

Title

Developing a multivariate prediction model of antibody features associated with protection of malaria-infected pregnant women from placental malaria

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Abstract

Background: *Plasmodium falciparum* causes placental malaria, which results in adverse outcomes for mother and child. *P. falciparum* infected erythrocytes that express the parasite protein VAR2CSA on their surface can bind to placental chondroitin sulfate-A. It has been hypothesized that naturally acquired antibodies towards VAR2CSA protect against placental infection, but it has proven difficult to identify robust antibody correlates of protection from disease. The objective of this study was to develop a prediction model using antibody features which could identify women protected from placental malaria.

Methods: We used a systems serology approach with elastic net-regularized logistic regression, Partial Least Squares Discriminant Analysis and a case control study design to identify naturally acquired antibody features mid pregnancy that were associated with protection from placental malaria at delivery in a cohort of 77 pregnant women from Madang, Papua New Guinea.

Results: The machine learning techniques selected six out of 169 measured antibody features towards VAR2CSA that could predict (with 86% accuracy) whether a woman would subsequently have active placental malaria infection at delivery. Selected features included previously described associations with inhibition of placental binding and/or opsonic phagocytosis of infected erythrocytes, and network analysis indicated that there are not one but multiple pathways to protection from placental malaria. **Conclusions:** We have identified candidate antibody features which could accurately identify malaria-infected women as protected from placental infection. It is likely that there are multiple pathways to protection against placental malaria.

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Introduction

The burden of *Plasmodium falciparum* malaria is greatest in young children and pregnant women (World Health Organisation, 2019). Among women with lifelong malaria exposure, pregnant women have increased susceptibility to malaria, and complications include maternal mortality, severe maternal anaemia, miscarriage and stillbirth, and impaired fetal growth or prematurity leading to low birth weight delivery (Steketee et al., 2001, Guyatt and Snow, 2001, Desai et al., 2007, Moore et al., 2017a, Moore et al., 2017b). The prevalence, intensity and consequences of infection decline with increasing gravidity (Guyatt and Snow, 2001).

The susceptibility of pregnant women to *P. falciparum* malaria is in part due to the ability of infected erythrocytes (IEs) to sequester in the maternal blood spaces of the placenta (Rogerson et al., 2007), where IEs that express VAR2CSA adhere to chondroitin sulphate-A (CSA), a glycosaminoglycan chain on syndecan-1 expressed by the placental syncytiotrophoblast (Salanti et al., 2004, Ayres Pereira et al., 2016). VAR2CSA is a unique 350 kDa protein that is a member of the *P. falciparum* erythrocyte membrane protein 1 (*PfEMP1*) family of variant surface antigens. *PfEMP1*s are encoded by *var* genes and are composed of multiple Duffy binding-like (DBL) domains and cysteine-rich interdomain regions (CIDR). VAR2CSA has six DBL domains, with CSA adhesion being strongly associated with the DBL2 domain, which is present in the N-terminal region of the protein (Doritchamou et al., 2013).

Two constructs based on this region have entered clinical trials as vaccine candidates to prevent placental malaria (Mordmuller et al., 2019, Sirima et al., 2020).

Antibodies to VAR2CSA, in its native form on IE or expressed as recombinant protein, develop in a parity-dependent manner, block adhesion to CSA and opsonise IE for phagocytosis by monocytic cells (Fried et al., 1998, Ricke et al., 2000, Salanti et al., 2004, Keen et al., 2007). Some studies report associations between measures of single antibody features and protection from malaria complications such as placental infection, impaired fetal growth, prematurity or anaemia (Duffy and Fried, 2003, Staalsoe et al., 2004, Feng et al., 2009). However, identifying targets and features of protective antibody responses to malaria is challenging, because exposure to infection is accompanied by the production of antibody to many parasite antigens, only a fraction of which contributes to protective immunity (Crompton et al., 2010). The large size and multi-domain structure of VAR2CSA adds complexity to the identification of targets and features of protective immune response. In a recent systematic review, antibody responses to VAR2CSA were often correlated with exposure to placental malaria, rather than protection from infection (Cutts et al., 2020), although some studies indicate an association between antibody to IE and protection from low birth weight in subsets of pregnant women (Duffy and Fried, 2003, Staalsoe et al., 2004, Feng et al., 2009). (Cutts et al., 2020)

To identify antibody responses which protect women from placental malaria, an alternative approach that examines multiple antibody features and controls for exposure may be needed. Placental malaria mostly occurs when antigenic variation (Roberts et al., 1992) leads to a parasite, that has infected a pregnant woman, expressing VAR2CSA. These parasites can flourish by sequestering in the placenta in women who lack VAR2CSA specific immunity (Hviid, 2004). By contrast, some pregnant women with peripheral blood infection remain free of placental malaria, and we postulate that these women have antibody responses that specifically protect them against VAR2CSA and placental malaria.

Antibody measures towards the IE have traditionally focused on the quantity of bound antibodies and their ability to inhibit IE binding to CSA (Cutts et al., 2020), but there is increasing interest in how antibodies that recognise IE engage with innate immune cells and activate complement (Aitken et al., 2020), which are both determined by biophysical features of the Fab and Fc regions of antibody (reviewed in (Arnold and Chung, 2018)). In recent years, detailed functional and biophysical characterisation of antibody responses has led to the identification of specific antibody determinants that correlate with vaccine-induced protection from HIV (Chung et al., 2015), control of latent tuberculosis infection (Lu et al., 2016), transplacental transfer of antibody (Martinez et al., 2019, Jennewein et al., 2019) and correlates of vaccine-induced protection in human malaria challenge models (Suscovich et al., 2020). This approach, which has been termed systems serology, is well suited to placental malaria, because there is a clear relationship between a single antigen (VAR2CSA) and a specific pathology (the sequestration of parasites in the placenta, reviewed in (Ataide et al., 2014)).

The objective of this study was develop a prognostic model using antibody features to identify women protected from placental malaria. In this prospective study we identified features, functions and targets of naturally acquired antibody to VAR2CSA that contribute to protection against placental malaria. Pregnant Papua New Guinean women who were participating in a trial of intermittent preventive treatment against malaria (Unger et al., 2015) were categorised at delivery

as currently uninfected, having placental malaria, or having a non-placental infection. Using plasma samples collected at enrolment in mid-pregnancy we performed extensive profiling of antibody responses to VAR2CSA recombinant proteins and IEs expressing VAR2CSA. Machine learning techniques revealed that six of 169 antibody features measured mid pregnancy were able to discriminate between women who had placental malaria and non-placental infection at delivery with high accuracy. Five of the six leading antibody features we found were related to inhibition of placental sequestration and/or opsonisation for phagocytic clearance.

Materials and Methods

Experimental Model and Subject Details

Human subjects

Study participants were recruited between November 2009 and August 2012 as part of a randomized controlled trial of Intermittent Preventive Treatment in Pregnancy (IPTp) (Unger et al., 2015) (ClinicalTrials.gov [NCT01136850](https://clinicaltrials.gov/ct2/show/study/NCT01136850)), in which pregnant women received either three courses of sulphadoxine pyrimethamine and azithromycin or one course of sulphadoxine pyrimethamine and chloroquine. Women were recruited at 14-26 gestation weeks and followed up until delivery. All women presenting for their first antenatal visit at one of the nine participating health centres in Madang Papua New Guinea were invited to participate in the original cohort study. Exclusion criteria included a gestation of > 26 weeks, haemoglobin <6d/dl with symptomatic anaemia, previous serious adverse reactions to the IPTp study medications, permanent disability or chronic medical conditions, known multiple pregnancy, age <16 years or known unavailability to followup (Unger et al., 2015). Demographic data collected at enrollment included maternal gravidity, age, residence and bed net use. Peripheral blood collected at 14-26 gestation weeks, before initiation of IPTp, was used in the antibody assays. Peripheral blood collected at delivery was used to prepare blood smears for malaria microscopy and to extract DNA for qPCR for malaria parasites (Unger et al., 2015). Placental biopsies were formalin fixed and paraffin embedded. Giemsa stained sections of placental biopsies were examined for malaria infection (Lufele et al., 2017). Women were selected for inclusion in this case control study based on the presence of *P. falciparum* infected erythrocytes (IEs) in peripheral blood and/or placenta at delivery. Groups included women with no evidence of infection in peripheral blood by PCR or light microscopy nor on examination of placental histology at delivery (n=50); women with active placental malaria characterized by detection of *P. falciparum* IEs by placental histology (n=50); and women with *P. falciparum* infection by light microscopy and/or PCR in the peripheral blood but with no IEs detected by placental histology examination (n=27). Sample size was based on published work which used a similar approach to investigate antibody responses to infectious diseases (Lu et al., 2016), in addition sample size for the non-placental infection group was also limited by sample availability as few women met the criteria for inclusion. All clinical data was collected and grouping of women by outcome was completed prior to measurements of antibody features included in the model. The three groups were frequency matched for primigravidity, IPTp regime receipt, bed net use, rural residency and age. See Table 1 for details on clinical characteristics of participants.

Parasite Cell Lines

P. falciparum IE of the parasite lines CS2 and 3D7 were cultured as previously described (Chandrasiri et al., 2014). Cultures were synchronized as needed by sorbitol lysis (Lambros and Vanderberg, 1979) and IE were regularly selected for expression of knobs by gelatin flotation (Goodyer et al., 1994). Cell cultures were mycoplasma negative (tested for mycoplasma using the MycoAlert kit (Lonza, Mount

Waverley, Australia) as per manufacturer's instructions). For the binding inhibition assays, the parent lines of CS2 and 3D7 (FCR3 and NF54 respectively) were used, cultured and selected for CSA adhesion as described previously (Nielsen and Salanti, 2015).

Monocyte Cell Line THP-1

THP-1 cells were mycoplasma negative and cultured as previously described (Ataide et al., 2010).

Primary Leukocytes

Neutrophils were isolated from fresh venous blood collected in lithium heparin vacutainers (BD, Scoresby, Australia) using the EasySep™ Direct Human Neutrophil Isolation Kit (STEMCELL™ Technologies, Tullamarine, Australia) as per manufacturer's instructions. Neutrophil purity was assessed by cell morphology using light microscopy of Giemsa stained smears of the isolated cells, and viability was assessed using trypan blue exclusion. Monocytes were isolated from both fresh venous blood, collected in lithium heparin vacutainers (BD), as well as from buffy coats supplied from the Australian Red Cross Blood Service. Monocytes were isolated by negative selection using the RosetteSep™ Human Monocyte Enrichment Cocktail (STEMCELL™ Technologies) as per manufacturer's instructions. Monocyte purity was assessed by staining (anti-CD14 antibody, BioLegend, San Diego, CA) and measuring CD14⁺ cells by flow cytometry. Monocytes were either frozen in foetal bovine serum (FBS) in 20% dimethyl sulfoxide (DMSO) in liquid nitrogen for later use or used immediately after isolation. Natural Killer (NK) cells were isolated from fresh venous blood collected in sodium heparin vacutainers (BD). NK cells were isolated by negative selection using the RosetteSep™ Human NK Enrichment Cocktail (STEMCELL™ Technologies) as per manufacturer's instructions.

Method Details

Samples and Controls

Plasma samples of study participants were obtained from venous blood collected into sodium heparin vacutainers upon enrollment into the trial at 14-26 weeks' gestation and plasma was separated by centrifugation and stored at -80°C until used. To generate a positive control, plasma samples from pregnant women in Malawi with high levels of IgG which recognizes IEs of the *P. falciparum* line CS2 (a VAR2CSA-expressing and chondroitin sulfate A (CSA)-binding line (Elliott et al., 2005b)), were pooled. Negative controls were sera from individual Melbourne donors obtained from the Australian Red Cross Blood Service or plasma from Melbourne donors collected in lithium heparin vacutainers (BD). All controls were kept at -80°C until use. Antibody features were measured after grouping of women based on outcome. Researchers measuring antibody features were blinded to outcome data of individual samples, this was done by allocating all samples a number (which was not associated with clinical outcomes and which was different from the original cohort study number), samples were decoded after acquisition of the antibody feature was complete. For some experiments IgG was purified from plasma and serum using Melon Gel purification kits (Thermo Fisher Scientific, Scoresby, Australia) as per manufacturer's instructions. Purified IgG was quantified before use with a human IgG ELISA development kit (MabTech, Preston, Australia) as per manufacturer's instructions.

VAR2CSA DBL Recombinant Antigens

Twelve recombinant proteins consisting of subunits or Duffy binding-like domains (DBL) of VAR2CSA were used in experiments (see Supplementary File 1 for a table detailing proteins used). For some

experiments, proteins were biotinylated using EZ-link Sulfo NHS-LC-Biotin kit (*Thermo Fisher Scientific*) as per manufacturer's instructions.

Infected Erythrocytes (IE)

The CSA-binding phenotypes of CS2 and 3D7 IEs were monitored by measuring binding of trophozoite stage IEs in static binding assays to recombinant human CD36 (R&D systems, Noble Park, Australia) and CSA (Sigma-Aldrich, Macquarie Park, Australia) as previously described (Yosaatmadja et al., 2008). 3D7 was selected for a CSA-binding phenotype by flow cytometry. Purified trophozoite stage 3D7 IEs were incubated with fluorescein isothiocyanate (FITC)-labelled CSA 100 µg/mL (Creative PEGWorks, Durham, NC) for 30 min at 37°C. Purified IE incubated with CSA-FITC and 3D7 without CSA-FITC were used to set the gates. IEs were then gated based on forward and side scatter and sorted based on FITC. For some assays trophozoite stage IE were purified by Percoll gradient and stained with dihydroethidium (DHE) 25 µg/mL (Sigma-Aldrich). For the binding inhibition assays, FCR3 and NF54 IE were selected for a CSA-binding phenotype by panning on BeWo cells as previously described (Nielsen and Salanti, 2015).

Antibody Features to VAR2CSA by Multiplex

Multiplex assays were used to detect plasma levels of antigen-specific antibodies (Abs) towards VAR2CSA DBL domains. Features assessed included total IgG, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM as well as antibody engagement with complement (C1q) and FcγRs (FcγRI, FcγRIIa, FcγRIIIa and FcγRIIIb). To conduct the multiplex assays, DBL domains (Supplementary File 1) of the VAR2CSA protein were coupled to Bio-Plex magnetic carboxylated microspheres (Bio-Rad) as per manufacturer's instructions, blocked with PBS plus 0.1% bovine serum albumin (BSA), then stored in PBS-0.05% sodium azide at -80°C.

On the day of the assay the DBL-coupled microspheres were resuspended at 1×10^4 beads/mL in 1:100 dilution of plasma in PBS-1% BSA and 50 µL were aliquoted into each well of a 96-well round bottom plate (Greiner Bio-One, Kremsmünster, Austria). Plates were incubated on a shaker overnight at 4°C, then centrifuged and washed with PBS-0.1% Tween® 20 using a magnetic plate-washer (Bio-Plex Pro wash station). The anti-human Ab detectors for IgG, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 or IgM conjugated with phycoerythrin (PE) (SouthernBiotech, Birmingham, AL) were added, and the mixture was incubated for 2 h on a plate shaker. C1q (MP Biomedicals), FcγRI (R&D Systems), and FcγRIIIb (R&D Systems) were biotinylated using EZ-Link® Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) as per manufacturer's instructions and then labelled streptavidin-PE (SouthernBiotech), before being added to samples. For FcγRIIa and FcγRIIIa, the beads/plasma samples mixture was incubated with biotinylated recombinant dimeric FcγRIIa and FcγRIIIa ectodomains (Wines et al., 2016) for 1 h to allow binding of FcγRs to antigen-specific IgG, washed, and incubated with streptavidin-PE for 1 h. After washing with PBS and resuspending in xMAP sheath fluid (Life Technologies, Scoresby, Australia), the plates were read on a Bio-Plex MAGPIX™ multiplex reader (Bio-Rad, Gladesville, Australia), and analyzed on Bio-Plex Manager software (Bio-Rad). A single measurement of each sample for each antibody feature was done. Data were excluded if the number of beads acquired was too low. Readouts were expressed as the raw median fluorescence index (MFI).

Detection of Antibody binding to the IE

Binding of IgG and IgG subclass antibodies to the surface of CS2 and CSA-selected 3D7 IEs was measured as previously described, all samples were run in duplicate (Aitken et al., 2010, Elliott et al., 2005b).

ADCP of VAR2CSA DBL-coated Beads by THP-1

Antibody-dependent cellular phagocytosis (ADCP) of beads coated with VAR2CSA DBL domains and opsonized with plasma or purified IgG was assessed using THP-1 cells. Individual DBL domains (Supplementary File 1) were biotinylated and incubated with Neutravidin 1 μ m beads at a ratio of 1 μ L of beads (stock 1% v/v) to 3 μ L of 1 mg/mL of the respective DBL domain. Beads and proteins were incubated overnight at 4°C on a roller. After incubation, beads were washed twice in PBS-0.1% BSA and then resuspended at 0.01% v/v in PBS-0.1% BSA with 0.02% sodium azide. For the phagocytosis assay a sterile 96-well U bottom plate (Corning, Mulgrave, Australia) was blocked with high salt PBS buffer (PBS plus 0.5 M NaCl with 1% BSA 0.05% Tween®20) for 30 min at room temperature. Then 20 μ L of plasma diluted 1:800 in high salt PBS buffer and 10 μ L of beads was added and incubated for 1 h at room temperature. The plate was spun at 4700 x g for 3 min, supernatant was replaced with 30 μ L of leukocyte media (RPMI-1640 with 10% FBS and Penicillin-Streptomycin-Glutamine (all from Gibco, Scoresby, Australia)), then 5×10^4 THP-1 cells in 50 μ L of leukocyte media were added and incubated for 40 min at 37°C in 5% CO₂. After incubation, cells were washed and resuspended in cold PBS-4% paraformaldehyde (PFA). Phagocytosis was measured by gating on THP-1 using FSC and SSC parameters. Phagocytic score was calculated as (% of THP-1 cells positive for beads x geometric MFI of the bead-positive THP-1 cells). All samples were run in duplicate.

ADCP of IE by THP-1

ADCP of IE opsonized with plasma by THP-1 cells was assessed as previously described (Ataide et al., 2010). Phagocytosis of IE opsonized with purified IgG was measured as previously described (Ataide et al., 2010) with the modification that IE were opsonized with purified IgG at a concentration of equivalent to a 1:20 dilution of plasma. All samples were run in duplicate.

ADCP of VAR2CSA DBL-coated Beads by Monocytes

Monocyte phagocytosis of beads coated with VAR2CSA DBL domains and opsonized with plasma was assessed. Beads were coated with DBL domains as described above, except that neutravidin 1 μ m beads were incubated with 6 μ L of 1 mg/mL of each DBL domain. To measure ADCP, a sterile 96-well U bottom plate (Corning) was blocked with salt PBS buffer (PBS plus 0.25 M NaCl and 1% BSA) for 30 min at room temperature. 40 μ L of plasma (diluted 1:300 in salt PBS buffer) was added to each well along with 20 μ L of beads (0.01% v/v) and incubated for 1 h at room temperature. The beads were then washed and resuspended in 60 μ L of leukocyte media. In a separate 96-well U bottom plate, 5×10^4 monocytes in leukocyte medium were plated out per well and rested at 37°C for 1 h prior to phagocytosis. 30 μ L of the opsonized beads were then added to each well with monocytes. The monocytes and beads were co-incubated for 50 min at 37°C with 5% CO₂. After incubation, the cells were washed and resuspended in cold PBS-2% PFA. Phagocytosis was measured by gating monocytes on the FSC and SSC. All samples were run in duplicate and assays were repeated with three different primary cell donors. For each donor a phagocytic score was calculated (% of monocytes cells positive for beads x geometric MFI of the bead positive monocytes) and then the average of the phagocytic scores for the three donors was taken.

ADCP of IE by Monocytes

ADCP of opsonized IE by primary monocytes was assessed. A sterile 96-well U bottom plate (Corning) was blocked with PBS-1% BSA. In each well 30 μ L of diluted plasma (1:10) along with 3.3 μ L of DHE stained IE in leukocyte media (1.65×10^7 IE/mL) was mixed and left for 1 h at room temperature, before being washed and resuspended in 50 μ L RPMI-1640 with 25 mM HEPES, 0.5% Albumax (w/v), 5% heat inactivated human serum (HIHS). In a separate 96-well U bottom plate, 5×10^4 monocytes in leukocyte medium were plated out per well and rested at 37°C for 2 h prior to phagocytosis. Then 25 μ L of the opsonized IE were added to each well with monocytes. The monocytes and IE were co-incubated for 40 min at 37°C with 5% CO₂. After incubation, the cells were washed and resuspended in cold PBS-2% PFA. Phagocytosis was measured by gating on monocytes using the FSC and SSC parameters. All samples were run in duplicate and assays were repeated with three different primary cell donors. For each donor a phagocytic score was calculated (% of monocytes cells positive for DHE x MFI of the DHE positive monocytes) and then the average of the phagocytic scores for the three donors was used.

ADNP of VAR2CSA DBL-coated Beads

Antibody-dependent neutrophil phagocytosis (ADNP) of beads coated with VAR2CSA DBL domains and opsonized with plasma was assessed. Beads were coated with DBL as described for primary monocytes. Neutravidin 1 μ m beads were incubated with 6 μ L of 1 mg/mL of the respective DBL domain. To quantitate ADNP, 12 μ L of plasma (diluted 1:100 in high salt PBS) was added to each well of a sterile 96-well U bottom plate (Corning) along with 6 μ L of beads (0.01% v/v) and incubated for 1 h at room temperature. The beads were then washed and resuspended in 30 μ L of leukocyte medium. In a separate 96-well U bottom plate, 3×10^4 neutrophils in leukocyte medium were plated out per well, 15 μ L of the opsonized beads were then added to each well. The plate was incubated for 1 h at 37°C in 5% CO₂, then the cells were washed and resuspended in cold PBS-2% PFA. Phagocytosis was measured by gating neutrophils on the FSC and SSC. All samples were run in duplicate and assays were repeated with three different primary cell donors. For each donor a phagocytic score was calculated (% of monocytes cells positive for beads x geometric MFI of the bead positive monocytes) and then the average of the phagocytic scores for the three donors was used.

ADNP of IE

ADNP of IE opsonized with plasma was assessed. 30 μ L of diluted plasma (1:10) and 3.3 μ L of DHE-stained IE in neutrophil medium (1.65×10^7 IE/mL) were mixed in a sterile 96-well U bottom plate (Corning), incubated for 1 h at room temperature, before being washed and resuspended in 50 μ L of leukocyte medium. In a separate 96-well U bottom plate, 2.5×10^4 neutrophils in 50 μ L of leukocyte medium were plated out per well, 25 μ L of the opsonized IE were then added to each well, and the plate was incubated for 1 h at 37°C with 5% CO₂. After incubation, the cells were washed and resuspended in cold PBS-2% PFA. Phagocytosis was measured by gating neutrophils on the FSC and SSC. All samples were run in duplicate and assays were repeated with three different primary cell donors. For each donor a phagocytic score was calculated (% of monocytes cells positive for beads x geometric MFI of the bead positive monocytes) and then the average of the phagocytic scores for the three donors was used.

ADRB using VAR2CSA DBL Domains

Antibody-dependent respiratory burst (ADRB) was measured using an assay of reactive oxygen species (ROS) production. Firstly 25 μ L of 4 μ g/mL of individual DBL domains (Supplementary File 1) in PBS were coated on 96-well, white, flat bottom plates (NUNC MaxiSorp flat bottom; Thermo Fisher Scientific) and left overnight at 4°C. The liquid was removed, and plates were then washed with PBS and blocked with PBS-0.1% BSA for 1 h at room temperature. Then 25 μ L of each diluted sample (1:10 in PBS) was added to a single well and incubated at room temperature for 2 h, the plates were then washed twice with PBS. Neutrophils were resuspended in Hanks' buffered saline solution at 2×10^6 /mL and 20 μ L of neutrophils were added to each well followed by 20 μ L luminol horse radish peroxidase (HRP) solution (PBS with 33 ng/mL of HRP and 4 mM luminol; all from Sigma-Aldrich)). The plate was spun briefly to settle the contents and read immediately on a FLUOstar plate reader. Luminescence was measured in each well for 1 sec every 2 min for 1 h and was calculated as the average luminescence 5 min either side of the peak of the curve. Luminescence score was standardized to the no serum controls which were run on every plate. All assays were repeated with three different primary cell donors and the average luminescence score from the three donors was used.

ADRB to IE

To measure ADRB in response to IEs, 96-well U bottom plates (Corning) were coated with PBS-1% FBS for 1 h. 1 μ L of plasma from each donor was aliquoted into a single well along with 20 μ L of purified IEs at 2×10^7 cells/mL in PBS-1% FBS were mixed and incubated for 1 h at room temperature. The IE were then washed and resuspended in 20 μ L of PBS. 10 μ L of neutrophils in Hanks' buffered saline solution at 2×10^6 /mL, 10 μ L of luminol HRP solution and 5 μ L of opsonized IEs were added to each well of a 384-well white, flat bottom plate (NUNC MaxiSorp flat bottom; Thermo Fisher Scientific). The plate was spun briefly to settle the contents and then read immediately on a FLUOstar plate reader. (BMG LABTECH, Morington, Australia). Luminescence was measured as described above. Luminescence score was calculated as the % of the positive plasma control, which was run on every plate. All assays were repeated with three different primary cell donors and the average luminescence score from the three donors was used.

ADCC using VAR2CSA DBL Domains

Antibody-dependent cellular cytotoxicity (ADCC) assays using human NK cells were modified for use with DBL antigens (Supplementary File 1) (Jegaskanda et al., 2013, Lu et al., 2016). NUNC MaxiSorp flat bottom plates (Thermo Fisher Scientific) were coated with DBL proteins (200 ng/well) at 4°C for 12 h. After washing with PBS, the plate was blocked with PBS-1% BSA for 1 h. Purified IgG (0.5 mg/mL) was added to each well and incubated at 37°C for 2 h. NK cells (0.25×10^6 cells/mL), anti-CD107a-allophycocyanin (APC)-H7 (BD) brefeldin A (10 mg/mL; Sigma-Aldrich), and GolgiStop (BD) were added to each well, and the plates were incubated for 5 h at 37°C. NK cells were then stained for surface markers using anti-CD16-BrilliantTM violet (BV)-605 (BD), anti-CD56-BrilliantTM ultraviolet (BD), and anti-CD3-peridinin-chlorophyll-protein (PerCP; BD), and then stained intracellularly with anti-IFN γ -PE (BD) and anti-TNF α -BV-785 (BD) after fixation (10% PFA) and Perm B solutions (Thermo Fisher Scientific). NK cells were analyzed via flow cytometry and defined as CD3⁻ and CD56^{high}CD16[±] and CD56^{low}CD16^{high}. Boolean gates (FlowJo) were used to include all NK cells that expressed degranulation marker CD107a or produced cytokines, IFN γ and TNF α . Levels of CD107a, IFN γ and TNF α expression or production were calculated as % of NK cells positive for APC-H7, PE and BV-785

respectively. All assays were repeated with three different primary cell donors and the average scores from the three donors was used.

CSA Binding Inhibition Antibody to IE

CSA binding inhibition was measured as previously described (Nielsen and Salanti, 2015). The assays were all run twice and averages of the two runs were used.

IgG levels to non-pregnancy specific antigens

IgG levels towards schizont extract and MSP-1 were measured by ELISA as previously described (Barua et al., 2019) using goat anti-human IgG biotinylated (Mabtech 3820-4-250).

Quantification and Statistical Analysis

Processing of Data

Prior to analysis the data were processed. The right-skewness of the distribution of the features was reduced by log-transformation ($\log(x+1)$). Four antibody features that had negative values were right-shifted to have their minimum at zero prior to log-transformation. Next, the distributions of the features were centered and scaled to have zero mean and unit standard deviation.

Demographic and clinical characteristics of the pregnant women were described using the mean (SD) for continuous variables and frequency (%) for categorical variables.

Univariate Analysis

Antibody levels for individual antibody features were compared between groups using the Welch's *t*-test. The fold-change in the volcano plot was defined as the exponential of difference between the means of log-transformed and standardized data across the groups. Antibody features were correlated using Pearson pairwise correlation and correlation networks were plotted using the qgraph package (Epskamp et al., 2012). Associations between select antibody features and gravity were investigated using linear regression.

Identification of Key Antibody Features

Non-infected women were excluded from the multivariate analyses. Multivariate Imputations by Chained Equations (van Buuren and Groothuis-Oudshoorn, 2011) with predictive mean matching was used to impute any missing values (0.82% of observations). The imputation process was repeated five times and the median of the imputed values across the five generated imputed datasets was finally used for each missing value. An elastic net-regularized logistic regression model was initially used to identify the subset of antibody features that best discriminate between the pregnant women with non-placental infection and women with placental infection. Elastic net (Zou and Hastie, 2005) allows to attain sparsity (keeping only a small subset of important variables), but unlike the Least Absolute Shrinkage and Selection Operator (LASSO) penalization, groups of highly correlated important variables can as well be selected (a property of ridge regression). The parameter α , which alters the nature of penalization between ridge regression and LASSO, was set to 0.5 to achieve a balance between sparsity and group selection; the final selected antibody features did not alter when α was varied over [0, 1], as detailed below. We used a resampling approach in which the elastic net-regularized logistic regression (ENLR) model was repeatedly fitted to subsets of data, as used by Gunn et al (Gunn et al., 2018). This provided a means to take account of the uncertainty in the feature selection by ENLR, and to identify the features that are consistently

selected when the model is fitted to different resampled data. The selection stability of an antibody feature was defined as the proportion of times that it was picked in the selected set of important features by elastic net when the model was repeatedly fitted to 50,000 resampled subsets of data (5000 repeats of 10-fold cross-validation). In each of the 5000 repetitions, the dataset was divided into 10 folds, of which 9 folds were used to perform an inner 10-fold cross-validation to find the best value of λ (shrinkage parameter) that maximizes the Area Under the Receiver Operating Characteristic (AUROC) and in turn select the antibody features; using other performance measures such as Binomial deviance gave the same ranking of the features according to selection frequencies. In each resampling iteration, the majority group (50 women with placental malaria) was down-sampled to have an equal number of observations in both groups, thus avoiding overfitting the model to the majority group. The resampling process was repeated where α was also tuned over the set $\{0, 0.25, 0.5, 0.75, 1\}$ (instead of only $\alpha = 0.5$) in addition to λ and the results showed that the final top frequently selected variables do not change (See Figure 2-figure supplement 1).

Partial Least Squares Discriminant Analysis (PLSDA) (Barker and Rayens, 2003) was subsequently applied on the results of the ENLR to reassert the set of selected antibody features and find a minimal set of antibody features that accurately classifies the pregnant women; two components were selected for the PLSDA model (exception: one component was used where the model included only one feature). The antibody features were added to the PLSDA model one by one, from the highest to lowest selection frequency as estimated by the ENLR, and the minimal set of features beyond which the accuracy did not improve significantly were selected as the final set of key antibody features (Gunn et al., 2018); 500 repeats of 10-fold cross validation were performed to compute the accuracies. The statistical significance of the results was assessed by comparing the performance of the model with that of two random permutation tests (null cases): 1) the PLSDA model was fitted to six randomly selected antibody features and the performance was computed for 500 repeats of 10-fold cross-validation resampling; 2) 100 datasets were generated by randomly permuting the group labels (PM and NPI) and the same analysis performed for the original data set (i.e. building PLSDA models using the top six frequently selected antibody features found by resampling of elastic net) was repeated for each dataset.

All of the computations were performed in the R software (R Core Team, 2019). The resampling was performed using the caret package (Kuhn, 2008). The glmnet package (Zou and Hastie, 2005) was utilized for implementing the ENLR. The PLSDA method was implemented via the PLS package (Mevik and Wehrens, 2007) and some visualizations of the results of the PLSDA method were carried out using the mixOmics package (Rohart et al., 2017).

Ethics

Collection and use of plasma samples from women in PNG was approved by the PNG Institute of Medical Research Institutional Review Board, the PNG Medical Research Advisory Council and the Melbourne Health Human Research Ethics Committee. All participants provided informed written consent. The use of blood products from donors in Melbourne for isolation of primary cells, culture of parasites and leukocytes and for use as negative controls was approved by the Melbourne Health Human Research Ethics committee and the University of Melbourne Human Research Ethics committee.

Results

Cohort description

We measured antibody features in plasma samples collected at enrollment (mean 21 weeks' gestation) in 127 women and assessed the influence of antibody features on the presence of placental malaria at delivery. All women resided in malaria exposed areas and 17 were positive for *P. falciparum* at enrolment (by PCR and/or light microscopy). Women were selected based on *P. falciparum* infection status at delivery, using histology to define placental malaria (Bulmer et al., 1993). The cohort comprised 50 women who subsequently had no *P. falciparum* parasites detected on placental histology and were PCR negative for peripheral parasitemia at delivery (non-infected), 50 women who had *P. falciparum* parasites in the placenta by histology (placental malaria) and 27 women who had no *P. falciparum* parasites in the placenta by histology but who had peripheral blood parasitemia by PCR and/or light microscopy (non-placental infection) at delivery. Of the latter group, 10 were also PCR positive in placental blood, consistent with free circulation of early-stage parasites throughout the body. Sulfadoxine pyrimethamine (SP) and azithromycin was more efficacious against placental malaria than SP-chloroquine (Unger et al., 2015), but antimalarial regimes were similarly distributed between groups. The three groups were frequency matched for primigravidity, IPTp regime receipt, bed net use, rural residency and age and all three groups had similar clinical characteristics at enrollment (Table 1).

Measurement of antibody features

We initially studied antibody to an array of recombinant VAR2CSA DBL proteins (see figure 1-figure supplement 1 for a schematic of VAR2CSA). Thirteen features of antibody towards recombinant forms of each of the six DBL domains were measured by multiplex in the 127 women. In order to consider antigenic diversity (Benavente et al., 2018) four of the DBLs were also represented by an additional recombinant protein from a heterologous parasite strain (see Supplementary File 1 for details of recombinant proteins). Antibody features measured by multiplex included antibody isotypes (IgG, IgA, IgM) and subclasses (IgG1, IgG2, IgG3, IgG4, IgA1, IgA2), and the ability of antigen-specific antibody to bind Fcγ Receptors (FcγR) and complement protein C1q (which assesses the ability of antibodies to activate the classical complement cascade). Thus, using samples from the 127 women and measuring 13 antibody features directed at ten DBL domains, 15,340 out of 16,510 possible data points were acquired (figure 1-figure supplement 2 for a flow chart of data acquisition).

Levels of antibody features measured by multiplex were compared between non-infected and placental malaria, and between non-placental infection and placental malaria groups. Volcano plots summarizing the two comparisons of the multiplex data are in Figure 1 (see Supplementary File 2 for individual univariate analyses). Notably, levels of antibody to DBL domains at enrollment were similar between non-infected women at delivery and those with placental malaria (Figure 1A), whereas multiple antibody features were higher in women with non-placental infection than in placental malaria, indicating potential roles in protection (Figure 1B). Antibody features which were higher in women with non-placental infection compared to those with placental malaria included DBL2-specific IgG3, IgA2, IgG2 and IgG4 (IgG3.DBL2(ID1-ID2a).FCR3, IgA2.DBL2.1010, IgG2.DBL2(ID1-ID2a).FCR3 and IgG4.DBL2.1010 respectively) as well as DBL3-specific antibodies which bind FcγRIIIb (FcγRIIIb.DBL3.FCR3) and DBL5-specific IgG2 and IgG4 (IgG2.DBL5.0466 and IgG4.DBL5.0466). Thus, we selected DBL2, DBL3 and DBL5 for assessment of functional antibody features using cell-based

assays, in order to provide insights to the mechanistic contribution of these enhanced antibody populations. To investigate whether the higher antibody levels seen in the non-placental infection group were just correlates of exposure, we also measured IgG levels to non-pregnancy specific *P. falciparum* antigens merozoite surface protein-1 (MSP-1) and schizont extract (which are associated with exposure (Barua et al., 2019)) by ELISA and compared the levels between groups. IgG levels to neither MSP-1 nor schizont extract were higher in women without placental malaria (Supplementary File 2), suggesting that differences in antibody to pregnancy specific antigens were not simply due to exposure. As antibody responses did not significantly differ between non-infected and placental malaria groups, further data analysis was restricted to those 77 women with non-placental infection or with placental malaria.

We next measured responses to native protein on the surface of IEs of *P. falciparum* lines CS2 (which is isogenic with FCR3) and 3D7 that had been selected for high levels of CSA adhesion and studied whether antibody-opsonised IE and beads coated with recombinant DBL domains could activate leukocytes or promote phagocytosis to identify which antibody features associated with innate immune cells play important roles in parasite clearance or killing. We adapted existing cellular assays to measure antibody-dependent cellular phagocytosis (ADCP) of DBL protein-coated fluorescent beads and IE using THP-1 cells (Ackerman et al., 2011, Teo et al., 2015) and to measure NK cell activation following IgG binding to DBL proteins in plate-based assays (Jegaskanda et al., 2014, Lu et al., 2016). We also developed novel assays of ADCP by primary monocytes, and antibody-dependent neutrophil phagocytosis (ADNP) using primary human neutrophils, with IE and DBL-coated beads as their targets, as well as assays to measure antibody dependent respiratory burst (ADRB) with IEs or DBL proteins coated onto plates as their targets. Thus up to nine different cellular responses towards DBL2, DBL3 and DBL5 were measured. Additionally, six features of antibody to variant surface antigens (VSA) of two strains of CSA-binding IE were measured, namely IgM, IgG, and IgG subclass binding to VSA, and the ability of antibodies to block CSA binding was assessed (see Supplementary File 3 for a table listing all antibody features measured). With 39 variables for 77 women, 2997 measurements (99.8% of possible 3,003) were acquired. When added to the multiplex data, 169 variables with a total of 12,906 measurements (99.2% of 13,013) were used in subsequent analyses (see figure 1-figure supplement 1 for the flow chart). None of the women had any data missing for the outcome variable, 11 out of 77 women had data missing for one or more of the 169 predictor variables.

Identifying antibody features at enrollment which differentiate women with non-placental infection and placental malaria at delivery

We used a combination of elastic net-regularized logistic regression (ENLR) and Partial Least Squares Discriminant Analysis (PLSDA) to identify a set of antibody features that could best differentiate women with non-placental infection from those who had placental malaria at delivery (Gunn et al., 2018). Initially, the ENLR model was repeatedly fitted to randomly resampled subsets of the data and the selection frequency and the effect size (odds ratio) of the antibody features were calculated (Figure 2); only the top 20 antibody features with highest selection frequency are shown. The results show the antibody features that were consistently selected (Figure 2A) and have a high effect on discriminating the group of pregnant women (Figure 2B). The frequently selected antibody features were positively associated with non-placental infection (Figure 2B) and the effect of the antibody features on the odds of belonging to the non-placental infection group increased as the selection frequency increased.

A minimal subset of antibody features which could accurately separate the two groups was then identified by adding the antibody features into a PLSDA model one by one (based on the selection frequency; Figure 2A). The mean AUROC and accuracy of classifying women with non-placental infection do not increase significantly after the inclusion of the six top features (Figure 3A). The performance of the model with these six selected features was then compared with the results of two random permutation tests (Figure 3B). The results show that the model with the six selected features performed significantly better than the PLSDA models with randomly selected features. The AUROC is also significantly higher than that of the permutation test where the group labels were randomly permuted (Figure 3B). The AUROC for the 6 features corresponds to a median accuracy of 0.86 (Figure 3-figure supplement 1) and means that the model with these six antibody features can accurately classify, on average, 86% of the women.

The segregation of the pregnant women and the loadings on the components of the PLSDA model for the six selected features are illustrated in Figures 3C and 3D. The loading components illustrate that the segregation of the observations by the PLSDA was largely driven by the top two variables identified by ENLR, DBL2 specific IgG3 (IgG3.DBL2(ID1-ID2a).FCR3) and ADCP of the CSA-binding IE CS2 by THP1 (THP1.Phago.CS2).

As immunity against placental malaria increases with gravidity (Fried et al., 1998), we also conducted a univariate linear regression for these six selected variables with gravidity (Supplementary File 4-table 1). Ability of antibodies to inhibit binding of IE to CSA (CSA.Binding.Inhibition.FCR3) was the only antibody feature with evidence of a positive association with gravidity in this cohort ($p=0.046$). In addition, as a small subset of women were infected with *P. falciparum* at enrollment (by light microscopy and/or PCR, $n=17$, Table 1), to investigate the influence of these early infections on the identified features we conducted a sub-analysis, excluding these infected women, comparing the six identified antibody features between non-placental and placental malaria cohorts (Supplementary File 4-table 2). Importantly, four of the six antibody features were significantly higher in the non-placental infection cohort ($p\leq 0.013$), and the remaining two features trended to be higher in the non-placental cohort ($p\leq 0.064$). This suggested that the associations seen with these six variables was not greatly affected by the presence of the individuals with infections at enrolment.

Closer inspection of the features using a correlation network of the 77 women provided information about the relationships between different antibody features (Figure 4). Distinct clustering of some antibody features was apparent; for example IgA1, IgA2, IgG4 and IgM to recombinant DBL domains formed four distinct clusters. Similar functions sometimes also clustered together (e.g. primary monocyte and THP1 cell phagocytosis of DBL domain-coated beads). Multiplex measurement of IgG, IgG1, IgG3 and ability of antibody to bind FcγRs and C1q for all the different DBLs formed one large cluster highlighting the collinearity of many the antibody features measured towards recombinant proteins. Importantly, the six features identified by ENLR+PLSDA were spread throughout the correlation network and were not well correlated with each other (Figure 4 & Figure 4-figure supplement 1).

Antibody features selected highlight the importance of functional antibodies towards the infected erythrocyte and DBL2

The six selected features included IgG3 to DBL2 (IgG3.DBL2(ID1-ID2a).FCR3), ADCP and ADNP of CSA-binding IE (THP1.Phago.CS2 and Neutrophil.Phago.CS2 respectively), binding inhibition of IE to CSA (CSA.Binding.Inhibition.FCR3), IgA2 to DBL2 (IgA2.DBL2.1010) and IgG3 to CSA binding IE (IgG3.3D7). For all six features antibody levels were significantly higher in the non-placental infection group compared to the placental malaria group (Figure 5A-F), but the patterns of antibody responses distinguishing the two groups were not uniform. For example, some women in the non-placental infection group had no measurable levels of THP1.phago.CS2, IgG3.3D7 or IgG2.DBL5.0466 antibody. This indicated that there are multiple mechanisms for antibodies to confer protection. To investigate this further we constructed a heat map of the Z-score of each feature against individual women and compared the groups with non-placental infection and placental malaria (Figure 5G). Within these two cohorts there was significant variation in individual profiles and no evidence for a unique and consistent profile of antibody functions in all individuals with non-placental infection. Overall, the women with non-placental infection had significantly higher levels of most of the identified antibody features, 81% of women with non-placental infection had a Z-score >0 for at least four antibody features (compared to 26% of those with placental malaria). Looking at just the three functional measures, 52% of women with non-placental infection had elevated antibodies (a Z-score >0) which both promoted IE phagocytosis by THP-1 cells and/or neutrophils and inhibited binding of IE to CSA, compared to 18% of women with placental malaria (Figure 5G).

Discussion

We used a systems serology approach to broadly characterise antibody responses to the VAR2CSA protein that mediates protection from placental malaria. This involved measuring a wide range of antibody features to this protein followed by employing the machine learning techniques elastic net-regularized regression and PLSDA to identify those antibody features that protect pregnant women with *P. falciparum* infection from placental malaria. The combination of a prospective study design and a systems serology approach provided an unbiased way to identify the key features of the antibody response to VAR2CSA that mediates protection. Using this approach, we identified six antibody features of 169 tested which were able to correctly differentiate, on average, 86% of the pregnant women with placental malaria and non-placental infection. The antibody features identified fell into broad functional groups: antibody that blocks placental binding of IEs and opsonising antibody that result in phagocytic clearance of IEs (see Figure 6 for a schematic of mechanisms of protection of the six selected antibody features).

We hypothesised that pregnant women who had *P. falciparum* peripheral blood parasitemia at delivery but who did not have placental sequestration were protected from placental malaria by antibody immunity targeting VAR2CSA, and we sought to determine the characteristics of this protective antibody response. We frequency matched groups on participant characteristics such as gravidity and urban or rural residence to minimise confounding by these variables that are known to affect exposure and immunity. Antibody features at enrolment did not differ between women with placental malaria and those who were uninfected at delivery, but they did differ between infected women with and without placental malaria at delivery.

Unlike previously published studies (Aitken et al., 2010, Fried et al., 2018), the inclusion of an infected group without placental malaria allowed us to accurately control for exposure to the parasite at the time point of outcome. The lack of differences in antibody features between the non-infected and placental malaria groups was quite striking and mirrors previous observations

(reviewed by (Cutts et al., 2020)). It may in part be attributable to the heterogeneous nature of the non-infected group which probably includes both women who are exposed but protected by antibody and women who have not been exposed to malaria in pregnancy and therefore lack pregnancy-specific antibodies. It is possible that the non-infected group are protected by antibodies towards other antigens. That differences were seen only when comparing the two infected groups clearly highlights the importance for controlling for exposure when studying antibody responses and protection from malaria. In Cameroon, multigravid women studied in early pregnancy who did not go on to have placental malaria had a greater breadth and intensity of antibody responses to DBL domains than those with placental malaria at delivery (Tutterrow et al., 2012), and higher avidity antibodies to full length VAR2CSA, suggesting a broad, high affinity IgG response to DBL proteins may also correlate with protection.

In this study, fitting the ENLR model to resampled subsets of data allowed us to rank antibody features by their selection frequency. We generated a ranking of the 20 most frequently selected features, and then used PLSDA to determine a minimal set of antibody features that classify the pregnant women with a high accuracy. These six features were selected by ENLR more than 70% of the time and the inclusion of these six features in the PLSDA showed an ability to accurately distinguish 86% of the women in the two groups. Notably this ability to distinguish the two groups and did not increase if more than six features were included in the model. The six antibody features that best distinguished the groups were all more common in women with non-placental infection, and they included previously identified and novel responses.

Among the features we identified as associated with protection against placental malaria were adhesion inhibition and cytophilic antibodies that mediate ADCP of IE. Ability to block IE binding to CSA has been associated with protection against placental malaria, maternal anaemia, low birth weight or prematurity in subsets of pregnant women (Duffy and Fried, 2003, Ndam et al., 2015). IgG1 and IgG3 antibody responses to VAR2CSA are dominant, and mediate opsonic clearance of IE (Tebo et al., 2002, Elliott et al., 2005a, Megnekou et al., 2005, Keen et al., 2007, Damelang et al., 2019). ADCP using THP-1 cells has been correlated with protection from malaria or its complications in pregnant women and in adults (reviewed in (Teo et al., 2016)). ADNP of IE, which has not previously been systematically studied in malaria immunity also correlated with placental infection status. Antibodies that block adhesion and mediate ADCP by THP-1 cells or ADNP are important correlates of protection that should be included in future studies of naturally acquired and vaccine-induced antibody to malaria in pregnancy.

Although only 11% of features measured antibody to IE, four of the six top antibody features involved assays using IE. Similarly, both features towards recombinant proteins involved the DBL2 domain, which is the principal DBL domain involved in placental adhesion. The overrepresentation of both IE and DBL2 in the selected features supports the notion that antibody features directed towards functionally important targets are key to protection from placental malaria.

Novel features identified using the system serology approach include the antibody feature IgA2. Of note, IgA2 levels in serum are generally low (most serum IgA is IgA1) (Macpherson et al., 2008). Interestingly a recent study looking for correlates of malaria protection from vaccine induced immunity also identified IgA2 as a variable which helped predict those protected from *Plasmodium falciparum* infection (Suscovich et al., 2020). IgA2 can elicit myeloid cell effector functions, including opsonic phagocytosis (mediated by Fc α RI)(Breedveld and van Egmond, 2019) and as it recognised

the CSA binding DBL domain DBL2, it may also inhibit IE binding to CSA. However, since IgA2 levels in serum are low we hypothesise that rather than being directly involved in protection IgA2 might instead be a marker of a protective antibody response. Further investigation of VAR2CSA specific B cells and whether a protective response is associated with increased IgA2 is warranted.

Our analysis suggests features determined by measuring antibody to VAR2CSA-expressing IE are important indicators of protection from placental malaria. Four features measured antibody to IE, using representatives of the dominant 3D7-like and FCR3-like clades (Patel et al., 2017, Benavente et al., 2018) whose N-terminal regions are used in two VAR2CSA-based pregnancy malaria vaccines (Sirima et al., 2020, Mordmuller et al., 2019). While three of these four assays targeted FCR3 or the isogenic CS2 parasite line, studies of global diversity in VAR2CSA to date have included few sequences from PNG (Benavente et al., 2018), and the relative importance of immunity to specific clades may vary by region. Vaccination offered limited heterologous protection (Sirima et al., 2020) and it is unclear whether effective naturally acquired protective immunity needs to target multiple clades, or different clades in different regions.

Antibody responses to VAR2CSA domains serve as excellent markers of exposure to malaria in pregnant women (Cutts et al., 2020), but few were selected as correlates of protection in our analysis. This may reflect the fact that the responses to different VARCSA domains were generally highly correlated, as revealed by their clustering together in the centre of our network analysis, or it might be because of the longevity of antibody which promotes ADCP of IE compared to that of antibody responses to recombinant proteins (Teo et al., 2014, Teo et al., 2015, Chandrasiri et al., 2014). Antibody to VAR2CSA recombinant proteins, and particularly to specific VAR2CSA peptides, appears to be a good correlate of exposure and transmission intensity (Fonseca et al., 2019), but these responses appear to be less useful as correlates of protection.

The correlation network showed close correlations between different antibody responses to recombinant DBL domains (which predominantly located in the central cluster) but the protective antibody features were dispersed, suggesting that distinct pathways contribute to development of protection against placental malaria. The analysis of functions identified in the selected features further supports this, with most protected women (and few unprotected women) having significant antibody that promoted both binding inhibition to CSA and phagocytosis, however no consistent pattern of antibody functions in protected women was seen. Similar associations have been seen in the relationships between antibody features and protection from viral pathogens and there is a growing awareness that polyfunctional antibody responses, rather than a single functional correlate, contribute to protection from disease (Gunn et al., 2018, Chung et al., 2014). None of the antibody features targeted more than one variant of the protein or parasite strain. One possible explanation is that epitopes recognised by antibodies mediating each function may vary between strains (as suggested by (Doritchamou et al., 2019). This could in turn mean that protective functional features could vary with different populations (as there is variation in global distribution of the different VAR2CSA domain clades (Benavente et al., 2018)). Future studies should ideally include multiple VAR2CSA clades, and study diverse populations.

We used systems serology to identify protective features of the naturally acquired antibody response to VAR2CSA, a PfEMP1 variant which is specifically associated with placental malaria, complementing a recent study which used systems serology to identify functional antibody correlates of vaccine induced immunity, in subjects vaccinated with RTS,S (Suscovich et al., 2020). A

systems serology approach could also be used to identify the characteristics of protective immunity to other malaria syndromes, such as cerebral malaria which is often associated with a subset of PfEMP1s that are potential vaccine candidates (reviewed in (Jensen et al., 2020). It will be important to determine whether protective features of the cerebral malaria associated PfEMP1 antibody response analogous to the ones that we identified in this study can be identified.

We were interested in measuring antibodies to placental binding parasites and we defined placental malaria by the detection of IE in the placenta by histology because previous studies have shown that these are the infections associated with adverse pregnancy outcomes (Menendez et al., 2000, Rogerson et al., 2003). Ring stage parasites in the peripheral blood will circulate through the placenta and in some cases these could be detected by PCR of placental blood, however we did not consider these submicroscopic infections as placental malaria as they were not shown to be sequestering there.

Study strengths include the application of 169 novel and established assays; minimal missing predictor and no missing outcome data, the ability to control for exposure with the identification of pregnant women with *P. falciparum* infection with, and without, placental malaria; the prospective study design; and the use of native antigens on IEs to complement the data we acquired using recombinant protein. Together, this allowed us to discover determinants of naturally acquired protective immunity. Weaknesses include the lack of inclusion of full length VAR2CSA in our Luminex and bead-based assays, and an imbalance in numbers between women with parasitemia but no placental infection relative to the other groups. Samples from all women in the former group were selected for this study (and statistical analysis controlled for this imbalance between groups), but this group is relatively rare, with placental infection in the absence of peripheral infection being substantially more common (Desai et al., 2007). High levels of collinearity between some variables make it difficult to dissect out individual protective responses. Demonstration that our selected variables correlate with lack of placental malaria in other cohorts would further strengthen the findings. Antibody glycosylation (Lu et al., 2016) is an additional feature that could be included in future studies.

The present study identified leading candidate antibody features that mediate naturally acquired protection from placental malaria and demonstrated we could use these features to identify women in this cohort protected from placental malaria with high accuracy (though this should be validated in a second cohort). Our results suggest that multiple pathways exist that lead to development of protective immunity against placental malaria. Next steps will include validating this set of features in other sample sets from different geographical settings and also determining whether these antibody features predict protection from adverse pregnancy outcomes such as low birth weight. The immune responses identified are relevant to further development of pregnancy malaria specific therapies such as vaccines or monoclonal antibodies and possibly to the identification of protective antibody against malaria in non-pregnant populations. Multiple antibody functions and characteristics may be required to develop robust protective immunity to placental malaria.

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Table 1: Clinical characteristics of the three groups of pregnant women at the time of antibody feature measurement at enrollment (14-26 weeks' gestation) and also at delivery.

	Non-infected at delivery N=50		Placental malaria at delivery N=50		Non-placental infection at delivery N=27	
Enrollment						
Mean age (years), SD	24.5	5.3	24.0	5.0	23.1	4.4
Residence, N(%)						
Rural	37	(74.0)	38	(76.0)	18	(66.7)
Non-rural	13	(26.0)	12	(24.0)	9	(33.3)
Ethnicity, N(%)						
Sepik	6	(12.0)	11	(22.0)	3	(11.1)
Madang/Morobe	39	(78.0)	30	(60.0)	22	(81.5)
Highlander	3	(6.0)	5	(10.0)	1	(3.7)
Other	2	(4.0)	4	(8.0)	1	(3.7)
Formal Schooling, N(%)	46	(92.0)	46	(92.0)	25	(92.6)
Smoking, N(%)	9	(18.0)	11	(22.0)	6	(22.2)
Betel nut user, N(%) [†]	41	(82.0)	41	(82.0)	24	(88.9)
Alcohol, N(%)	2	(4.0)	2	(4.0)	2	(7.4)
Gravidity, N(%)						
Primigravidae	26	(52.0)	29	(58.0)	14	(51.9)
Secundigravidae	8	(16.0)	7	(14.0)	8	(29.6)
Multigravidae	16	(32.0)	14	(28.0)	5	(18.5)
IPTp Regime, N(%)						
SPCQ	27	(54.0)	30	(60.0)	18	(66.7)
SPAZ	23	(46.0)	20	(40.0)	9	(33.3)
Mean gestational age (days), SD	145.9	31.4	147.5	31.3	152.2	19.8
Mean maternal weight (kg), SD ^a	54.7	13.1	53.5	8.2	54.1	7.4
Mean maternal height (cm), SD ^b	154.3	5.9	154.6	6.9	154.4	6.0
Bed net use, N(%)	34	(68.0)	40	(80.0)	21	(77.8)
Hb (g/dL), Mean SD ^c	9.7	1.2	9.3	2.0	9.4	1.2
Light microscopy positive for Pf, N(%)	2	(4.0)	4	(8.0)	4	(14.8)
PCR positive for Pf, N(%)	4	(8.0)	5	(10.0)	5	(18.5)
Delivery						
Placenta	0	(0)	50	(100)	0	(0)
Light microscopy positive for Pf N(%)						
Peripheral blood	0	(0)	10	(20)	13	(48.2)
light microscopy positive for Pf, N(%)						
Peripheral blood PCR positive for Pf, N(%)	0	(0)	13	(26)	21	(77.8)
Placental blood PCR positive for Pf, N(%) ^d	1	(2.0)	10	(20)	10	(37.04)
Birthweight (g), SD	3062	546	2827	501	2840	416
Gestation at delivery (days), SD ^e	278	18	279	16	280	13
Mean Hb (g/dL), SD	10.1	2	9.5	1.9	10.2	1.4

Abbreviations: SD, standard deviation; Hb, hemoglobin; PCR, polymerase chain reaction; IPTp, Intermittent preventive treatment in pregnancy; SPAZ, Sulphadoxine pyrimethamine - azithromycin; SPCQ, sulphadoxine pyrimethamine – chloroquine.

a 1 participant with missing data on betel nut use in placental malaria.

b 1 participant with missing data on weight in the non-infected group.

c Missing Hb data, 5 in non-infected group, 4 in placental malaria group, and 2 in the non-placental infection group.

d Missing placental PCR data in 5 non-infected, 8 placental malaria, 4 non-placental infection women.

e 1 participant with missing data on gestation length at delivery in the non-infected group.

Figure Titles and Legends

Figure 1: Individual antibody features to recombinant VAR2CSA DBL domain proteins measured by multiplex comparing women with placental malaria at delivery to (A) non-infected women and (B) women with non-placental infection. Fold change (\log_2 transformed), characterizing the magnitude of difference between the antibody levels of two groups (x-axis) are plotted against the $-\log_{10}$ p-value, characterizing the statistical significance of the difference (y-axis). The vertical dotted lines ($\log_2(2)$ and $\log_2(0.5)$) mark a threshold for a two-fold change and the horizontal dotted lines ($\log_{10}(0.05)$) mark the statistical significance threshold ($P \leq 0.05$, Welch's *t*-test). Antibody features beyond the thresholds are shown in blue and labelled. DBL domain, Duffy binding like domain.

Figure 1-figure supplement 1: Diagram of the *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) VAR2CSA on the surface of the infected erythrocyte. DBL2 which is involved in binding to chondroitin sulfate-A is highlighted in light purple. DBL, Duffy binding like domain; ID, interdomain region; TM, transmembrane segment; ATS acid terminal segment.

Figure 1-figure supplement 2: Flow chart of data acquisition and analysis. NPI, non-placental infection; PM, placental malaria; NI, Non-infected; IE, infected erythrocyte; PLSDA, Partial Least Squares Discriminant Analysis.

Figure 2: Antibody features that are influential in distinguishing between malaria infected women with and without placental infection identified by the elastic net-regularized logistic regression model. Resampling (5000 repeats of 10-fold cross-validation) was used to obtain the selection frequencies and the odds ratios. (A) The top 20 antibody features are ranked in ascending order of selection frequency. (B) Box plots of the estimated odds ratios, an odds ratio > 1 indicates the antibody feature is positively associated with non-placental infection at delivery. Boxplots are median, IQR, whiskers (the lowest data point that falls between Q1 and $1.5 \times Q1$ IQR, the highest data point that falls between Q3 and $1.5 \times Q3$ IQR). IQR, Interquartile range.

Figure 2-figure supplement 1: Selection frequencies estimated across all α values {0, 0.25, 0.5, 0.75, 1} using the resampling of ENLR. See Section: Identification of Key Antibody Features in methods.

Figure 3: Selecting a minimal set of antibody features by PLSDA. (A) Performance of PLSDA at classifying women as having placental malaria or non-placental infection when the features (ranked by selection frequency using the elastic net; Figure 2) are added one by one to the model from highest to lowest rank (500 repeats of 10-fold cross-validation were performed to estimate accuracy for each model). The three lines represent the accuracy of classification of all women in the cohort (black), those with non-placental infection (blue) and those with placental malaria (red). The vertical dashed line denotes the cutoff beyond which the accuracy does not change significantly by adding more antibody features (used for selecting the minimal set of features). (B) Comparing the performance of the ENLR+PLSDA applied on the original data of the top six variables (rightmost boxplot) with two permutation tests (null cases): 1) the PLSDA model was fitted to six randomly selected antibody features and the performance was computed for 500 repeats of 10-fold cross-validation resampling; 2) 100 datasets were generated by randomly permuting the group labels (PM

and NPI) and the same analysis performed for the original data set (i.e. building PLSDA models using the top six frequently selected antibody features found by resampling of elastic net) was repeated for each dataset (C) Segregation of women with non-placental infection (blue) and placental malaria (red) using the scores of the two components of the PLSDA model with data from the selected six antibody features. The background colors show the predicted classification of the women for all the possible score values in the depicted range. (D) Feature loadings on the components of the PLSDA of the six selected antibody features (see Figure 3-source data 1 for more details about the factor loadings and group prediction using the PLSDA). Boxplots are median, IQR, whiskers (the lowest data point that falls between Q1 and $1.5 \times Q1$ IQR, the highest data point that falls between Q3 and $1.5 \times Q3$ IQR). IQR, Interquartile range. AUROC: area under the receiver operating characteristic curve.

Figure 3-figure supplement 1: Accuracy of PLSDA at classifying women as having placental malaria or non-placental infection when the features (ranked by selection frequency using the elastic net; Figure 2) are added one by one to the model from highest to lowest rank (500 repeats of 10-fold cross-validation were performed to estimate accuracy for each model). The three lines represent the accuracy of classification of all women in the cohort (black), those with non-placental infection (blue) and those with placental malaria (red). The vertical dashed line denotes the cutoff beyond which the accuracy does not change significantly by adding more antibody features (used for selecting the minimal set of features.)

Figure 3-source data 1: PLSDA prediction model. A PLSDA model with two components and using the six selected antibody features can be formed for prediction of the group of the pregnant women. The estimated loading factors of the model for the two components (shown in Figure 3 of the manuscript) are listed here. The *softmax* technique was used to normalize the scores for each class (placental malaria and non-placental infection) which works as the probability of an observation belonging to a certain class (Kuhn, 2008). The predicted class is the one with the largest model prediction, or equivalently, the largest class probability.

Figure 4: Correlation network of antibody features. Correlation network of all antibody features in both women with non-placental infection and those with placental malaria. The six selected antibody features do not cluster together. Antibody features with similar functions (denoted by the same letter) tend to correlate with each other. Blue, positive correlation; red, negative correlation; line width and closeness of variables increase with increasing correlation coefficients; only significant correlations (after Bonferroni correction for multiple comparisons) are shown. Selected antibody features identified by elastic net are highlighted and labelled. See supplementary File 3 for full list of feature names.

Figure 4-figure supplement 1: Correlation matrix of the six selected antibody features from women with non-placental infection at delivery and women with placental malaria. Colour and size of dots represent estimates of the correlation coefficient (r) (the further the correlation coefficient from 0 the larger the dot).

Figure 5: Distribution of antibody features in women with non-placental infection and placental malaria. (A-F); Levels of each of the selected antibody features in individual women in the two groups **(G)** No single antibody feature was present in all individuals with non-placental infection (or was absent in all those with placental malaria). Errors bars are mean (SD), P-values derived from

Welch's *t*-test. NPI, non-placental infection; PM, placental malaria; IE, infected erythrocyte. Z-score, distribution of the features were centered and scaled to have zero mean and a standard deviation of 1.

Figure 6: The six selected antibody features may protect women from placental malaria by (A) inhibiting infected red blood cells from binding CSA and sequestering in the placenta and/or (B) promoting phagocytosis of infected red blood cells by monocytes and/or neutrophils. Selected features which may inhibit placental sequestration include IgG3 to the whole infected red blood cell and IgG3 and IgA2 to VAR2CSA's CSA binding domain DBL2. Selected features which may promote parasite clearance by antibody dependent phagocytosis include IgG3 to the whole infected red blood cell and to DBL2, IgA2 to DBL2 and neutrophil and monocyte phagocytosis of whole infected red blood cells. CSA, chondroitin sulfate A; ADNP, antibody dependent neutrophil phagocytosis; ADCP, antibody dependent cellular phagocytosis; VAR2CSA, a parasite protein expressed on the surface of the infected red blood cell, made up of Duffy binding like domains (DBL).

Supplementary Files

Supplementary file 1: Table: Recombinant VAR2CSA DBL Domain Proteins Used to Measure Antibody Features

Supplementary file 2: Table: Univariate analysis non-infected v placental malaria and non-placental infection v placental malaria

Supplementary file 3: Table: Antibody feature code, name and description

Supplementary file 4: Table 1: Association between selected antibody features and gravidity^a in 77 women (women with placental malaria or non-placental infection at delivery) & Table 2: Univariate analysis of selected antibody features placental malaria v non-placental infection, in women uninfected at enrolment

Data set used for figures 1-6 has been uploaded to Dryad: doi:10.5061/dryad.wpzgmsbkx

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Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
strain, strain background (Plasmodium falciparum, CS2)	CS2	(Chandrasiri et al., 2014)		
strain, strain background (Plasmodium falciparum, 3D7)	3D7	(Chandrasiri et al., 2014)		Selected for Chondroitin sulfate A (CSA) adhesion
strain, strain background (Plasmodium falciparum, FCR3)	FCR3	(Nielsen and Salanti, 2015)		Selected for CSA adhesion (parent of CS2)
strain, strain background (Plasmodium falciparum, NF54)	NF54	(Nielsen and Salanti, 2015)		Selected for CSA adhesion (parent of 3D7)
biological sample (<i>Homo-sapiens</i>)	Plasma	(Unger et al., 2015)		
biological sample (<i>Homo-sapiens</i>)	Primary monocytes	This paper		Freshly isolated cells (see methods - Primary Leukocytes)
biological sample (<i>Homo-sapiens</i>)	Primary neutrophils	This paper		Freshly isolated cells (see methods - Primary Leukocytes)
biological sample (<i>Homo-sapiens</i>)	Primary NK cells	This paper		Freshly isolated cells (see methods -

				Primary Leukocytes)
cell line (<i>Homo-sapiens</i>)	THP1	(Ataide et al., 2010)	RRID: CVCL_0006	Monocytic cell line
peptide, recombinant protein	DBL1-7G8	(Avril et al., 2011)	MV-1398	Parasite line 7G8
peptide, recombinant protein	DBL2(ID1-ID2a)-FCR3	(Doritchamou et al., 2016)	MV1942	Parasite line FCR3
peptide, recombinant protein	DBL2(ID1-ID2a)-FCR3	(Mordmuller et al., 2019)		Parasite line FCR3
peptide, recombinant protein	DBL2-isolate	(Doritchamou et al., 2016)	MV 1940	Parasite Isolate 1010
peptide, recombinant protein	DBL3- FCR3	(Nielsen et al., 2009)	MP1028	Parasite line FCR3
peptide, recombinant protein	DBL3- 7G8	(Avril et al., 2011)	MV-1914	Parasite line 7G8
peptide, recombinant protein	DB4-FCR3	(Fried et al., 2013)	MP2369	Parasite line FCR3
peptide, recombinant protein	DBL4-isolate	(Doritchamou et al., 2016)	MV1700	Parasite Isolate I 0711
peptide, recombinant protein	DBL5-3D7	(Avril et al., 2011)	1218	Parasite line 3D7

peptide, recombinant protein	DBL5-7G8	(Avril et al., 2011)	1269	Parasite line 7G8
peptide, recombinant protein	DBL5-isolate	(Doritchamou et al., 2016)	MV 1749	Parasite Isolate I 0466
peptide, recombinant protein	DBL6-IT4	(Avril et al., 2011)	MV-1137	Parasite line IT4
peptide, recombinant protein	MSP-1	(Barua et al., 2019)		
biological sample (<i>Plasmodium falciparum</i>)	Schizont extract	(Barua et al., 2019)		
Antibody	Goat anti Human IgG (polyclonal)	Mabtech	3820-4-250	(1:2000)
Antibody	mouse anti- human IgG- PE (polyclonal)	SouthernBiotech	9040-09 RRID: AB_2796601	(1:77)
Antibody	mouse anti- human IgG1- PE (monoclonal)	SouthernBiotech	9052-09 RRID:AB_2796621	(1:77)
Antibody	mouse anti- human IgG2- PE (monoclonal)	SouthernBiotech	9070-09 RRID:AB_2796639	(1:77)
Antibody	mouse anti- human IgG3- PE (monoclonal)	SouthernBiotech	9210-09 RRID: AB_2796701	(1:77)

Antibody	mouse anti-human IgG4-PE (monoclonal)	SouthernBiotech	9200-09 RRID:AB_2796693	(1:77)
Antibody	mouse anti-human IgA1-PE (monoclonal)	SouthernBiotech	9130-09 RRID:AB_2796656	(1:77)
Antibody	mouse anti-human IgA2-PE (monoclonal)	SouthernBiotech	9140-09 RRID:AB_2796664	(1:77)
Antibody	mouse anti-human IgM-PE (monoclonal)	SouthernBiotech	9020-09 RRID:AB_2796577	(1:77)
Antibody	Rabbit anti-human IgG (polyclonal)	DAKO	A0425	(1:100)
Antibody	mouse anti-human IgG1 HP6069 (monoclonal)	Merck Millipore	411451	(1:50)
Antibody	mouse anti-human IgG2 HP6002 (monoclonal)	Merck Millipore	MAB1308	(1:50)
Antibody	mouse anti-human IgG3 HP6050 (monoclonal)	Sigma	I7260.2ml	(1:50)
Antibody	mouse anti-human IgG4 HP6023 (monoclonal)	Merck Millipore	MAB1312-K	(1:50)
Antibody	Goat anti-mouse IgG alexafluor 647 (polyclonal)	Life technologies	A-21235 RRID: AB_2535804	(1:500)

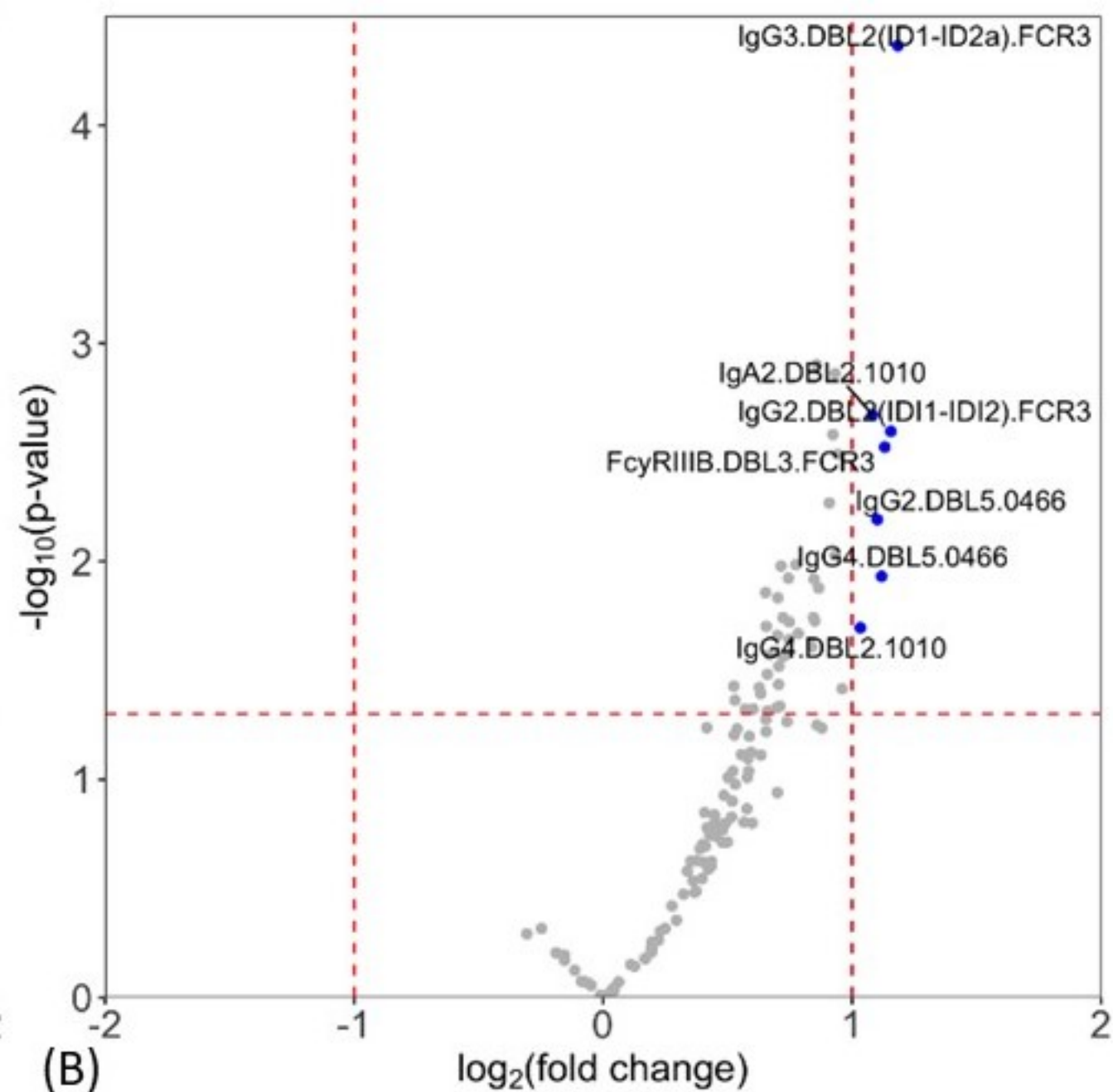
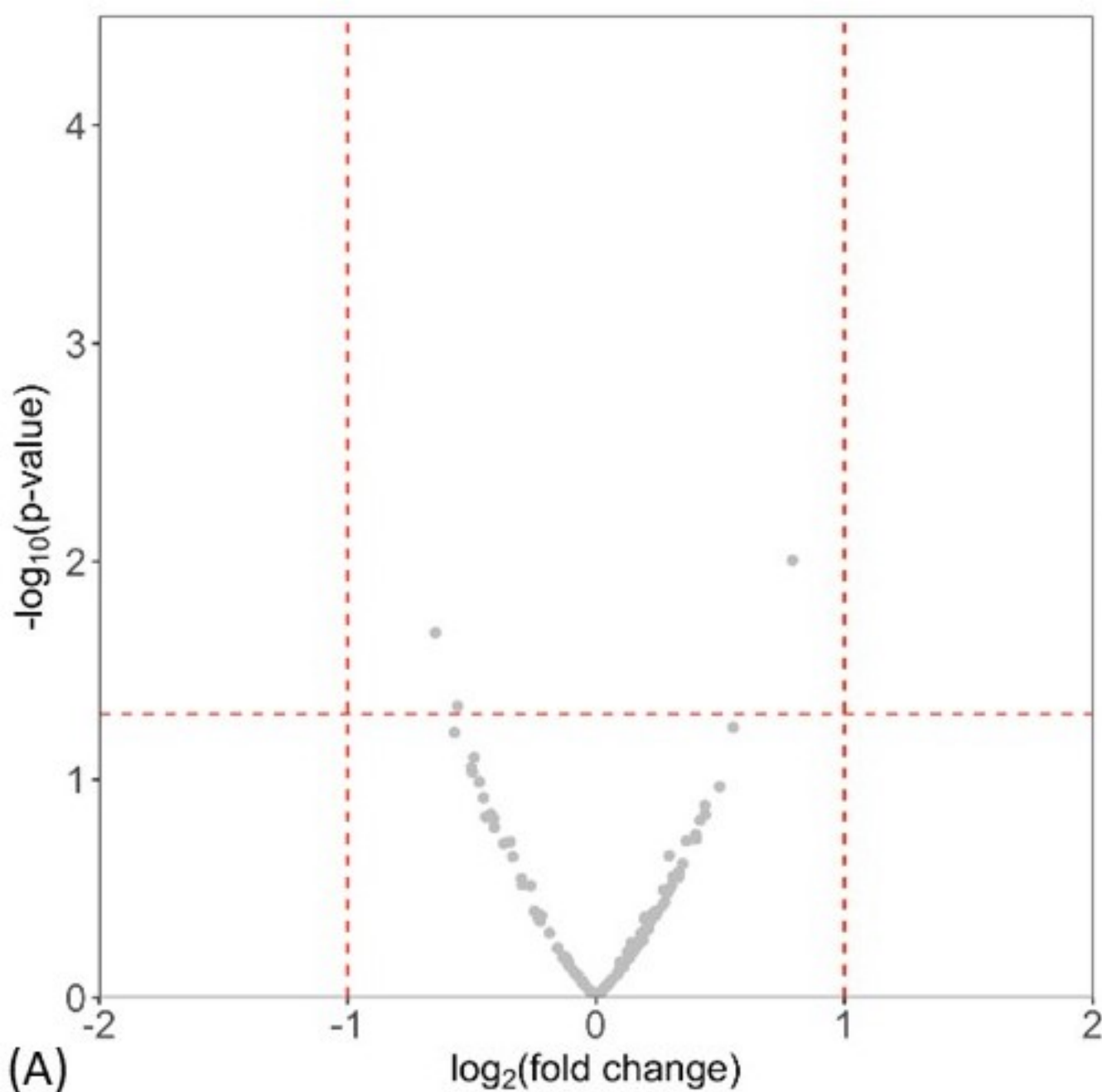
Antibody	Donkey anti-rabbit 647 (polyclonal)	Life technologies	A-31573 RRID: AB_2536183	(1:500)
peptide, recombinant protein	C1q	MP Biomedicals	80295-33-6	1.3µg/ml
peptide, recombinant protein	FcγRI	R&D Systems	1257-FC	
peptide, recombinant protein	FcγRIIa	(Wines et al., 2016)		
peptide, recombinant protein	FcγRIIIa	(Wines et al., 2016)		
peptide, recombinant protein	FcγRIIIb	R&D Systems	1875-CD	
Antibody	Mouse anti-human CD16-Brilliant™ violet 605 (monoclonal)	BD	563172	(1:50)
Antibody	Mouse anti-human CD56-Brilliant™ ultraviolet 737 (monoclonal)	BD	347344	(1:800)
Antibody	Mouse anti-human CD3-peridinin-chlorophyll-protein (monoclonal)	BD	552127	(1:200)
Antibody	Mouse anti-human IFNγ-PE (monoclonal)	BD	554701	(1:200)

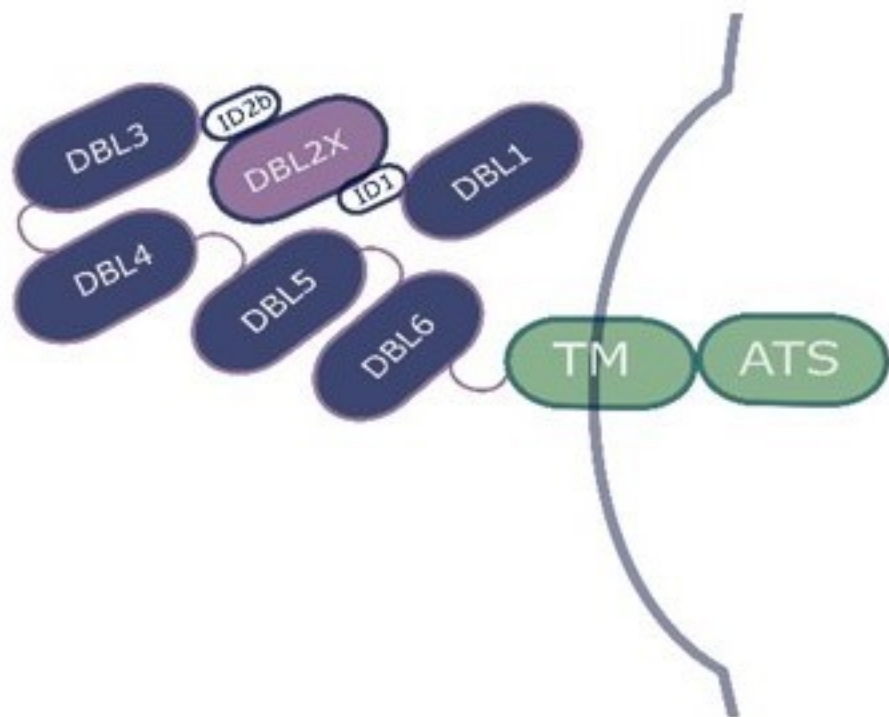
Antibody	Mouse anti-human TNF α -BV-785 (monoclonal)	Biolegend	502947 RRID: AB_2565857	(1:200)
software, algorithm	R software, caret package	R software	(Kuhn, 2008)	
software, algorithm	R software, glmnet package	R software	(Zou and Hastie, 2005)	
software, algorithm	R software, PLS package	R software	(Mevik and Wehrens, 2007)	
software, algorithm	R software, mixOmics package	R software	(Rohart et al., 2017)	
software, algorithm	R software, qgraph package	R software	(Epskamp et al., 2012)	
Commercial assay or kit	EasySep™ Direct Human Neutrophil Isolation Kit	STEMCELL™ Technologies	19666	
Commercial assay or kit	RosetteSep™ Human Monocyte Enrichment Cocktail	STEMCELL™ Technologies	15068	
Commercial assay or kit	RosetteSep™ Human NK Enrichment Cocktail	STEMCELL™ Technologies	15065	
Commercial assay or kit	Melon Gel purification kits	Thermo Fisher Scientific	45212	

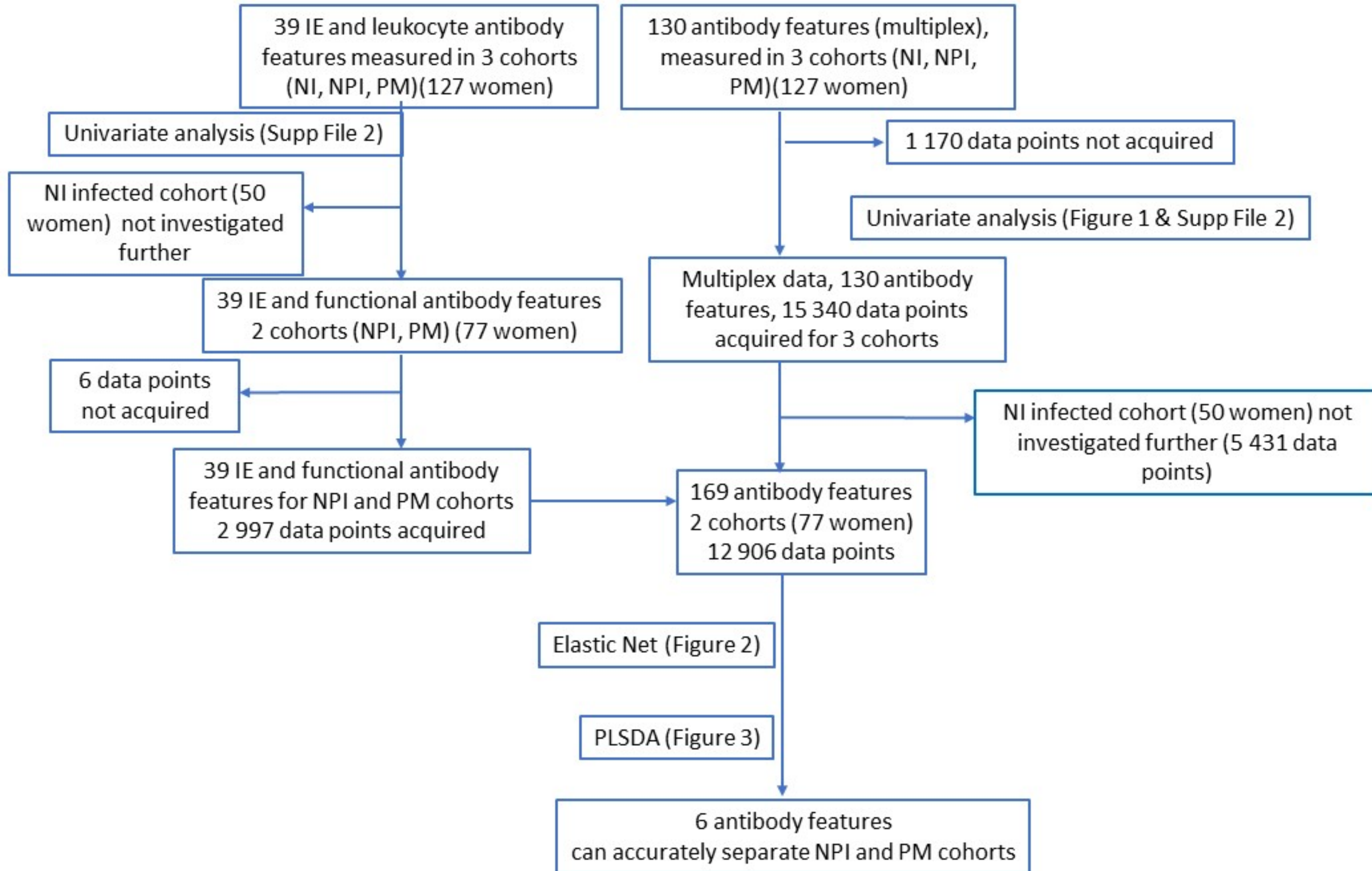
Commercial assay or kit	EZ-link Sulfo NHS-LC-Biotin kit	Thermo Fisher Scientific	24520	
other	Bio-Plex magnetic carboxylated microspheres	Bio-Rad	#MC100xx-01	
other	streptavidin-PE	SouthernBiotech	7105-09	

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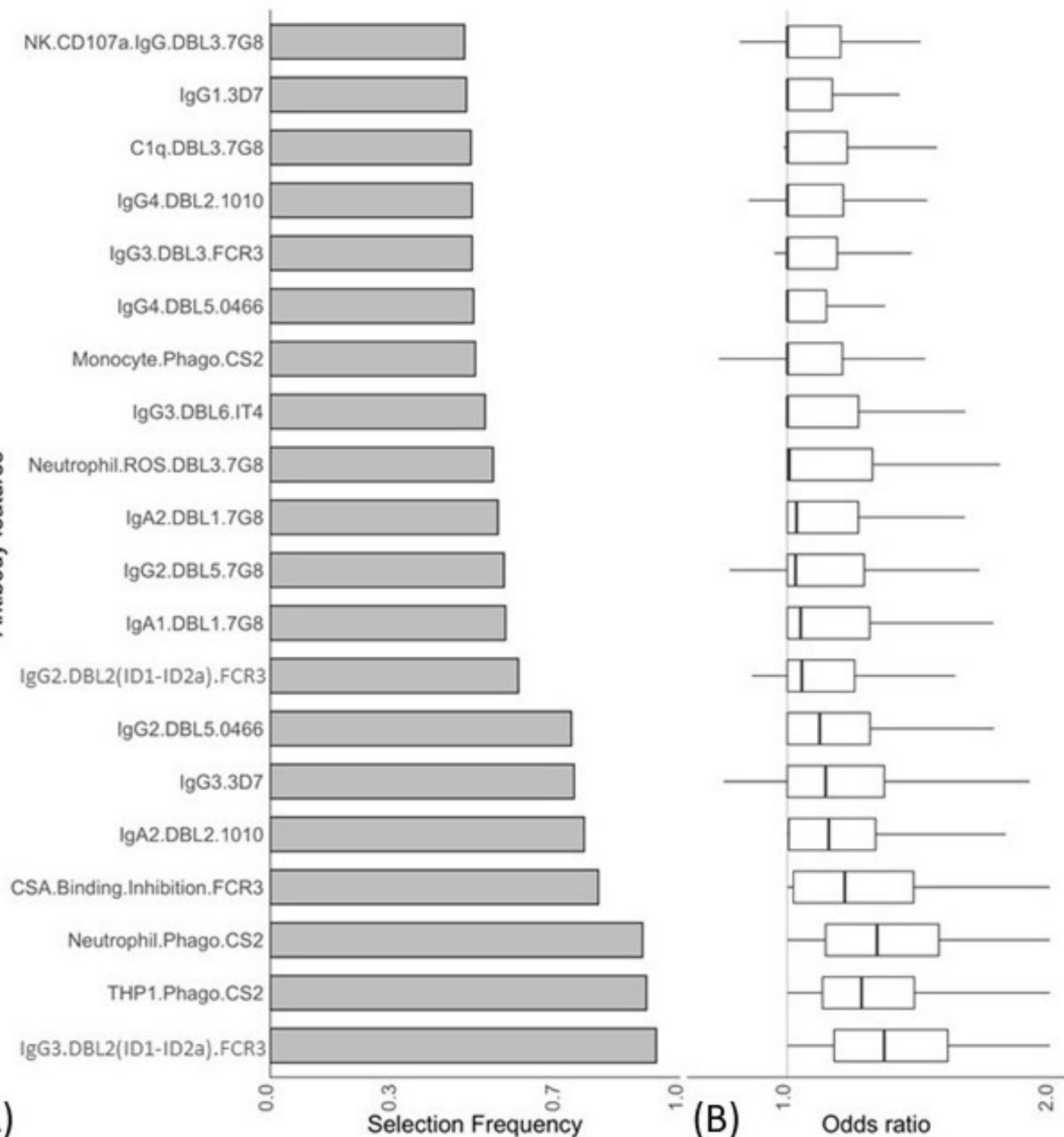
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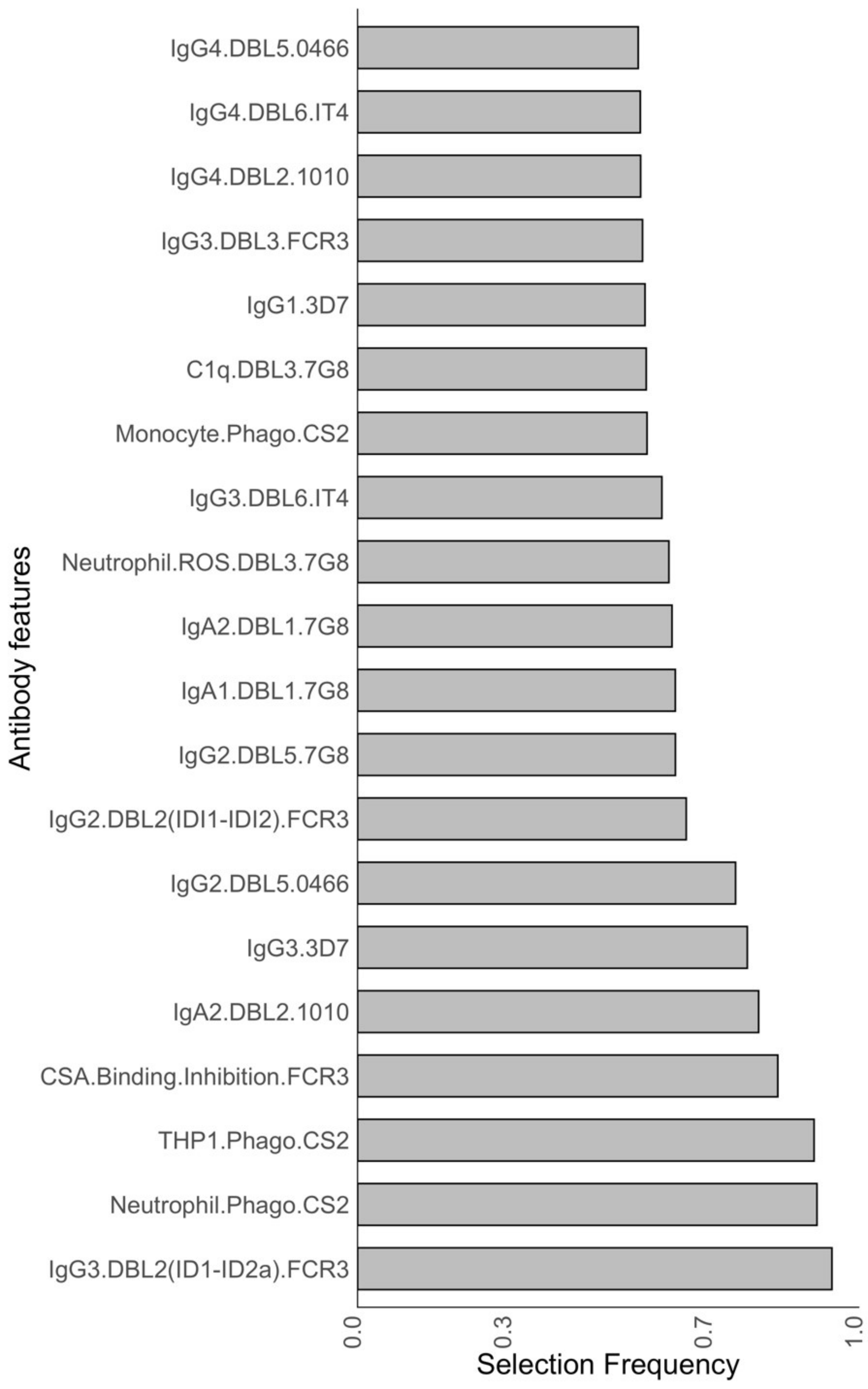


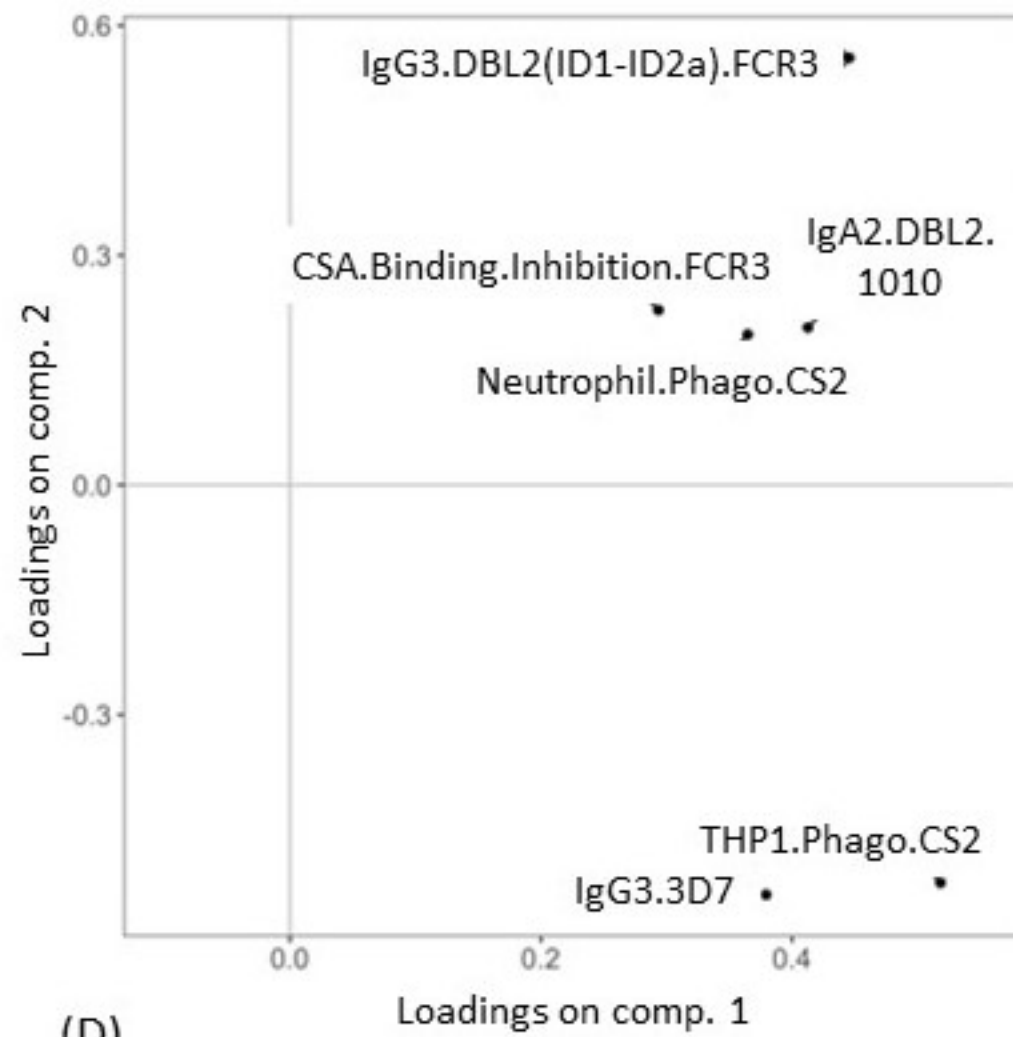
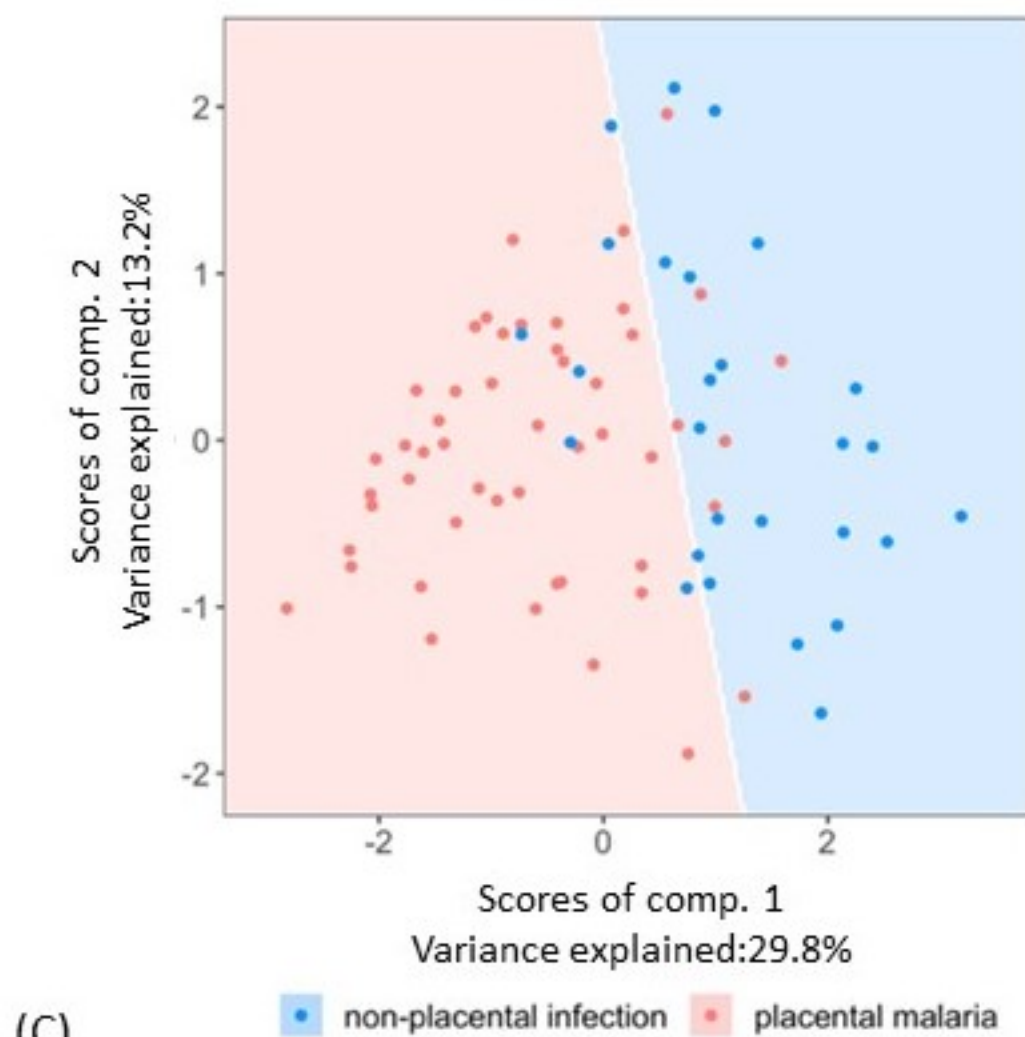
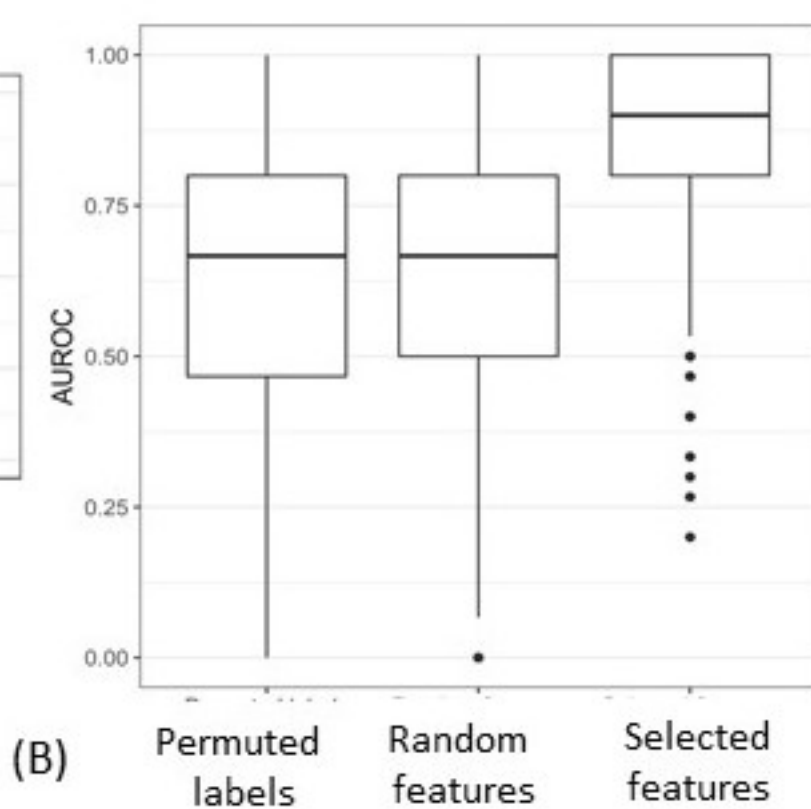
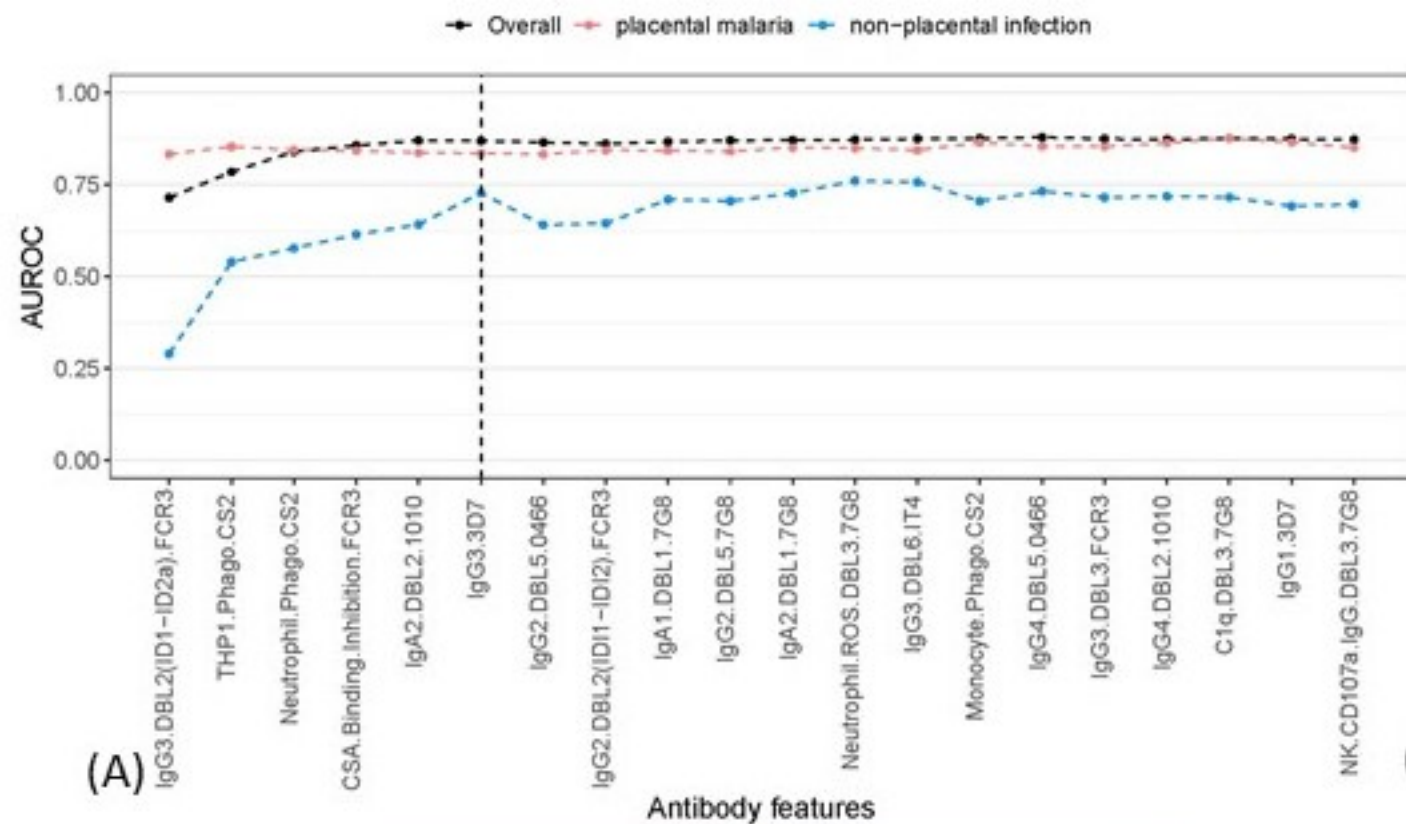


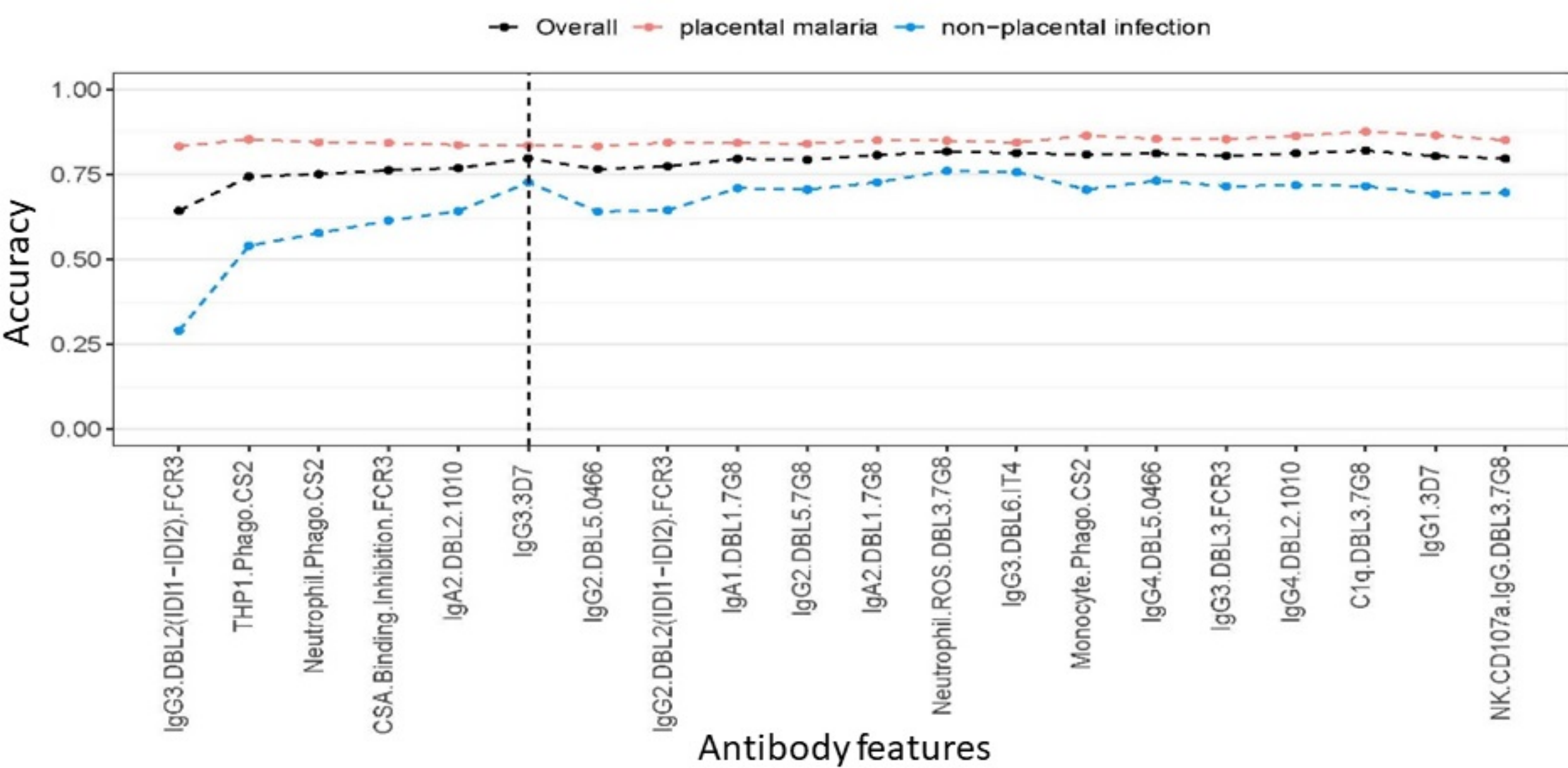


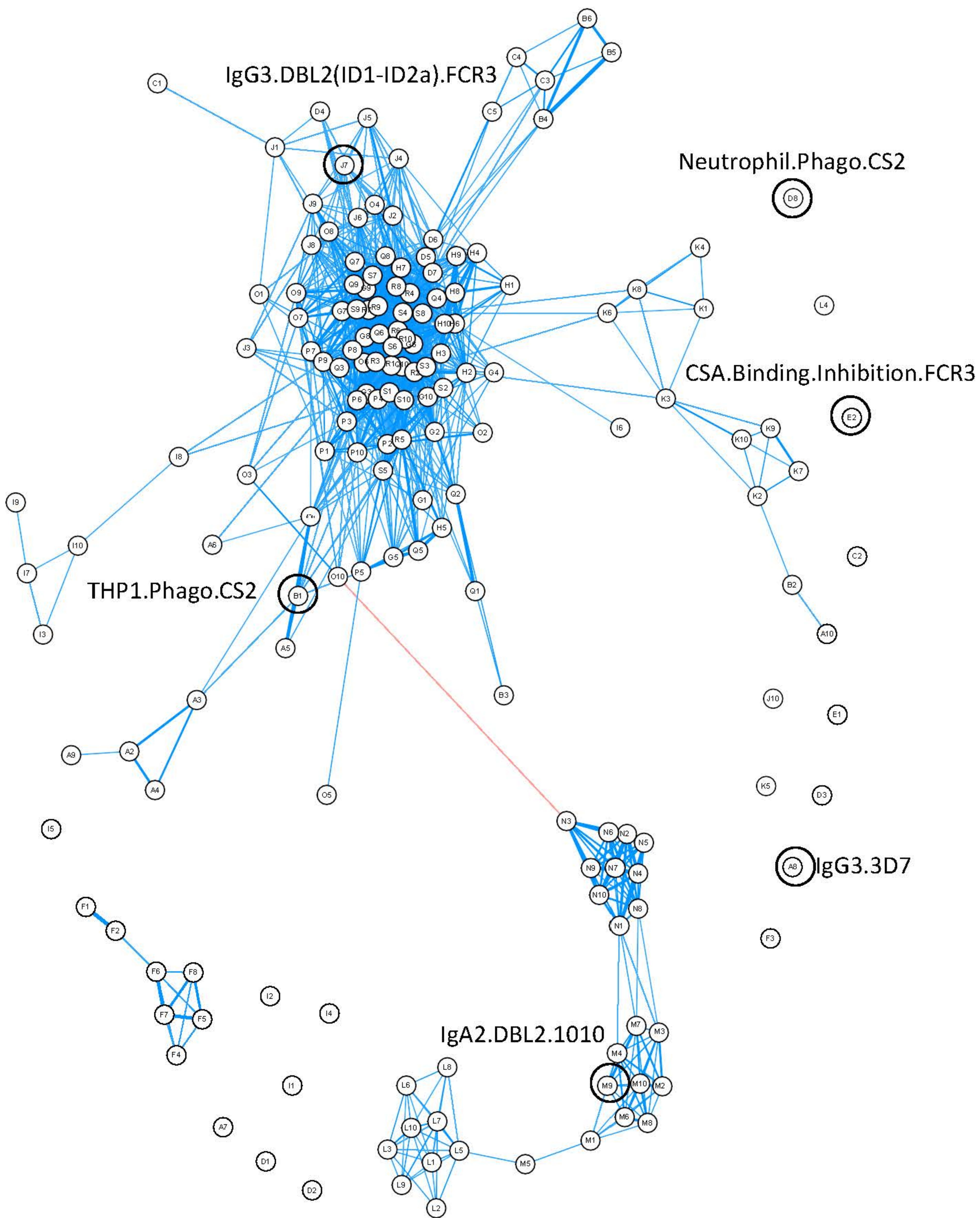
Antibody features

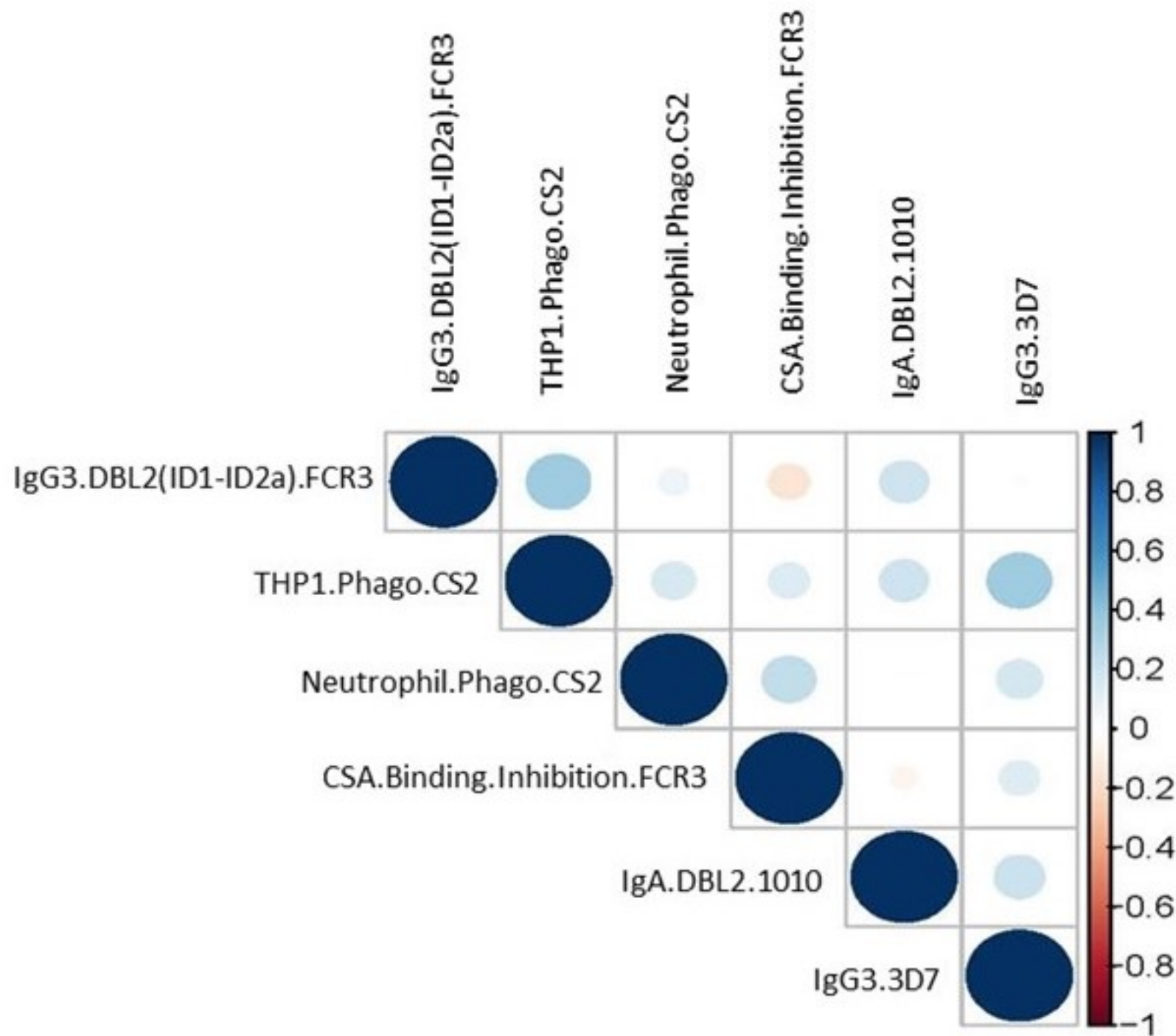


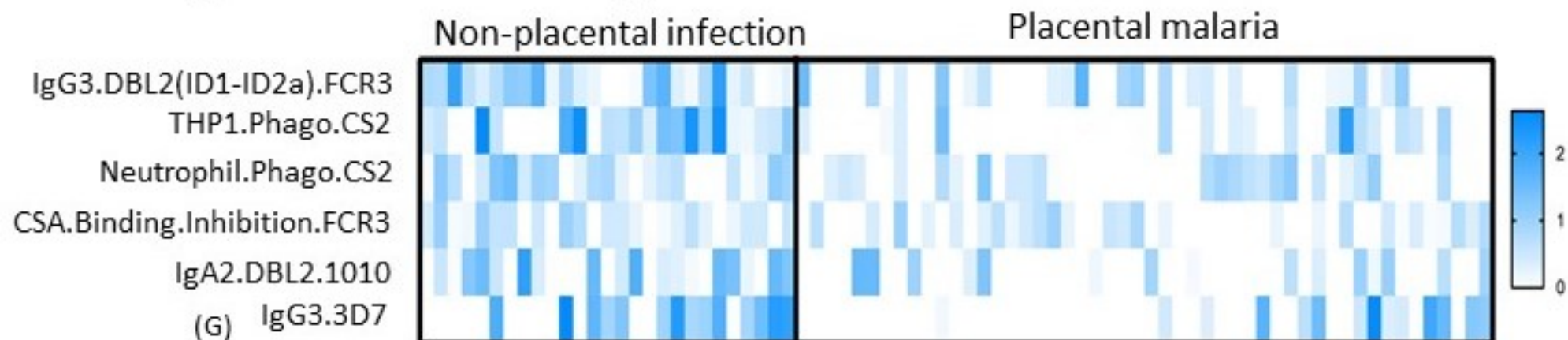
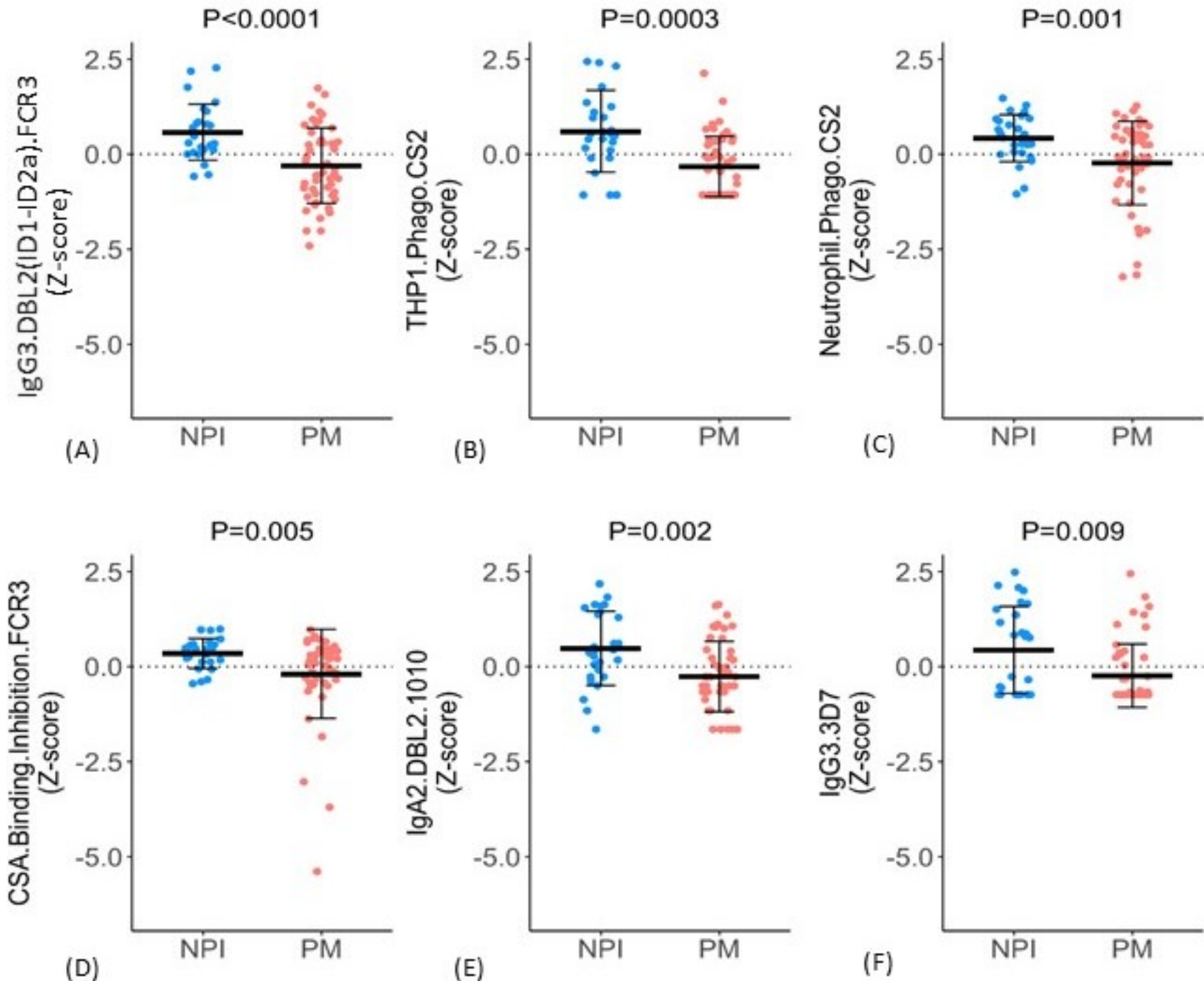




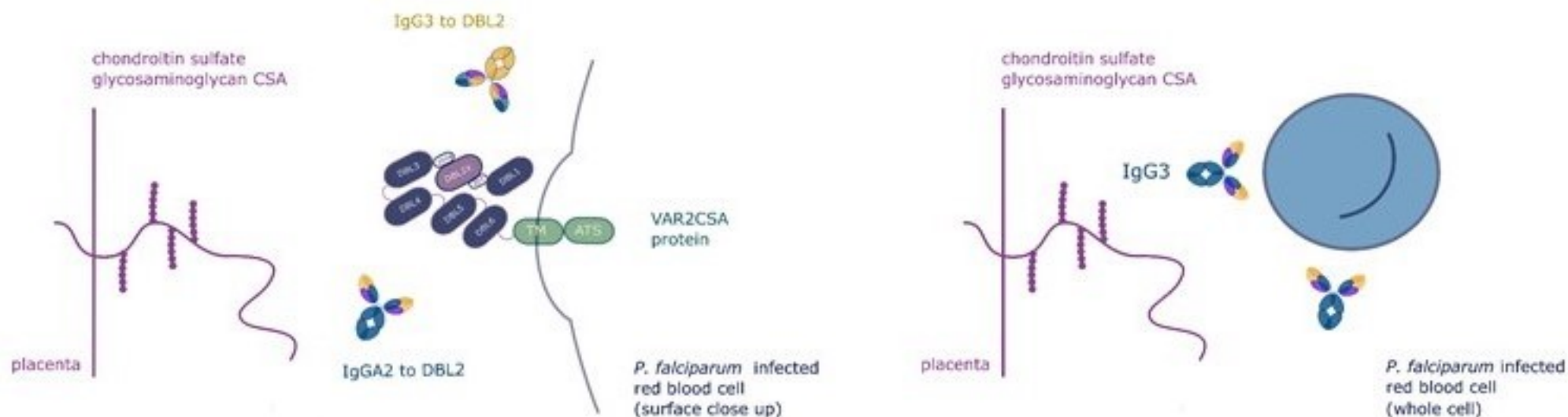








A) Antibody features that inhibit binding of *P. falciparum* infected erythrocytes to placental CSA



B) Antibody features that induce phagocytosis of *P. falciparum* infected erythrocytes

