Journal of Blood Medicine

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ORIGINAL RESEARCH

Influence of α_2-Macroglobulin, Anti-Parasite IgM and ABO Blood Group on Rosetting in *Plasmodium falciparum* Clinical Isolates and Their Associations with Disease Severity in a Ghanaian Population

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Purpose: The severity of *Plasmodium falciparum* infections is associated with the ability of the infected red blood cells to cytoadhere to host vascular endothelial surfaces and to uninfected RBCs. Host blood group antigens and two serum proteins α_2-macroglobulin (α_2M) and IgM have been implicated in rosette formation in laboratory-adapted *P. falciparum*. However, there is only limited information about these phenotypes in clinical isolates.

Methods: This was a hospital-based study involving children under 12 years-of-age reporting to the Hohoe Municipal Hospital with different clinical presentations of malaria. Parasite isolates were grown and rosette capabilities and characteristics were investigated by fluorescence microscopy. α_2M and IgM were detected by ELISA.

Results: Rosette formation was observed in 46.8% (75/160) of the parasite isolates from all the blood groups tested. Rosettes were more prevalent (55%) among isolates from patients with severe malaria compared to isolates from patients with uncomplicated malaria (45%). Rosette prevalence was highest (30%) among patients with blood group O (30%) and B (29%), while the mean rosette frequency was higher in isolates from patients with blood group A (28.7). Rosette formation correlated negatively with age ($r = -0.09$, $P= 0.008$). Participants with severe malaria had a lower IgM concentration (3.683±3.553) than those with uncomplicated malaria (5.256±4.294) and the difference was significant ($P= 0.0228$). The mean concentrations of anti-parasite IgM measured among the clinical isolates which formed rosettes was lower (4.2 ±3.930 mg/mL), than that in the non rosetting clinical isolates (4.604 ±4.159 mg/mL) but the difference was not significant ($P=0.2733$). There was no significant difference in plasma α_2M concentration between rosetting and non rosetting isolates ($P=0.442$).

Conclusion: *P. falciparum* parasite rosette formation was affected by blood group type and plasma concentration of IgM. A lower IgM concentration was associated with severe malaria whilst a higher α_2M concentration was associated with uncomplicated malaria.

Keywords: *Plasmodium falciparum*, severe malaria, uncomplicated malaria, rosettes, blood group, alpha-2 macroglobulin, immunoglobulin M

Introduction

Despite the recent decline in *P. falciparum* infections globally, malaria continues to affect the African continent with most of the disease burden and mortalities occurring in this region.[1] Case incidence of malaria among children under 5 years-of-age was estimated to be 225/1000 individuals at risk in 2019, which was a slight decline from the year 2000 estimate. Malaria deaths in children less than 5 years declined between year 2000 and 2019 by 17% worldwide.[1]

Received: 24 August 2021
Accepted: 7 March 2022
Published: 18 March 2022

Plasmodium falciparum continues to be the dominant species in the sub-region, where the climate favors the *Anopheles* vector.[2] *P. falciparum* is virulent and is responsible for the majority of malaria mortality and severe morbidity.[1,3]

The pathogenesis of *P. falciparum* malaria is directly linked to the ability of the parasite to express variant surface antigens (VSA) on the surface of IRBCs, which allows the parasites to establish long-lasting chronic infection.[4] Since the publication of the full genome of *P. falciparum* three major families of variant genes have been studied extensively.[5,6] The proteins expressed by these variant genes include Erythrocyte Membrane Protein 1 (PfEMP1); RIFIN (Repetitive Interspersed family of polypeptides) proteins; STEVOR (Sub telomeric variable open reading frame) proteins.[7] In *P. falciparum* infection, the VSA expressed is mainly for evading the host immune responses by allowing IRBCs to sequester in tissues, thereby avoiding their removal by the spleen.[8,9] These parasite ligands (RIFINs, STEVOR, PfEMP1) have all been studies and known to be released onto IRBC at specific times in the parasite life cycle. PfEMP1 is transcribed at the ring stage and exported to the surface of the IRBC at the late trophozoite stage whilst RIFINs and STEVORs are transcribed and expressed later in the pigmented trophozoite and schizont stages.[10–12]

The ability of the IRBC to attach to uninfected RBCs is another binding process, known as rosetting, which has been found to be associated with severe malaria in African children.[13–15] Some host factors including complement receptor 1 (CR1), heparan sulphate (HS), blood group antigens and recently host serum proteins such immunoglobulin M (IgM) IgM and α_2M have been identified to be associated with rosette formation.[15–17] For instance, polymorphism in CR1, thalassemia and red blood cells such as the AS trait has been shown to confer protection against severe malaria.[18–20] Another important biological role of PfEMP1 is their affinity for binding to blood group A and B antigens, which has also been linked to severe malaria.[21] Various studies have shown the involvement of IgM as one of the serum proteins involved in the attachment of rosette formation.[22,23] Whilst IgM remains the largest and first immunoglobulin to appear in response to malaria antigens, the structure of IgM and its pentameric nature make it an effective immune effector molecule.[16,24] Previous studies have implicated serum IgM in rosette formation in children and their contribution to the development of severe malaria.[15,25]

Another serum protein identified to be involved in rosette formation is α_2M, which is a non-immunoglobulin molecule and one of the most abundant proteins found in peripheral blood circulation in humans.[16,25] α_2M is a protease inhibitor, which is produced by the liver and locally synthesised by macrophages, adrenocortical cells and fibroblast and part of the innate immune system.[26] In humans, α_2M has been demonstrated to play an important role in maintaining homeostasis of cytokines and growth factors.[27] Increased α_2M plasma levels have consistently been seen during periods of life characterized by growth, development, and differentiation such as embryogenesis, pregnancy, and childhood.[26,28]

Laboratory-adapted parasites strains[16] and patient parasite isolates[25] have previously been used to show that serum α_2M works in synergy with IgM to increase avidity of binding multiple PfEMP1 molecules on the IRBC surface to mediate rosetting. This study seeks to investigate further the associations of anti-parasite IgM and α_2M plasma concentrations, as well as the effect of ABO blood group types on rosette formation among field isolates and their association with severity of malaria infection.

Materials and Methods

Ethical Statement

Ethical clearance for this study was obtained from by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, Ghana (Study number 026/13-14) and the Ethical Review Committee of the Ghana Health Service (MOH; file GHS-ERC 08/05/14).

The study complied with the Helsinki declaration for conducting research involving human subjects. Written informed consent was obtained from each parent/guardian of all the study participants.

Study Design and Population

The study was cross-sectional and involved 206 children less than 12 years of age who were admitted to or attended the outpatient department (OPD) of the Hohoe Municipal Hospital with different malaria pathologies (uncomplicated malaria and severe malaria). In this study, severe malaria was defined as a child presenting with signs and symptoms of malaria and in the presence of *P. falciparum* asexual parasitaemia with one or more of either impaired consciousness (Blantyre coma score of <3), acidosis, hypoglycaemia, hyperparasitaemia, renal impairment (acute kidney injury), severe malarial anaemia (haemoglobin concentration of <5 g/dl), Jaundice and others as defined by the Physician in-Charge. Uncomplicated malaria was also defined as children presented with both microscopy and RDT positive for malaria but with no features of severe malaria.

Rosetting is defined as the binding of uninfected red blood cells to a centrally *Plasmodium falciparum* infected red blood cell. Different categories of resetting include small rosette defined as the binding of an infected RBC by 2 to 3 uninfected RBCs. A medium rosette is a *Plasmodium falciparum* infected RBC bound to 4 or 5 uninfected RBCs. A large rosette is an infected RBC bound by more than 5 uninfected RBCs. In this study, rosette frequency is defined as the percentage of total mature-parasite-infected cells forming rosettes against two hundred cells infected with matured parasites whilst rosette prevalence referred to proportion of the total parasite isolates collected throughout the study period that formed rosettes.

Sample Collection

In brief, the study was explained to the parents/guardians after which informed consent was sought from them. Approximately 3 mL of whole blood was collected after rapid diagnostic test (RDT) gave a positive result. In addition, the presence of parasites when viewed under a light microscopy confirmed malaria infection and a PCR test was used to confirm the *Plasmodium falciparum* malaria cases and differentiation of species of mixed infection cases. Haemoglobin levels were measured using automated haematology analyser (Sysmex KX-21N) and blood groups determined using standard protocols as described elsewhere.[29] The blood samples were centrifuged and separated into infected red blood cells and plasma. The plasma was stored in a −80°C freezer until ready to be used for the measurement of IgM and α_2 M levels by ELISA. Approximately, 200uL of the patients' pellet RBC was e used for short term in vitro cultures in the patients' own blood and the rest stored in liquid nitrogen for long term storage.

ABO and Rhesus Blood Grouping

Approximately three drops (20 µL) of whole blood were put on a clean white tile. The blood was individually mixed with an equal volume of anti-A, anti-B or anti-D (Fortress Diagnostic Limited, UK.) after which, the tile was rocked gently for 5–10 minutes. The blood drops were observed for agglutination. The observation of agglutination in the blood drops indicated the presence of rhesus (Rh) antigen corresponding to the anti-sera used. Lack of agglutination for both A and B indicated the blood group O.

Parasitemia Estimation by Light Microscopy

Thick and thin blood smears were prepared on microscopic glass slide for all RDT confirmed malaria positive samples. The thick blood smear was prepared by placing a drop of blood on the slide and spreading it out evenly in a circular motion. On the same slide, a thin blood smear was also prepared by placing a drop of blood on the slide and using another slide held at 45 degrees to the drop on the first slide. The thin film side was immersed in methanol (VWR, USA) to fix the cells and dried whilst the thick film was only air dried. The dried slides were stained with 10% Giemsa stain (Merck, Germany) for 15 minutes. Slides were washed under running water and air dried. A drop of immersion oil (Sigma Aldrich, USA) was put on the slide and observed under a light microscope at 100X objective lens. The parasitaemia was calculated as the number of parasites divided by the number of white blood cells all multiplied by 8000 and the species differentiation was done to confirm that sample was positive for *P. falciparum*.

Short Term in vitro Cultivation of Fresh *Plasmodium falciparum* Clinical Isolates

The *Plasmodium* parasite isolates were maintained in short term culture (one cycle) in the erythrocytes of the participants with RPMI-1640 medium supplemented with 1% L-glutamine, (Sigma Aldrich, USA, Cat no. R8758) (50 µg/m gentamycin (Thermo Fisher, Cat no. 15750060), 50 mg/L hypoxanthine (Sigma Aldrich, UK, H9377), and 10% heat-inactivated type AB^+ human serum (Sigma Aldrich, USA, Cat no. H6914). Cultures were incubated at 37 °C with a gas mixture of 5% O_2, 2% CO_2, balanced with N_2 (Air Liquide, UK, Cat No. 4687). The parasites were maintained until the late trophozoite and schizont stage when the rosette capability was assessed using a fluorescent microscope.

Rosette Formation Analysis by Fluorescence Microscopy

Rosetting was assessed in the first cycle of the in vitro growth when parasites reached late trophozoite stages. In brief, aliquot of 100 ul of the parasite culture was picked from the 5mL culture flask into a 1.5mL tube followed by the addition of 2ul of 25ug/mL ethidium bromide and incubated for 15 minutes at 37°C. About 10uL of the ethidium bromide-stained parasites were pipetted onto a fluorescence microscope slide and covered with a coverslip and read under fluorescence and at X40 magnification using Olympus BX 43 (Olympus, Japan). Rosettes were counted per 200 infected red blood cells and images recorded. Isolates rosettes were then categorized into small, medium and large based on the number of uninfected RBCs bound to a centrally IRBC. In isolates with different sized rosettes, the predominant rosette type is used to categorise it.

Measurement of Alpha-2-Macroglobulin Concentration Using ELISA

The plasma concentration of α_2M was measured using a commercial ELISA kit (RAB 0600: Sigma Aldrich) following the manufacturer's instructions and protocols. In brief, samples (diluted at 1:2000), controls, and standards were added to pre-coated plates at 200 µL/well. Plates were incubated for two and half hours at room temperature with gentle shaking at 200rmp. Plates were washed with 300uL of wash buffer and detection antibody was added at 100 µL/well and incubated for one hour at RT. Streptavidin solution was added at 100µL/well and incubated for 45 minutes. The plates were developed with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate reagent at 100 µL/well for 30 minutes in the dark. Reaction was stopped with 50 µL of Stop solution and plate read immediately at 450 nm.

Measurement of Plasma Anti-Parasite IgM Concentrations Using ELISA

The plasma concentration of anti-parasite IgM was measured using indirect ELISA technique. High binding 96 - well ELISA plates (Nunc Maxisorp, Thermo Fisher Scientific) were coated with soluble crude malaria antigen (0.5 X 10^6 schizonts/ well) at 50ul/well and incubated at 4°C overnight. Plates were retrieved and washed 3X with 200ul/well of washing buffer (PBST; 1X PBS supplemented with 0.05% Tween 20 at pH 7.2) and blocked with 200ul per well blocking buffer (5% non-fat dry milk (Marvel, UK) in PBS) and incubated for 1 hour at room temperature on a shaker (150rpm). Plasma and controls were added at 50ul/well diluted 100X in assay buffer (1% milk/PBS). Positive control (pool of hyperimmune Ghanaian adults) and negative controls (pool of unexposed Europeans) were added alongside the samples in their respective wells for each plate. The plates were incubated for 1 hour at room temperature. Anti-parasite IgM was detected using 100ul/well of goat anti-human IgM conjugated Horseradish Peroxidase (HRP-IgM) (Invitrogen, USA) at a dilution of 1:1000 for 1 hour. Plates were developed with 50L/well TMB for 10 minutes and stopped with 50ul/well of 0.2M H_2SO_4. The optical density was read at 450nm Biotek plate reader (VT, USA) and converted to concentrations using ADAMSEL (version b040; Ed Remarque).

Statistical Analysis

Spearman rank correlation was performed to analyse the associations between the clinical variables and the concentration of anti-parasite IgM or α_2M. Clinical isolates were stratified as rosetting and non rosetting and differences between the clinical variables were compared by students *t*-test and Mann Whitney test where appropriate. A linear regression analysis was used to determine the relationship between host variables and rosette formation. $P<0.05$ were considered statistically significant. All the data collected was analysed using Graph pad prism 5.0 (Graph pad Software, Inc).

Results

Clinical Characteristics of Study Population

A total of 230 children were enrolled into the study over a two-year period. In the first year, 116 participants were recruited whilst the second year had 114. At the end of recruitment, the data base was cleaned to exclude all participants who were positive by RDT but had no parasites present in their sample after light microscopy. Most of them had taken medication before hospital visit and their in vitro cultures were not successful. Based on our criteria for participant enrollment, malaria cases were carefully classified into either severe or uncomplicated. There were more males (110) than females (96) in the study and the mean age of the children was 5.0 years (7 months −11 years). The mean haemoglobin level was 9.3g/dL (3.6–13.9g/dL) for the general population. When this was stratified, anaemia was seen in 90 (43.6%) whilst severe anaemia was seen in 4 (1.9%) of the study participant. In 52 (25%) of the participants, parasite density (parasitemia) was above 100,000 parasites /ul. Most of the participants were of blood group O type (94/206). The clinical characteristics of participants with severe cases was compared to those with uncomplicated cases. The mean age for the uncomplicated malaria was 5 years whilst for the severe cases it was 3.8 years. Haemoglobin level was lower in the severe cases (8.4g/dL) than the uncomplicated (10.3g/dl). There was no significant difference in the mean parasitemia between the uncomplicated and the severe cases (P = 0.31). Patients with severe disease had a lower body weight than those with uncomplicated malaria (Table 1).

Rosette Capabilities Among *P. falciparum* Clinical Isolates

Rosetting assays were run on parasite isolates when they were at the trophozoite stage at a parasitemia of >2%. Infected RBC are shown as bright spots not bound by uninfected RBCs (Figure 1A). Rosettes are shown as IRBCs (bright spot) bound by two or more uninfected RBCs as observed under the fluorescence microscope (Figure 1B). Rosettes were categorized as small (Figure 1C), medium (Figure 1D) or large, defined by the number of uninfected RBCs bound to the IRBC. Out of the 206 parasite isolates put into short term culture, 160 were successful and were used for the rosetting assay. Rosette formation was observed in 75 (46.8%) of the clinical isolates comprising 42 from the severe malaria category and 33 from the uncomplicated malaria category but the difference in rosette formation capabilities was not statistically significant (P=0.3). Rosetting rate of 56% (42/75) among parasites from severe disease cases was higher than those found among uncomplicated malaria cases (44%, 33/75). Majority (30) of the samples had large rosettes followed by those with small rosettes (24) with 21 of them forming medium rosettes (Figure 2).

Table 1 Clinical Characteristics of Study Participants

Parameter	Severe Malaria N = 79 Mean (±SD)	Uncomplicated Malaria N =127 Mean (±SD)	P values
Sex			
Male n (%)	47 (22.8%)	65 (31.5%)	
Female n (%)	32 (15.5%)	62 (30.1%)	
Age (years)	3.79 (±1.97)	5.0 (±2.93)	0.006
Haemoglobin (g/dl)	8.4 (±2.29)	10.3 (±1.95)	**0.0001**
Body Weight (kg)	14.7 (±4.92)	17.1 (±8.07)	**0.014**
Body Temperature (°C)	38.7 (±1.44)	38.6 (±1.23)	0.530
α_2M conc (mg/mL)	2.27 (± 0.16)	1.916 (± 0.1814)	0.149
Parasitemia (Parasites /ul)	77,274 (± 14,124)	58,196 (± 12,292)	0.3110
Glucose (mmol/dl) ±SD	6.207 ± 0.1730	7.135 ± 0.2609	**0.0041**
Blood Group Type n (%)			
A	18 (8.73%)	29 (14.1%)	
B	32 (15.5%)	25 (12.1%)	
AB	4 (1.9%)	4 (1.9%)	
O	44 (21.4%)	50 (24.3%)	

Notes: Values are represented as mean and standard deviation or proportions between groups. Values highlighted represent comparison with significant P-values < 0.05. Significant P-values are highlighted in bold.
Abbreviations: N, number; SD, standard deviation.

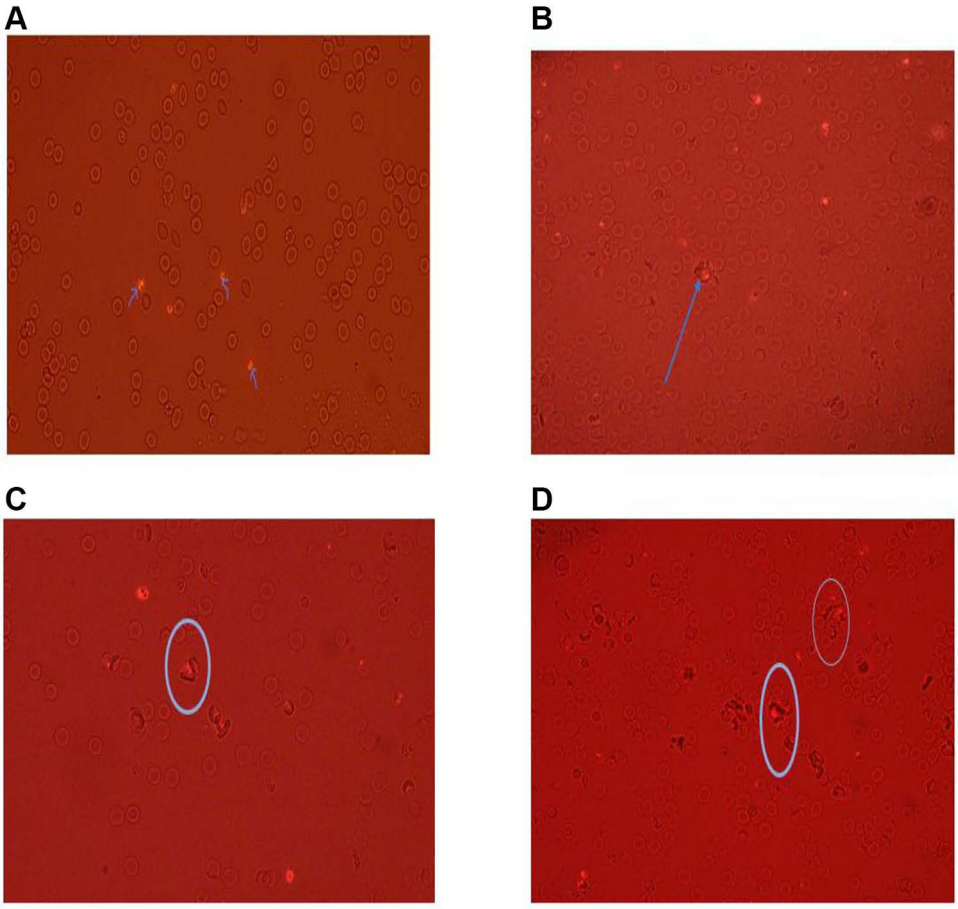

Figure 1 Micrographs of rosetting parasites. (**A**) infected RBC with parasite showing as bright spots, (**B**) infected RBC bound by uninfected RBC, (**C**) small rosette with 2 or 3 uninfected RBC and (**D**) large rosette with >3 uninfected RBCs.

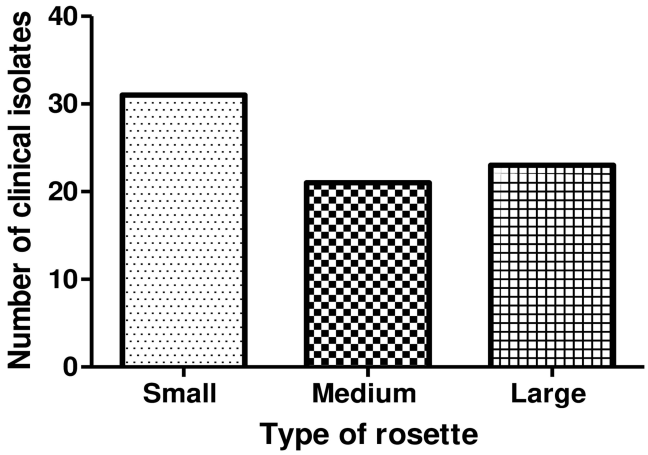

Figure 2 Categorizing Parasite Isolates by their rosetting capabilities. Large rosettes = infected RBC bound by > 5 uninfected RBCs, Medium = bound by 3–5 uninfected RBCs, Small rosettes = bound by 2 or 3 uninfected RBC.

Interestingly, when the rosettes were categorized according to the source of the parasite isolate, whether from severe malaria or uncomplicated malaria, it was observed that small rosettes were from parasites collected from severe malaria patients and the difference was statistically significant (P=0.015) (Table 2).

Table 2 Rosette Categorization in Severe and Uncomplicated Malaria Isolates

Rosette Categorization	SM	UM	P value
Large rosettes	14	16	0.3
Medium rosette	10	11	0.2
Small rosette	18	6	**0.015**
Total	42	33	

Note: Significant P-values are highlighted in bold.
Abbreviations: SM, severe malaria isolate UM, uncomplicated malaria isolate.

Rosette Formation Was Observed in All Blood Group Types in Different Proportions

Rosette formation was observed in all blood groups with rosettes frequently seen in isolates from blood group O followed by blood group B type. Rosetting frequency was highest in parasite isolates from blood group A. There was no statistical difference in mean rosetting frequency among the blood groups (P=0.5). Rosette characteristics were different among the blood groups.

Large rosettes (bound by >5 uninfected RBC) were seen in blood group types A and B whilst group O showed smaller rosettes (Figure 3).

Rosetting Capabilities of *P. falciparum* Clinical Isolates from Different Disease Categories in Different Blood Group Types

Rosette formation was seen in all four blood group types in different proportions, but the number of rosettes did not differ significantly between disease categories (Figure 4). The chance of finding a rosette (rosette prevalence) among the clinical isolate was higher in blood group O type (30.1%) and blood group B type (29.8%). On average, blood group A had the highest mean frequency (rosettes per 200 IRBC) of 28.7 rosettes per isolate as compared to 16.7 rosettes per isolate observed among blood group O type isolates. The highest number of rosettes per isolate was recorded in blood group B followed by A with the least in blood group AB. Generally, there was rosette formation among parasites from all disease categories grown in all blood group types with the only exception being among blood group AB where resetting was not observed in the severe malaria group (Figure 4).

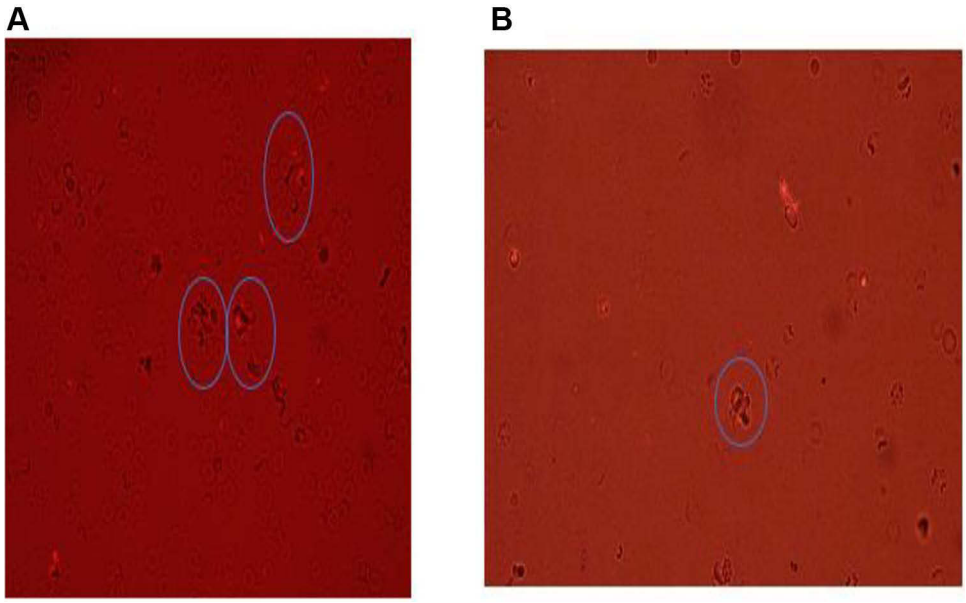

Figure 3 Micrograph showing parasite isolate rosettes from (**A**) blood group A type rosette and (**B**) blood group O type. (**A**) Large rosettes seen in blood group type B (**B**) Small rosettes seen in blood group type O.

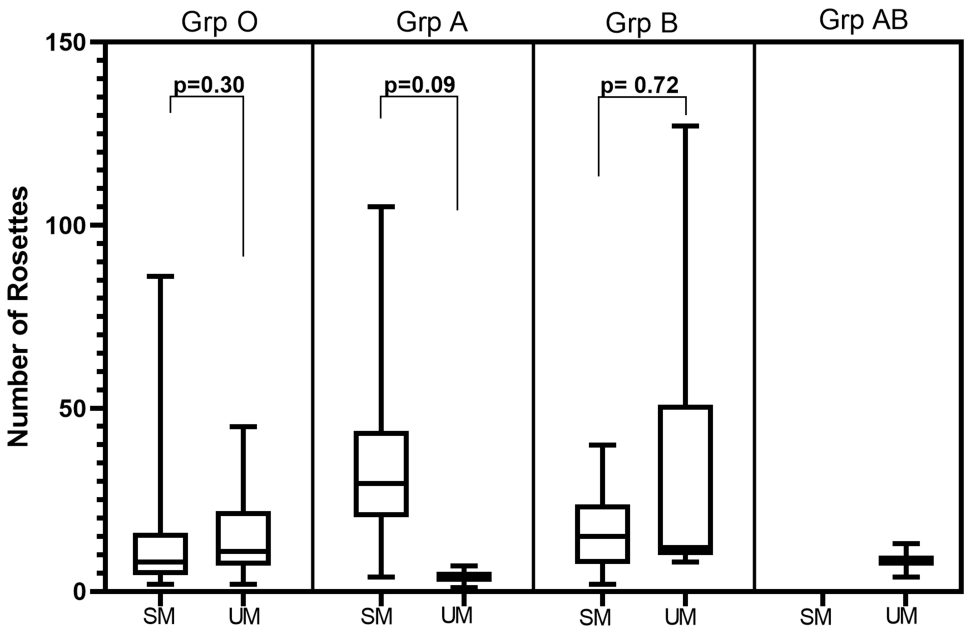

Figure 4 Association between number of rosettes and disease categories in different ABO blood group types. The middle line represents the median and the lower and upper bars are the minimum and maximum number of rosettes.
Abbreviations: SM, severe malaria; UN, uncomplicated malaria; Grp, group.

Host Factors That Affect Rosette Formation

When the measured clinical variables were analysed between rosetting and non rosetting clinical isolates, it was observed that parasitemia was higher among rosetting isolates compare to non rosetting isolates (P=0.002) (Table 3). The age of the participant was not associated with rosetting although resetting was commonly found among parasite isolates from younger children than parasites from older children (p<0.9) (Table 4). The relationship between rosette formation and measured parameters were assessed using a linear regression analysis.

Parasitemia and Rosette Formation

When the measured clinical variables were analysed between rosetting and non rosetting clinical isolates, it was observed that parasitemia was higher among rosetting isolates as compare to non rosetting isolates (P=0.002) (Table 3).

Association of Plasma Concentrations of α_2M on Rosette Formation and Malaria Severity

The α_2M concentration weakly correlated positively with rosette frequencies (r=0.080 but the difference was not significant (p=0.52) (Figure 5A). Although α_2M concentration was found to be higher in uncomplicated malaria (mean = 2.27 mg/mL) than in severe malaria (mean=1.92 mg/mL), (P = 0.2), the difference was not statistically significant. A significantly higher α_2 M concentration was observed in patients with a higher parasitemia (P= 0.02) (Table 4).

Table 3 Effect of Patient Variables on Rosetting and Non Rosetting Isolates

Parameter	Mean Values Non Rosetting	Mean Values Rosetting	P-values
Malaria parasites (parasites/uL)	65,403	74,014	**0.002**
Haemoglobin (g/L)	9.621	10.19	0.4
Age	4.695	4.698	0.2

Note: Significant P-values are highlighted in bold.

Table 4 Association Between Patients' Parameters and Plasma Levels of α_2M Concentration

Parameter	r	P
Age (years)	4.752e-005	0.9
Glucose (mmol/L)	0.0006348	0.8
Haemoglobin levels (g/dl)	0.0003071	0.9
Disease category	0.02139	0.2
Parasitemia (parasite/uL)	0.123	**0.02**
Weight	0.003972	0.6

Notes: Spearman rank correlation analysis of patient parameters and the concentration of α_2M. Significant P values have been highlighted. Plasma alpha 2 macroglobulin concentration in study participants of varying clinical presentations and rosette formation. Significant P values are highlighted in bold.

Also, in correlation analysis of non resetting parasite isolates, only parasitemia was found to correlate with α_2 M concentration (P= 0.04) whilst age and hemoglobin levels did not show any significant association (Table 5).

To determine the effect of α_2M concentration on rosette formation, the mean plasma concentration of α_2M was compared between rosette formation by isolates from severe and uncomplicated malaria patients. It was observed that the

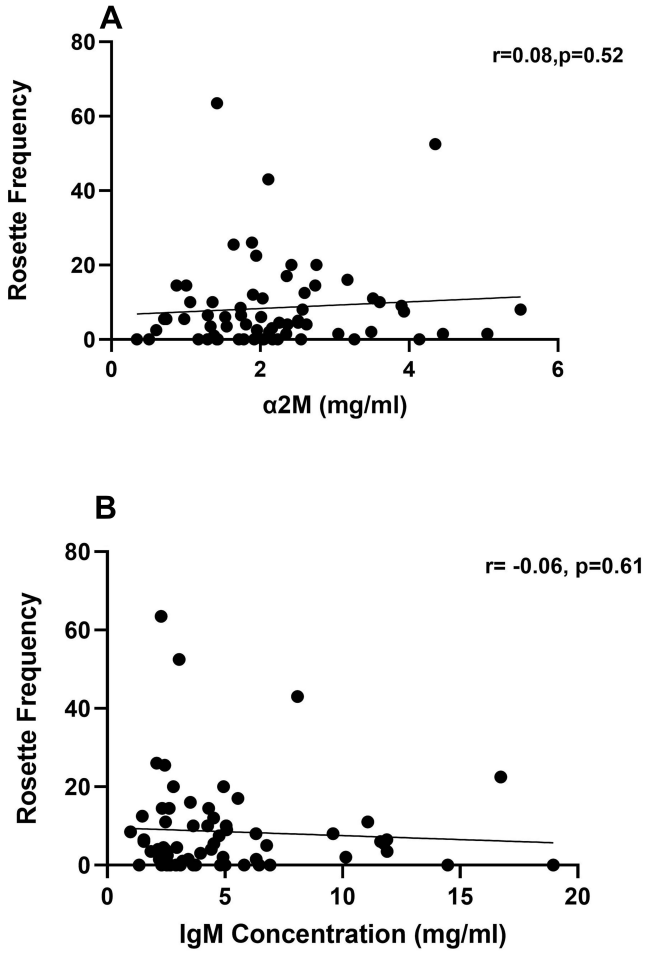

Figure 5 Scatter plot showing the relationship between rosette frequency and plasma a2M and IgM concentrations. (**A**) scatter plot of rosette frequencies and plasma A2M concentrations and (**B**) scatter plot of rosette frequencies and plasma anti-parasite IgM concentrations.

Table 5 Association of α_2M Concentration on Non Rosetting Isolates

	r	P value
Age (years)	0.091	0.6
Haemoglobin levels (g/dL)	0.223	0.2
Parasitemia (IRBCs/μL)	0.334	**0.04**

Note: Spearman Rank correlation analysis of non rosetting isolates only. Significant P values are highlighted in bold.

Table 6 Association of Anti-Parasite IgM Concentration on Clinical Variables Among Rosetting Parasite Isolates

	Spearman r value	P value
Haemoglobin	0.3012	**0.02**
Number of rosettes	0.0039	0.5
Parasitemia	0.1260	0.2
Age	0.1793	0.1

Notes: Spearman Rank correlation of anti-parasite IgM and measured parameters among the rosetting isolates only. Significant values are highlighted in bold.

mean concentration of rosetting isolates [2.08 mg/mL (0.3mg/mL - 5.4mg/mL)] was similar to that of non rosetting isolates [2.02mg/mL (0.3mg/mL - 4.9mg/mL)] (P=0.3534) (Figure S1).

Association of Plasma Concentration of Anti-Parasite IgM on Rosette Formation and Disease Severity

The anti-parasite IgM concentrations did not correlate with rosette frequencies among all the isolates studied (Figure 5B) although a weak negative correlation was observed it was not statistically significant (r=−0.06, p=0.61). Also, the mean plasma concentration of anti-parasite IgM among the study population was 4.5 mg/mL (±4.0). Males had higher mean anti-parasite IgM levels (4.6±4.4 mg/mL) compared to the females (4.2±3.7 mg/mL), P<0.05. Participants with severe malaria had a lower mean anti-parasite IgM concentration (3.7±3.6mg/mL) as compared to those with uncomplicated malaria (5.3±4.3) and the difference was statistically significant (P=0.02) (Figure S2).

The mean plasma concentration of anti-parasite IgM measured among patients with rosetting forming isolates was lower at 4.2 (±3.930) as compared to the mean anti-parasite IgM concentration of (4.604 (±4.159) from patients whose parasite isolates did not form rosettes.

In correlation analysis, among the rosetting clinical isolates, anti-parasite IgM concentration was affected by only haemoglobin levels (P=0.02) whilst all the other variables were not significantly affected by the anti-parasite IgM concentration (Table 6).

Discussion

Studies have shown that among patients, rosetting rate is dependent on the disease category of either severe or uncomplicated and that among severe malaria, typically therefore, rosette formation is more pronounced in severe malaria than in uncomplicated malaria cases. In this study, rosette formation was observed in 46.8% (75/160) of the parasite isolates tested. This level of rosetting is lower as compared to higher rosetting rates found in other studies,[13] but not unexpected as this could be as a result of the relatively fewer number of isolates obtained from the participants with severe malaria in the study. Even so, rosetting rate was higher (56%) among the severe malaria cases compared to the uncomplicated (44%). The difference was however not statistically significant. This agrees with other studies which

showed that among severe malaria cases, there was higher rate of rosetting compared to uncomplicated malaria, though rosette formation was found in all categories of disease in different proportions.[13,25,30,31]

A number of reasons could account for the lower rosetting rate observed among the parasite isolates. Barragan et al showed that among children from Kenya and Gabon, repeated malaria attacks in high malaria transmission areas resulted in exposure to antigens which prompted an antibody mediated response that recognises many rosetting antigens.[32] These anti-rosetting antibodies produced in response to repeated exposure to parasite ligand has been linked to a reduced rosetting rates and gradual acquisition of immunity in children.[33] In support of this, others found high levels of antibodies to a parasite ligand (NTS-DBL1α) known to be associated with rosetting in children in malaria endemic areas[34,35] which resulted in a reduced level of rosetting in the clinical isolates from that endemic community.

Parasite ligand PfEMP1 being expressed by *var* genes have the ability to produce clonally different phenotypes of the genes (antigenic variation) with different binding affinities that protect the parasite from immune clearance.[36] Other factors such as host immunity, genetics and polymorphisms in certain host factors may be involved in the development of severe malaria aside rosetting.[37]

Rosette formation was enhanced by blood group A antigens and the age of the child. This is consistent with a study done elsewhere which supports the high frequency of rosetting found in isolates from blood group A type.[21] Though rosette formation was prevalent in groups O and B, it was blood group A isolates that produced the largest rosettes supporting similar findings in other studies.[21] Remarkably, small rosettes were associated with severe disease compared to large ones but this is contrary to other studies where larger rosettes were found to be associated with severe malaria.[14,38] The dominant blood group in our study area was blood group O and this blood group has been associated with formation of small sized rosette and in essence protect against severe disease.[39]

Parasitemia also affected rosette formation and there is conflicting data on the effect of parasitemia on rosetting among African children. There is conflicting data on the effect of parasitemia on rosetting among African children. Whilst some studies found positive correlation between parasitemia and rosetting,[14,40,41] other studies show no association of rosette formation with high parasitemia.[42,43] Our findings agree with studies by Marsh et al, as well as the Heddini and later Rowe which showed a positive correlation between parasitemia (parasite densities) and rosetting in African isolates.[13,41,42] A previous study conducted in India which investigated DBL-α (parasite ligand that support rosetting) diversity in severe disease in Odisha population showed significantly higher parasitemia among rosetting isolates.[44] These findings implicate rosette formation to contribute to elevated parasite densities that favor invasion of the host cells from bursting schizont.[14] Therefore, the elevated parasitemia seen among the rosetting isolate is probably advantageous to ensuring the survival and replication of the parasite.[12,45]

Our study showed a lower anti-parasite IgM concentration among rosetting isolates and this was positively correlated with rosette frequency consistent with findings by others that showed a positive association between rosette frequency and IgM concentrations in Kenyan children.[15,46] Conversely, higher anti-parasite IgM concentrations were found among patients whose parasite isolate did not form rosettes and this supports recent findings that show that high IgM concentrations among children in endemic areas is protective against severe malaria.[16,47,48]

A higher mean α_2M serum concentration was found among uncomplicated malaria patients than severe malaria patients, who had a lower mean α_2M concentration. Similar observations were made in a study in Thailand where higher α_2 M concentrations were found in women with malaria parasites compared to women who did not have parasites.[49] Increased α_2M concentration has also been shown in children suffering from "chagasis" (*Trypanosoma cruzi* infection) in Bolivia.[50]

A high α_2M concentration among the non rosetting isolates contradicts findings elsewhere which showed otherwise.[16] However, α_2M concentration was positively associated with parasitemia among non rosetting isolates only, a phenomenon not observed among the rosetting isolates studied. During malaria infections, merozoites use proteolytic enzymes in re-invading red blood cells whilst schizont degradation of hemoglobin involves the use of these same proteolytic enzymes. Therefore, α_2M is produced by the body in response to high proteolytic enzymes to binds irreversibly to these proteolytic enzymes and protecting the tissues from tissue damage.[51] The findings also shows that α_2M may elicit high parasitemia in non rosetting isolates and therefore α_2M may be used as a biological marker for high parasitemia in malaria infected individuals.

Parasite isolates from patients with high α_2M concentrations had more rosettes than those from patients with low α_2 M concentration. This confirms the role of α_2M in enhancing rosette formation by increasing the avidity of binding of PfEMP1 molecules on the surface of the IRBC consistent with others studies.[16,25]

This may confirm the role of this serum protein in supporting high parasite growth in lieu of severe disease. Increases in α_2M concentration has been documented in diseases that result in high plasma and tissue proteinase levels such as cystic fibrosis rheumatoid arthritis and pancreatitis.[50] During *Plasmodium* infections, are used in the rapturing and re-invading of red blood cells and this process generates high amounts of proteinases that effectively trigger high production of α_2M to remove the excess proteinases.[51]

Conclusion

This study has shown that rosette formation in *P. falciparum* clinical isolates is affected by the blood group type of the patient with isolates from blood group A type producing the highest number of rosettes per isolates.

A low IgM concentration was associated with severe disease whilst a high α_2M concentration was associated with uncomplicated malaria. In relation to disease outcome, blood group A type and low anti-parasite IgM concentration increased the risk of severe malaria among participants.

Abbreviations

α_2M, alpha 2 macroglobulin; CR1, complement receptor 1; ELISA, enzyme linked immunosorbent assay; ERC, ethical review committee; GHS, Ghana health service; HRP, horse radish peroxidase; HS, heparan sulphate; IgM, immunoglobulin M; IRBC, infected red blood cells; MOH, ministry of health; NHS, normal human serum; OD, optical density; OPD, out patient department; PBS, phosphate buffered saline; PBST, phosphate buffered saline 0.1% Tween 20 detergent; PfEMP1, plasmodium falciparum erythrocyte membrane protein 1; RBC, red blood cells; RDT, rapid diagnostic test; RIFIN, *P. falciparum*-encoded repetitive interspersed families of polypeptides; RPMI, Roswell Parks Memorial Institute; SM, severe malaria; STEVOR, subtelomeric variable open reading frame; TMB, 3,3′,5,5′-tetramethylbenzidine; UM, uncomplicated malaria; VSA, variant surface antigens.

Data Sharing Statement

The datasets analysed in this study are available from the corresponding author on reasonable request.

Acknowledgments

The authors are grateful to the parents and guardians of the children involved in the study. Special thanks also go to the management and staff of the Hohoe Municipal Hospital as well as the staff of the Immunology Department, NMIMR. Mr Alex Danso-Coffie is thanked for his logistic and technical support.

Author Contributions

All authors made significant contribution to the work, whether in the conception, study design, implementation, acquisition of data, analysis or interpretation. All the authors were involved in the drafting, revising and critical reviewing of the article. They all agreed on the journal to which the article has been submitted and agreed to be accountable for all aspects of the work.

Funding

Michael F. Ofori and Lars Hviid are supported by the Danish Research Council for Development Research (Grant No. 17-02-KU). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Disclosure

The authors report no conflicts of interest in this work.

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