration between scientists in Benin and in Europe. During the project, we transferred knowhow from Europe, a clinical trial site was constructed and research personnel were exchanged. This impact on other activities at UAC and will build up capacity for testing of other vaccines at this field site.

SME impact: ExpreS2ion strives to become a leading provider of solutions for cost effective production of complex antigens. This project served as an independent verification of ExpreS2ion's ability to deliver on complex vaccine projects and it has driven the company's development of the necessary infrastructure. The project enabled ExpreS2ion to optimize its technology for increased product yields at lower cost. This strengthened ExpreS2ion's competitive position in its core market of vaccine production. As part of this project, ExpreS2ion expanded its facilities to include a fully equipped downstream purification laboratory. Downstream capabilities are an integral part of vaccine process development and this therefore strengthened and expanded ExpreS2ion. Strong ties to CMOs form a central part of ExpreS2ion's expansion strategy, which requires the building of a CMO network capable of

helping ExpreS2ion offer a complete vaccine production solution as well as creating co-marketing opportunities. Process transfer and management of the outsourced GMP manufacturing further strengthened ExpreS2ion's business model of offering a complete vaccine production solution through collaboration with CMOs.
European and International added value: The research has led to enhancement of already established networks of collaboration between centers of scientific excellence in EU Member States and partner institutions in low income countries. Development of a VAR2CSA vaccine has been a European priority for years and the major milestone of testing the vaccine in humans is now within reach. The project emphasized the role of the EU as the world leading capacity within blood stage malaria vaccine development. In particular, UCPH benefitted from a close collaboration with UAC and IRD in our research to develop malaria vaccine for children. Parallel activities enhanced the possibilities in using the Expres2 platform to produce other malaria vaccine candidates from partner institutions – to the benefit of both the SME and the University partners.

Impact of exploitable foreground: developing of virus like particle vaccine platforms:
An accessible, cost effective and versatile virus like delivery platform that allows display of complex recombinant antigens in an ordered and high density manner is currently not available. Hence, clinical development is either based on recombinant soluble antigens or alternative display methods, making it difficult to evaluate and compare the candidacy of target antigens. Our aim is to address this issue by developing and making available a versatile VLP delivery platform that can be used to conjugate any complex antigen thereby reducing eminent the risk of failure in clinical testing associated with sub-optimal titers. This will impact upon preclinical development and antigen selection as well as clinical trial outcome.
The overall strength of development of the VLP platforms is the possibility to produce with a minimal effort and alteration, any recombinant vaccine candidate into a VLP. This is especially potent with respect to induce antibodies but may also address the potency of T-cell responses. The preclinical development of the MSA VLP platform was partly developed through a Gates Foundation funded project at UCPH and is therefore part of BMGF Global Access policies. We aim to continue the "Global Access" development by making the vaccine delivery platform available for both the malaria and neglected disease vaccine research community. The impact of this will be an increase in the number and quality of vaccine candidates to proceed into further development and clinical testing.
In our pre-clinical work we have found from 8-fold (VAR2CSA) to 800 fold (cancer self-antigens) increases in antibody titers using our VLP technology. The proposed further clinical development of the PAMVAC vaccine will strengthen research into the strategic goals of the malaria vaccine technology roadmap. At the later stages of clinical development the delivery platform will potentially provide a reduction of final cost of employing a vaccine, as VLP display of antigens potentially reduces the vaccine dose 10 fold and potentially reduce the number of immunizations from three to one due to generation of long lasting immune response. Ultimately, this could provide a 30-fold reduction in the cost-per-dose when mass administering vaccines.
The VLP platform will also provide proof of concept for a system to enable screening and validation of novel vaccine targets. Applying the VLP platform to early stage screening of target vaccine candidates enables a systematic approach to prioritizing antigens with less effort as the need for screening with several different extrinsic adjuvants to find the most optimal one, is no longer required. The reason for this is that displaying the target antigens on a VLP potentially transcends the need for extrinsic adjuvants. This is important as extrinsic adjuvants may divert immunological responses, especially B-cell responses from certain epitopes. The VLP technology allows comparison of antigens in an unbiased screening system; therefore, by increasing the

inherent immunogenicity of vaccine candidates, reducing the number of pre-clinical animal screening-groups by at least two-fold depending on the number of extrinsic adjuvants to be tested, which is normally more than two. This potentially improves the cost associated with small animal immunizations in pre-clinical screening programs by more than two fold, thus an increasing number of antigens can be screened for the same cost, eventually increasing the chance of discovering an effective vaccine. Importantly reducing the number of small animals also has ethical impacts.

Societal impacts of a VLP platform technology

One of the long term goals of developing a VLP platform is to identify a pediatric malaria vaccine. Malaria is one of the leading causes of death in children under five years in sub-Saharan Africa. Worldwide there are an estimated 214 million cases of malaria in 2015 and hundreds of thousand deaths. In order to protect against malaria children under five must be targeted. If lifelong or extended duration of protection against malaria cannot be achieved, which presently is the most likely scenario, the annual target population via the EPI in sub-Saharan Africa alone is 30 million infants annually. The target population in South-East (SE) Asia would be more than 40 million infants. With regard to transmission blocking malaria vaccines the annual target population that serves as a parasite reservoir would consist of several billion individuals living in mainly in Africa, South America and SE Asia. Repeated vaccination is not the preferred option as it is costly, but this should be compared to the current cost of malaria control (over 2 billion dollars annually), malaria case management (several hundred million dollars annually), and reduced labor productivity [116].

A malaria vaccine would benefit from demonstrating applicability with children who are already part of the World Health Organization (WHO) Expanded Program on Immunization (EPI) currently reaching more than three quarters of the target population. This would however require that malaria vaccination does not interfere with other vaccinations during early childhood and that the number and interval between immunizations can align with already existing programs.

Other Impacts

The VLP technology may be useful to combat not only infectious diseases. We have, as a proof of concept, used cancer and asthma associated self-antigens as examples of how the VLP technology may be applied (the varieties of other applications are too many to be accounted for here). Cancer vaccines: There is an urgent need for novel strategies to combat cancer, both in the developed part of the world and, in particular, in less developed countries where the majority of the population does not benefit from the currently available therapies, based on drug conjugates or immunotherapy, which are often very expensive. The VLP technology could be used to develop cost-effective therapeutic cancer vaccines targeting antigens, which has been demonstrated to be overexpressed in the vast majority of ovarian cancers, breast cancers, cervical cancers, renal cell cancers, bladder cancers and colorectal cancers thus constituting a major market. In addition targeting antigens such as the PD-L1 antigen on tumour cells potentiates antitumor immune responses. Our VLP technology may remove obstacles that have made cancer vaccines fail because of the inability to induce a potent immune response against self-antigens. The establishment of the vaccine platform in this project will therefore serve to establish proof-of-principle for a broad applicability VLP technology in the fight against cancer. This may pave the way for the development of vaccines as a cost effective alternative to current monoclonal antibody therapy that suffers from major shortcomings in a chronic setting: most notably, generation of anti-antibodies and high cost of goods.

Chronic diseases: As a proof of concept, we have demonstrated both breaking of self-tolerance against IL5 and furthermore shown induction high affinity antibodies using the VLP platform.

This may lead to better antibody treatments or even vaccines to treat eosinophilic airway inflammation. It is currently estimated that more than 240 million people live with asthma worldwide. Around 10% of all asthma patients are estimated to have severe asthma and a sub population of these patients have eosinophilic airway inflammation. The over-production of eosinophils is known to cause inflammation in the lungs that can affect the airways, limiting breathing and increasing the frequency of exacerbations. As interleukin-5 (IL-5) is the main promoter of eosinophil growth and activation, as well as being essential in signalling the movement of eosinophils from the bone marrow into the lung, administering of IL-5 specific monoclonal antibodies (Mepolizumab) is a possible treatment, which is close to market approval by GSK.