JCI The Journal of Clinical Investigation

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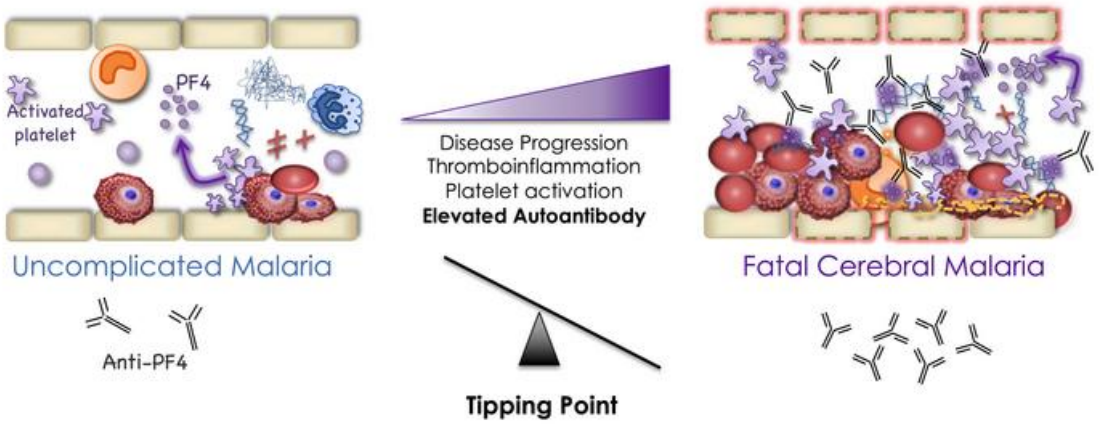
Iset M. Vera, … , Karl B. Seydel, Kami Kim

J Clin Invest. 2024. https://doi.org/10.1172/JCI176466.

Clinical Medicine | In-Press Preview | Infectious disease | Microbiology

Graphical abstract



Title: Pro-thrombotic autoantibodies targeting Platelet Factor 4/polyanion are associated with pediatric cerebral malaria.

Authors:

Iset M. Vera[1], Anne Kessler[2], Visopo Harawa[3,4,5], Ajisa Ahmadu[3], Thomas E. Keller[1], Stephen T.J. Ray[3,5,6,7], Terrie E. Taylor[5,8], Stephen J. Rogerson[9,10], Wilson L. Mandala[3,4,11], Morayma Reyes Gil[12#], Karl B. Seydel[5,8*], and Kami Kim[1*]

Affiliations:

1 Division of Infectious Disease and International Medicine, Department of Internal Medicine, University of South Florida, Tampa, FL, USA

2 Center for Genomics and Systems Biology, Department of Biology, New York University, New York, NY, USA

3 Malawi-Liverpool Wellcome Trust Clinical Research Programme, Blantyre, Malawi

4 Biomedical Department, University of Malawi College of Medicine, Blantyre, Malawi

5 Blantyre Malaria Project, Kamuzu University of Health Sciences, Blantyre, Malawi

6 Oxford Vaccine Group, Department of Paediatrics, University of Oxford, Oxford, UK

7 Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, UK

8 Department of Osteopathic Medical Specialties, Michigan State University, East Lansing, MI, USA

9 Department of Medicine (RMH), The University of Melbourne, Melbourne, Australia

10 Department of Infectious Diseases, Doherty Institute, The University of Melbourne, Melbourne, Australia.

11 Academy of Medical Sciences, Malawi University of Science and Technology, Thyolo, Malawi

12 Department of Pathology, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY, USA

#Current affiliation: Robert J. Tomsich Pathology & Laboratory Medicine Institute, Cleveland Clinic, Cleveland, OH USA

*Correspondence to Karl B. Seydel (KBS) or Kami Kim (KK)

KBS: Karl B. Seydel, MD, PhD, Michigan State University, College of Osteopathic Medicine, 909 Wilson Rd., West Fee Hall, Rm B-305, East Lansing, MI 48824; Blantyre Malaria Project, PO Box 32256, Blantyre, Malawi BT3, +265 99 945 2989, seydel@msu.edu

KK: Kami Kim, MD, USF Infectious Disease Research, 3720 Spectrum Boulevard, Suite 304, Tampa, FL 33612, +1 813 974 5891, kamikim@usf.edu

Conflict of interest statement: “The authors declare that no conflict of interest exists. Funding sources had no role in study design, data collection, analysis, decision to publish, or preparation of this manuscript.”

ABSTRACT

Background. Features of consumptive coagulopathy and thromboinflammation are prominent in cerebral malaria (CM). We hypothesized that thrombogenic autoantibodies contribute to a procoagulant state in CM.

Methods. Plasma from children with uncomplicated malaria (UM, n=124) and CM (n=136) was analyzed by ELISA for a panel of 8 autoantibodies including anti-Platelet Factor 4/polyanion (anti-PF4/P), anti-Phospholipid, anti-Phosphatidylserine, anti-Myeloperoxidase, anti-Proteinase 3, anti-dsDNA, anti-Beta-2-Glycoprotein I (β2GPI), and anti-Cardiolipin. Non-malarial coma (NMC, n=49) and healthy controls (HC, n=56) were assayed for comparison. Associations with clinical and immune biomarkers were determined using univariate and logistic regression analyses.

Results. Median anti-PF4/P and anti-PS IgG levels were elevated with malaria infection relative to HC ($p<0.001$) and NMC (PF4/P: $p<0.001$). Anti-PF4/P IgG levels were elevated in CM (median=0.27, IQR: 0.19-0.41) compared to UM (median=0.19, IQR: 0.14-0.22, p=<0.0001). Anti-PS IgG levels did not differ between UM and CM ($p=0.39$). When CM cases were stratified by malaria retinopathy (Ret) status, levels of anti-PF4/P IgG correlated negatively with peripheral platelet count in Ret+ CM cases ($R_s= 0.201$, $p=0.04$) and associated positively with mortality (OR=15.2, 95% CI: 1.02 – 275, $p=0.048$). Plasma from CM patients induced greater platelet activation in an ex-vivo assay relative to plasma from UM patients ($p=0.02$), and the observed platelet activation was associated with anti-PF4/P IgG levels ($R_s= 0.293$, $p=0.035$).

Conclusions. Thrombosis mediated by elevated anti-PF4/P autoantibodies may be one mechanism contributing to the clinical complications of CM.

Keywords. Cerebral Malaria, autoimmune thrombotic thrombocytopenia, anti-phosphatidylserine, procoagulant autoantibodies, anti-PF4 (CXCL4) antibodies.

INTRODUCTION

Immunothrombosis is a host defense mechanism where the innate and adaptive immune systems work in conjunction with the hemostatic system (platelets and endothelial cells) to control infection (1). Although it is a protective mechanism, immunothrombosis can quickly advance to pathogenic thromboinflammation resulting in a life threatening pro-coagulant state if unregulated. Hallmark features of thromboinflammation include thrombocytopenia with intravascular coagulation and thrombi formation, uncontrolled activation of innate immune effectors such as neutrophils and monocytes, and endothelial dysfunction (2). Thromboinflammation is a complication observed in non-infectious inflammatory conditions including cardiovascular and autoimmune diseases (5, 6). Thromboinflammatory characteristics are observed in a variety of infections, including those that often lead to sepsis (e.g., *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium* bacteremia), and viral infections such as Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2), Human Immunodeficiency Virus (HIV), Influenza virus type A, and Dengue Virus (DENV) (3, 4), and is also a prominent pathophysiological characteristic in cerebral malaria (CM) (7-13).

CM, caused by infection with *Plasmodium falciparum* parasites, is a neurovascular syndrome associated with decreased consciousness, coma, and seizures and is a leading cause of death in children under the age of five in sub-Saharan Africa (14). Children with CM, as defined by the World Health Organization (WHO), present with *P. falciparum* infection and are comatose with no other evident etiology of coma. Despite intravenous anti-malarial treatment, death still occurs in ~15-20% of cases, suggesting that host processes contribute to poor outcomes. *P. falciparum* infected erythrocytes (iRBC) sequester and aggregate in the microcapillaries of lungs, kidneys, liver, intestines, and brain causing obstruction and inflammation (8, 15-17). The initial activation of platelets by iRBCs and subsequent activation and recruitment of monocytes and other immune effectors compromises intravascular integrity (8, 9, 15).

Under inflammatory conditions, damage associated molecular patterns (DAMPs) are produced, becoming potential sources of self-recognition by the immune system (18). Pathogenic autoantibodies

that recognize DAMPs can form immune complexes and engage various immune effectors such as complement and Fc receptors, or directly crosslink immune receptors to activate and promote a feedback loop of inflammatory cell activation (19). For example, antibodies that recognize the neutrophil proteins Proteinase 3 (PR3) and Myeloperoxidase (MPO) are associated with vasculitis and have been shown to stabilize extruded chromatin content from neutrophils called neutrophil extracellular traps (NETs) (20, 21). In addition to entrapping extracellular pathogens, NETs serve as scaffolds for platelet aggregation and clotting that when stabilized by antibodies enhance thrombogenesis (22-24). In cases of Heparin Induced Thrombocytopenia (HIT), autoantibodies targeting the pleiotropic platelet effector Platelet Factor 4 (PF4) form immune complexes that bind the platelet Fc gamma Receptor IIA (FcγRIIA) resulting in platelet activation and further release of autoantigen (PF4) and other pro-coagulant molecules (25-28). Coronavirus disease 2019 (COVID-19) caused by SARS-CoV2 is also often complicated by hypercoagulation, and autoantibodies specific for phospholipids, neutrophil proteins, and platelet factors are associated with disease severity and clinical outcome (22, 23, 29-32).

Autoantibodies may contribute to CM pathogenesis via similar mechanisms where systemic and focal inflammatory conditions, including dysregulated platelet activation, NET release, and vascular wall injury offer sources of self-antigens (8, 11, 18, 33-36). Data from both animal models of malaria and humans infected with malaria have suggested that self-reacting antibodies to phosphatidylserine (anti-PS) and to double stranded DNA (anti-dsDNA) are associated with complications of malaria including anemia and acute kidney injury (37-42).

We hypothesized that procoagulant self-reactive antibodies are elevated in patients with CM and contribute to disease pathogenesis. We measured and compared antibody profiles in Malawian pediatric malaria patients presenting with uncomplicated malaria (UM) or CM and evaluated associations with markers of disease severity and thromboinflammation.

RESULTS

Patient characteristics.

Demographic and clinical characteristics comparing the four participant groups – cerebral malaria (CM, N=136), uncomplicated malaria (UM, N=124), non-malarial coma (NMC, N=49), and healthy controls (HC, N= 56) are in Table 1. The CM group was divided into (i) retinopathy positive (Ret+ CM, N=100) and retinopathy negative (Ret- CM, n=36), and (ii) survived (Ret+ CM: N=87; Ret- CM: N=27) or deceased (Ret+ CM: N=13; Ret- CM: N=9) categories (Figure 1, Table 1). Abnormal retinal pathology in children presenting with WHO-defined CM improves the specificity of the clinical case definition (43, 44). Ret- CM cases are more heterogeneous, likely representing a mix of milder CM cases and cases of other coma etiology with malaria co-infection.

All groups were analyzed for statistical differences in demographic or clinical characteristics relative to Ret+ CM. Children with Ret- CM had lower levels of PfHRP2 (469 vs. 1496 ng/mL, p=0.001), but higher platelet counts (148 vs. 60 x 10^3 cells/μl, p<0.0001) compared to Ret+ CM children (Table 1). UM cases presented with increased hemoglobin (HgB) levels (10.0 vs 7.9 g/dL; p=0.0001), increased platelet count (304 x 10^3 vs. 60 x 10^3 cells/μl; p<0.0001), and lower PfHRP2 levels (60 vs. 1496 ng/mL, p<0.0001) relative to Ret+ CM children.

The NMC control patients had a significantly higher HgB (10 vs. 8 g/dL, p<0.0001) and platelet count (330 vs. 60 x 10^3 cells/μl, p<0.0001) relative to Ret+ CM (Table 1). Among the cases of children presenting in coma (NMC and CM), mortality rates were comparable (NMC, 12% vs. Ret+ CM, 13%): p=0.897, NMC vs. Ret- CM, 25%: p=0.128). NMC patients had a lower median Blantyre Coma Score, with a higher proportion of patients having a BCS score equal to or less than 1 compared to Ret+ CM children (60% vs. 41%, p=0.04; Table 1).

Analysis of Prothrombotic autoantibodies in malaria patients.

In this analysis, levels of a select panel of circulating antibodies in plasma samples were compared between UM and CM patients. Patient plasma was analyzed via ELISA for a panel of IgG autoantibodies (anti-PL, anti-PS, anti-Cardiolipin (anti-CL), anti-β2GPI, anti-DNA, anti-PF4/P, anti-MPO, and anti-PR3) associated with thrombogenesis (Supplemental Figure S1). We also quantified levels of circulating immune complexes (CIC) in plasma samples as antibody-antigen immune complexes

contribute to immunopathology in various autoimmune diseases (45) (Supplemental Figure S2). Of the eight autoantibodies analyzed, only antibodies against the PF4-polyanion antigen (anti-PF4/P) were significantly elevated in CM compared to UM cases (median OD: 0.27 [0.19-0.41] vs. 0.19 [0.14-0.22]; p<0.0001) (Supplemental Figure 1H). Levels of the remaining autoantibodies analyzed were not significantly different between UM and CM (Supplemental Figure S1A-G; Table 2). In some malaria patients, anti-PF4/P IgG levels were elevated above the assay clinical cutoff point (OD=0.4) for HIT diagnosis, with a higher proportion of levels above the cutoff point observed in CM relative to UM (27% of CM vs. 1.6% of UM; Figure 1H, Table 2). Levels of circulating immune complexes (CIC) above the assay clinical cutoff point (CIC > 4μg/mL) were also observed in some individuals, but neither the prevalence nor the median values differed significantly between UM and CM groups (74% of CM, 4.84 pg/mL [4.0-7.3] vs. 73% of UM, 5.45 pg/mL [3.9-7.9]; Supplemental Figure S2.A). Correlation analyses among the various antibody profiles in the Ret+ CM group demonstrated antibodies associated with anti-phospholipid syndrome (APS) correlated with one another, namely anti-PL, anti-PS, and anti-Cardiolipin (Supplemental Figure S3; R_s range=0.48-0.84, p<0.005-0.0005). Anti-PS IgG levels correlated positively with anti-MPO and anti-PR3 IgG levels (PR3: R_s=0.48, p=0.0008, MPO: R_s=0.53, p<0.0002; Supplemental Figure S3) but not with anti-PF4/P. Anti-PF4/P antibodies did not correlate with the APS antibody panel. Anti-PF4/P IgG correlated with levels of antibodies to the neutrophil effector proteins anti-Proteinase 3 (anti-PR3; R_s=0.39, p=0.005) and anti-Myeloperoxidase (MPO; R_s=0.51, p=0.0002) (Supplemental Figure S3).

Anti-PF4/P IgG levels are elevated in pediatric cerebral malaria.

We quantified the IgA/IgM (combined) isotypes of anti-PF4/P antibody levels in patient plasma and observed elevated levels above the cutoff (OD>0.4) of clinical significance (UM 52%, CM 48%) but saw no difference in median IgM/A levels between UM and CM cases (median OD: 0.414 vs. 0.388, p=0.97) (Figure 2A). We focused our analysis on the IgG isotype which is considered the clinically relevant isotype (26) and found that levels of anti-PF4/P IgG were elevated in UM compared to healthy controls (HC, median OD=0.139 [0.12-0.17]; p=0.032) but did not differ significantly from levels observed in non-

malarial coma controls (NMC) (median OD=0.16 [0.12-0.22]; p=0.52). Relative to both HC and NMC patient plasma, the levels of anti-PF4/P IgG in CM plasma were significantly elevated (CM vs. HC: p<0.0001; CM vs NMC: p<0.0001) (Figure 2B).

When stratified by retinopathy status and outcome, we observed no difference in median PF4/P IgG levels between Ret+ CM survivors (median OD=0.250 [0.19-0.43]) and Ret- CM survivors (median OD=0.298 [0.19-0.37]; p=0.23). However, we observed elevated median levels of anti-PF4/P IgG in Ret+ CM fatal cases (median OD=0.381 [0.25-0.55] compared to both Ret+ CM survivors (median OD 0.25 [0.19-0.40]; p=0.04) and Ret- fatal cases (median OD=0.215 [0.14-0.29]; p=0.008; Figure 2C). We quantified levels of PF4/P IgG in available convalescent plasma of surviving CM patients (Figure 2D; 30d). PF4/P IgG levels decreased with convalescence (acute CM: OD=0.27 [0.19-0.41] vs. 30d convalescent: OD=0.186 [0.14-0.24]; p=0.0005). Levels of anti-PF4/P IgG in convalescence were similar to those of HC controls (HC vs. CM 30d median OD: 0.16 vs. 0.18; p=0.16; Figure 2D).

For a clinical HIT diagnosis, neutralization with high dose heparin (HDH; 100 U/mL) is used as an additional verification of heparin dependent anti-PF4/P antibody specificity (46). If neutralization does not occur, the result is considered equivocal, indicating that antibodies to a PF4/polyanion or PF4 antigen are present in circulation, but their binding is not dependent on heparin or a polyanion for binding (47). When we tested neutralization of IgG binding to HIT antigen with HDH (100U/mL), neutralization (determined as >50% inhibition) was significant relative to the control in 46% of samples (p<0.0001; Figure 2E). Cell-free DNA (cfDNA), another naturally occurring polyanion (48), is elevated in malaria and associated with disease severity and survival status in children with CM (36). Since none of our patients received prophylactic heparin treatment, we tested and confirmed that DNA neutralizes anti-PF4/P IgG binding (Figure 2F, Supplemental Figure S4) in a similar pattern to that of HDH (Pearson Rho=0.80; p<0.0001; Figure 2G).

Anti-PF4/P antibodies are associated with markers of thromboinflammation.

Markers of parasite burden such as peripheral blood parasitemia (Ret+ CM IgG: R_s=0.204, p=0.043), *P. falciparum* cell free DNA (Pf cfDNA; UM IgG: R_s=0.323, p=0.0002; Ret+ CM: R_s=0.286,

p=0.004), and total parasite load (PfHRP2; UM: R_s=0.317, p=0.005) were positively correlated with anti-PF4/P IgG levels (Supplemental Table S1). Neutrophil activation and NET release are pro-thrombotic pathogenic processes that are associated with elevated levels of anti-PF4/P IgG in HIT (49-52). Markers of neutrophil activation and NETosis include Myeloperoxidase (MPO), a neutrophil effector molecule embedded within NETs, and cfDNA, a marker and degradation byproduct of neutrophil DNA release (53). In UM cases, we observed a positive correlation of anti-PF4/P IgG with MPO (R_s= 0.268, p=0.032) and total cfDNA (R_s=0.248, p=0.006) (Supplemental Table S1). Anti-PF4/P IgG correlated positively with the inflammation marker soluble Suppressor of Tumorigenicity 2, sST2, in both UM (R_s=0.336, p=0.028) and Ret+ CM (R_s=0.321, p=0.018) (Supplemental Table S1; Figure 3A).

Among markers associated with platelet activation and coagulation, we observed a positive correlation only in UM between anti-PF4/P and the marker for active coagulation, D-dimers (R_s= 0.270, p= 0.039) (Supplemental Table S1) (7). In Ret+ CM, an inverse correlation was observed between anti-PF4/P IgG and soluble CD40 Ligand (sCD40L), which is exposed to the platelet surface upon activation and subsequently shed (54) (R_s=-0.231, p=0.032; Figure 3D). Furthermore, we observed an inverse correlation between anti-PF4/P IgG and circulating platelet count in Ret+ CM (R_s=-0.201, p=0.048; Supplemental Table S1, Figure 4A). (55-57). Anti-PF4/P antibodies have not been described in association with anemia, but we observed an inverse correlation in UM cases between anti-PF4/P and Hemoglobin (HgB: R_s=-0.225, p=0.015) and packed cell volume (PCV: R_s=-0.200, p=0.029) (Supplemental Table S1).

Since the pathogenic function of HIT-like anti-PF4 IgG is primarily mediated through immune complexes that engage FcγRIIa receptors on platelets and monocytes (58, 59), we analyzed the associations of CIC in Ret+ CM with anti-PF4 IgG and soluble markers of thrombosis. Levels of CIC were positively correlated with anti-PF4/P IgG in Ret+ CM patients (R_s=0.252, p=0.024; Supplemental Figure S2B, Supplemental Table S2). We observed a positive association between CIC and soluble ST2 levels (R_s=0.326, p=0.046), MPO levels (R_s=0.340, p=0.002), soluble CD62p levels (R_s=0.445, p=0.001), and Pf cfDNA levels (R_s=0.280, p=0.009) in Ret+ CM. Similar to anti-PF4/P IgG, we also

observed a negative correlation between CIC and markers of anemia (PCV: (R_s=-0.256, p=0.040; HgB: R_s=-0.313, p=0.01) in UM cases (Supplemental Table S2).

Elevated levels of anti-PF4/P IgG in Ret+ CM cases are associated with fatal outcome.

To better understand how sST2 and sCD40L plasma levels relate to anti-PF4/P IgG levels in CM, we plotted the values in relation to the following malaria case classifications: UM, Ret+ CM survived, or Ret+ CM fatal. Levels of sST2 were elevated according to malaria disease severity with the highest median levels observed in Ret+ CM fatal cases ($517x10^3$ pg/mL [443-$768x10^3$]), followed by Ret+ CM survived cases ($260x10^3$ pg/mL [166-$399x10^3$]), and UM $83x10^3$ pg/mL [39-$193x10^3$]; Figure 3B). Moreover, sST2 levels in Ret+ CM cases were negatively associated with circulating platelet counts (R_s=-0.322, p=0.016; Figure 3C). In a similar analysis, we observed that the median levels of sCD40L were higher in Ret+ CM survivors (3228 pg/mL [1695-4323]) compared to UM cases (2239 pg/mL [1465-3548]; p=0.05; Figure 3E). The plasma levels of sCD40L in Ret+ CM fatal cases (2486 pg/mL [2202-3879]) were lower compared to survivors, although this observation was not statistically significant (Figure 3E). When we plotted sCD40L levels in Ret+ CM against circulating platelet count, we observed a positive association (R_s=0.227, p=0.037; Figure 3F) indicating that in Ret+ CM, lower levels of sCD40L in patient plasma are concomitant with thrombocytopenia. Logistic regression analysis of concurrent clinical diagnoses or outcome with anti-PF4/P IgG levels in Ret+ CM revealed a significant positive association with death as an outcome (OR=15.2, 95% CI=1.02 – 275; p=0.048; Table 3) consistent with a role for anti-PF4/P in CM pathogenesis.

PF4-dependent platelet activation induced by CM patient plasma is associated with anti-PF4/P IgG levels and thrombocytopenia.

In HIT, a follow up platelet activation test confirms a positive diagnosis when anti-PF4 IgG levels exceed the clinical cutoff. Diagnostic evaluation assays employed for HIT and vaccine-induced thrombotic thrombocytopenia (VITT) demonstrate the ability of patient plasma to activate normal platelets (60, 61). Relative to UM patient plasma, the level of platelet activation with CM plasma in the presence of PF4 was significantly elevated (UM: mean=17% ± 17 vs. CM: mean=30.4% ± 12.5, p=0.04)

(Figure 4B). Platelets incubated with plasma from HIT+ patients (mean = 50.6% ± 14.2) or the agonist adenosine diphosphate (ADP, 10μM) served as positive controls. When high dose heparin (HDH) was added to neutralize the plasma-platelet interaction, we observed a decrease in the proportion of CD62p+ activated platelets for UM + HDH (3.3% ± 7; p=0.02), CM + HDH (20.6% ± 17.5; p=.02) and HIT + HDH (26.46% ± 18.2; p=0.05). (Figure 4B).

Platelet activation in the ex-vivo functional assay was positively associated with PF4/P IgG levels in patient plasma (R_s=0.293, p=0.035; Figure 4C) and trended in the direction of negatively correlating with the associated peripheral platelet count observed in the malaria patients (R_s=-0.243, p=0.09; Figure 4D). In the subset of samples used in the ex-vivo platelet activation assay, we also observed (as shown in Figure 4A) a negative association between peripheral platelet count and corresponding PF4/P IgG plasma levels (R_s= -0.371, p=0.036; Figure 4E).

The platelet activation observed when patient plasma was incubated with exogenous PF4 and normal platelets was not associated with anti-PS IgG levels (Figure 4F), indicating specificity of the platelet activating properties induced by the patient plasma in the presence of exogenous PF4.

Anti-PS antibodies are elevated in pediatric malaria infections.

We quantified anti-PS IgM levels and found no difference between median levels in UM and CM (Figure 5A). Comparing anti-PS IgG levels in UM and CM cases to HC and NMC controls, we observed that anti-PS levels were significantly elevated in malaria infection as compared to non-malarial controls for both UM and CM cases (HC: 0.61 pg/mL [0.43-.85] vs. UM: 2.6 pg/mL [1.2-2.2], p<0.0001; NMC: 0.91 pg/mL [0.65-1.74] vs. UM: p<0.0001; HC vs. CM: 2.2 pg/mL [1.2-3.4], p<0.0001; NMC vs. CM: p<0.0001) (Figure 5B). We stratified the CM patient group by retinopathy status and fatal outcome but did not observe significant differences in anti-PS antibody levels in either comparison (p>0.9999, Figure 5C).

Correlation analysis shows that anti-PS IgG is positively associated with markers of platelet activation including CD62p (UM: R_s= 0.524, p <0.0001; Ret+ CM R_s=0.367, p=0.006) and CD40L (UM: R_s=0.336, p=0.005; Ret+ CM: R_s=0.253, p=0.034) (Figure 5D & 5E; Supplemental Table S3). One

proposed pathogenic prothrombotic function of anti-PS in diseases like antiphospholipid syndrome (APS) and COVID-19 is through the activation of neutrophils, specifically the induction of NET release (NETosis) (22, 62). We observed a positive association between anti-PS IgG and MPO (R_s=0.497, p=0.005), host cfDNA (R_s=0.407, p=0.017), and total cfDNA (R_s=0.405, p=0.0004) in UM. In contrast, in Ret+ CM, host and total cfDNA levels correlated negatively with anti-PS IgG (host cfDNA: R_s=-0.365, p=0.001; total cfDNA: R_s=-0.230, p=0.048) (Supplemental Table S3).

Studies in malaria infection animal models and those involving *Plasmodium*-infected human subjects have reported an association of elevated levels of anti-PS with anemia through the direct targeting of anti-PS antibodies to the exposed phosphatidylserine lipid on erythrocytes during infection (37-39, 63, 64). We analyzed anti-PS IgG levels with markers of anemia and did not observe any correlations with packed cell volume (PCV) or hemoglobin levels (HgB) in either UM or Ret+ CM pediatric cases (Figure 5F & 5G, Supplemental Table S3). We also did not observe significant associations between anti-PS IgG levels in Ret+ CM pediatric cases that presented with a concurrent clinical complication of severe anemia (SMA), respiratory distress (RD), jaundice, or with death as an outcome (Supplemental Table S4).

DISCUSSION

Autoimmune activity contributing to prothrombotic and inflammatory processes in the context of systemic infections has become increasingly appreciated (30, 65). Here, we evaluated whether prothrombotic autoimmune processes play a role in the pathogenesis of pediatric malaria. Of a panel of eight autoantibodies, only anti-PF4/P IgG, the primary pathologic agent of the clinical syndrome HIT (26), were elevated in the plasma of children with CM relative to UM. Compared to healthy pediatric community controls and sick comatose children (non-malaria coma controls) infected with non-*Plasmodium* infectious agents, anti-PF4 IgG levels were elevated in malaria (UM and CM) suggesting that anti-PF4/P antibody production may be specific to *Plasmodium* infection. We observed a significant decline in anti-PF4/P antibody levels in convalescence, like what is observed in HIT, VITT, or COVID-

19 where antibody levels are transiently and acutely elevated under antigen exposure and inflammatory conditions with a rapid decline in recovery (26).

Parallels in the clinical presentation between CM and other “anti-PF4 disorders”, such as HIT and VITT, include severe thrombocytopenia, endothelial dysfunction, evidence of consumptive coagulopathy, and microvascular thrombosis (7, 10, 26). We observed a positive association between anti-PF4/P IgG levels and markers of thromboinflammation including neutrophil activation and NETosis (*i.e.,* cell free DNA and the neutrophil effector, MPO) and active coagulation (D-Dimers) in UM cases. Plasma from CM patients had a greater capacity to activate normal platelets in vitro in a PF4-dependent manner compared to UM patient plasma. PF4-dependent (as opposed to heparin dependent) platelet activation is typical in cases of “autoimmune or spontaneous HIT” such as VITT (31). Heparin-independent anti-PF4 antibodies, such as those of VITT were shown to have greater binding strengths to PF4 molecules which enhanced thrombus formation by immune complexes (66). Altogether, the data suggests that in UM, processes such as NETosis (MPO and cfDNA) and active coagulation (D-Dimers) are an early event in infection which correspond to elevated anti-PF4/P IgG levels.

In malaria, thrombocytopenia is associated with retinopathy, disease progression, and a worse clinical outcome (12, 67). In our analyses of Ret+ CM cases, we observed a negative association between anti-PF4/P IgG levels with peripheral platelet count and a positive association with mortality. We further showed that soluble ST2 is elevated in Ret+ CM with the highest levels observed in fatal Ret+ CM cases. ST2 levels are also positively associated with anti-PF4/P IgG levels and negatively associated with peripheral platelet count. ST2 is an interleukin-1-like receptor to the alarmin interleukin 33 (IL-33), associated with neuroinflammation and vascular dysfunction (68-70), and was recently described as a prognostic marker of neurological sequelae in children with CM (55).

We also observed a negative association between plasma levels of anti-PF4/P IgG and sCD40L, an inflammatory immune modulator that bridges the adaptive and innate immunity and whose soluble levels are primarily derived from activated platelets (71). Interestingly, in Ret+ CM patients,

318 sCD40L levels, considered a marker for platelet activation, wane with reducing levels of peripheral
319 platelets. Although not significant in our analysis, sCD40L median levels in fatal cases were reduced
320 relative to those observed in Ret+ CM survivors, providing a potential link between a state of
321 consumptive coagulopathy in fatal Ret+ CM cases and pathogenic anti-PF4/P antibodies.

322 In HIT, anti-PF4/P antibody production is induced when PF4 tetramers released from platelets
323 form complexes with heparin, a long polyanion, revealing a neoantigen on PF4 (72). PF4 molecules
324 adhere to cell surfaces (58, 73) and interact with polyanions to form PF4/P antigen (74). Available
325 polyanions include cell free DNA (cfDNA) from NETs or other damaged/necrotic cells (48, 49), heparan
326 or chondroitin sulfate glycosaminoglycans on endothelial cells and monocytes (58, 75), procoagulant
327 vWF strands secreted by endothelial cells (76), gram-negative bacterial cell wall components (77), and
328 polyphosphates released by platelets (78). Cell sources of polyanions that can form HIT-like antigens
329 are abundant in malaria (34-36, 79-83). As the endothelium is an active site of CM pathogenesis (84), it
330 is likely that PF4/P antigen readily forms on the injured vascular wall(s) of CM children promoting
331 immune complex aggregation and localized endothelial and immune cell activation. Additionally,
332 during infection, platelets bind to iRBCs, deploying PF4 which accumulates and destroys the parasite
333 within the erythrocyte (85-87).

334 Antibodies bound to PF4 can also form circulating immune complexes (CIC) with soluble
335 polyanions such as cfDNA or vWF strands in the presence of anti-PF4/P antibodies. CIC levels were
336 substantially elevated in about 75% of malaria cases tested here and positively correlated with anti-
337 PF4/P, as well as with MPO, parasite cfDNA, soluble ST2, and soluble CD62p in Ret+ CM. Thus, CIC
338 could mediate pathogenic processes of anti-PF4/P immune complexes in malaria patients through
339 engagement of Fc receptors, such as FcγRIIA, or through opsonization.

340 Of the antibodies in the APS panel, we focused on anti-PS IgG given multiple independent
341 reports of elevated levels in adult and pediatric cases of malaria caused by multiple infecting species
342 including *P. vivax* and *P. falciparum (37-39, 63, 88)*. Anti-PS antibodies are also considered a risk

343 factor for thrombosis and associated with disease severity in cases of systemic lupus erythematosus
344 (SLE), Anti-phospholipid Syndrome, COVID-19, and cardiovascular disease (89-95). PS is a
345 phospholipid found on the inner leaflet of plasma membranes during basal conditions that is “flipped” to
346 the outer leaflet of the plasma membrane under disease conditions to alert the system of stress or a
347 breach (96). Major sources of PS include apoptotic or necrotic cells, neutrophils undergoing NETosis,
348 and activated platelets (96, 97). In *Plasmodium* infection, PS is also found on microvesicles from iRBCs
349 (98, 99), as well as on the surface of iRBCs, and uninfected erythrocytes (39). Prior work
350 characterizing anti-PS IgG’s role in anemia pathogenesis, both *in vitro* and *in vivo*, has focused on
351 destruction of PS-exposed uninfected erythrocytes that in multiple independent studies and various
352 patient cohorts have been associated with acute and post-acute malarial anemia (38, 39, 100, 101). In
353 our pediatric CM cohort, we did not observe an inverse correlation of anti-PS IgG levels with markers of
354 anemia (e.g., HgB and PCV) or a concurrent diagnosis of severe anemia which is clinically defined as
355 HgB nadir levels <=5 g/dl. Although seemingly contradictory to previous studies, these results may
356 indicate that different clinical presentations of severe malaria (i.e., SMA vs. CM) vary in their etiology
357 and pathophysiology.

358 To date, no reports have linked anti-PS antibody function with thrombosis and/or pro-coagulant
359 processes in pediatric CM. We observed positive associations between anti-PS IgG levels and markers
360 of NETosis (e.g., MPO and cfDNA) in UM but not in Ret+ CM. We also observed a positive association
361 between anti-PS IgG levels with soluble CD62p and CD40L, markers of platelet activation, in both UM
362 cases and Ret+ CM cases, suggesting that elevated anti-PS antibodies may be involved in platelet
363 activation. Unlike anti-PF4/P antibody levels, anti-PS IgG levels were not associated with cerebral
364 malaria, which supports a recent study of Ugandan pediatric cases of severe malaria, including CM
365 (64). Further, we observed an inverse correlation between anti-PS IgG and both cfDNA (host and total)
366 and PfHRP2, two markers strongly associated with worse outcomes in CM, in Ret+ CM cases (12, 36,
367 102-104). Anti-PS antibodies could have a pathogenic or protective role depending on the disease
368 context and the tissue microenvironment (105), just as NETs and neutrophils are important in host

defenses but are also linked to pathogenic states including thromboinflammation. Antisera of SLE patients containing anti-PS antibodies can be protective, recognize malaria antigens, and inhibit *in vitro* parasite growth (106). Further functional studies will be needed to explore the role, if any, of anti-PS IgG in prothrombotic processes in malaria.

Elevated levels of anti-PS IgG observed in malaria are produced by Tbet+ atypical memory B cell (atMBC) in response to IFNγ and parasite DNA via TLR-9, both of which are associated with malarial anemia (63, 101). Similarly, anti-PF4/P antibodies are produced from rapidly responding marginal zone B-cells through TLR signaling in response to blood infections when antigen is presented via complement (107). The positive correlation we observed between Pf cfDNA and anti-PF4/P suggests another link between parasite derived DNA and expansion of autoantibody secreting B-cells via TLR9. Whether Tbet+ atMBCs are a subset of marginal zone B cells that respond to parasite cfDNA viaTLR-9 to produce anti-PF4/P antibodies is not known. Rapidly responding, germinal center-independent Tbet+ B cells were tracked to the splenic marginal zone during viral infections (108), and a TLR9/IFNγ dependent expansion of Tbet+ B cells in the marginal zone of spleens was observed in a rodent model of malaria re-infection (109, 110). In-depth immunophenotyping of autoantibody secreting B cells will be necessary to understand anti-PF4/P and anti-PS antibody production in pediatric malaria patients.

An inherent limitation in our analysis is the retrospective nature for all patient/case classifications. We were limited by a lack of longitudinal measurements of hemoglobin and platelet counts. Studies evaluating the importance of anti-PS or anti-PF4/P antibodies have shown stronger correlations with nadir measurements of blood counts (31, 37, 38). We also did not have post recovery clinical data to associate autoantibody levels to post-malaria anemia or post-acute thrombosis. Association of autoantibodies in post-recovery complications has been described for malaria (37, 39), as well as other infections, including post-recovery complications of COVID-19 (111-114).

Overall, our study points to a role for prothrombotic autoantibodies in pediatric cerebral malaria. Induction of autoantibodies, such anti-PF4/P, in a subset of malaria patients, may be one of multiple

mechanisms that tilts an uncomplicated malaria case towards CM. Understanding the underlying pathologic immune processes of malaria thromboinflammation is imperative for the establishment of clinical interventions, generation of adjunct or prophylactic therapeutics, and improved patient prognoses. Our findings lay the groundwork for further investigations into autoimmune mechanisms contributing to thromboinflammatory processes in pediatric cerebral malaria.

METHODS

Sex as a biological variable

The study design aimed to consent patients irrespective of sex, such that the patient recruitment would be unaffected by selection biases towards one sex or another. Our study examined both male and female pediatric patients, and our analysis on key variables presented here did not find differences with relation to sex.

Ethics statement and Study Approval

The studies included in this manuscript were approved by the institutional review boards at the University of Malawi College of Medicine, the University of South Florida, and Michigan State University. Informed consent was obtained from the parent or legal guardian of all pediatric study participants or directly from the study participant (American healthy adult volunteers or patients) prior to enrollment. Our study involved both male and female children and similar findings are reported for both sexes.

Patient cohort and sample collection.

Uncomplicated malaria patients (UM, N=124) were recruited during consecutive malaria seasons (January – June) in 2016-2017 and met the following criteria: children aged 1-12 years old presenting to the Accident and Emergency (A&E) Department at Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi with a history of fever, positive smear score for peripheral *P. falciparum* parasitemia as calculated by thick blood smears (parasites/100 fields) (115), consciousness, and no overt signs of compromised health, malnutrition, or progression to severe malaria. UM patients were treated as outpatients within the A&E Department of QECH and medicated according to national

protocols (116). Cerebral malaria (CM, N=136) patients were recruited during consecutive malaria seasons (January – June) in 2015-2017 (Figure 1, Table 1). Children aged six months to 12 years, were admitted to the Pediatric Malaria Research Ward at QECH and met criteria for WHO-defined CM (*i.e., P. falciparum* parasitemia, coma that is not resolved despite treatment for hypoglycemia and/or seizures, and exclusion of other identifiable causes of coma). The Blantyre coma score (BCS), a clinical assessment on a scale of 0 (no consciousness) to 5 (consciousness), was used to determine level of consciousness with scores ≤2 indicating a comatose state (103, 117). Retinal fundoscopy was performed by a trained ophthalmologist and stratified the CM cases into either Ret- CM (N=36; i.e., normal ocular fundi) or Ret+ CM (N=100; i.e., observation of retinal whitening, hemorrhages, and/or abnormal vessels). Children who recovered from CM (N=39) were re-assessed 30-days post admission, and convalescent plasma was collected for analysis.

Healthy controls (HC, n=56) aged 7 months to 11 years were recruited from Ndirande Health Centre (Figure 1, Table 1) during well checkups. They presented with no symptoms and were negative for *Plasmodium* infection by thick blood smears. Children with non-malarial coma (NMC, n=49) were admitted to the QECH Pediatric Research Ward as part of an ongoing prospective study (CHildhood Aetiologies of Severe Encephalopathy; CHASE) (Figure 1, Table 1). The NMC cases were recruited between February 2018 and April 2020 and included children aged three months to 14 years who were negative for *P. falciparum* infection by microscopy and RDT. They presented with fever, a deep coma (BCS ≤2), and a suspected central nervous system infection not of malarial etiology.

For UM, CM, NMC, and HC, 4 mL of blood was drawn into citrate anticoagulant tubes and 0.5 mL of blood was drawn into EDTA tubes at the time of study enrollment. The number of samples used in each assay is reported within the text, figures, or figure legends. For some assays, 4 mL of citrated plasma from adult American volunteers or patients was collected following approved IRB protocols.

Autoantibody ELISA analysis

Plasma or serum samples were analyzed for the presence of anti-Proteinase 3 (anti-PR3), anti-Myeloperoxidase (anti-MPO), anti-phosphatidylserine (anti-PS), anti-Cardiolipin (anti-CL), anti-

447 phospholipid (anti-PL), and anti-Beta-2-glycoprotein I (anti-β2GPI) IgG antibodies using commercially
448 available enzyme-linked immunosorbent assay (ELISA) kits (Orgentec Diagnostika GmbH) designed for
449 *in vitro* diagnostic use. Anti-PS IgM antibody levels were also measured. Briefly, samples were diluted
450 1:100 in diluent buffer per the manufacturer's protocol, detected with HRP-conjugated anti-human IgG
451 secondary antibody followed by incubation with 3,3', 5,5'- Tetramethylbenzidine (TMB) chromogenic
452 substrate. Plasma antibody concentrations (U/mL) were determined using optical density and a
453 standard curve. Levels were considered clinically relevant and positive at pre-determined
454 recommended cut-off levels ≥10U/mL for anti-CL, anti-PL, anti-PS, anti-β2GPI and ≥5U/mL for anti-PR3
455 and anti-MPO.

456 Levels of complement protein C1q-associated circulating immune complexes (CIC-C1q) were
457 quantified in plasma using MicroVue's CIC-C1q enzyme immunoassay (EIA) according to the
458 manufacturer's instructions (Quidel Corporation). CIC levels were determined with a validated standard
459 curve and a clinical cutoff of 4μg/mL per kit specifications. Control samples were added to all plates for
460 reference and included pooled plasma from SLE patients (118) and plasma from healthy American
461 adult volunteers.

462 Total anti-dsDNA IgG was measured using a modified ELISA protocol from a published protocol
463 (118). Briefly, plates were coated overnight with salmon sperm DNA (Thermo Fisher Scientific). Plasma
464 from each participant was added to the plate at a 1:50 dilution and incubated for 2h at room
465 temperature. Alkaline-phosphatase-conjugated goat anti-human IgG (Southern Biotech) was added for
466 1h and developed with a phosphatase substrate (MilliporeSigma). Positive and negative controls were
467 the same as those described for the for CIC-C1q EIA. All UM, CM, SLE, and HC ODs were normalized
468 to the average of two human SLE cases with known, high levels of anti-dsDNA Ab. The cutoff was
469 determined by the mean plus three standard deviations of the HC samples.

470 Antibody (IgG or IgM/A) levels specific to PF4-complexed to polyanions (PF4/P) in patient
471 samples were determined using a clinical diagnostic immunosorbent assay for Heparin Induced
472 Thrombocytopenia (PF4 Enhanced Assay, Immucor). Positive tests were defined as samples with OD ≥

0.400 according to manufacturer's pre-determined cutoff. To determine polyanion neutralization, 100U/mL of unfractionated high molecular weight heparin (HDH) or 200µg/mL of dsDNA was added to each diluted plasma sample prior to incubation in a coated ELISA plate. Percent neutralization was determined as described in manufacturer's protocol and calculated as [1-(OD sample w/ polyanion) ÷ (OD sample alone) × 100].

All ELISA/EIA assays used in this manuscript, internal positive and negative controls and standard curve calibrators were validated with optical density values that complied with kit specifications. Measurements were made on a Molecular Devices SpectraMax M2 (USF) or a Biotek ELx800 (University of Malawi) plate reader, and values were converted to U/mL based on a four-parameter logistic regression (4PL) analysis (GraphPad Prism 9).

Soluble thrombosis marker quantification.

Plasma IL-8 was measured using Cytokine Bead Arrays (BD Biosciences). The data obtained from this analysis were partially previously published (36, 119). Briefly, plasma samples were incubated with capture beads and processed according to the manufacturer's protocol. Data was acquired on a CyAn ADP flow cytometer (Beckman Coulter) and analyzed with BD FCAP software v3.0 (BD Biosciences).

D-dimers, Suppression of Tumorigenicity 2 protein (sST2), P-selectin (CD62p), and Cluster of Differentiation 40 Ligand (CD40L) were analyzed using a custom magnetic bead based Luminex assay (R&D Systems® Luminex® Assays). Diluted plasma (1:2) was incubated with Luminex beads overnight (18 hours) at 4°C and processed by washing and subsequently incubating with biotin-conjugated antibody and streptavidin as stipulated by kit protocol. Bead counts were acquired on a MagPix system (Luminex Corp), and concentrations were calculated using their xPONENT software.

Plasma concentrations of Myeloperoxidase (MPO) were measured by commercial ELISA (Human MPO ELISA kit, R&D Systems). Sample dilutions were modified (manufacturer suggested 1:10) to a 1:50 dilution to allow for a range of readings within the standard curve. OD measurements

were taken at 450nm using a Molecular Devices SpectraMax M2 (USF) or Biotek ELx800 (University of Malawi) plate reader, and values were converted to pg/mL based on the corresponding standard curve.

Total cell free DNA levels (Total cfDNA) were measured in plasma samples by commercial fluorometer (Invitrogen Qubit 4 Fluorometer; Qubit™ dsDNA Quantification Assay) as previously described (36). Levels of host or parasite-derived cfDNA were determined with a probe-based qPCR method as previously described (36).

P. falciparum Histidine Rich Protein 2 (PfHRP2) was measured by ELISA according to validated methods (102) using a commercial kit (Cellabs, Brookvale, Australia).

Ex-vivo platelet activation by P-selectin expression assay (PEA)

Platelet activation was assessed using a modified assay based on published methods (60, 61). Blood from healthy adult volunteers was collected in sodium citrate tubes and processed to collect platelet rich plasma (PRP) by low force centrifugation at 100g for five minutes at room temperature. PRP (3µl) from two to three donors was incubated with 10µl of patient plasma in the presence of human PF4 (hPF4, 15µg/mL) with or without high dose unfractionated heparin (HDH, 200U/mL), or saline only (no treatment) in a total activation volume of 20µl. PRP samples were incubated for 60 minutes at room temperature. Samples were then stained with anti-human CD41-APC (clone P2, Beckman Coulter) and anti-human CD62p-PE (clone CLBThromb/6, Beckman Coulter) for 20 minutes, fixed with 1% paraformaldehyde for 10 minutes at room temperature, and analyzed by flow cytometry (Beckman Coulter CytoFLEX). The positive control for maximum activation was PRP treated with 10µM adenosine diphosphate (ADP) for eight minutes prior to staining with antibodies and fixation with 1% PFA, as described above. PRP incubated with normal platelet poor plasma (PPP) served as the negative control and was used to normalize sample values. Platelets were identified by forward and side scatter and gated by CD41-positive staining. CD41+ platelets were then analyzed by histogram for CD62p staining (Supplemental Figure S5). The following equation determined the fold-change reactivity to PF4 treatment on platelets incubated with patient plasma: % relative platelet activation = ((PF4 treated – no treatment)/(PF4 treated)) * 100.

Statistical analysis

Descriptive and univariate analyses were performed in GraphPad Prism 9 or SPSS 28. Mann-Whitney non-parametric analysis and parametric t-test with Welch's correction were used for unpaired comparisons. Wilcoxon signed rank was used for paired data. Kruskal–Wallis with Dunn's post-hoc test was used for multiple comparisons. The resultant data are presented as median ± IQR or Spearman rho (R_s). To adjust for multiple comparisons when performing a correlation matrix analysis, we carried out the two-stage step-up method Benjamini, Krieger, and Yekutieli (120) with a Q value set to 1. Chi-squared tests were used for categorical data analyses and are presented as n (% of group). Spearman correlation analyses were utilized for associations between continuous variables. Logistic regression was used to identify associations with binary variables, such as clinical risk factors for severe malaria anemia (SMA), respiratory distress (RD), lactic acidosis (LA), jaundice, and death. All statistical analyses are two-tailed and p-values of 0.05 or less were considered statistically significant.

Data Availability.

The underlying values for plotted graphs within the main text and the supplemental material are available in the Supporting Data Values file.

Author Contributions

KK, AK, MRG, and IMV conceptualized the study. IMV, AK, and VH performed experiments. Data was analyzed by IMV and TK performed and advised on statistical regression analyses. AK and VH collected and processed the CM and UM patient samples for BMP. SJR and WM supervised recruitment of UM cases. AA processed patient samples for CHASE. KS and TT recruited patients, provided clinical data for CM patients, and provided intellectual input. SR recruited patients and provided clinical data for CHASE. IMV and AK wrote the manuscript with guidance from corresponding authors (KK and KBS) with edits and input from all authors.

Acknowledgements/Funding

549 We are indebted to the patients and their families for their consent to participate in our studies. We give
550 thanks to the staff of the Blantyre Malaria Project for sharing facilities and aiding with sample collection
551 and processing. We are grateful to the staff supervised by Dr. Morayma Reyes Gil of the Hematology
552 Clinical Laboratory, Montefiore Medical Center, Bronx, NY (Julissa Pena, Mayra Almonte, Leli Serrano,
553 and Geovanna Cruz) for their assistance and technical advice with HIT diagnostic tests. We also thank
554 Samantha Chalmers and Chaim Putterman for SLE control samples and help with the anti-dsDNA
555 antibody assays. This work was supported by a National Health and Medical Research Council of
556 Australia Project Grant APP1061993 (SJR), NIH National Center for Advancing Translational Science
557 (NCATS) Einstein-Montefiore CTSA TL1TR001072 (AK), NIH 5U01AI126610 (TT), and the Burroughs
558 Wellcome Fund (AK). The authors report no conflicts of interest.

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FIGURES

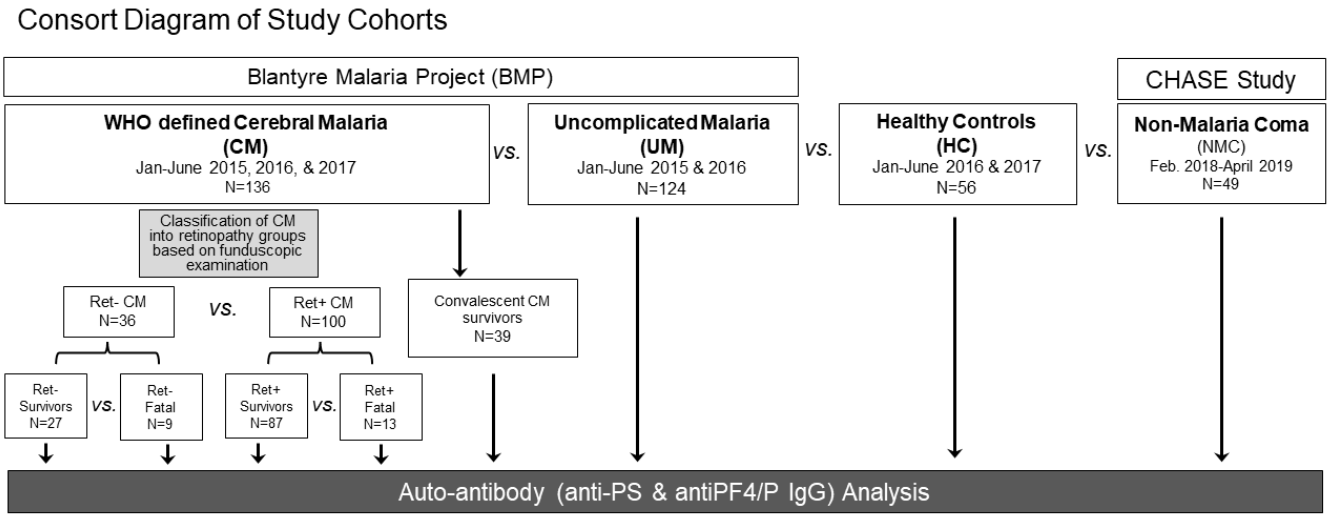

Figure 1. Consort diagram of patient recruitment.

Patient Recruitment for Blantyre Malaria Project (BMP) included uncomplicated malaria (UM) and cerebral malaria (CM) cases. Non-malarial coma controls (NMC) were recruited under the Childhood Aetiologies of Severe Encephalopathy study (CHASE). CM patients were stratified by retinopathy (negative: Ret- or positive: Ret+) and by outcome (Survivors or Fatal). Convalescent CM survivors returned for follow-up assessment at 30-days post admission. Healthy Controls (HC) were recruited from Ndirande Health Centre while attending routine well visits. Plasma samples were collected and analyzed for autoantibody analysis.

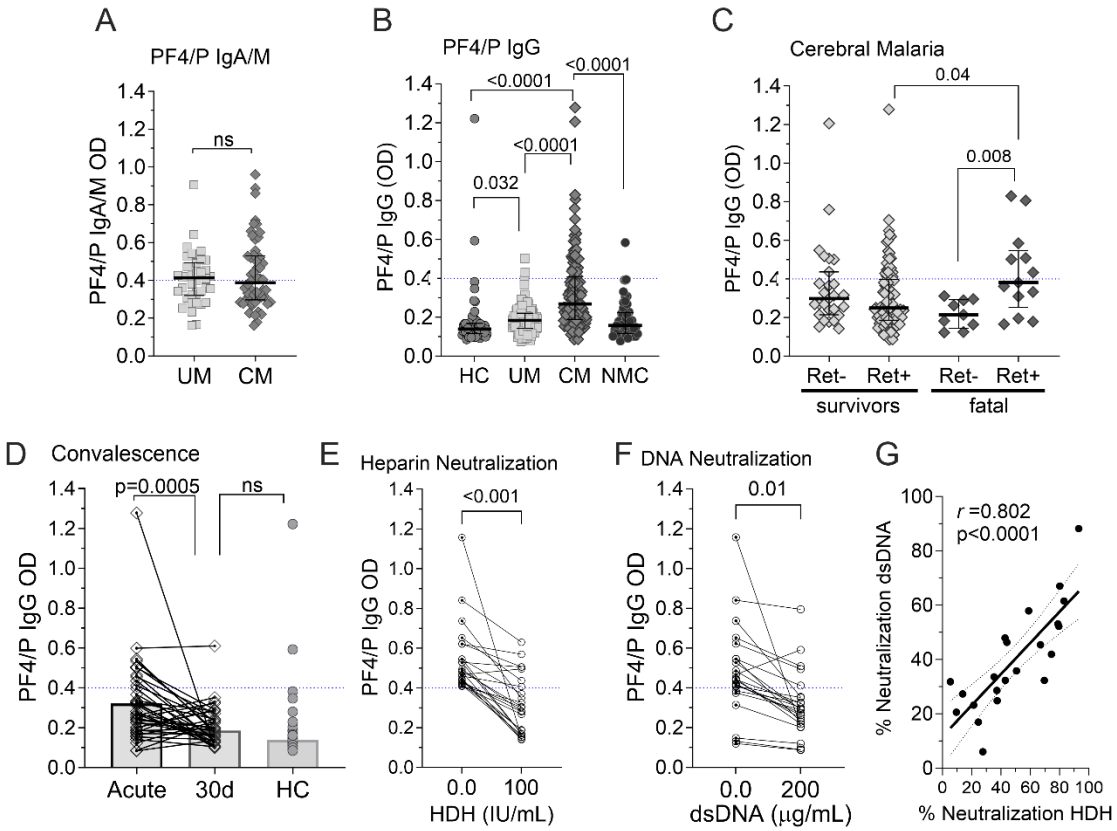

Figure 2. Anti-PF4/P IgG levels are elevated in pediatric cerebral malaria.

A) Plasma levels of anti-PF4/P IgM/IgA antibodies in uncomplicated malaria (UM, n=38) vs. cerebral malaria (CM, n=54) cases. B) Plasma levels of anti-PF4/P IgG in healthy controls (HC, n=56) vs. uncomplicated malaria (UM, n=124) vs. cerebral malaria (CM, n=136) vs. non-malarial coma controls (NMC, n=49). C) Plasma levels of anti-PF4/P IgG in CM survivors (Ret- CM, n=27); Ret+ CM n=87) and CM fatal cases (Ret- CM, n=9; Ret+ CM n=13). D) Pair-wise comparison of anti-PF4/P IgG in CM acute vs. convalescent plasma at 30 days post admission (30d) (n=39). E-F) Pair-wise analysis of neutralization of anti-PF4/P IgG binding in patient plasma with E) 100U/mL of high dose heparin (HDH, n=24) or F) 200μg/mL of dsDNA (n=24). In panels A-C, the assay cutoff threshold of OD>0.4 is depicted by a dashed blue line. Shown are median levels ± interquartile ranges, statistical significance determined by Mann-Whitney test (A, D), Kruskal-Wallis test with Dunn's multiple comparisons (B-C), and parametric paired t-test (E-F). G) Pearson correlation analysis between neutralization of anti-PF4/P binding by dsDNA vs. HDH (n=22). Shown within graph is the Pearson rho coefficient (r) and associated p-value.

Figure 3.

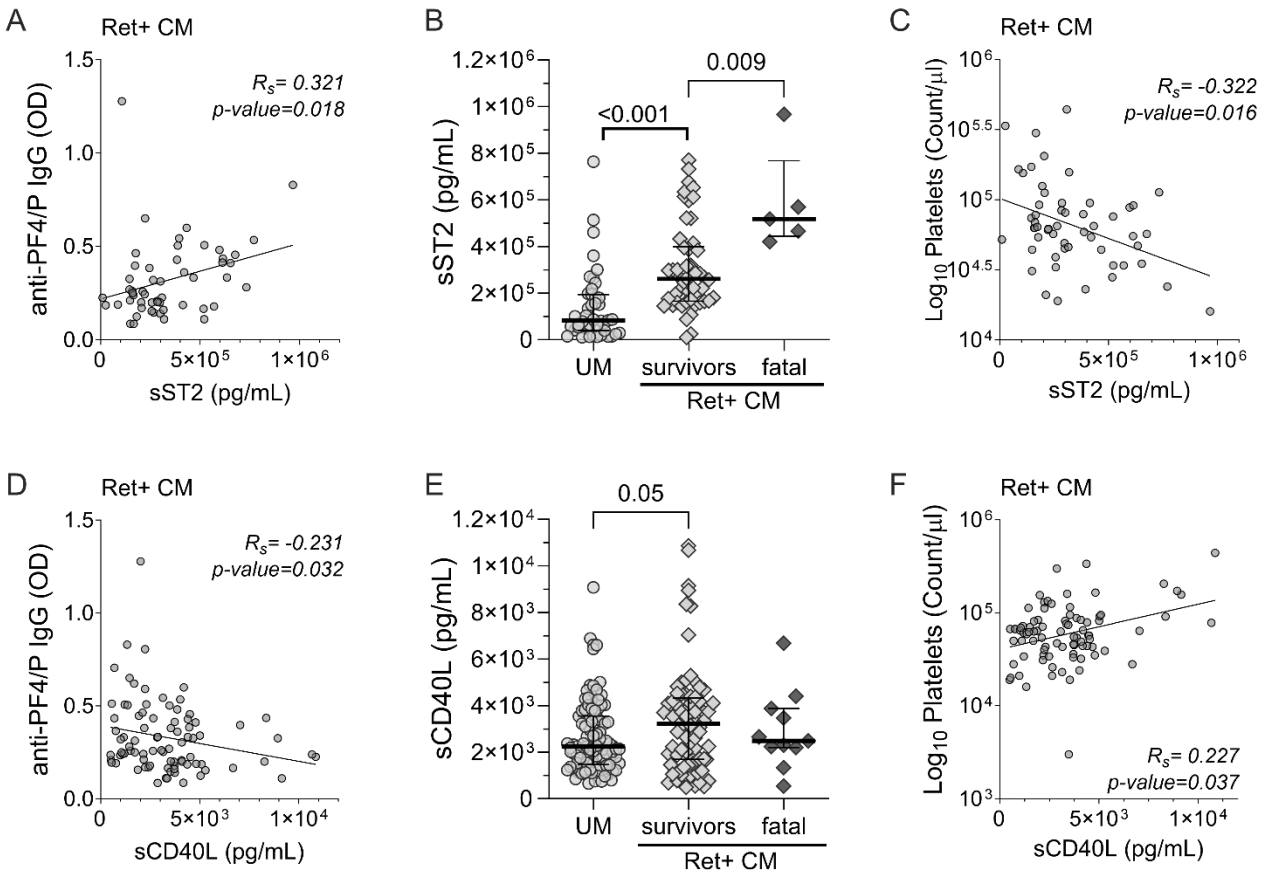

Figure 3. Soluble ST2 and CD40L levels, markers of thromboinflammation, link anti-PF4/P IgG levels in Ret+ CM with thrombocytopenia and disease outcome.

A) Spearman correlation analysis between anti-PF4/P IgG levels and soluble suppression of tumorigenicity-2 (sST2) plasma levels in Ret+ CM cases (n=54). B) Plasma levels of sST2 for UM (n=43), Ret+ CM survivors (n=50, and Ret+ CM fatal (n=5) cases. C) Spearman correlation analysis between peripheral platelet count and sST2 plasma levels in Ret+ CM cases (n=55). D) Spearman correlation analysis between anti-PF4/P IgG levels and soluble CD40 Ligand (sCD40L) plasma levels in Ret+ CM cases (n=86). E) Plasma levels of sCD40L for UM (n=100), Ret+ CM survivors (n=76), and Ret+ CM fatal (n=11) cases. F) Spearman correlation analysis between peripheral platelet count and sCD40L plasma levels in Ret+ CM cases (n=85). For A, C, D, and F the Spearman rho (R_s) coefficient and associated p-value are shown within graph. For B and E, the median with interquartile range is shown for each sample set as a horizontal bar and error bars. Statistical significance for B and E was determined by Kruskal-Wallis test with Dunn's multiple comparisons.

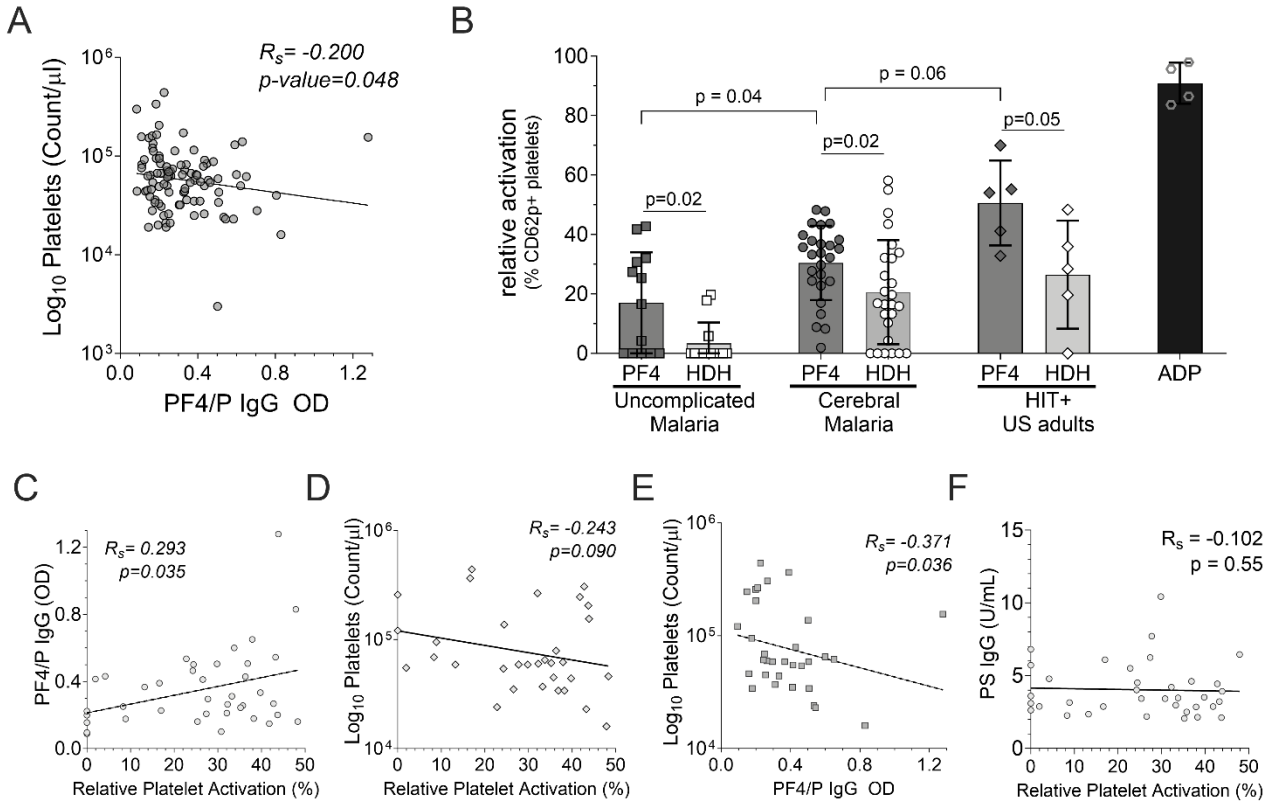

Figure 4. PF4/P IgG antibody levels in cerebral malaria patient plasma are associated with decreased circulating platelets and platelet activation.

A) Spearman correlation analysis of circulating platelet levels in Ret+ CM patients versus PF4/P IgG levels (N= 98) in plasma. Values were log transformed for linear regression analysis. B) Heterologous platelet activation assay showing relative activation levels (%CD62p/CD41) of donor platelets when incubated with plasma from either UM (N=13), CM (N=26), or HIT+ patient plasma (N=5) in the presence of recombinant hPF4 (15µg/mL), recombinant hPF4 + high dose heparin (200U/mL, HDH). Shown for each data point is the mean relative activation from 3-independent experiments. Treatment with adenosine diphosphate (ADP, 10 µM), serves as internal positive control of maximal platelet activation. Statistical significance between hPF4 treated clinical groups was determined by Kruskal-Wallis test with Dunn's multiple comparisons. Analysis within a clinical group for hPF4 treated vs. hPF4+HDH treated was determined by Welch's t-test. Bar graph represents the mean ± the standard deviation. C) Spearman correlation analysis of the relative platelet activation (x-axis) in the subset of samples from (B) plotted against corresponding PF4/P IgG levels (n=39) in patient plasma, Spearman correlation analysis of the relative platelet activation (x-axis) against (D) circulating platelet counts (n=32), and (F) against anti-PS IgG levels (n=36) in patient plasma. E) Spearman correlation of PF4/P IgG plasma levels (x-axis) plotted against circulating platelet counts (n=32) for the subset of samples from (B). Spearman rho coefficient (R_s), associated p-value, and calculated linear regression (dashed line) are shown within the graph (A, C-F).

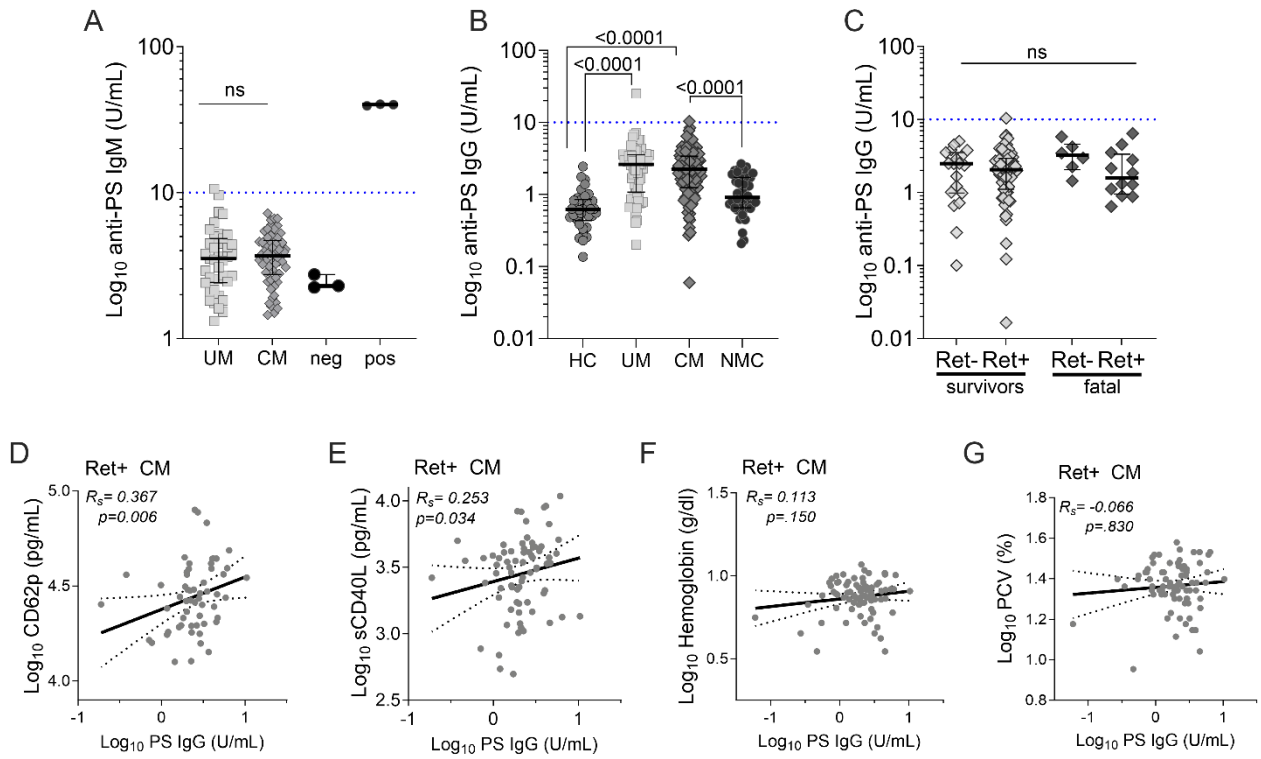

Figure 5. Anti-PS antibodies are elevated in malaria, but do not vary with severity.

A) Plasma levels of anti-phosphatidylserine (anti-PS) IgM antibodies measured in uncomplicated (UM, n=39) vs. cerebral malaria (CM, n=58). Shown are internal negative (neg, n=3) and positive (pos, n=3) controls. Dotted blue line across the y-axis at 10 U/mL represents the pre-determined assay clinical cutoff. B) Plasma levels of anti-PS IgG in uncomplicated malaria (UM, n=74) vs. CM (n=108) vs. healthy controls (HC, n=34) vs. non-malarial coma (NMC, n=48). C) Plasma levels of anti-PS IgG in CM stratified by retinopathy (ret- or ret+) status and outcome (survivors or fatal). Ret- survivors: n=19; Ret+ CM survivors: n=63; Ret- CM fatal: n=6; and Ret+ CM fatal: n=12. Shown are median levels ± interquartile ranges, statistical significance determined by Mann-Whitney test for (A), Kruskal-Wallis test with Dunn's multiple comparisons for (B) and (C). Spearman correlation analysis of soluble markers in Ret+ CM patient plasma associated with platelet activation (D-E) or anemia (F-G) in relation to circulating levels of anti-PS IgG. Values were converted to Log form to accommodate non-normal distribution. D) CD62p vs. anti-PS IgG (N=58), E) sCD40L vs. anti-PS IgG (N=73), F) Hemoglobin vs. anti-PS IgG (N=77), G) Packed Cell Volume (PCV) vs. anti-PS IgG (N=79). Shown within scatter plots are linear regression curve with 95% confidence interval in dotted line and the calculated spearman Rho (R_s) and associated p-value.

TABLES

Table 1. Demographic and clinical characteristics of malaria patients, coma control patients, and healthy controls.

Clinical Parameters	Cerebral Malaria			Uncomplicated Malaria		NMC controls		Healthy Controls	
	Ret+ (n=100)	Ret- (n=36)	p[a]	UM (n=124)	p[a]	NMC (n=49)	p[a]	HC (n=56)	p[a]
Age in months [IQR]	52 [36, 83]	40 [23-60]	0.037	44 [24, 82]	ns	66 [30, 108]	ns	48 [20, 96]	ns
Male (%)	53 (53)	17 (47)	ns	53 (59)	ns	28 (56)	ns	31 (55)	ns
Parasitemia (10^3/μL) [IQR]	6 [0.5, 108]	3.4 [1, 117]	ns	n/d	—	na	—	na	—
PfHRP2 (ng/mL) [IQR]	1496 [581, 5251]	469 [286, 1048]	<0.001	60 [21, 190]	<0.001	na	—	na	—
HGB (g/dL) [IQR]	8 [6.9, 9.1]	8 [7, 10]	ns	10 [9, 11]	<0.001	10 [8.4, 12]	<0.001	12 [11, 13]	<0.001
PCV (%) [IQR]	24 [21, 28]	27 [21, 32]	ns	34 [29, 36]	<0.001	34 [28, 39]	<0.001	n/d	—
Platelets (10^3/μL) [IQR]	60 [36, 84]	148 [60, 235]	<0.001	304 [232, 362]	<0.001	330 [199, 530]	<0.001	n/d	—
Blantyre Coma Score[b] [IQR]	2 [1,2]	2 [1, 2]	ns	UM	—	1 [1, 2]	0.04	na	—
% BCS score ≤1	41	42	ns	n/d	—	60	0.04	n/d	—
% BCS score 2	59	38	—	n/d	—	38	—	n/d	—
% BCS score 3	0	0	—	n/d	—	2	—	n/d	—
SMA	28 (28)	4 (11)	0.04	n/d	—	n/d	—	na	—
Respiratory distress	22 (22)	12 (33)	ns	n/d	—	n/d	—	na	—
Jaundice	8 (8)	0 (0)	ns	n/d	—	n/d	—	na	—
Mortality	13 (13)	9 (25)	ns	0 (0)	—	6 (12)	ns	na	—

a. P-values (p) between indicated group and Ret+ CM, Wilcoxon rank-sum for continuous measures with values shown as median [IQR] or chi-square test for categorical measures with values shown as n (%). not significant (ns, p>0.05).
b. Blantyre Coma Score (BCS), scale 0-5; minimum score of 0 (poor consciousness), maximum score of 5 (fully responsive), score < 4 (abnormal response).
IQR, interquartile range; UM, uncomplicated and by definition - no clinical symptom of severity; n/a, not applicable; n/d, no data available. NMC – Non-malarial coma controls; PfHRP2 - P. falciparum Histidine Rich Protein 2; HGB - hemoglobin; PCV - packed cell volume; SMA- severe malarial anemia.

Table 2. Prevalence of positive autoantibody levels.								
Antibody (IgG)	Cutoff value[b]	Uncomplicated Malaria			Cerebral Malaria			UM vs. CM
		% above cutoff	N	Median (IQR)	% above cutoff	N	Median (IQR)	p-value[a]
Phospholipid	10 U/mL	2	46	2.84 (2.1-3.7)	0	69	2.71 (2.0-3.6)	ns
Cardiolipin	10 U/mL	0	44	3.21 (2.5-4.2)	1.4	69	3.17 (2.4-3.9)	ns
Phosphotidylserine	10 U/mL	0	73	2.61 (1.1-3.6)	1	105	2.25 (1.2-3.4)	ns
β2 Glycoprotein I	8 U/mL	10	48	2.92 (2.4-5-0)	8	64	2.69 (2.2-3.9)	ns
PF4/P	0.4 OD	1.6	124	0.184 (0.14-0.22)	27	136	0.27 (0.19-0.43)	<0.0001
dsDNA	0.375 OD[c]	2.6	77	0.122 (0.10-0.15)	0	135	0.11 (0.10-0.13)	ns
Myeloperoxidase	5 U/mL	0.3	44	1.80 (1.6-2.0)	4.4	68	1.82 (1.6-2.1)	ns
Proteinase 3	5 U/mL	0	46	1.76 (1.6-2.0)	0	69	1.82 (1.6-2.1)	ns

a. P-value determined by Fishers Exact contingency test. b. Cutoff based on manufacturer's clinical assay predetermined levels. c. anti-dsDNA is in-house assay (118), cut off OD=0.375, represents mean + 3x SD of negative control.

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Table 3. Regression analysis of Anti-PF4/P IgG with clinical outcomes/complications in Ret+ CM.									
Variable	Odds Ratio	OR 95% CI	Beta	Beta 95% CI	Beta p-value	AUC	AUC p-value	N total analyzed	N with complication
Severe Malaria Anemia	1.95	0.18 - 18.6	0.67	-1.69 to 2.93	0.561	0.56	0.324	100	28
Respiratory Distress	8.96	0.84 - 114	2.19	-0.176 to 4.73	0.073	0.64	0.040	100	22
Jaundice	5.98	0.18 - 128	1.79	-1.72 to 4.85	0.256	0.64	0.195	100	8
Death	15.18	1.02 - 275	2.72	0.02- 5.6	0.048	0.67	0.045	100	13

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