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Antibodies against Plasmodium falciparum erythrocyte membrane protein-1 that correlate with protection from severe malaria in children

Isobel S Walker
University of Melbourne
Ali Haghiri
University of Melbourne
Wina Hasang
University of Melbourne
Amaya Ortega Pajares
Burnet Institute
Daniel Getacher Feleke
University of Melbourne
Laurens Manning
The University of Western Australia
Timothy M E Davis
The University of Western Australia
Ivo Mueller
Walter and Eliza Hall Institute
Oscar H Lloyd Williams
University of Melbourne
Megha Rajasekhar
University of Melbourne
Julie A Simpson
University of Melbourne
Thomas Lavstsen
University of Copenhagen
Louise Turner
University of Copenhagen
Yvonne Adams
University of Copenhagen
Anja TR Jensen
University of Copenhagen
Rebecca W Olsen

University of Copenhagen
Michael Duffy
University of Melbourne
Janavi S Rambhatla
University of Melbourne
J Alexandra Rowe
University of Edinburgh
Stanley E. Otoboh
University of Edinburgh
Aaron S Meyer
Massachusetts Institute of Technology
Eizo Takashima
Ehime University
Takafumi Tsuboi
Ehime University
Elvin Lufele
Papua New Guinea Institute of Medical Research
Moses Laman
Papua New Guinea Institute of Medical Research
Amy W Chung
University of Melbourne
Elizabeth H Aitken
University of Melbourne
Stephen J Rogerson
sroger@unimelb.edu.au

University of Melbourne

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Abstract

Background

Plasmodium falciparum erythrocyte membrane protein-1 (PfEMP1) are multidomain variant antigens on *P. falciparum*-infected erythrocytes (IE) that mediate vascular sequestration. Expression of specific PfEMP1 are upregulated in severe malaria (SM), and IgG against them has been linked to protection. The Fc features of PfEMP1-specific antibodies that correlate with protection from severe malaria are not well defined. A combination of PfEMP1 antibody targets and Fc features may distinguish between children with SM and UM.

Methods

We measured antibodies against 28 PfEMP1 antigen domains in Papua New Guinean children with SM (n = 141) or UM (n = 68), at presentation and eight weeks convalescence. We measured IgG, IgG subclasses, Fc receptor and C1q binding, and measured antibody dependent THP-1 cell and neutrophil phagocytosis (ADNP) of IEs.

Results

At presentation, UM was positively associated with IgG, IgG1, IgG3 and FcγRIII binding antibodies, against domains that bind endothelial protein C receptor (EPCR), intercellular adhesion molecule-1 (ICAM-1) or CD36, and with ADNP of IE that bind ICAM-1 and CD36 (two-fold difference in mean antibodies, p-value range 0.03 to 10^{-10}). Associations were most notable when compared to children with severe anaemia. By convalescence, FcγRIIIb and FcγRIIb binding antibodies declined while C1q binding antibodies increased. Using Elastic Net Regression modelling, functional antibodies targeting diverse PfEMP1 domains distinguished between SM or UM with 84% accuracy (77–89%), whereas using only EPCR binging variants achieved 66% (57–74%). Using Tensor-structured decomposition, variation between SM and UM was driven by functional antibodies against all PfEMP1 domains.

Conclusions

IgG1 and IgG3 antibodies that engage Fc receptors and target EPCR, ICAM-1 and CD36 binding domains, and ADNP of IE, may protect from SM. Accurate classification of SM and UM requires functional antibody measures to a combination of PfEMP1 domains of multiple binding phenotypes, and deficiencies in antibodies vary between SM presentations. Interventions to prevent SM or biomarker panels to predict protective immunity may need to include a breadth of PfEMP1 variant targets.

Background

Severe *Plasmodium falciparum* malaria causes around 600,000 deaths each year and encompasses a range of clinical presentations, including severe anaemia, hyperparasitaemia, impaired consciousness (cerebral malaria), metabolic acidosis, respiratory distress and renal impairment (1). The adhesion of *P. falciparum* infected erythrocytes (IE) to endothelial cells in the microvasculature is central to severe malaria pathogenesis, allowing the parasite to sequester, obstruct microvascular flow and evade splenic clearance while also causing localized inflammation. Adhesion is mediated by *P. falciparum* erythrocyte membrane protein-1 (PfEMP1) proteins, expressed on the IE surface. Importantly, previous studies suggest that antibodies targeting PfEMP1 drive protective immunity against severe malaria (reviewed in (2)), and therefore PfEMP1 proteins may represent useful targets for therapeutics or serological biomarkers.

Parasites can switch between expression of different PfEMP1. These are encoded by ~ 60 *var* genes per genome and subject to frequent ectopic recombination, creating extensive antigenic diversity. PfEMP1 are composed of two to eight Duffy Binding Like (DBL) domains and one or two Cysteine-rich Interdomain Regions (CIDR), classified into subclasses DBL α0.1-2, β1–13, γ1–18, δ1–9, ε1–14, ζ1–6, pregnancy associated malaria domains (pam) 1–5, and CIDR α1–8, β1–7, δ1–2, γ1–12, based on amino acid sequence homology (3). Each parasite carries a similar repertoire of PfEMP1, which divide into four main mutually exclusive receptor specificities defined by their N-terminal domains, and are linked to the clinical outcome of infection. Expression of PfEMP1 with CIDRα1 domains that bind to endothelial protein C receptor (EPCR) (4, 5) is consistently associated with parasites causing severe malaria in young children and non-immune adults (6–10), whereas expression of PfEMP1 with CIDRα2–6 domains that bind to Cluster of Differentiation 36 (CD36) (11), the most common binding phenotype, is not associated with severe malaria (12, 13). All parasites also encode PfEMP1 with CIDRβ/γ domains that mediate binding of IE to uninfected erythrocytes (rosetting), a phenotype associated with severe childhood malaria (14, 15), as well as the atypical VAR2CSA PfEMP1, which binds placental chondroitin sulphate A (CSA) and mediates pregnancy-associated malaria (16, 17). In addition, a subset of PfEMP1s bind intercellular adhesion molecule-1 (ICAM-1) via DBLβ domains in combination with CD36 or EPCR (18, 19). Together, these binding phenotypes enable sequestration across multiple vascular beds including brain, heart, lung, kidney, intestines and placenta (20, 21). Dual EPCR–ICAM-1 binding variants are likely favoured under conditions of inflammation-driven upregulation of ICAM-1 and other endothelial adhesion molecules in severe malaria, (19, 22, 23). Other PfEMP1 domains expressed in severe malaria may contribute to cytoadhesion through as yet unidentified receptor interactions (24).

Despite its high sequence diversity, evidence indicates that antibodies targeting a subset of PfEMP1 domains protect against severe disease (reviewed in (2)). In high malaria transmission settings, IgG antibodies against IEs (25, 26) and PfEMP1 domains (27–30) associated with severe malaria are acquired in early childhood and the risk of severe disease decreases with age. Antibodies targeting PfEMP1 could protect against malaria by reducing IE binding to endothelial cells (4, 31–33), or through engaging Fc receptors on leukocytes to induce functions that kill the IE, such as antibody dependent cellular phagocytosis (ADCP), antibody dependent neutrophil phagocytosis (ADNP) or antibody dependent cellular cytotoxicity (ADCC) (34–37). In the small number of studies of PfEMP1-specific

antibody Fc features and functions in severe malaria in children, ADNP of ICAM-1 and EPCR-binding IE was associated with protection from cerebral malaria (38) and ADCP of EPCR-binding IE or DBLβ-coated beads was associated with protection from severe malaria (36, 39).

Investigating antibody Fc features and functions (40) can reveal correlates of protective immunity that may be useful for therapeutic development or provide biomarkers of protective immunity. In malaria in pregnancy, ADCP against placental malaria-associated PfEMP1 VAR2CSA was a better correlate of protective immunity against placental infection that IgG titres alone (34, 41). Using a systems serology approach to measure the Fc features of antibodies to multiple PfEMP1 target antigens, we previously identified a combination of antibody responses that could distinguish between cerebral and uncomplicated malaria in children from Malawi (38). In the present study, we applied a similar approach to identify targets and Fc features of antibody to PfEMP1 that distinguish between children from Papua New Guinea (PNG) with severe and uncomplicated malaria at presentation to hospital, and to investigate how these antibody measures change during convalescence.

Methods

Clinical samples

Plasma samples were collected as part of an observational study of severe paediatric illness, conducted in PNG from 2006 – 2009 (42). Children aged 0.5-10 years were recruited at presentation to Modilon General Hospital, Madang, Madang Province. Malaria infection was detected by microscopy and species was confirmed by nested polymerase chain reaction (nPCR). Severe malaria was defined as >1000 parasites per microlitre with presentation with one or more of: impaired consciousness or coma (Blantyre Coma Score (BCS)<5); prostration; multiple convulsions (>2 seizures in 24 h); hyperlactatemia (blood lactate >5 mmol/L); severe anaemia (haemoglobin <50 g/L); dark urine; hypoglycaemia (blood glucose ≤2.2 mmol/L); jaundice; respiratory distress; persistent vomiting; abnormal bleeding; and signs of shock (42). For the present study, children with severe malaria were further categorized into four subgroups: hyperparasitaemia (>100,000 parasites/mL), cerebral malaria (BCS <3), severe anaemia (haemoglobin <50 g/L) and metabolic acidosis (bicarbonate <12.2 mmol/L). Uncomplicated malaria was defined as *P. falciparum* infection with fever without any criteria for severe malaria, treated as an outpatient. Blood samples were collected by venepuncture at recruitment and 8 weeks later during convalescence. Plasma was separated, stored at -70 °C and shipped on dry ice to Melbourne, Australia, for analysis. Samples were selected for analysis based on the available volume, and confirmation of *P. falciparum* by light microscopy and/or nPCR at enrolment. Samples were heat inactivated at 50 °C, 45 min. A pooled positive control plasma was made from six malaria-exposed pregnant women from Malawi that were collected as part of a previous study (Madanitsa et al. 2016). Sera from 20 malaria naïve individuals were used as negative controls.

Recombinant proteins

The study included 28 recombinant PfEMP1 protein domains or domain combinations (cassettes) (Table 2). Eight were derived from *var* gene sequences upregulated in severe or uncomplicated malaria in Papua, Indonesia (24,28) and expressed in the wheat germ cell free system (WGCFS) (43). Ten were DBLβ domains derived from laboratory adapted parasite lines, including domains predicted to be ICAM-1 binding and non-ICAM-1 binding, and one dual binding double domain protein (CIDRα1.6-DBLβ3), and were expressed in *Escherichia coli (E. coli)* (32,33). Six were CIDRα1 domains binding EPCR and four were CIDRα2-8 domains binding CD36, derived from laboratory-adapted parasite lines and expressed in *Baculovirus*- insect cells (5,11). MSP2 and AMA1 derived from a Papuan patient with severe malaria were included as surrogate markers of cumulative exposure and were expressed in WGCFS (28). Tetanus toxoid from *Clostridium tetani* (Sigma-Aldrich, Missouri, United States) and simian immunodeficiency virus viral capsid protein p27 (Sino Biological, China) were respectively used as experimental positive and negative controls in the multiplex.

Multiplex immunoassays

Luminex® MagPlex® magnetic carboxylated microspheres were coupled with recombinant proteins as previously described in detail (44). Antigen-specific antibodies were detected by phycoerythrin (PE) conjugated fluorescent detector antibodies, as previously described (38,44), using the FLEXMAP3D (Luminex®). Antigen-specific IgG, IgG1, IgG2, IgG3 and IgG4 were detected with mouse anti-IgG-PE (JDC-10), mouse anti-IgG1-PE (HP6001), mouse anti-IgG2-PE (HP6002), mouse anti-IgG3-PE (HP6050), mouse anti-IgG4-PE (HP6025); all from Southern Biotech, AL, USA. Antigen-specific antibody binding to Fc receptors and C1q were detected by fluorescent tetramers; tetramers were formed by pre-conjugating a 1:4 molar ratio of streptavidin-R-PE Conjugate (ThermoFisher, MA, USA) with biotinylated human FcgRIIa (H167), FcgRIIIa (V176), FcgRIIb or FcgRIIIb (NA1), all from Acrobiosystems, China or recombinant C1q protein (MP Biochemicals, California, USA).

Parasite culture and selection

Laboratory adapted parasite lines were selected for expression of six PfEMP1 with binding phenotypes of interest (Additional File 1, Fig. 1). 3D7 IE were enriched for expression of PFD1235w, predicted to bind to ICAM-1 and EPCR, and IT4 IEs were enriched for IT4VAR13, predicted to bind to ICAM-1 and CD36, as previously described (38). By flow cytometry, 36-52% of IT4VAR13 IE were recognized by a rabbit polyclonal antibody to DBLβ3 of IT4VAR13, and 44-55% of PFD1235w IE were recognized by a mouse monoclonal antibody to DBLβ3 of PFD1235w (45). For ADCP, IT4 parasite line was enriched for IT4VAR19 by Fluorescence-Activated Cell Sorting using a rabbit polyclonal antibody to anti-DBLα of IT4VAR19. For ADNP, IT4 parasite lines were transfected with a blasticidin resistance gene and grown in the presence of blasticidin S to select for IT4VAR19 (46). By flow cytometry, the percentage of IEs recognized by the anti-DBLα of IT4VAR19 antibody after selection was >60% for parasites used in ACDP assays and >80% for ADNP assays. R29 was enriched for the rosetting phenotype by selection of IE that pellet after 40 min in 0.75% (v/v) gelatin, followed by selection of IE that remain in suspension after 40 min in gelatin with heparin lithium, 100 units/mL. By microscopy, 50-60% of IE were in rosettes. P6A1

was confirmed to bind to recombinant CD36, but not ICAM-1 or CSA, by microscopy, as previously described (47). E8B were selected for ICAM-1 binding by panning on recombinant ICAM-1, as previously described (48) and confirmed to bind to both recombinant ICAM-1 and CD36, but not CSA, by microscopy.

Antibody dependent cellular and neutrophil phagocytosis

ADCP of IE was assessed using the THP-1 monocytic cell line as previously described (49). ADNP of IE was assessed in primary neutrophils isolated from two healthy Melbourne donors, as previously described (38). Rabbit anti-human red blood cell polyclonal antibody (Cappel, MP Biochemicals, Ohio, USA) or pooled plasma of PNG adults were used as a positive control. The DHE positive gate was set to <2% positive events using a plasma-free control. Percentage ADNP was defined as the proportion of DHE positive events out of single cell events, relative to the positive control on the same plate.

Statistical analyses

For multiplex immunoassays, median fluorescence intensity (MFI) was collected from >30 bead events per antigen. Variables that correlated with experimental replicates with a Pearson's correlation coefficient r^2 >0.8 were accepted, except for c1q detector variables, where a r^2 >0.65 was accepted. Variables with <20% missing data were accepted. Antibody features for some antigens were not measured due to antigen unavailability for the assay. Based on these conditions, 11 variables were removed or unavailable prior to analysis: IgG1 targeting DBLe9, IgG2 targeting DBLd1 (SM9), DBLd1 (SM6), and DBLd1 (UM2), and c1q targeting DBLβ6 (N32), CIDRα1DBLβ3, CIDRα2.4, DBLe9, DBLd1 (SM6), DBLd1 (SM9), and DBLz4 (SM15). Individuals had <20% missing data across all antibody measures. MFI of the plasma free control was subtracted from each variable and negative MFI were assigned the value one. A pooled positive control on each plate was used to adjust for variation across plates. For ADCP and ADNP, technical replicates with ≥5 points difference and ≥20% variance were removed. Missing values (1.7% of total combined dataset) were imputed by MICE (50), using the average of 5 imputed datasets. Combined multiplex, ADCP and ADNP data was $\log_e(x+1)$ transformed, mean centred and scaled to 1 standard deviation, to adjust for right skewing.

To produce volcano plots, linear regression was performed between clinical presentation (severe and uncomplicated malaria) and age as exposure co-variates, and individual antibody measures as the outcome, using the lm() function in base R (version 4.1.2). For time point comparison between paired samples, a Paired T-test was performed between acute and convalescence antibody measures. Significant differences were defined by an unadjusted p-value < 0.05 and a geometric mean ratio > 2-fold difference (shown on log2 scale).

To identify PfEMP1 antibody measures that best distinguish between severe and uncomplicated malaria we used elastic net regularized logistic regression (ENLR), as previously described (34,38). Merozoite antigens MSP2 and AMA1 were not included in this analysis. The α regularization parameter was set to 0.5. The glmnet (V4.1-9) and caret (V7.0-1) packages were used to perform 5000 repeats of 10-fold

cross validation, where each repeat contained an inner cross validation on the training data set to tune the best value of l, based on the AUROC (area under the receiver operating characteristic curve). Due to class-imbalance, randomized down-sampling on the severe malaria group was performed on each repeat. The frequency that antibody measures were selected for in the 'best' 5000 models were recorded. Partial least squares discriminant analysis (PLSDA) was then used to determine the classification accuracy (AUROC), when antibody measures were added one by one in order of selection frequency in the ENLR, using 500 repeats of 10-fold cross validation for each combination of variables. After the addition of the 14^{th} feature, the overall classification accuracy of the model did not improve. The classification accuracy was determined by 500 repeats of PLSDA with 10-fold cross validated resampling for the 14 most frequently selected measures by ENLR, for 14 randomly selected measures on each repeat, and for randomly permuted clinical groups on each repeat. To determine if classification could be achieved with EPCR binding variants alone, the classification accuracy was also determined by 500 repeats of PLSDA with 10-fold cross validated resampling for all antibody Fc measures targeting CIDRα1.4, FcgRIIIb measures targeting the seven CIDRα1 proteins (including two-domain protein, CIDRα1.6DBLβ3, Table 2), and IgG targeting the seven CIDRα1 proteins. For correlation networks, Spearman's correlations were determined between the 14 antibody measures selected by ENLR and all other antibody measures. A cut off Spearman's correlation coefficient of r_s>0.5 or <-0.5, and p-value < 0.05 was used for plotting.

We applied Tensor Structured Decomposition to reduce the data dimensionality and visualize the major patterns of variation across timepoint, antigens, detectors and individuals. Canonical polyadic decomposition (CPD) of multiplex data was performed using the TensorLy package in Python (v 0.9.0) and the workflow in Tan *et al* (51,52). A 4-dimensional tensor was constructed from the original dataset (*timepoint-by-antigens-by-detectors-by-individuals*). The rank of the decomposition was found to be three by evaluating the variance of the original dataset that could be explained by the reconstructed tensor (R2X), without causing redundancy (i.e., identical biological interpretations from two of the components). Weightings of the original factors to each component are presented as heatmaps (Fig. 3). Factor components were scaled to fall within the range of -1 to 1. Weightings from the individuals axis were compared between severe and uncomplicated malaria by Mann Whitney U-test and p<0.05 was considered significant (no correction for multiple comparisons). Differences between individual weights across more than two clinical groups was compared by Kruskal Wallis test and p<0.05 was considered significant.

Results

Characteristics of children with malaria included in study

The study included samples collected at presentation from 68 children with uncomplicated malaria and 141 children with severe malaria, and paired follow-up samples taken eight weeks later for 40 children with uncomplicated malaria and 75 with severe malaria (Table 1). Children with severe malaria and severe subgroups (hyperparasitaemia, severe anaemia, metabolic acidosis, cerebral malaria) were

younger than children with uncomplicated malaria (Additional File 1, Supplementary Table 1), consistent with age reflecting cumulative exposure and acquisition of immunity. Therefore, age was included as a covariate for linear regression and elastic net logistic regression (ENLR) analysis. There was no difference in IgG to AMA1 or MSP2 between children with severe or uncomplicated malaria to indicate difference in prior exposure (Additional File 1, Supplementary Fig. 2). Amongst children with severe malaria, a greater percentage were female and had both parents born in Madang province, compared to children with uncomplicated malaria. Amongst children with severe malaria, 88 (62%) had more than one recorded severe malaria feature (Additional File 1, Supplementary Table 2).

[TABLE 1 – see end of document]

Children with severe malaria have lower levels of antibodies to PfEMP1 compared to children with uncomplicated malaria, at presentation and in convalescence

The study measured antigen-specific IgG, IgG1, IgG3, IgG4, and antigen-specific antibody binding to FcγRIIa, FcγRIIIa, FcγRIIb, FcγRIIIb and C1q for 28 recombinant PfEMP1 antigens (Table 2). ADNP of three parasite lines and ADCP of five parasite lines were also measured.

in Papua, Indonesia. Sequences available elsewhere (24,28).

[TABLE 2 – see end of document]

Volcano plots show uncomplicated malaria was associated with higher antibody measures against PfEMP1 antigens, at both the acute and convalescent timepoints (Fig. 1; Additional File 1, Supplementary Table 3; Additional File 2). At the acute timepoint, 23 antibody measures were higher in uncomplicated malaria ($p<0.05$) with mean antibody levels at least two-fold greater (Fig. 1A). These included nine readings against EPCR-binding domains (IgG, IgG1, IgG3 and FcgRIIIb, FcgRIIIa and C1q), five against CD36-binding domains (IgG1, IgG2, FcgRIIb), and four targeting ICAM-1-binding domains (IgG, IgG3, FcgRIIIb). ADNP of IT4VAR13 IE (expressing PfEMP1 that binds ICAM-1 and CD36) was significantly higher in uncomplicated malaria. ADNP of IT4VAR19 IE (expressing PfEMP1 that bind EPCR) were also positively associated with uncomplicated malaria ($p<0.001$) but did not reach our threshold for effect (two-fold mean difference). Only one antibody measure was positively associated with severe malaria.

At convalescence, 37 antibody measures were positively associated with uncomplicated malaria (Fig. 1B). These included six antibody measures targeting EPCR binding domains (FcgRIIb, FcgRIIIb, FcgRIIIa, C1q), three targeting CD36 binding domains (all FcgRIIb), three targeting ICAM-1 binding domains (FcgRIIb, FcgRIIIa), and eight targeting domains with unknown binding phenotypes (IgG, IgG2, IgG3, FcgRIIIb, FcgRIIIa, C1q). Three antibody measures were associated with severe malaria (all C1q).

Figure 1. Associations between clinical presentation and antibody responses at two time points. Linear regression between clinical presentation and age (as co-variates) with antibody responses at

presentation (A, 68 uncomplicated malaria and 141 severe malaria), and at convalescence (B, 40 uncomplicated malaria and 75 severe malaria). X-axes represent geometric mean ratio (GMR) of antibody titres in uncomplicated compared to severe malaria, shown on a $\log_2$ scale. Vertical dotted lines represent two-fold change in GMR in uncomplicated malaria or in severe malaria, respectively. Y-axes represent unadjusted p-values from linear regression, shown on a $-\log_{10}$ scale. Above horizontal line at 1.3 represents $p<0.05$. C,D) Paired t-test between acute and convalescence antibody measures amongst individuals with C) uncomplicated malaria and D) severe malaria. X-axes represent GMR of antibody titres at convalescence compared to acute timepoints, shown on a $\log_2$ scale. Dotted vertical lines represent two-fold change in GMR at convalescence and at presentation, respectively. Y-axes represent unadjusted p-values from paired t-test, shown on a $-\log_{10}$ scale. Above horizontal line represents $p<0.05$. Triangles are responses targeting EPCR-binding proteins (CIDRα1) and circles are non-EPCR-binding proteins. Corresponding geometric mean ratio (GMR) and P values for all antigens shown in Additional File 2. Significant differences summarized in Additional File 1, Supplementary Table 3.

FcgRIIIb and FcgRIIb binding antibodies decline while C1q binding antibodies increase over the course of an episode of malaria

Specific antibody measures changed between acute and convalescent timepoints amongst children with uncomplicated malaria (Fig. 1C) and severe malaria (Fig. 1D, Additional File 1, Supplementary Table 4 and Additional File 2). Amongst children with uncomplicated malaria, 19 antibody measures increased from acute to convalescent timepoints, 15 of which were C1q binding antibodies targeting a range of antigens, including EPCR binders (Fig. 1C). Twenty-seven antibody measures decreased from between the timepoints, 13 of which were FcgRIIIb-binding antibodies and seven of which were FcgRIIb-binding antibodies (Fig. 1C). ADNP of IE from two parasite lines (IT4VAR13 and PFD1235w) also decreased.

Amongst children with severe malaria, 23 antibody measures increased from acute to convalescent timepoints, eighteen of which were C1q binding antibodies targeting a range of antigens, including EPCR binders (Fig. 1D). Thirty-five antibody measures decreased between the two timepoints (Fig. 1D), including 19 measures of FcgRIIb binding antibodies and 12 FcgRIIIb binding antibodies.

We hypothesized that children with severe malaria would have lower levels of antibodies to pathogenic PfEMP1 variants at presentation and would acquire them subsequently if infected with these parasite variants. Amongst children with severe malaria, three antibody measures that were lower in severe malaria compared to uncomplicated malaria at presentation (Fig. 1A) were increased between acute and convalescence timepoints (Fig. 1D): IgG1 targeting EPCR binding proteins (CIDRα1.4 and CIDRα1.6) and IgG2 targeting a CD36 binding protein (CIDRα2.4). There were no significant differences between severe and uncomplicated malaria for these five measures during convalescence (Fig. 1B).

Children with severe anaemia or hyperparasitaemia have distinct antibody profiles compared to uncomplicated malaria

Children with severe malaria presented with a range of clinical syndromes (Table 1). Children with severe anaemia (n = 34), hyperparasitaemia (n = 59), cerebral malaria (n = 17) and/or acidosis (n = 15) were compared to children with uncomplicated malaria at the acute timepoint, with adjustment for differences in age (Fig. 2). One hundred and sixty-four (164) antibody measures were elevated in children with uncomplicated malaria compared to severe anaemia, including IgG to all proteins and ADNP of IT4VAR13 IE (Fig. 2A).There were no antibody measures elevated in severe anaemia compared to uncomplicated malaria. Fewer differences were observed between children with hyperparasitaemia, acidosis and/or cerebral malaria, compared to uncomplicated malaria. Five antibody measures were elevated in uncomplicated malaria compared to cerebral malaria, targeting ICAM-1 and CD36-binding antigens (Fig. 2B). Eight antibody measures were elevated in hyperparasitaemia compared to uncomplicated malaria (Fig. 2C), all of which were IgG4 targeting a range of antigens. Three antibody measures were elevated in uncomplicated malaria compared to acidosis. ADNP of IE that bind ICAM-1 and CD36 (IT4VAR13) was the only common antibody measure elevated in uncomplicated malaria across all four severe subtypes (Fig. 2A-D).

Figure 2. Differences in antibody responses between severe malaria clinical subtypes and uncomplicated malaria. Linear regression between clinical presentation and age (as co-variates) with antibody responses at presentation. Severe malaria clinical subtypes were A) severe anaemia (n = 34), B) cerebral malaria (n = 17), C) acidosis (n = 15) and D) hyperparasitaemia (n = 59), and all uncomplicated malaria samples were included (n = 68). X-axes represent geometric mean ratio (GMR) of antibody titres in uncomplicated compared to severe malaria subtype, shown on a log2 scale. Vertical dotted lines represent two-fold change in GMR in uncomplicated malaria or in severe malaria subtype, respectively. Y-axes represent p-value from linear regression, shown on a $-\log_{10}$ scale. Above horizontal line represents $p<0.05$. Antibody responses (points) are coloured by detector. Triangles are responses targeting EPCR binding proteins (CIDRα1) and circles are non-EPCR binding proteins. Corresponding GMR and P values for all antigens shown in Additional File 2.

Differences in severe and uncomplicated malaria are driven by cytophilic antibodies targeting all PfEMP1 antigens

Tensor structured decomposition was used to identify broad patterns of variation in antibody measures between individuals with severe and uncomplicated malaria (Fig. 3). The original data were arranged as a ‘tensor’ (cube) with 4-modes: antigens, detectors (Fc features), individuals (severe and uncomplicated) and timepoints (acute/convalescence). The tensor is reduced into new variables called ‘components’, where each component represents a unique pattern of variation that can be visualized across the 4-modes (Fig. 3A-D). A three-component model could be reconstructed into a tensor that explains 56% of the variation in the original data, with limited redundancy in the components (Fig. 3E).

Within each component, the contribution of the antigens, detectors, individuals and timepoints, called ‘weightings’, are scaled between -1 and 1, where 0 indicates no contribution. Weightings are represented as colours (Fig. 3A-D). The sign/orientation of the weightings is arbitrary and does not directly

correspond to the direction of the underlying data. Weightings from individuals (Fig. 3D) were compared between severe or uncomplicated malaria groups, within each component (Fig. 3F). Component 1 showed the greatest variation between the groups (Fig. 3F, Mann-Whitney U test p = 0.04). Component 2 and Component 3 represent unique variation in the data that did not differ between severe and uncomplicated malaria (Fig. 3F).

Component 1 captured unique variation that was broadly similar across antigens, shown in the lack of variation in the antigen weightings (colours) (Component 1, Fig. 3B). Component 1 represents variation driven by IgG, IgG1, IgG3, and FcgRIIa and FcgRIIIa detectors, shown as higher weightings (bolder colours) for these detectors (Component 1, Fig. 3C). The unique pattern of variation in antibody responses represented by Component 1 was driven by responses at the acute timepoint (Component 1, Fig. 3A). Together, Component 1 demonstrates unique variation between children with severe and uncomplicated malaria that is driven by cytophilic antibodies targeting all PfEMP1 at the acute timepoint.

Figure 3. Tensor structured decomposition showing trends in antibody responses in paired acute and convalescent samples. Heatmaps represent decomposed components across four factors A) time, B) antigen, C) detector and D) subject, for a three-component model. Component weights are scaled from -1 to 1 across each factor to represent difference, not direction. E) Percentage variance reconstructed (R2X) with number of components used in the tensor decomposition model. F) To identify components that represent variation in children with severe and uncomplicated malaria, individual's component weights from D were compared by Mann Whitney U test (p-values), for each component. Component weights from children with severe malaria shown in red (n = 75) and uncomplicated in blue (n = 40).

Antibody Fc measures against diverse PfEMP1 can classify severe and uncomplicated malaria with up to 84% accuracy

To identify the antibody measures at the acute timepoint that could best distinguish between children with severe and uncomplicated malaria we applied ENLR feature selection followed by Partial Lease Squares Discriminant Analysis (PLSDA) (Fig. 4). Twenty (20) measures were selected in more than 70% of 5000 model iterations (Fig. 4A), and stepwise addition of measures in order of selection frequency showed minimal increase in classification accuracy beyond 14 measures (Fig. 4B). A PLSDA model fitted with the 14 selected measures could classify children as having severe or uncomplicated malaria with 84% accuracy (AUROC; 95% CI 77 – 89%, Fig. 4D). The 14 measures with randomly permuted outcome groups (severe or uncomplicated) could classify children into groups with 46% accuracy (AUROC; 95% CI 37 – 54%), and using 14 random measures could classify the children into groups with 59% accuracy (AUROC; 95% CI 50 – 68%). Of the 14 selected measures, seven were associated with uncomplicated malaria: IgG2 targeting CIDRα2.4, IgG4 targeting DBLβ3, FcgRIIb binding antibodies targeting DBLβ3 and DBLβ13, FcgRIIIb binding antibodies targeting CIDRα2.4 and DBLβ3, and c1q targeting CIDRα1.4 (Fig. 4E and 4F). Seven of the selected measures were associated with severe malaria: IgG4 targeting CIDRα1.1,

FcgRIIb binding antibodies targeting DBLβ5, DBLβ6, DBLβ3 and DBLz4, and C1q binding antibodies targeting DBLβ5 and CIDRα1.5 (Fig. 4E and 4F).

Figure 4. Identifying a minimum combination of antibody measurements that predict severe or uncomplicated malaria at presentation. A) Top 20 most frequently selected variables across 5000 repeated elastic net logistic regression (ENLR) models of antibody measurements with clinical presentation (uncomplicated malaria compared to severe malaria), adjusted for age. B) Log adjusted odds ratio of most frequently selected variables. Odds ratio >1 is associated with uncomplicated malaria (UM) and <1 with severe malaria (SM). C) Performance (Area under the receiver operator curve, AUROC) of partial least squares regression (PLSR) to predict clinical presentation after the addition of antibody measures to the model from left to right (x-axis). Dotted lines indicate mean AUROC to classify severe malaria (red, SA), uncomplicated malaria (blue, MA) and both groups (black). Vertical dashed line indicates plateau of overall AUROC and left of dashed line are the 14 measures selected for performance evaluation by PLSR. D) Performance (AUROC) of 14 measures selected from ENLR (Selected features), 14 selected measures with the group randomly shuffled as severe or uncomplicated malaria (Randomly permuted groups) and resampled sets of 14 randomly selected measures (Random features). Boxes show median and interquartile range (IQR, Q1, Q3) and whiskers range from (Q1-1.5×IQR) to Q3+1.5×IQR). E) Individual scores for component 1 and 2 from PLSR of 14 antibody measures with clinical outcome (severe = red, uncomplicated = blue). F) Variable loadings on component 1 and 2 from PLSR of 14 antibody measures with clinical outcome. G) Performance (AUROC) of FcgRIIIb binding antibodies targeting seven CIDRα1 domains, IgG targeting seven CIDRα1 domains and 10 antibody measures targeting CIDRα1.4.

A combination of multiple antigen phenotypes is required to classify uncomplicated and severe malaria

We assessed whether antibody measures to domains from a single binding phenotype, CIDRα1 proteins that bind to EPCR, could be used to accurately classify the groups as severe or uncomplicated malaria (Fig. 4G). A PLSDA model fit with IgG binding antibodies targeting seven EPCR binding CIDRα1 proteins could classify the groups with 66% accuracy (AUROC; 95% CI 57 – 74%), and FcgRIIIb targeting the same proteins could classify the groups with 64% accuracy (AUROC; 95% CI 55 – 72%). Fitting the model using all 11 measures of Fc features of antibodies against a single EPCR binding protein (CIDRα1.4) could classify the groups with 64% (AUROC; 95% CI 55 – 72%) accuracy.

Correlations between ENLR selected antibody measures and other antibody measures

The 14 measures selected by ENLR correlated with 118 of the 297 antibody measures but were not highly correlated with one another (Fig. 5A). In some cases, ENLR selected measures clustered by antigen type (Fig. 5C), rather than feature (Fig. 5B): ENLR selected FcgRIIb and FcgRIIIb binding antibody measures correlated with IgG, IgG1, FcgRIIa and FcgRIIIa binding antibodies (Fig. 5B) targeting similar protein phenotypes (Fig. 5C). In other cases, ENLR selected measures clustered by feature, rather than antigen type: ENLR selected IgG4 measures formed a unique cluster with other IgG4 measures targeting diverse protein phenotypes (Fig. 5B and 5C). There was no clear clustering pattern for ENLR selected

c1q binding antibody measures (some c1q measures clustered by antigen type, others clustered together with other c1q measures, Fig. 5B and 5C).

Figure 5. Antibody measures (nodes) that correlate with the 14 measures selected by ENLR. A) selected antibody measures identified as coloured nodes. B) Nodes colour coded by antibody feature and C) antigen groups. Proximity of nodes and thickness of connecting lines represent strength of correlation between antibody measures determined by Spearman's correlation coefficient r_s. All correlations are positive. Only correlations with $r_s > 0.5$ and $p<0.05$ are shown, and only measures that correlate with the 14 selected measures are shown. Decode for numbers on nodes and $r_s$values for correlation matrix are provided in Additional File 2.

Discussion

Defining the targets and Fc features of antibodies associated with protective immunity is a critical step towards the development of novel biomarker panels and immunotherapeutics for malaria. In this study, we used systems serology to identify Fc features of antibodies to specific PfEMP1 antigens that could distinguish children with severe malaria from those with uncomplicated malaria.

Antibody responses that are lower in children with severe malaria compared to uncomplicated malaria at presentation might represent protective responses. Previous studies have found evidence that children with severe malaria had lower levels of IgG targeting PfEMP1 proteins that bind EPCR at presentation and/or showed boosting of these responses in convalescence (27, 28, 36, 53, 54). Our study extends this by identifying cytophilic IgG1 and IgG3 responses and antibody engagement with FcγRIIIb, FcγRIIIa and C1q as potentially protective attributes of antibody to one or more CIDRα1 domains, within a broader antibody profile. Of the 22 antibody measures that were higher in uncomplicated malaria at presentation, many targeted CIDRα1.4 (six features) and CIDRα1.6b (four features) (Fig. 1).

Uncomplicated malaria was also positively associated with IgG, IgG3 and FcγRIIb- and FcγRIIIb-binding antibody to at least one ICAM-1 binding domain at presentation. IgG targeting ICAM-1 binding domains has been associated with protection from severe and uncomplicated malaria (54–58). While some studies have identified protective associations with DBLβ3 specific IgG (38, 39, 59), two other studies found DBLβ specific IgG1, IgG3 or FcγRIIIb-binding antibodies to be protective against severe or cerebral malaria (38, 39). Like the present study, these studies highlight the benefit of broader antibody profiling and the importance of cytophilic antibody responses for protection.

Multiple antibody measures (IgG, IgG1, IgG2 and FcγRIIIb binding antibodies) targeting a CD36-binding domain (CIDRα2.4) were elevated in uncomplicated malaria at presentation. While expression of *var* genes encoding CD36-binding PfEMP1 is not associated with severe malaria, antibodies targeting CD36 binding PfEMP1 have been associated with protection from both severe and uncomplicated malaria, in line with our findings (53, 57).

Finally, antibodies to two domains of unknown function (DBLβ13 and DBLδ1) were higher in uncomplicated malaria at presentation. The contribution of antibodies targeting a variety of previously under-investigated domains to immunity from severe malaria requires further study (Rambhatla et al., 2022; Tessema et al., 2019; Walker et al., 2024).

Emerging data suggest that ADNP of IE is important in protection from severe malaria. Neutrophils bind IgG1 and IgG3 on the IE, probably via both FcγRIIIb and FcγRIIa activation (60), and the latter is key to phagocytosis of IE (61). Children with uncomplicated malaria had higher cytophilic IgG1 and IgG3 as well as FcγRIIIb binding antibodies to recombinant domains, and their plasma caused significantly more ADNP of ICAM-1 + CD36 binding IE compared to children with severe malaria or severe malaria subgroups. ADNP of EPCR binding IE was also higher in uncomplicated malaria compared to severe malaria ($p < 0.001$) but did not reach our threshold for significance (two-fold mean difference). In previous studies, antibodies were shown to enhance ADNP of ICAM-1 binding IE (35) and both FcγRIIIb binding antibodies and ADNP of ICAM-1 + EPCR binding IE have been associated with protection from cerebral malaria (38). In pregnant women, ADNP of VAR2CSA expressing IE has been associated with protection from placental malaria (34). While neutrophil activation may be detrimental in localized environments such as within the brain (62), ADNP appears to be an important mechanism for clearance of IE.

There were changes in many of the antibody measures in both severe and uncomplicated malaria between presentation and convalescence. FcγRIIIb and FcγRIIb binding antibodies and ADNP decreased, and C1q binding antibodies increased. Changes may reflect antibody glycosylation states, which can alter FcγR and C1q affinity (63, 64). Changes in C1q might also correlate with changes in IgM, which we did not measure in the present study. Apart from the FcγRIIIb and FcγRIIb decrease and C1q increase described, the most striking finding amongst children with severe malaria was increased IgG1 binding antibodies to CIDRα1 proteins. That children with severe malaria had lower levels of antibodies to EPCR-binding domains at presentation and showed increased responses at convalescence supports a role of EPCR-binding PfEMP1 in severe malaria pathogenesis and of antibodies against them in protection, and is consistent with a previous study (27).

Severe malaria comprises multiple, overlapping syndromes including severe anaemia, cerebral malaria, hyperparasitaemia and acidosis, and many children in this cohort had more than one syndrome (Additional File 1, Supplementary Table 2). Severe anaemia was common and IgG, IgG1, IgG2, IgG3, FcγRIIb, FcγRIIIa and FcγRIIIb responses to the majority of antigens (and a subset of C1q binding responses) were positively associated with uncomplicated malaria compared to severe anaemia. The most significantly different responses (all $p < 0.0001$) were directed against CIDRα1.4, CIDRα1.6b, a dual CIDR1αDBLβ domain, other DBLβ domains and CD36-binding CIDRα2–6. Previous studies have shown expression of non-CD36 binding PfEMP1s is upregulated in severe malarial anaemia (Lavstsen et al., 2012; Warimwe et al., 2009). However, children with severe anaemia have been observed to lack antibodies against multiple PfEMP1 variants (65) and against variants associated with severe malaria (54, 66, 67). Our data suggests that reduced antibody responses to PfEMP1 overall, rather than to

specific PfEMP1 phenotypes, is associated with severe anaemia. Anaemia can impair memory B cell formation and B cell proliferation *in vitro*, possibly influencing antibody titres in the severe malarial anaemia group (68). Future studies should determine whether correction of severe anaemia can improve naturally acquired or vaccine induced immunity against malaria, as has been demonstrated in other diseases (69).

Children with hyperparasitaemia showed a unique antibody profile, with higher antigen specific IgG4 responses compared to uncomplicated malaria. The factors that cause hyperparasitaemia are not well defined but might include attenuated splenic clearance due to sequestration in the microvasculature (70), iron availability (71) and red blood cell polymorphisms (72). IgG4 can interfere with IgG1 and IgG3-mediated phagocytosis in Sars-CoV-2 (73), and a lack of cytophilic response in this group might promote less parasite clearance, in line with the observed lack of ADNP and ADCP of IE in children with hyperparasitaemia compared to those with uncomplicated malaria (Fig. 2).

Biomarkers of immunity may be able to accurately classify children as having severe or uncomplicated disease. Using ENLR we identified a subset of 14 antigen-specific antibody measures that distinguished between severe and uncomplicated malaria with 84% accuracy, whereas randomly selected measures gave 59% accuracy. As the accuracy improved marginally by adding each additional antibody measure to the model it is likely that each measure captures distinct variation across individuals. When we included only IgG or FcγRIIIb responses to EPCR-binding domains, predictive accuracy was 64–66%, highlighting the importance of having antibodies to a breadth of PfEMP1 domains.

Correlation networks and tensor structure decomposition also suggest that including a broad range of antigen targets is likely required to predict predictive immunity from severe malaria. Of the selected measures, IgG1, IgG3, and FcγRIIa/b and FcγRIIIa/b receptor binding antibody measures correlated highly with each other, and antigen phenotypes formed unique clusters. This clustering suggests cytophilic antibody measures (IgG1, IgG3, FcγRII and FcγRIII) to different antigen phenotypes (EPCR, ICAM-1, CD36, non-binding), rather than a single phenotype, are needed to describe the variation in the clinical groups. The need for measuring a broad antibody response is also reflected in the tensor decomposition (Fig. 3), in which component 1 differed between individuals with severe and uncomplicated malaria and represented cytophilic antibodies across all antigens, rather than a specific subset of antigens.

The findings of this study have important potential implications for understanding of PfEMP1-mediated protective immunity against severe malaria. Antibodies targeting a single PfEMP1 binding phenotype, such as EPCR binding, were associated with protection from severe malaria but were not sufficient alone to predict protection. If severe disease is caused by parasites that express PfEMP1 to which the host lacks antibodies (74), and protective immunity is associated with a breadth of antibodies acquired with age (25, 75), interventions to prevent severe malaria or biomarker panels designed to predict protective immunity may need to include a breadth of PfEMP1 variant targets.

Strengths of this study include the use of multiple examples of PfEMP1 domains of key binding phenotypes, and detailed measures of PfEMP1 specific antibody Fc features that were correlated to clinical outcomes and infection timepoints. Another strength was the use of multiple parasite infected erythrocytes expressing PfEMP1 of key binding phenotypes, including the recently developed parasite line modified to have stable expression and high of *IT4VAR19*. Limitations of this study include potential differences in prior exposure amongst the groups and variation in the duration of infection prior to enrolment. Lack of pre-infection samples limited our ability to identify pre-existing protective antibody responses. We had relatively few cases of cerebral malaria or acidosis, which may have limited our ability to confirm our previous observations regarding cerebral malaria (38). Children with severe malaria and uncomplicated malaria were not matched for host polymorphisms that protect from severe malaria in PNG (42, 76–79), which may explain some of the variation in the groups that is not explained by antibodies measured. Recombinant antigens were produced in different expression systems and may not have included all PfEMP1 domains relevant to severe disease in this cohort. For some proteins, less than 25% of children were seropositive for IgG, therefore differences may be driven by a small number of individuals (Additional File 1, Supplementary Fig. 3). IE used in phagocytosis assays expressed at least 40% purity of the selected PfEMP1, therefore other PfEMP1 may be contributing to antibody responses measured. We have not assessed functional antibodies targeting merozoite stage antigens or other variant surface antigen families such as rifins that may also contribute to protective immunity (80, 81).

Conclusions

This detailed study of the antibody response to PfEMP1 in children with severe and uncomplicated malaria identifies important antibody features and targets that differ between groups including, but not restricted to, EPCR, ICAM-1 and CD36 binding domains. Severe malarial anaemia and hyperparasitaemia showed distinct antibody profiles compared to uncomplicated malaria. Cytophilic antibodies IgG1 and IgG3 that bind well to Fc receptors and induce cellular immune functions like ADNP are important for protective immunity. In this cohort, differences in functional antibody against a breadth of antibody targets, rather than responses to a single PfEMP1 domain type or phenotype, best differentiated severe from uncomplicated malaria.

Abbreviations

ADCC
Antibody dependent cellular cytotoxicity
ADCP
Antibody dependent cellular phagocytosis
ADNP
Antibody dependent neutrophil phagocytosis
AMA1
Apical membrane antigen 1
AUROC

Area under the receiver operating characteristic curve
BCS
Blantyre Coma Score
C1q
Complement component 1q
CIDR
Cysteine-rich Interdomain Region
CPD
Canonical polyadic decomposition
CSA
Chondroitin sulphate A
DBL
Duffy Binding Like
DHE
Dihydroethidium
E. coli
Escherichia coli
EPCR
Endothelial protein C receptor
ENLR
Elastic net regularised logistic regression
FcγR
Fc gamma receptor
GMR
Geometric mean ratio
ICAM
1-Intercellular adhesion molecule-1
IE
Infected erythrocytes
IgG
Immunoglobulin G
IQR
Interquartile range
IRB
Institutional Review Board
MFI
Median fluorescence intensity
MRAC
Medical Research Advisory Committee
MSP2

Merozoite surface protein 2
nPCR
Nested polymerase chain reaction
PE
Phycoerythrin
PfEMP1
Plasmodium falciparum erythrocyte membrane protein-1
PLSDA
Partial least squares discriminant analysis
PNG
Papua New Guinea
R^2X
Variance reconstructed
SM
Severe malaria
UM
Uncomplicated malaria
VAR2CSA
Variant surface antigen 2-chondroitin sulphate A
WGCFS
Wheat germ cell-free system

Declarations

Ethics approval and consent to participate

Ethics approval was granted by the PNG Institute of Medical Research Institutional Review Board (IRB number 1103), the Medical Research Advisory Committee of the PNG Health Department (MRAC number 11.12) and the Human Research Ethics Committee of University of Melbourne (Reference number 2024-28460-53448-3). Written, informed consent was obtained from guardians of all participants.

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Author Contribution

Conceptualization, S.J.R., E.H.A., I.S.W.; investigation, I.S.W., W.H., A.O.P., D.G.F., J.R; formal analysis, I.S.W., A.H., O.L.W; validation, A.W.C., A.M., M.R, J.S; writing—original draft, I.S.W., S.J.R.; writing—review and editing, I.S.W, S.J.R., E.H.A, O.L.W., resources, L.M., T.D., I.M., M.L., T.L., L.T., Y.A., M.D., R.W. O., A.T.J.R., J.A.R., E.T., T.T.; supervision, J.A.S., S.J.R., E.H.A.; funding acquisition, S.J.R., E.H.A., A.W.C., I.S.W., S.D., J.A.S. I.S.W. drafed the manuscript and prepared figures 1,2,4 and 5. I.S.W and O.L.W. prepared figure 3. All authors read and approved the final manuscript.

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Data Availability

All data generated or analysed during this study are included in this published article and supplementary materials.

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Tables

Table 1. Characteristics of children with malaria included in study

Characteristic	UM acute	UM conv.	SM acute	SM conv.
n	68	40	141	75
Age, median [IQR] months	50 [34-62]	49 [30-59]	43 [30-60]	41 [30-59]
Age group, n (%)				
0-60 months	49 (72%)	30 (75%)	110 (78%)	59 (79%)
60-90 months	19 (28%)	10 (25%)	31 (22%)	16 (21%)
Sex, n (%)				
Female	25 (45%)	20 (50%)	65 (46%)	37 (49%)
Male	31 (55%)	20 (50%)	76 (54%)	38 (51%)
Birthplace of parents, n (%)				
Madang	50 (88%)	34 (85%)	105 (74%)	57 (66%)
Non-Madang	7 (12%)	6 (15%)	36 (26%)	30 (34%)
Blantyre Coma Score, n (%)				
0-2			17 (12%)	
3			8 (6%)	
4			21 (15%)	
5			93 (67%)	
Coinfection with vivax, n (%)	3 (9%)		20 (14%)	14 (19%)
Parasite density, median [IQR] per µL				
Pf	23058 [6879-78667]	0 [0-829]	76165 [13347-177543]	0 [0-0]
Pv	0 [0-0]	0 [0-0]	0 [0-0]	0 [0-0]
Hyperparasitaemia, n (%)	0 (0%)		59 (42%)	
Haemoglobin, median [IQR][A]	90 [78-103]	107 [101-112]	75 [50-90]	110 [97-117]
Severe anaemia, n (%)	0 (0%)	0 (0%)	34 (24%)	0 (0%)
Metabolic acidosis n (%)			15 (11%)	
Hyperlactaemia, n (%)			27 (20%)	
Respiratory distress, n (%)			14 (10%)	

Other severe etiology, n (%)	45 (32%)

A – haemoglobin summary statistics were calculated from 63 uncomplicated acute, 37 uncomplicated convalescent and 69 severe convalescent samples with available data. UM: uncomplicated malaria. SM: severe malaria. Conv: convalescence. IQR: interquartile range. Hyperparasitaemia (>100,000 parasites /mL); severe anaemia (Hb <50 g/L); metabolic acidosis (bicarbonate < 12.2 mmol/L); cerebral malaria (BCS <3). Hyperlactaemia (blood lactate >5 mmol/L).

Table 2. Recombinant PfEMP1 proteins probed in multiplex assays

Domain	Parasite line & variant name	Group	Predicted receptor	Expression system[A, B, C]
DBLβ3	3D7 PF110521/M1	A	ICAM-1[A]	*E. coli* [B]
DBLβ3	3D7 PFD1235w/M6	A	ICAM-1 [A]	*E. coli* [B]
DBLβ1	Dd2 VAR32/M9	A	ICAM-1 [A]	*E. coli* [B]
DBLβ3	IT4 VAR13/N27	B	ICAM-1	*E. coli* [B]
DBLβ7	Dd2 VAR52/N30	A		*E. coli* [B]
DBLβ6	BT1983 JQ691647/N32	A		*E. coli* [B]
DBLβ3	HB3 VAR34/KOB58843	B	ICAM-1	*E. coli* [B]
DBLβ5	HB3 VAR21/ KOB63129	B	ICAM-1	*E. coli* [B]
DBLβ5	Dd2 VAR01/ AA75396	B	ICAM-1	*E. coli* [B]
CIDRα1.6-DBLβ3	3D7 PFD1235w	A	EPCR & ICAM-1 [A]	*E. coli* [B]
CIDRα1.1	IT4 VAR06	A	EPCR	Insect cells [C]
CIDRα1.4	IT4 VAR22	A	EPCR	Insect cells [C]
CIDRα1.5	1965 X1965_2	A	EPCR	Insect cells [C]
CIDRα1.6b	GA019 ERS010031	A	EPCR	Insect cells [C]
CIDRα1.7	P2083-1	A	EPCR	Insect cells [C]
CIDRα1.8a	GA026 ERS010178_NODE_17	A	EPCR	Insect cells [C]
CIDRα2.1	IT4 VAR30	B	CD36	Insect cells [C]
CIDRα2.2	IT4 VAR24	B	CD36	Insect cells [C]
CIDRα2.4	IT4 VAR33	B	CD36	Insect cells [C]
CIDRα3.3	IT4 VAR26	B	CD36	Insect cells [C]
DBLd1	3D7 SM6	B		WGCFS [D]
DBLd1	3D7 SM9	B		WGCFS [D]
DBLe9	3D7 SM12	B		WGCFS [D]

DBLz4	3D7 SM15	B	WGCFS [D]
DBLe5	3D7 SM22	A	WGCFS [D]
DBLβ13	3D7 SM25	B	WGCFS [D]
CIDRg12	3D7 SM26	B	WGCFS [D]
DBLd1	3D7 UM2	B	WGCFS [D]
-	-	-	WGCFS [D]
-	-	-	WGCFS [D]

WGCFS: wheat germ cell free system. Other abbreviations: [A] Variant is predicted to also bind to EPCR, via a nearby CIDRα1 domain. [B] *var* sequences available elsewhere (32,33). [C] *var* sequence available elsewhere (5,11). [D] Protein derived from *var* gene sequences upregulated in severe or uncomplicated malaria

Figures

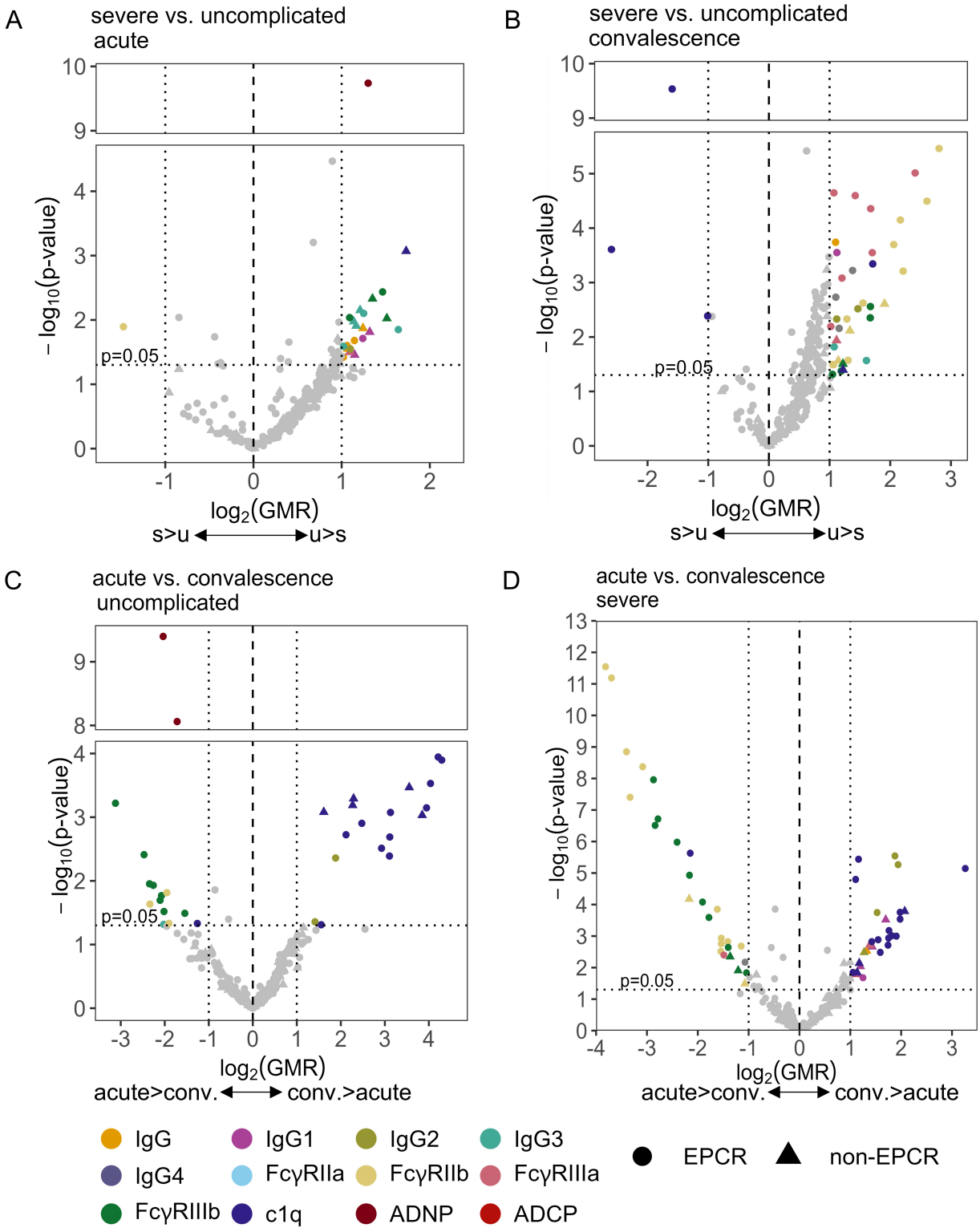


Figure 1

Associations between clinical presentation and antibody responses at two time points. Linear regression between clinical presentation and age (as co-variates) with antibody responses at presentation (A, 68 uncomplicated malaria and 141 severe malaria), and at convalescence (B, 40 uncomplicated malaria and 75 severe malaria). X-axes represent geometric mean ratio (GMR) of antibody titres in uncomplicated compared to severe malaria, shown on a $\log_2$ scale. Vertical dotted

lines represent two-fold change in GMR in uncomplicated malaria or in severe malaria, respectively. Y-axes represent unadjusted p-values from linear regression, shown on a $-\log_{10}$ scale. Above horizontal line at 1.3 represents $p<0.05$. C,D) Paired t-test between acute and convalescence antibody measures amongst individuals with C) uncomplicated malaria and D) severe malaria. X-axes represent GMR of antibody titres at convalescence compared to acute timepoints, shown on a $\log_2$ scale. Dotted vertical lines represent two-fold change in GMR at convalescence and at presentation, respectively. Y-axes represent unadjusted p-values from paired t-test, shown on a $-\log_{10}$ scale. Above horizontal line represents $p<0.05$. Triangles are responses targeting EPCR-binding proteins (CIDRα1) and circles are non-EPCR-binding proteins. Corresponding geometric mean ratio (GMR) and P values for all antigens shown in Additional File 2. Significant differences summarized in Additional File 1, Supplementary Table 3.

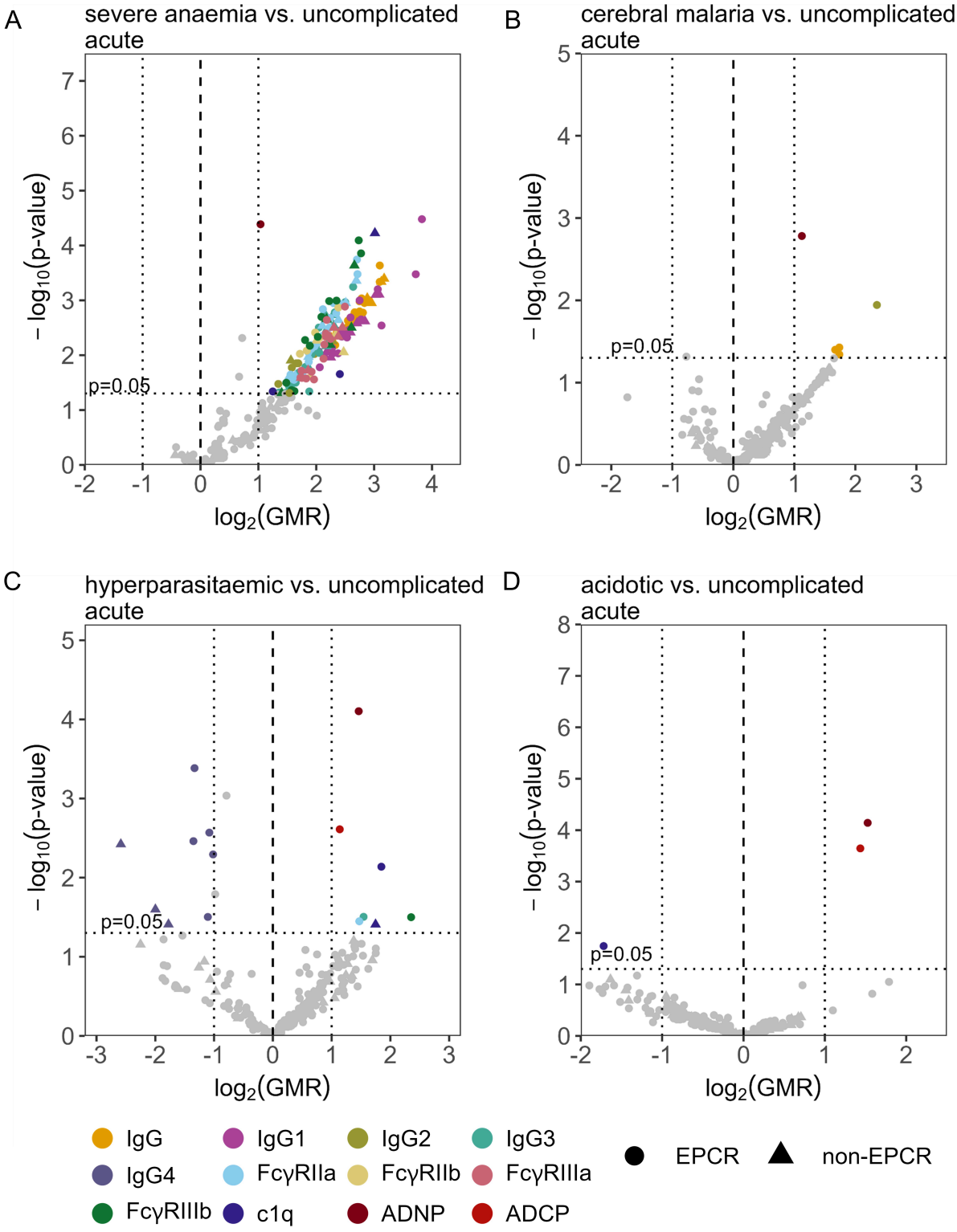


Figure 2

Differences in antibody responses between severe malaria clinical subtypes and uncomplicated malaria. Linear regression between clinical presentation and age (as co-variates) with antibody responses at presentation. Severe malaria clinical subtypes were A) severe anaemia (n = 34), B) cerebral malaria (n = 17), C) acidosis (n = 15) and D) hyperparasitaemia (n = 59), and all uncomplicated malaria samples were included (n = 68). X-axes represent geometric mean ratio (GMR) of antibody titres in

uncomplicated compared to severe malaria subtype, shown on a log2 scale. Vertical dotted lines represent two-fold change in GMR in uncomplicated malaria or in severe malaria subtype, respectively. Y-axes represent p-value from linear regression, shown on a $-\log_{10}$ scale. Above horizontal line represents p<0.05. Antibody responses (points) are coloured by detector. Triangles are responses targeting EPCR binding proteins (CIDRα1) and circles are non-EPCR binding proteins. Corresponding GMR and P values for all antigens shown in Additional File 2.

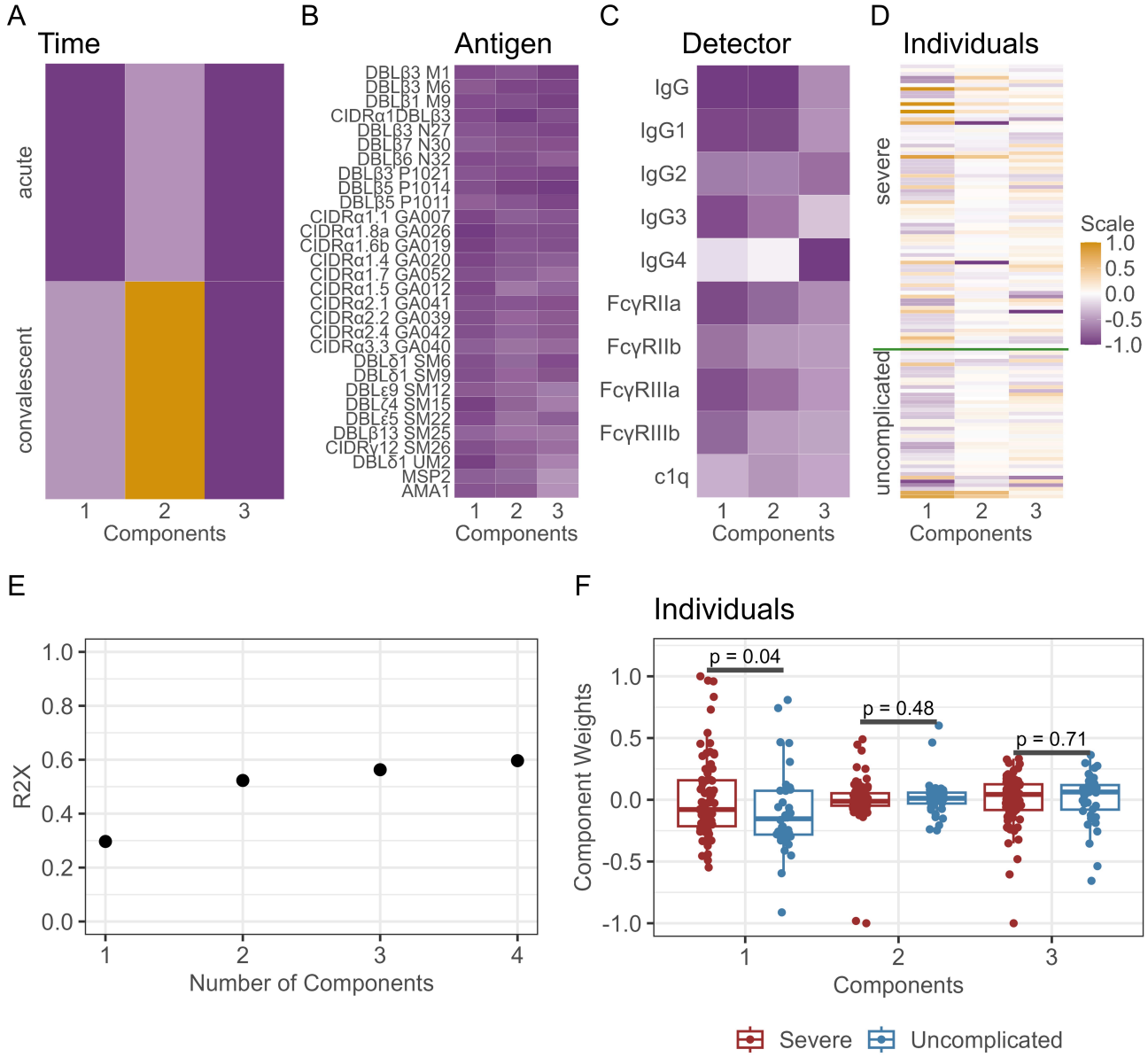


Figure 3

Tensor structured decomposition showing trends in antibody responses in paired acute and convalescent samples. Heatmaps represent decomposed components across four factors A) time, B) antigen, C) detector and D) subject, for a three-component model. Component weights are scaled from -1 to 1 across each factor to represent difference, not direction. E) Percentage variance reconstructed (R2X) with number of components used in the tensor decomposition model. F) To identify components that represent variation in children with severe and uncomplicated malaria, individual's component weights from D were compared by Mann Whitney U test (p-values), for each component. Component weights from children with severe malaria shown in red (n = 75) and uncomplicated in blue (n = 40).

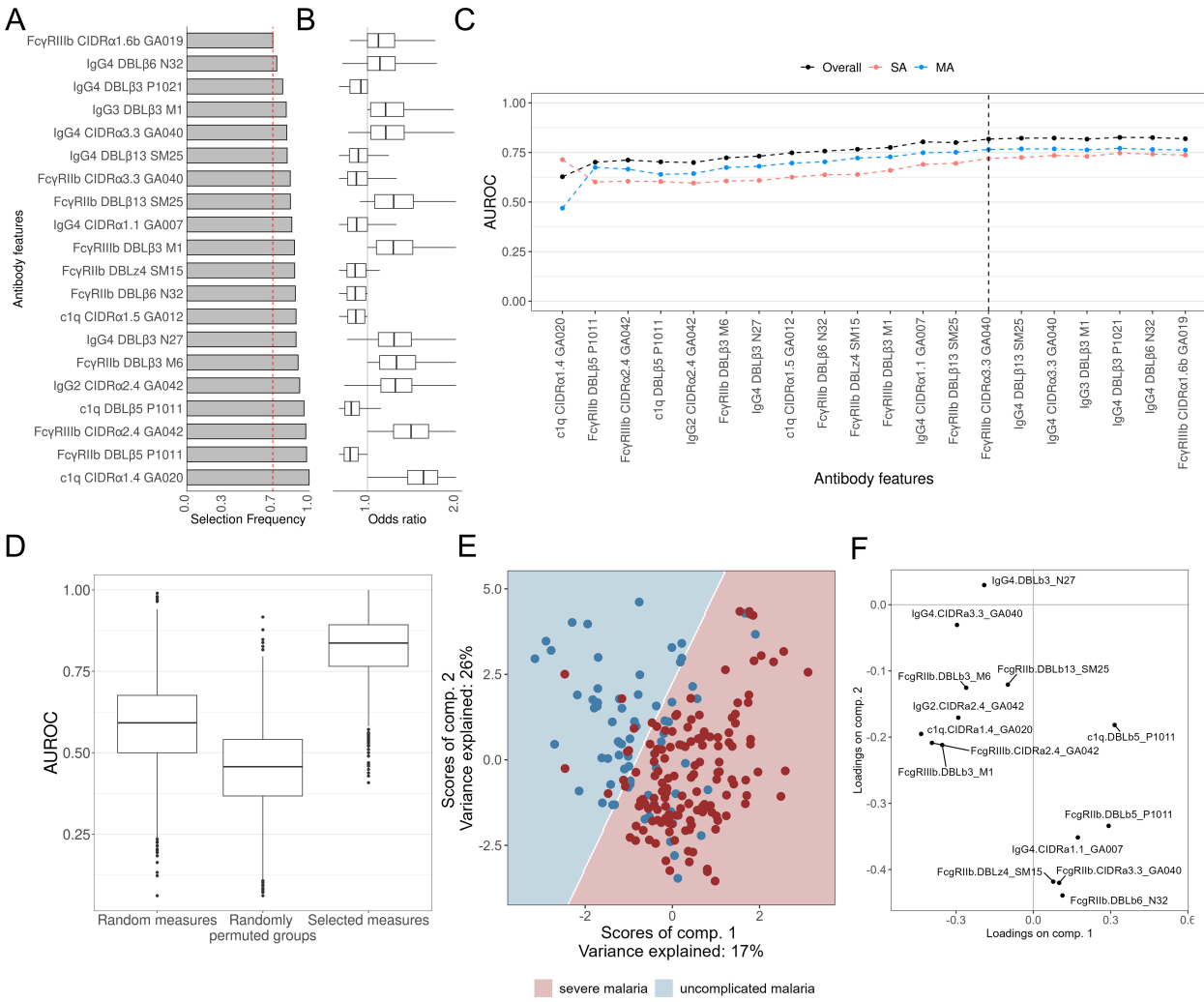


Figure 4

Identifying a minimum combination of antibody measurements that predict severe or uncomplicated malaria at presentation. A) Top 20 most frequently selected variables across 5000 repeated elastic net logistic regression (ENLR) models of antibody measurements with clinical presentation (uncomplicated malaria compared to severe malaria), adjusted for age. B) Log adjusted odds ratio of most frequently selected variables. Odds ratio >1 is associated with uncomplicated malaria (UM) and <1 with severe

malaria (SM). C) Performance (Area under the receiver operator curve, AUROC) of partial least squares regression (PLSR) to predict clinical presentation after the addition of antibody measures to the model from left to right (x-axis). Dotted lines indicate mean AUROC to classify severe malaria (red, SA), uncomplicated malaria (blue, MA) and both groups (black).

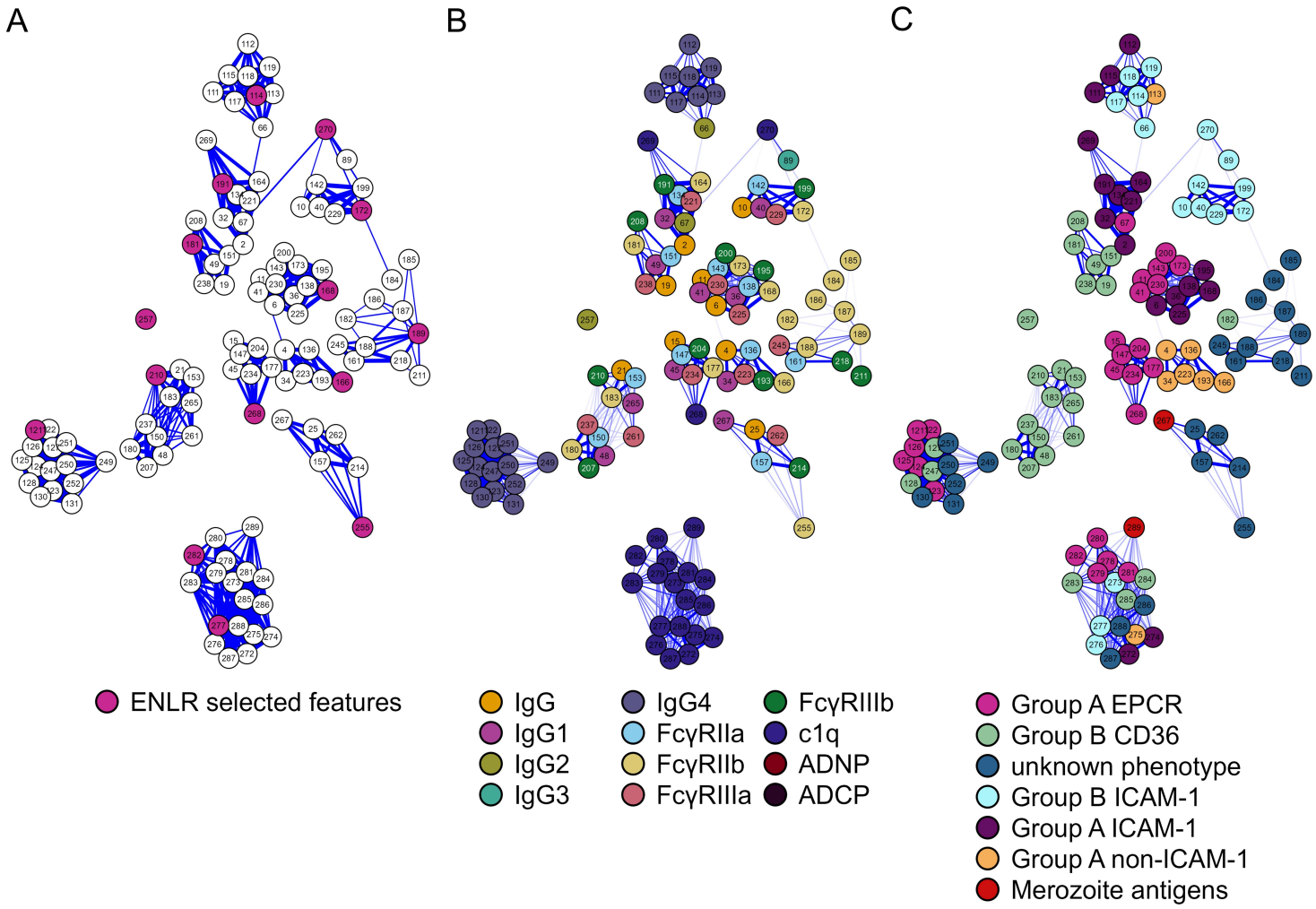


Figure 5

Antibody measures (nodes) that correlate with the 14 measures selected by ENLR. A) selected antibody measures identified as coloured nodes. B) Nodes colour coded by antibody feature and C) antigen groups. Proximity of nodes and thickness of connecting lines represent strength of correlation between antibody measures determined by Spearman's correlation coefficient r_s. All correlations are positive. Only correlations with $r_s > 0.5$ and $p<0.05$ are shown, and only measures that correlate with the 14 selected measures are shown. Decode for numbers on nodes and r_s values for correlation matrix are provided in Additional File 2.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile2.xlsx
- AdditionalFile1.pdf