# Malaria vaccine candidates displayed on novel virus-like particles are immunogenic and induce transmission-blocking activity

Authors/Affiliations:

Jo-Anne Chan[1,2], David Wetzel[3,4], Linda Reiling[1], Kazutoyo Miura[5], Damien Drew[1], Paul R Gilson[1], David A Anderson[1], Jack S Richards[1,6], Carole A Long[5], Manfred Suckow[3], Volker Jenzelewski[3], Takafumi Tsuboi[7], Michelle J Boyle[8], Michael Piontek[3] and James G Beeson[1,2,6]

[1]Burnet Institute, Melbourne, VIC, Australia

[2]Department of Immunology, Central Clinical School, Monash University, VIC, Australia

[3]ARTES Biotechnology GmbH, Langenfeld, Germany

[4]Technical University of Dortmund, Laboratory of Plant and Process Design, Dortmund, Germany

[5]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Disease, National Institutes of Health, Rockville, Maryland, USA

[6]Department of Medicine, University of Melbourne, VIC, Australia

[7]Proteo-Science Centre, Ehime University, Matsuyama, Ehime, Japan

[8]QIMR-Berghofer Medical Research Institute, Herston, QLD, Australia

Corresponding Author:

James Beeson

Burnet Institute

85 Commercial Road, Melbourne, VIC 3001, Australia

beeson@burnet.edu.au

Ph: 61-3-9282-2442

Fax: 61-3-9282-2100

## ABSTRACT

The development of effective malaria vaccines remains a global health priority. Currently, the most advanced vaccine, known as RTS,S, has only shown modest efficacy in clinical trials. Thus, the development of more efficacious vaccines by improving the formulation of RTS,S for increased efficacy or to interrupt malaria transmission are urgently needed. The RTS,S vaccine is based on the presentation of a fragment of the sporozoite antigen on the surface of virus-like particles (VLPs) based on human hepatitis B virus (HBV). In this study, we have developed and evaluated a novel VLP platform based on duck HBV (known as Metavax) for malaria vaccine development. This platform can incorporate large and complex proteins into VLPs and is produced in a *Hansenula* cell line compatible with cGMP vaccine production. Here, we have established the expression of leading *P. falciparum* malaria vaccine candidates as VLPs. This includes Pfs230 and Pfs25, which are candidate transmission-blocking vaccine antigens. We demonstrated that the VLPs effectively induce antibodies to malaria vaccine candidates with minimal induction of antibodies to the duck-HBV scaffold antigen. Antibodies to Pfs230 also recognised native protein on the surface of gametocytes, and antibodies to both Pfs230 and Pfs25 demonstrated transmission-reducing activity in standard membrane feeding assays. These results establish the potential utility of this VLP platform for malaria vaccines, which may be suitable for the development of multi-component vaccines that achieve high vaccine efficacy and transmission-blocking immunity.


## INTRODUCTION

Mortality caused by *Plasmodium falciparum* malaria is estimated at 216 million cases annually, with approximately 500,000 deaths occurring worldwide [1]. Despite on-going efforts, malaria control has stalled with little reduction of malaria cases observed in the past few years [1]. The spread of anti-malarial drug resistance together with insecticide resistance in parasite vectors has further escalated the need for an effective malaria

vaccine. Malaria vaccine strategies can be broadly classified into three approaches; pre-erythrocytic vaccines that target sporozoites and/or infected hepatocytes, blood-stage vaccines that generally target merozoites and antigens on the surface of infected red blood cells, and transmission-blocking vaccines that target the sexual stages of malaria or mosquito-stage antigens [2]. While vaccines targeting pre-erythrocytic stages and blood stages aim to directly prevent infection and disease, there is a growing focus on vaccines that can interrupt or reduce malaria transmission, highlighted by key global organisations including the World Health Organisation (WHO), Bill and Melinda Gates Foundation and PATH Malaria Vaccine Initiative [3]. The most advanced *P. falciparum* vaccine RTS,S (Mosquirix$^{TM}$) is based on the pre-erythrocytic stage of the parasite life cycle and is the only malaria vaccine to have completed phase III clinical trials [4] and is currently undergoing implementation trials in several African countries [1]. However, vaccine efficacy was low in young children [5] and antibodies induced by vaccination waned quickly in the year after immunisation [6]. The WHO and their partners have set an objective of developing a malaria vaccine with 75% efficacy [3]. To achieve this goal, future vaccine development may be dependent on novel strategies that induce sufficiently high levels of functional antibodies[2].

Transmission-blocking vaccines will need to induce a potent antibody response within the host to inhibit the downstream development of parasites in the mosquito vector after a blood meal [7]. This will prevent or reduce the subsequent spread of malaria parasites throughout an endemic population. However, the advancement of such vaccines remains hampered by the lack of knowledge and tools required to study the sexual, transmissible stages of *P. falciparum*. The progress of sexual-stage antigens as vaccine candidates is further limited, in part, by the difficulty to express high yields of properly folded recombinant antigens, and the requirement for vaccine approaches that generate high and sustained levels of antibodies for effective transmission-blocking activity. Leading vaccine candidates that are expressed during the *P. falciparum* sexual-stage include Pfs230 and Pfs25. Pfs230 is expressed on the surface of gametocytes that reside within the human host, while Pfs25 is expressed on the

surface of female gametes, zygotes and ookinetes in the mosquito vector [8]. Numerous studies have shown that individuals naturally exposed to malaria acquire antibodies that target Pfs230 (reviewed in [9]). Therefore, immunity afforded by vaccines based on Pfs230, and other major antigens expressed on the gametocyte surface, have the added benefit of potential for antibody boosting from natural malaria exposure. Pfs25 represents the only sexual-stage antigen that has completed human clinical trials [10-12], and Pfs230 is currently undergoing clinical trials. Antibodies generated through vaccination with Pfs25 have been reported to inhibit the development of parasites within the mosquito midgut [11-13], demonstrating the potential of vaccines to interrupt the transmission of malaria throughout a human population.

The development of potential vaccines requires the induction of strong and sustained immune responses in humans, which has been challenging to achieve. The classic approach of a subunit vaccine includes the delivery of a recombinant antigen formulated with the appropriate adjuvant (reviewed in [14]). Expression of antigens in virus-like particles (VLPs) have been successfully developed for the hepatitis B virus and human papilloma virus (reviewed in [15]). Key characteristic features of viruses such as their repetitive surface geometry and activation of specific immunological receptors are maintained by VLPs. However, they do not have the ability to undergo gene replication as they lack a viral genome [16]. Therefore, they have been established as a safe and effective vaccine delivery platform for use in humans (reviewed in [15]). Furthermore, the particle size of VLPs (ranging from 20–200nm) is optimal for immunogenicity and uptake by dendritic cells [17,18]. VLP-based vaccines elicit strong humoral and cellular responses, supported by their capacity to cross-link B cell receptors for successful B cell activation [19]. The use of VLPs to display and present antigens to the immune system appeared to generate a stronger cellular and humoral response compared to the soluble antigen alone [20,21]. The only VLP-based transmission-blocking vaccine designed for use in human clinical trials involves a chimeric, non-enveloped VLP expressing Pfs25 fused to the Alfalfa mosaic virus coat protein

[22]. However, although the vaccine induced Pfs25-specific antibodies in a clinical trial, there was limited inhibition of parasite transmission to mosquitoes [22], suggesting the need for improved vaccine formulations.

Here, we describe a novel platform that incorporates sexual-stage malaria vaccine candidates, Pfs230 and Pfs25, into a VLP scaffold that is based on the duck hepatitis B virus (DHBV) small surface protein (dS). In contrast to the human HBV platform, the DHBV delivery platform allows the expression of large and complex proteins [16], which are typical of malaria vaccine candidates. In this platform, resulting VLPs contain a lipid envelope that favours the optimal presentation of transmembrane and GPI-anchored proteins. *P. falciparum* sexual-stage antigens, Pfs25 and the domains Pfs230c [23] and Pfs230D1M [24] derived from the full-length Pfs230 protein, were genetically fused to the dS and the resulting fusion proteins were co-expressed with wild-type dS in methylotrophic yeast *Hansenula polymorpha* [16]. The successful display of antigens on the chimeric VLPs (referred to as Pfs25-dS/dS, Pfs230c-dS/dS and Pfs230D1M-dS/dS) was confirmed using Western blot analyses and visualized by electron microscopy and super resolution microscopy (presented in the accompanying manuscript by Wetzel D et al 2019, submitted) [25]. In this manuscript, we report the immunogenicity of these sexual-stage chimeric VLPs through animal immunisations and further characterized their functional antibody response through mosquito feeding assays.

## METHODS

### Production and purification of chimeric VLPs

The production and purification of the three different kinds of chimeric VLPs (Pfs230c-dS/dS, Pfs230D1M-dS/dS and Pfs25-dS/dS) was conducted as previously described [16,25]. Genes encoding the three fusion proteins Pfs230c-dS, Pfs230D1M-dS and Pfs25-dS were designed and codon-optimized for expression in *Hansenula polymorpha* (Genbank ref.: MH142260,

MH142261, MH142262). Pfs230c-dS consists of amino acids (aa) 443-1132 (630 aa fragment) from full-length Pfs230 [23] and the shorter variant Pfs230D1M-dS consists of aa 542-736 (194 aa fragment) from full-length Pfs230 [24]. The fusion protein Pfs25-dS is comprised of aa 23-193 (170 aa fragment) of the cysteine-rich Pfs25 protein fused to the dS. Each of the fusion proteins encoding genes were co-expressed with a gene encoding the wildtype dS (Genbank ref: MF510122) in a recombinant *Hansenula*-derived yeast cell line. The purification of the chimeric VLPs was based on a downstream process approved for human VLP vaccine production from yeast [26] including adaptations for the purification of VLPs containing the *P. falciparum* antigens [25]. The relative incorporation of the Pfs230-dS/dS or Pfs25-dS/dS fusion proteins into VLPs, compared to total protein, was estimated by densitometry analysis of Coomassie stained gels of VLPs, as described in the accompanying manuscript [25]. The relative incorporation rates were ~30% for Pfs230c-dS/dS VLPs, ~24% for Pfs230D1M-dS/dS VLPs, and ~3% for Pfs25-dS/dS VLPs.

**Expression of monomeric recombinant proteins**

The expression of monomeric recombinant Pfs230D1M was performed in HEK293F cells as previously described [27]. Briefly, a truncated form of Pfs230 containing the first 6-cys domain of Pfs230 has previously been expressed as a monomeric recombinant protein in *P. pastoris* termed Pfs230D1H [24]. To assess vaccine-induced antibodies to Pfs230, we expressed a modified form of monomeric recombinant Pfs230D1H in the mammalian HEK293 cell expression system, which we termed Pfs230D1M. Monomeric recombinant Pfs25 was expressed in a wheatgerm cell-free expression system as previously described [28].

**Measuring antibodies to chimeric VLPs by ELISA**

Chimeric VLPs or monomeric recombinant proteins were coated onto Maxisorp microtiter plates (Nunc) at 1µg/ml in PBS and incubated overnight at 4°C. Plates were blocked with 1% casein in PBS (Sigma-Aldrich) for 2h at 37°C before primary antibodies were added

(polyclonal mouse anti-Pfs230 or anti-Pfs25 antibodies; or rabbit antibodies against Pfs230 or Pfs25). Secondary HRP-conjugated antibodies (polyclonal goat anti-mouse IgG at 1/1000 or anti-rabbit IgG at 1/2500 from Millipore) were used to detect antibody binding. Colour detection was developed using ABTS or TMB liquid substrate (Sigma-Aldrich), which was subsequently stopped using 1% SDS (for ABTS) or 1M sulphuric acid (for TMB). PBS was used as a negative control and plates were washed thrice using PBS with 0.05% Tween in between antibody incubation steps. The level of antibody binding was measured as optical density at 405nm (for ABTS) or 450nm (for TMB).

**Measuring antibody affinity by ELISA**

Antibody affinity was assessed using standard ELISA, with the addition of an antibody dissociation step using increasing concentrations of ammonium thiocynate (in PBS). This was incubated for 20 min at room temperature, following incubation with antibody samples (R1917 and 1918 were tested at 1/800, R 1919 and R1920 were tested at 1/100). Colour detection was developed using ABTS (measured at optical density at 405nm).

**Measuring antibodies to the gametocyte surface by flow cytometry**

Mature stage V gametocyte-infected erythrocytes were generated as previously described [27,29] and treated with saponin to permeabilize the infected erythrocyte membrane. Gametocytes were subsequently incubated with whole serum from rabbits immunized with Pfs230c-dS/dS VLPs, followed by an AlexaFluor 488-conjugated donkey anti-rabbit IgG (1/500) with ethidium bromide to distinguish between infected and uninfected erythrocytes (1/1000), with washing between each step. Data was acquired by flow cytometry (FACS Verse, BD Biosciences) and analysed using FlowJo software. Antibody levels are expressed as the geometric mean fluorescence intensity (MFI; arbitrary units).

**Immunofluorescence Microscopy**

196 Thin blood smears of stage V 3D7 gametocyte-infected erythrocytes were fixed in 90%
197 acetone and 10% methanol for 5 min at -20°C as previously described [30]. Briefly, slides
198 were rehydrated in PBS and blocked with 6% BSA for 30 minutes before incubation with
199 primary antibodies (rabbit antibodies were tested at 1/100) for 2h. Slides were subsequently
200 incubated with the secondary AlexaFluor 488-conjugated IgG (1/1000; Thermo Scientific) for
201 1h. Slides were washed thrice in PBS between antibody incubation steps. Slides were
202 mounted in medium containing DAPI to label the parasite nucleus. Images were collected
203 using a Plan-Apochromat (100×/1.40) oil immersion phase-contrast lens (Carl Zeiss) on an
204 AxioVert 200M microscope (Carl Zeiss) equipped with an AxioCam Mrm camera (Carl
205 Zeiss). Images were processed using Photoshop CS6 (Adobe).
206
207 **Rabbit immunisations**
208 Pfs230c-dS/dS and Pfs230D1M-dS/dS VLPs were formulated with equal volumes of sterile
209 Alhydrogel aluminium hydroxide vaccine adjuvant (Brenntag, Denmark) and incubated on
210 the shaker for 5 min prior to immunisations. New Zealand White rabbits were immunised 2
211 weeks apart with 3 doses of chimeric VLPs. The total amount of protein and adjuvant used
212 in each immunisation is presented in Supplementary Tables S1-S3 for Pfs230c-dS/dS,
213 Pfs230D1M-dS/dS and Pfs25-dS/dS VLPs, respectively. Briefly, rabbits (R1864-R1871)
214 were immunised with two vaccine doses of Pfs230c-dS/dS VLPs formulated with and without
215 Alhydrogel. As standard doses, we used 20 µg or 100 µg to total VLP protein for
216 immunizations; the respective amount of Pfs230c protein is reported in Supplementary
217 Tables S1-S3. Rabbits were immunised with Pfs230D1M-dS/dS VLPs formulated with
218 Freund's adjuvant (R1917, R1918) or Alhydrogel (R1919, R1920) at a single vaccine dose
219 (of 100 µg to total VLP protein). Rabbits were immunised with Pfs25-dS/dS VLPs formulated
220 with Freund's adjuvant (R1825, R1826) at a single vaccine dose (of 100 µg to total VLP
221 protein). Animal immunisations and the formulation of Pfs25dS/dS VLPs with Freund's
222 Adjuvant were conducted by the Antibody Facility at the Walter and Eliza Hall Institute.
223 Rabbits were euthanised with a terminal dose of sodium pentobarbitone administered

intravenously. Animal immunisations were approved by the Animal Ethics Committee of the Walter and Eliza Hall Institute, Australia (#2017.018).

**Measuring transmission-blocking activity by standard membrane feeding assays**

IgG purification from individual rabbit serum samples was performed using Protein G columns (GE Healthcare) according to the manufacturer instructions and adjusted to a final concentration of 20 mg/ml in PBS. The standardized methodology for performing the standard membrane feeding assays (SMFA) was described previously [31]. Briefly, 16-18 days old gametocyte cultures of the *P. falciparum* NF54 line were mixed with purified test IgG at 7.5 mg/ml, and the final mixture, which contained human complement, was immediately fed to ~50 female *Anopheles stephensi* mosquitoes through a membrane-feeding apparatus. Mosquitoes were kept for 8 days and dissected to enumerate the oocysts in the midgut (n=40 per group for the negative control group, and n=20 for test groups). As the negative controls, total IgG purified from pre-immune rabbit sera were utilised. Only midguts from mosquitoes with any eggs in their ovaries at the time of dissection were analyzed. The human serum and red blood cells used for the SMFA were purchased from Interstate Blood Bank (Memphis, TN).

**Statistical analysis**

The best estimate of % inhibition in oocyst density (% transmission-reducing activity, %TRA), the 95% confidence intervals (95%CI), and *p*-values from single or multiple feeds were calculated using a zero-inflated negative binomial random effects model (ZINB model) described previously [32]. All statistical tests were performed in R (version 3.5.1), and *p*-values <0.05 were considered significant.

## RESULTS

**Sexual-stage vaccine candidates are displayed on chimeric VLPs**

Chimeric VLPs expressing sexual-stage *P. falciparum* antigens, Pfs230 and Pfs25, were produced and purified as described [25]. Antigens were incorporated into the VLP scaffold through genetic fusion of Pfs230 and Pfs25 with the dS protein. For the expression of the chimeric Pfs230 VLPs, we used 2 different constructs. Due to the large size and complexity of Pfs230, we did not attempt expression of full length Pfs230 in VLPs; instead we expressed two truncated forms based on published constructs that have demonstrated induction of transmission-blocking antibodies when used as recombinant protein vaccines in experimental animals. The first was a truncated construct of Pfs230, termed Pfs230c, which includes the first two 6-cysteine domains of Pfs230 [23], was used to generate fusion proteins for VLP formation. Pfs230c has been previously shown to elicit transmission-blocking antibodies [23]. The expression and purification of Pfs230c-dS VLPs was challenging [25]. Therefore, a shorter construct of Pfs230, termed Pfs230D1M (based on the construct described by [24]) which includes only the first 6-cysteine domain of Pfs230, was designed and fused to the dS antigen. Further, Pfs230D1M is a leading transmission-blocking vaccine candidate currently in clinical trials. Here, we evaluated the display of these sexual-stage antigens on the surface of native VLPs. We used specific antibodies generated against Pfs230 and Pfs25 to detect the expression of these antigens on the chimeric VLPs by ELISA (Fig 1). We found that a Pfs230-specific polyclonal antibody recognised the surface of Pfs230c-dS/dS (Fig 1A) and Pfs230D1M-dS/dS VLPs (Fig 1B). Similarly, a Pfs25-specific monoclonal antibody recognised the surface of Pfs25-dS VLPs (Fig 1C). These results confirm the expression of sexual-stage antigens on the surface of chimeric VLPs. There was no recognition of a plain dS VLP without a Pfs230 or Pfs25 fusion protein (Fig 1D).

**VLPs expressing sexual-stage antigens induced antibodies in rabbits**

To understand whether these chimeric VLPs displaying sexual-stage antigens were capable of eliciting an immune response, the VLP constructs were used to immunise rabbits (n=8 for Pfs230c-dS/dS, n=4 for Pfs230D1M-dS/dS, n=2 for Pfs25-dS/dS). Serum from immunised

rabbits had significant antibody recognition of monomeric recombinant Pfs230D1M (Fig 2A-D) or Pfs25 (Fig 2E, 2F) in a concentration-dependent manner by ELISA. For immunisations with Pfs230c-dS/dS VLP, we investigated two vaccine doses (20 or 100 µg of total VLP protein) formulated with or without Alhydrogel, which is an adjuvant suitable for clinical use. The higher dose appeared to induce a higher antibody response in rabbits, and formulation with the Alhydrogel adjuvant also contributed to higher antibody levels (Fig 2A, B). Given the findings with Pfs230c-dS/dS VLPs, immunisations with Pfs230D1M-dS/dS VLP were only performed at a single dose (using the higher dose of 100µg of total VLP protein), formulated with Alhydrogel or with Freund's adjuvant for comparison. Both formulations induced substantial antibodies to monomeric recombinant Pfs230D1M (Fig 2C, D); antibodies were higher for formulations with Freund's, which is a more potent adjuvant, but cannot be used clinically. For Pfs25-dS/dS VLPs, immunizations were only performed with Freund's adjuvant as a proof-of-principle, since Pfs25 has already been evaluated with different adjuvants using different platforms in published studies and has advanced into clinical trials [11,13].

**Rabbit antibodies induced by chimeric VLPs recognized native Pfs230 expressed on gametocytes**

We evaluated whether rabbit antibodies generated against Pfs230c-dS/dS VLPs were capable of recognizing native antigens expressed on the surface of mature stage V gametocytes by flow cytometry (Fig 3A). The majority of the rabbit sera had substantial antibody reactivity to the surface of mature gametocytes, suggesting that vaccine-induced antibodies recognize native Pfs230 (Fig 3A). This finding was confirmed using immunofluorescence microscopy, which showed that rabbit antibodies generated against Pfs230c-dS/dS VLPs labelled native Pfs230 expressed on the surface of fixed mature gametocytes (Fig 3B).

**Rabbit antibodies induced by chimeric VLPs have transmission-reducing activity**

306 To address the functional significance of rabbit antibodies generated against sexual-stage
307 chimeric VLPs, we examined the ability of these antibodies to inhibit mosquito infection
308 through standard membrane feeding assays (SMFA) in the presence of human complement
309 [31]. Functional transmission-blocking activity is defined as the reduction in oocyst count
310 compared to a negative control group. When antibodies against Pfs230c-dS/dS VLPs were
311 tested at 7.5mg/ml, none of them showed significant inhibition (Table 1). In contrast, 2 out of
312 4 rabbits immunized with Pfs230D1M-dS/dS VLPs demonstrated significant inhibition in
313 SMFA (Table 2). Antibodies generated against Pfs25-dS/dS VLPs from one of the two
314 rabbits also successfully blocked the development of oocysts within the mosquito midgut
315 (Table 3; 97.4% inhibition). It was interesting that the second rabbit did not significantly
316 inhibit transmission despite substantial induction of antibodies detected by ELISA,
317 suggesting that antibody titre and specificity may be important for functional activity.
318 Interestingly, there was no clear relationship between IgG levels and activity in SMFA by
319 rabbit antibodies generated to Pfs230D1M-dS/dS. While VLPs formulated with Freund's
320 adjuvant generated higher IgG reactivity to Pfs230, IgG generated using VLPs formulated
321 with alum tended to have stronger inhibitory activity in SMFA (Table 2). To understand these
322 differences further, we estimated the avidity of IgG binding to Pfs230D1M for different rabbit
323 antibodies (Supplementary Figure S1). This indicated no major difference in IgG avidity that
324 would explain difference in activity in SMFA, suggesting differences in functional activity may
325 be explained by differences in epitope targeting of antibodies.
326
327
328 **DISCUSSION**
329 Our work describes the immunogenicity of transmission-blocking malaria vaccine candidates
330 presented on the surface of VLPs using a novel platform based on the duck hepatitis B virus.
331 Leading sexual-stage antigens, Pfs230 and Pfs25 were engineered into chimeric VLPs and
332 their surface expression characterised using specific antibodies. Data is presented in the
333 accompanying manuscript [25] on the production and purification of these chimeric VLPs.

These showed that chimeric VLPs expressed proteins of expected size corresponding to Pfs25 and the respective Pfs230 domains, including the small surface protein dS. Imaging by electron microscopy identified homogenous, particulate structures corresponding to the chimeric VLPs and super resolution microscopy further confirmed the recognition of Pfs230-derived domains and Pfs25 on the chimeric VLP surface, and their colocalisation with the dS antigen, using specific polyclonal antibodies. In this study, we showed that the VLPs were reactive by ELISA with antibodies to Pfs230 or Pfs25, further demonstrating the display of malaria vaccine candidates on the VLP surface. In rabbit immunisation studies, we showed that the VLPs were immunogenic and were capable of inducing substantial antibody reactivity to monomeric recombinant Pfs230D1M or Pfs25 protein. Further, rabbit antibodies generated against Pfs230c-dS/dS and Pfs230D1M-dS/dS VLPs recognised the surface of native gametocytes by flow cytometry and this was also visualised by immunofluorescence microscopy. Importantly, rabbit antibodies against Pfs230D1M-dS/dS and Pfs25-dS/dS VLPs were capable of blocking mosquito infection measured through standard membrane feeding assays.

A conformational-dependent monoclonal antibody, 4B7, was used to characterise the display of antigen expressed by the Pfs25-dS/dS VLP. There were high levels of antibody recognition measured by ELISA to the Pfs25-dS/dS VLP, which correlated with the coating concentration of monomeric recombinant Pfs25. Similarly, using a Pfs230-specific polyclonal antibody, the display of antigen expressed by the Pfs230c-dS/dS and Pfs230D1M-dS/dS VLP was confirmed. This suggests that the Pfs25 and Pfs230 constructs had successfully been incorporated as fusion proteins into the chimeric VLPs. The incorporation rate of Pfs25-dS into VLPs was relatively low and optimization may be needed to increase incorporation rates prior to further evaluation of Pfs25 VLPs using this platform.

Our immunogenicity studies showed that the chimeric VLPs could elicit an immune response in rabbits. For immunisations with Pfs230c-dS/dS VLP, two dosing regimens were used in

362 the presence and absence of Alhydrogel. As the higher dose appeared to induce a better
363 antibody response in rabbits, and formulation with Alhydrogel was needed for high antibody
364 levels, immunisations with Pfs230D1M-dS/dS VLP were done at a single dose (using the
365 higher dose) and formulated with Alhydrogel. Pfs230D1M-dS/dS VLPs were also formulated
366 with Freund's adjuvant for comparison. VLPs formulated with Alhydrogel induced substantial
367 antibody responses, but responses were higher with Freund's adjuvant. This was expected
368 because Freund's is a more potent adjuvant than Alhydrogel. Our results suggest that
369 investigating other clinically-acceptable adjuvants that may induce higher antibody
370 responses than Alhydrogel would be valuable. Further, rabbits were immunised with Pfs25-
371 dS/dS VLP using only Freund's adjuvant as a proof-of-principle to evaluate immunogenicity
372 and transmission-blocking activity. Antibodies generated against the Pfs25-dS/dS VLP
373 recognised monomeric recombinant Pfs25 by ELISA. Together, these findings suggest that
374 immunisation with chimeric VLPs induced a good immune response in rabbits, further
375 supporting the potential for the use of this VLP platform to present malaria antigens for
376 vaccination. However, further detailed studies are needed to evaluate the immunogenicity
377 and functional activity of antibodies generated by antigens presented as VLPs compared to
378 monomeric recombinant proteins. In addition, we measured the ability of rabbit antibodies to
379 recognise the surface of native gametocytes by flow cytometry. We found that rabbit
380 antibodies generated against Pfs230c-dS/dS VLPs were capable of recognising the surface
381 of mature, stage V gametocytes. This was also confirmed by immunofluorescence
382 microscopy using fixed smears of mature gametocytes.
383
384 Functional antibody responses were evaluated using standard membrane feeding assays,
385 which are widely used to assess transmission-reducing activity [32]. Antibodies from rabbits
386 immunised with Pfs25-dS/dS VLPs and Pfs230D1M-dS/dS could strongly inhibit the
387 development of oocysts within the mosquito midgut, indicating the successful inhibition of
388 parasite transmission. However, inhibitory activity was variable between immunized rabbits.
389 While further studies with larger numbers of animals, and including mice or rats, will be

required to better understand variability in vaccine responses, the findings here are sufficient to establish a proof-of-concept for generating transmission-blocking activity using VLPs. Our findings support previous work that showed that rabbit antibodies against Pfs230D1M had transmission-reducing activity by SMFA [24]. Recent work has also reported that only constructs containing domain 1 of Pfs230 were capable of inducing transmission-reducing activity by SMFA, compared to constructs lacking that particular domain [33].

Further, the Pfs230D1M construct is currently undergoing phase I clinical trials. Together, these findings support the importance of Pfs230D1M in the induction of functional transmission-reducing antibodies. Of note, transmission-reducing activity was not observed for antibodies generated against Pfs230c-dS/dS VLPs. This could have been due to the sub-optimal folding of Pfs230c, such that key functional epitopes were not displayed, or important antibody-binding epitopes were masked. These factors potentially resulted in low purity and reduced yield of chimeric VLP and thus led to the lack of functional antibodies generated. Interestingly, there was no clear relationship between IgG reactivity levels quantified by ELISA and transmission-blocking activity in SMFA. This was particularly evident for Pfs230D1M-dS/dS; immunization with VLPs formulated with Freund's adjuvant generated higher IgG reactivity than using alum as the adjuvant, but IgG generated using alum tended to have greater activity in SMFA. There was no substantial difference in avidity between IgG induced using different adjuvants, which suggests the differences in transmission-blocking activity are most likely explained by differences in epitope-specificity of IgG. Future studies with a larger number of animals and immunization regimens may help further understand this.

In conclusion, we have demonstrated the successful display of sexual-stage antigens Pfs230 and Pfs25 on the surface of chimeric VLPs. We have established a proof-of-concept which showed that these VLPs generated significant immune responses that recognised homologous recombinant protein and native sexual-stage antigens expressed on gametocytes. Further, these antibodies had the ability to block the transmission of parasites

418 to mosquitoes through membrane feeding assays. Together, our results support the further
419 evaluation of chimeric VLPs as a novel delivery platform for leading malaria vaccine
420 candidates. Future studies to optimise antigen incorporation and presentation in VLPs and to
421 evaluate different adjuvants and dosing regimens for immunization will further inform the
422 potential utility of this strategy for malaria vaccine development.
423
424 **Author contributions statement**
425 Conceptualization, JC, DW, LR, PG, JR, DA, MS, MP, and JB; Methodology, JC, DW, LR,
426 DD, VJ, KM, TT and MP; Investigation, JC, DW, LR, DD and KM; Administration, VJ, JR,
427 DA, MP and JB; Manuscript preparation, JC, DW and JB (and critically reviewed by all
428 authors); Funding Acquisition, JB and MP. Supervision, JB, MP, CL, MB. All authors
429 approved the final manuscript.
430
431 **Acknowledgements**
432 The authors gratefully acknowledge Colleen Woods for ongoing advice and project
433 feedback, Heribert Helgers, Renske Klassen, Christine Langer, Ashley Lisboa-Pinto and
434 Thomas Rohr for technical and academic assistance.
435
436 **Funding**
437 Funding was provided by PATH Malaria Vaccine Initiative and the National Health and
438 Medical Research Council (NHMRC) of Australia (Senior Research Fellowship and Program
439 Grant to JB, Career Development Fellowship to MB). Burnet Institute is supported by funding
440 from the NHMRC Independent Research Institutes Infrastructure Support Scheme and a
441 Victorian State Government Operational Infrastructure grant. The SMFA work performed
442 here was supported was supported in part by the intramural program of the National Institute
443 of Allergy and Infectious Diseases/NIH. The funders had no role in study design, data
444 collection and analysis, decision to publish or preparation of the manuscript. ARTES
445 Biotechnology GmbH provided support in the form of salaries for authors (DW, MS, VJ and

446 MP) and generation of VLPs, but did not have any additional role in the study design, data
447 collection and analysis, decision to publish or preparation of the manuscript. The specific
448 roles of these authors are articulated in the 'Author Contributions' section.
449
450 **Competing Interests**
451 The authors DW, MS, VJ and MP are associated with ARTES Biotechnology GmbH which
452 owns the license for the VLP technology [34,35]. This does not alter our adherence to PLOS
453 ONE policies on sharing data and materials.
454
455

## REFERENCES

1. World Health Organization. World malaria report 2018. 2018: 1–210.

2. Beeson JG, Kurtovic L, Dobaño C, Opi DH, Chan J-A, Feng G, et al. Challenges and strategies for developing efficacious and long-lasting malaria vaccines. Sci Transl Med. American Association for the Advancement of Science; 2019;11: eaau1458. doi:10.1126/scitranslmed.aau1458

3. Malaria Vaccine Funders Group. Malaria Vaccine Technology Roadmap 2013. In: httpswww.malariavaccine.orgmalaria-and-vaccinesmalaria-vaccine- roadmap.

4. RTS,S Clinical Trials Partnership. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet. 2015;386: 31–45. doi:10.1016/S0140-6736(15)60721-8

5. Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C, et al. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. N Engl J Med. 2011;365: 1863–1875. doi:10.1056/NEJMoa1102287

6. White MT, Verity R, Griffin JT, Asante KP, Owusu-Agyei S, Greenwood B, et al. Immunogenicity of the RTS,S/AS01 malaria vaccine and implications for duration of vaccine efficacy: secondary analysis of data from a phase 3 randomised controlled trial. Lancet Infect Dis. 2015;15: 1450–1458. doi:10.1016/S1473-3099(15)00239-X

7. Sauerwein RW, Bousema T. Transmission blocking malaria vaccines: Assays and candidates in clinical development. Vaccine. 2015;33: 7476–7482. doi:10.1016/j.vaccine.2015.08.073

8. Schneider P, Reece SE, van Schaijk BCL, Bousema T, Lanke KHW, Meaden CSJ, et al. Quantification of female and male Plasmodium falciparum gametocytes by reverse

transcriptase quantitative PCR. Mol Biochem Parasitol. 2015;199: 29–33. doi:10.1016/j.molbiopara.2015.03.006

9. Stone WJR, Dantzler KW, Nilsson SK, Drakeley CJ, Marti M, Bousema T, et al. Naturally acquired immunity to sexual stage P. falciparum parasites. Parasitology. 2016;143: 187–198. doi:10.1017/S0031182015001341

10. Malkin EM, Durbin AP, Diemert DJ, Sattabongkot J, Wu Y, Miura K, et al. Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for Plasmodium vivax malaria. Vaccine. 2005;23: 3131–3138. doi:10.1016/j.vaccine.2004.12.019

11. Sagara I, Healy SA, Assadou MH, Gabriel EE, Kone M, Sissoko K, et al. Safety and immunogenicity of Pfs25H-EPA/Alhydrogel, a transmission-blocking vaccine against Plasmodium falciparum: a randomised, double-blind, comparator-controlled, dose-escalation study in healthy Malian adults. Lancet Infect Dis. 2018;18: 969–982. doi:10.1016/S1473-3099(18)30344-X

12. Wu Y, Ellis RD, Shaffer D, Fontes E, Malkin EM, Mahanty S, et al. Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. PLoS ONE. 2008;3: e2636. doi:10.1371/journal.pone.0002636

13. Talaat KR, Ellis RD, Hurd J, Hentrich A, Gabriel E, Hynes NA, et al. Safety and Immunogenicity of Pfs25-EPA/Alhydrogel®, a Transmission Blocking Vaccine against Plasmodium falciparum: An Open Label Study in Malaria Naïve Adults. PLoS ONE. 2016;11: e0163144. doi:10.1371/journal.pone.0163144

14. Draper SJ, Angov E, Horii T, Miller LH, Srinivasan P, Theisen M, et al. Recent advances in recombinant protein-based malaria vaccines. Vaccine. 2015;33: 7433–7443. doi:10.1016/j.vaccine.2015.09.093

503 15. Mohsen MO, Zha L, Cabral-Miranda G, Bachmann MF. Major findings and recent
504 advances in virus-like particle (VLP)-based vaccines. Semin Immunol. 2017.
505 doi:10.1016/j.smim.2017.08.014

506 16. Wetzel D, Rolf T, Suckow M, Kranz A, Barbian A, Chan J-A, et al. Establishment of a
507 yeast-based VLP platform for antigen presentation. Microb Cell Fact. 2018;17: 17.
508 doi:10.1186/s12934-018-0868-0

509 17. Grgacic EVL, Anderson DA. Virus-like particles: passport to immune recognition.
510 Methods. 2006;40: 60–65. doi:10.1016/j.ymeth.2006.07.018

511 18. Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics
512 and molecular patterns. Nat Rev Immunol. Nature Publishing Group; 2010;10: 787–
513 796. doi:10.1038/nri2868

514 19. Kündig TM, Senti G, Schnetzler G, Wolf C, Prinz Vavricka BM, Fulurija A, et al. Der p
515 1 peptide on virus-like particles is safe and highly immunogenic in healthy adults. J
516 Allergy Clin Immunol. 2006;117: 1470–1476. doi:10.1016/j.jaci.2006.01.040

517 20. Brune KD, Leneghan DB, Brian IJ, Ishizuka AS, Bachmann MF, Draper SJ, et al.
518 Plug-and-Display: decoration of Virus-Like Particles via isopeptide bonds for modular
519 immunization. Sci Rep. Nature Publishing Group; 2016;6: 19234.
520 doi:10.1038/srep19234

521 21. Keller SA, Bauer M, Manolova V, Muntwiler S, Saudan P, Bachmann MF. Cutting
522 edge: limited specialization of dendritic cell subsets for MHC class II-associated
523 presentation of viral particles. J Immunol. 2010;184: 26–29.
524 doi:10.4049/jimmunol.0901540

525 22. Chichester JA, Green BJ, Jones RM, Shoji Y, Miura K, Long CA, et al. Safety and
526 immunogenicity of a plant-produced Pfs25 virus-like particle as a transmission

527 blocking vaccine against malaria: A Phase 1 dose-escalation study in healthy adults.
528 Vaccine. 2018;36: 5865–5871. doi:10.1016/j.vaccine.2018.08.033

529 23. Williamson KC, KEISTER DB, Muratova O, Kaslow DC. Recombinant Pfs230, a
530 Plasmodium falciparum gametocyte protein, induces antisera that reduce the
531 infectivity of Plasmodium falciparum to mosquitoes. Mol Biochem Parasitol. 1995;75:
532 33–42.

533 24. Macdonald NJ, Nguyen V, Shimp R, Reiter K, Herrera R, Burkhardt M, et al.
534 Structural and Immunological Characterization of Recombinant 6-Cysteine Domains
535 of the Plasmodium falciparum Sexual Stage Protein Pfs230. J Biol Chem. 2016;291:
536 19913–19922. doi:10.1074/jbc.M116.732305

537 25. Wetzel D, Chan J-A, Suckow M, Barbian A, Weniger M, Jenzelewski V, et al. Display
538 of malaria transmission-blocking antigens on chimeric duck hepatitis B virus-derived
539 virus-like particles produced in *Hansenula polymorpha*. bioRxiv doi:10.1101/595538

540 26. Schaefer S, Piontek M, Ahn S, Papendieck A, Janowicz Z, Timmermans I, et al.
541 Recombinant hepatitis B vaccines—disease characterization and vaccine production.
542 In: G G, editor. Hansenula polymorpha—biology and applications. 2002. pp. 175–210.

543 27. Chan J-A, Drew DR, Reiling L, Lisboa-Pinto A, Dinko B, Sutherland CJ, et al. Low
544 Levels of Human Antibodies to Gametocyte-Infected Erythrocytes Contrasts the
545 PfEMP1-Dominant Response to Asexual Stages in P. falciparum Malaria. Front
546 Immunol. 2018;9: 3126. doi:10.3389/fimmu.2018.03126

547 28. Tsuboi T, Takeo S, Iriko H, Jin L, Tsuchimochi M, Matsuda S, et al. Wheat germ cell-
548 free system-based production of malaria proteins for discovery of novel vaccine
549 candidates. Infection and Immunity. American Society for Microbiology Journals;
550 2008;76: 1702–1708. doi:10.1128/IAI.01539-07

551 29. Dinko B, King E, Targett GAT, Sutherland CJ. Antibody responses to surface antigens
552 of Plasmodium falciparum gametocyte-infected erythrocytes and their relation to
553 gametocytaemia. Parasite Immunol. John Wiley & Sons, Ltd; 2016;38: 352–364.
554 doi:10.1111/pim.12323

555 30. Chan J-A, Howell KB, Reiling L, Ataíde R, Mackintosh CL, Fowkes FJI, et al. Targets
556 of antibodies against Plasmodium falciparum-infected erythrocytes in malaria
557 immunity. J Clin Invest. 2012. doi:10.1172/JCI62182

558 31. Miura K, Deng B, Tullo G, Diouf A, Moretz SE, Locke E, et al. Qualification of
559 standard membrane-feeding assay with Plasmodium falciparum malaria and potential
560 improvements for future assays. PLoS ONE. Public Library of Science; 2013;8:
561 e57909. doi:10.1371/journal.pone.0057909

562 32. Miura K, Swihart BJ, Deng B, Zhou L, Pham TP, Diouf A, et al. Transmission-blocking
563 activity is determined by transmission-reducing activity and number of control oocysts
564 in Plasmodium falciparum standard membrane-feeding assay. Vaccine. 2016;34:
565 4145–4151. doi:10.1016/j.vaccine.2016.06.066

566 33. Tachibana M, Miura K, Takashima E, Morita M, Nagaoka H, Zhou L, et al.
567 Identification of domains within Pfs230 that elicit transmission blocking antibody
568 responses. Vaccine. 2019;37: 1799–1806. doi:10.1016/j.vaccine.2019.02.021

569 34. Anderson D, Grgacic E. Viral vectors expressing fusion of viral large envelope protein
570 and protein of interest. 2004.

571 35. Grgacic E, Anderson D, Loke P, Anders R. Recombinant proteins and virus-like
572 particles comprising L and S polypeptides of avian hepadnaviridae and methods,
573 nucleic acid constructs, vectors and host cells for producing same. 2006.

574

**Table 1 Assessment of transmission-blocking activity of rabbit antibodies against Pfs230c-dS/dS VLPs by SMFA**

| Rabbit ID (+/- Adjuvant) | Protein content[1] | Antibody levels[2] | % inhibition[3] | 95% confidence intervals | *p* value |
|---|---|---|---|---|---|
| R1864 (-Alhydrogel) | 20µg VLP ~6µg Pfs230c-dS | 0.29 | -20.9 | -162.2 – 43.5 | 0.62 |
| R1865 (-Alhydrogel) | 20µg VLP ~6µg Pfs230c-dS | 0.30 | 6 | -100.0 – 57.2 | 0.85 |
| R1866 (+Alhydrogel) | 20µg VLP ~6µg Pfs230c-dS | 1.17 | -30.8 | -187.4 – 43.5 | 0.52 |
| R1867 (+Alhydrogel) | 20µg VLP ~6µg Pfs230c-dS | 1.40 | -34.8 | -193.4 – 37.7 | 0.46 |
| R1868 (-Alhydrogel) | 100µg VLP ~30µg Pfs230c-dS | 0.92 | -13.7 | -143.7 – 48.3 | 0.72 |
| R1869 (-Alhydrogel) | 100µg VLP ~30µg Pfs230c-dS | 2.16 | -28.9 | -182.5 – 41.8 | 0.53 |
| R1870 (+Alhydrogel) | 100µg VLP ~30µg Pfs230c-dS | 0.87 | -8.1 | -124.4 – 50.5 | 0.90 |
| R1871 (+Alhydrogel) | 100µg VLP ~30µg Pfs230c-dS | 1.88 | -16.6 | -156.6 – 48.0 | 0.70 |

[1]Refers to total VLP protein content and estimated incorporation rate of Pfs230c-dS

[2]Mean antibody levels by ELISA at 1/100 serum dilution (OD 405nm)

[3]% inhibition is the best estimate from 1 feed

All rabbit antibodies were tested at an IgG concentration of 7.5 mg/ml.

**Table 2 Assessment of transmission-blocking activity of rabbit antibodies against Pfs230D1M-dS/dS VLPs by SMFA**

| **Rabbit ID (Adjuvant)** | **Antibody levels[1]** | **% inhibition[2]** | **95% confidence intervals** | ***p* value** |
|---|---|---|---|---|
| R1917 (Freund's) | 3.84 | 43.7 | 4.1 – 68.0 | **0.03** |
| R1918 (Freund's) | 3.74 | 9.2 | -51.3 – 47.5 | 0.74 |
| R1919 (Alhydrogel) | 1.72 | 40.7 | -0.2 – 65.9 | 0.05 |
| R1920 (Alhydrogel) | 2.51 | 91.7 | 85.7 – 95.4 | **0.001** |

[1]Mean antibody levels by ELISA at 1/100 serum dilution (OD 405nm)

[2]% inhibition is the best estimate from 2 feeds

Protein content was 100µg to total VLP protein and 24µg Pfs230D1M-dS; all rabbit antibodies were tested at an IgG concentration of 7.5 mg/ml; *p* values that achieved statistical significance are presented in bold.

**Table 3 Assessment of transmission-blocking activity of rabbit antibodies against Pfs25-dS/dS VLPs by SMFA**

| **Rabbit ID (Adjuvant)** | **Antibody levels[1]** | **% inhibition[2]** | **95% confidence intervals** | ***p* value** |
|---|---|---|---|---|
| R1825 (Freund's) | 3.42 | 97.4 | 95.4 – 98.8 | **0.001** |
| R1826 (Freund's) | 2.91 | -1.4 | -69.4 – 40.5 | 0.96 |

[1]Mean antibody levels by ELISA at 1/100 serum dilution (OD 405nm)

[2]% inhibition is the best estimate from 2 feeds

Protein content was 100µg to total VLP protein and 3µg Pfs25-dS; all rabbit antibodies were tested at an IgG concentration of 7.5 mg/ml; *p* value that achieved statistical significance is presented in bold.

## FIGURES

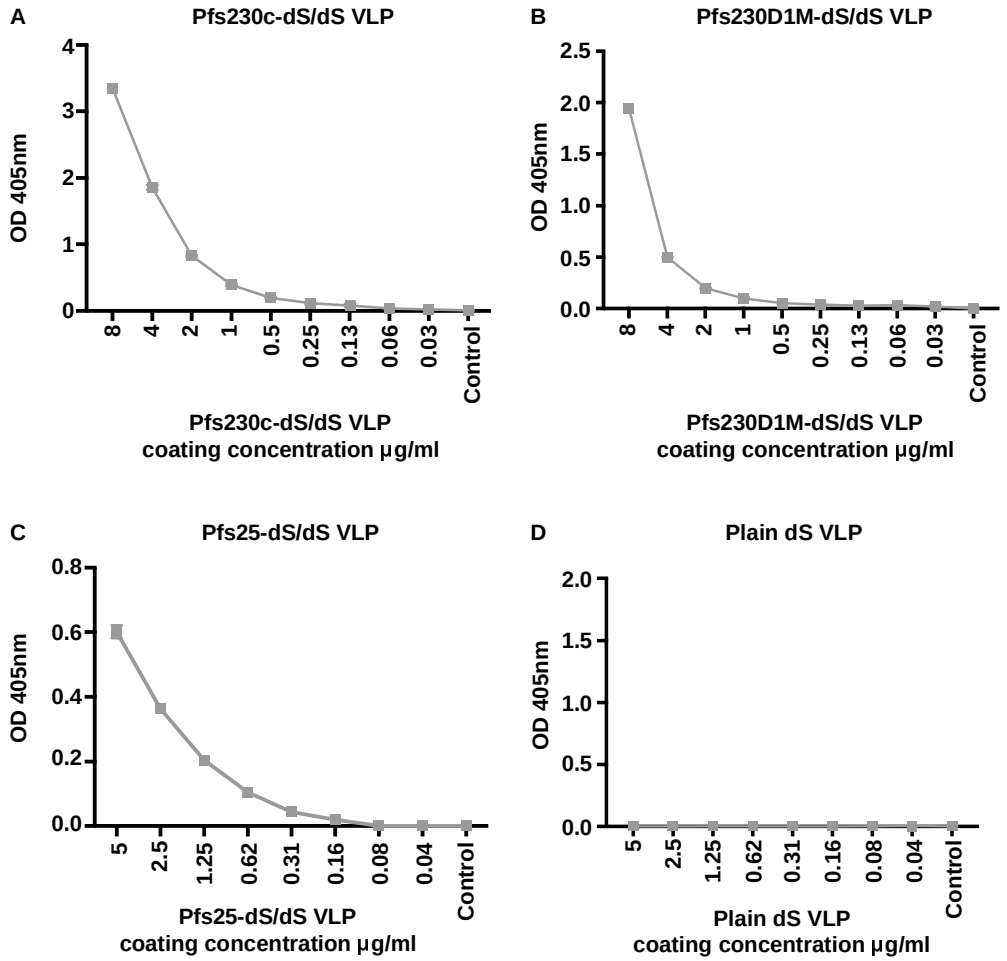
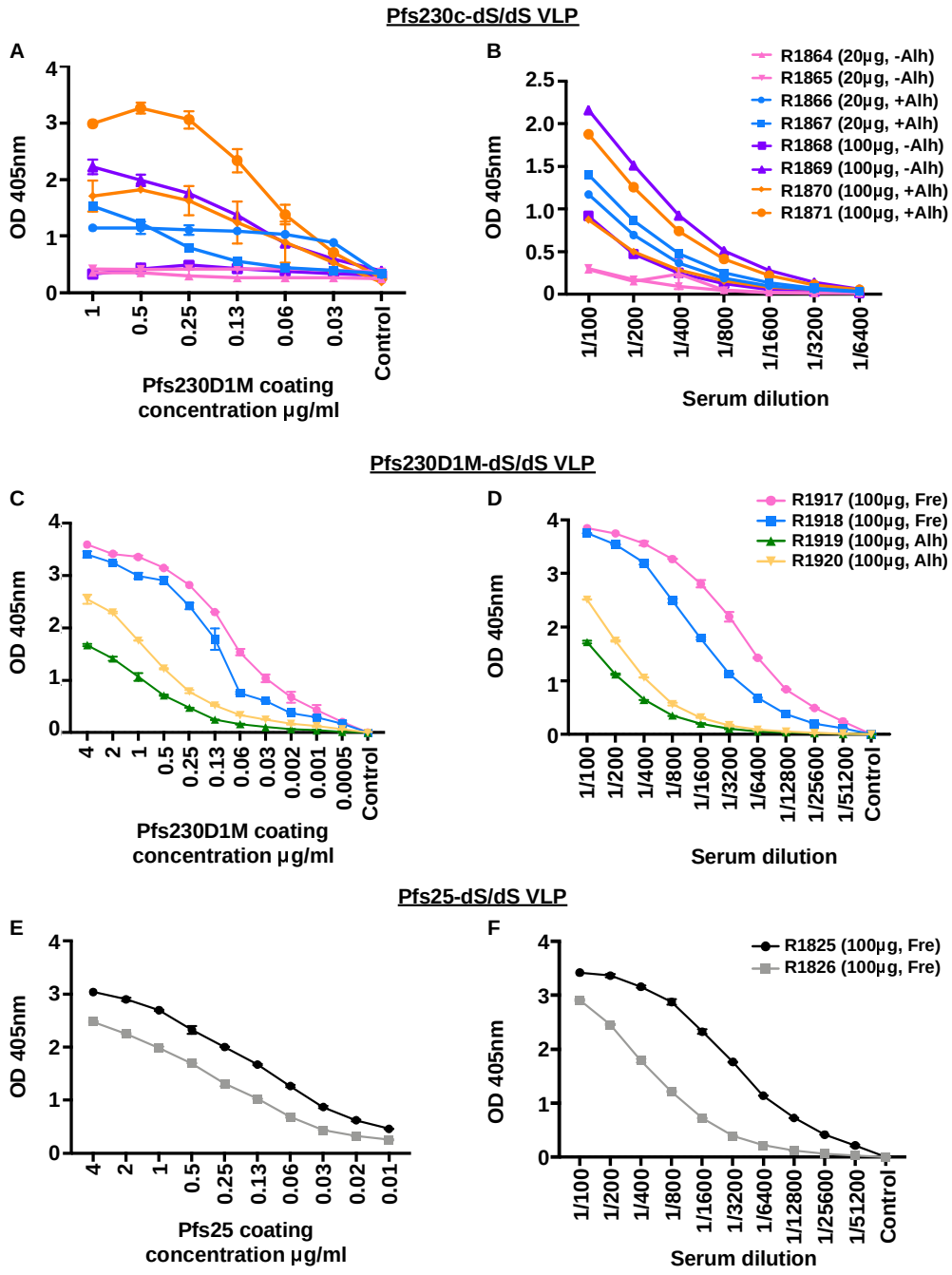



**Fig 1 Characterising the expression of sexual-stage antigens on chimeric VLPs**

Total antibody binding to (**A**) Pfs230c-dS/dS, (**B**) Pfs230D1M-dS/dS, (**C**) Pfs25-dS/dS and (**D**) plain dS VLPs were measured by ELISA. VLPs were coated at varying concentrations (µg/ml) and probed with either a Pfs25 or Pfs230-specific polyclonal antibody (1µg/ml). In (**D**), plain dS VLPs were probed with anti-Pfs230 antibody. The level of antibody binding is expressed as optical density (OD) measured at 405nm; symbols represent the mean and error bars represent the range between samples tested in duplicate.




611

612 **Fig 2 Immunogenicity of sexual-stage chimeric VLPs in rabbits**

613 The level of antibody binding to a titration of monomeric recombinant (**A**, **C**) Pfs230D1M and

614 (**E**) Pfs25 was measured in whole rabbit serum (1/100). Rabbits were immunised with

615 chimeric Pfs230c-dS/dS (**A**, **B**), Pfs230D1M-dS/dS (**C**, **D**) or Pfs25-dS/dS (**E**, **F**) VLPs.
616 Monomeric recombinant proteins were serially diluted from 4µg/ml.
617 The level of antibody binding to monomeric recombinant (**B**, **D**) Pfs230D1M and (**F**) Pfs25
618 (coated at 1µg/ml) was measured by titrating whole rabbit serum. Rabbits were immunised
619 with chimeric Pfs230c-dS/dS (**A**, **B**), Pfs230D1M-dS/dS (**C**, **D**) or Pfs25-dS/dS (**E**, **F**) VLPs.
620 Whole rabbit serum was serially diluted from 1/100.
621 For all graphs, antibody binding is expressed as optical density (OD) measured at 405nm;
622 symbols represent the mean and error bars represent the range between samples tested in
623 duplicate (n=8 for Pfs230c-dS/dS, n=4 for Pfs230D1M-dS/dS, n=2 for Pfs25-dS/dS). Rabbits
624 R1864-R1871 received Pfs230c-dS/dS VLPs formulated with and without Alhydrogel (**A**, **B**;
625 Alh). Rabbits R1917 and R1918 received Pfs230D1M-dS/dS VLPs formulated with Freund's
626 adjuvant (Fre), while R1919 and R1920 received Pfs230D1M-dS/dS VLPs formulated with
627 Alhydrogel (**C**, **D**). Rabbits R1825 and R1826 received Pfs25-dS/dS VLPs formulated with
628 Freund's adjuvant (**E**, **F**). Adjuvants and total VLP protein content used for immunisations
629 are also presented in Supplementary Tables S1-S3.
630

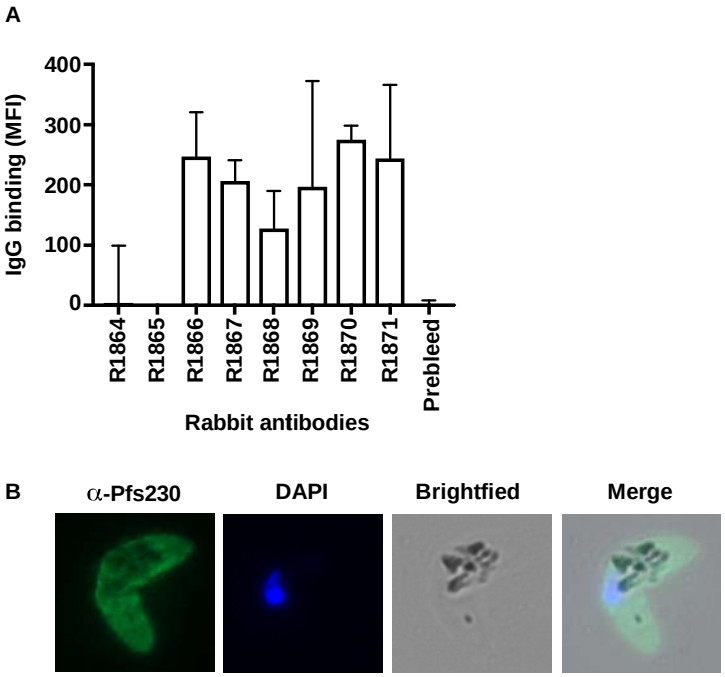



**Fig 3 Characterising the antibody function of rabbits immunised with chimeric VLPs**

**A**. Total antibody binding to the surface of native *P. falciparum* gametocytes (stage V gametocyte-infected erythrocytes were permeabilised with saponin) measured by flow cytometry. Whole serum from rabbits immunised with Pfs230c-dS/dS VLPs were used (n=8). Antibody levels are expressed as geometric mean fluorescence intensity (MFI); bars represent mean and range of samples tested in duplicate.

**B**. Immunofluorescence microscopy demonstrates the recognition of the native gametocyte surface by serum antibodies from rabbits immunised with Pfs230c-dS/dS VLPs (R1870; green). Cells were fixed with 90% acetone and 10% methanol, and DAPI was used to stain nuclear DNA (blue). Representative images taken of gametocytes labelled with R1870 are shown.

644 **SUPPLEMENTARY MATERIALS**

645 **Supplementary Table S1 Vaccine groups for Pfs230c-dS/dS VLP rabbit immunisations**

| Rabbit ID | Total VLP protein (µg) | Total Pfs230c-dS protein (µg) | Alhydrogel (with or without) |
|---|---|---|---|
| R1864, R1865 | 20 | 6 | - |
| R1866, R1867 | 20 | 6 | + |
| R1868, R1869 | 100 | 30 | - |
| R1870, R1871 | 100 | 30 | + |

646 Note: Estimated incorporation of Pfs230c-dS into VLPs was ~30%, determined by
647 Coomassie stained gels.

648

649 **Supplementary Table S2 Vaccine groups for Pfs230D1M-dS/dS VLP rabbit**
650 **immunisations**

| Rabbit ID | Total VLP protein (µg) | Total Pfs230D1M-dS protein (µg) | Adjuvant |
|---|---|---|---|
| R1917, R1918 | 100 | 24 | Freund's |
| R1919, R1920 | 100 | 24 | Alhydrogel |

651 Note: Estimated incorporation of Pfs230D1M-dS into VLPs was ~24%, determined by
652 Coomassie stained gels.

653

654 **Supplementary Table S3 Vaccine groups for Pfs25-dS/dS VLP rabbit immunisations**

| Rabbit ID | Total VLP protein (µg) | Total Pfs25-dS protein (µg) | Adjuvant |
|---|---|---|---|
| R1825, R1826 | 100 | 3 | Freund's |

655 Note: Estimated incorporation of Pfs25-dS into VLPs was ~3%, determined by Coomassie
656 stained gels.

657

**Supplementary Fig S1**

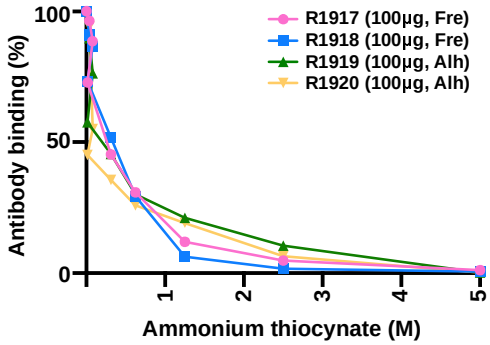



**Fig S1 Avidity of rabbit antibodies to bind monomeric recombinant Pfs230D1M**

The avidity of antibody binding to monomeric recombinant Pfs230D1M was assessed in serum from rabbits immunised with Pfs230D1M-dS/dS VLPs. The affinity of rabbit antibodies to bind monomeric recombinant Pfs230D1M was similar, irrespective of adjuvant formulation used for rabbit immunisations. Rabbits R1917 and R1918 received Pfs230D1M-dS/dS VLPs formulated with Freund's adjuvant (Fre), while R1919 and R1920 received Pfs230D1M-dS/dS VLPs formulated with Alhydrogel (Alh). Antibody affinity was assessed by the dissociation of antibodies using increasing concentrations of ammonium thiocynate. Antibody binding is defined as the OD of ammonium thiocynate-treated samples/OD of untreated samples x100.