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Genotypic and antigenic diversity of *Plasmodium falciparum* merozoite proteins in Malawian children with severe and uncomplicated malaria

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Abstract

Background Malaria disease presentation and severity could be influenced in part by differential virulence among *Plasmodium falciparum* genotypes and/or by the multiplicity of infection (MOI).

Methods Using polymorphic merozoite surface proteins MSP-1 and MSP-2, the genetic and serological diversity of *P. falciparum* parasites infecting children with different clinical presentations of malaria was characterized during two transmission seasons in Malawi. *Plasmodium falciparum* isolates were obtained from 93 patients with cerebral malaria (CM), 50 with severe anaemia (SMA), 26 with CM and SMA, and 92 with uncomplicated malaria (UM).

Results There was more parasite genetic diversity amongst patients with SMA (mean ± SD 2.70 ± 1.20) than amongst those with CM (2.25 ± 0.89) ($p < 0.01$). The MSP-1 dimorphic K1-type was more frequent in parasites from SMA cases but almost absent in parasites from CM patients ($p = 0.03$). The MSP-1 MAD20 block 2 type was less common in CM than in UM patients ($p < 0.05$). Presence of MSP-2 FC27-type parasites was associated with SMA (66%) whereas MSP-2 IC1-type parasites were associated with CM (61.5%) ($p < 0.025$). The DNA sequence polymorphisms were largely reflected in antigenic diversity as defined by distinct serological recognition of anti-MSP-1 and MSP-2 antibodies.

Conclusions Patients with CM, SMA and UM could be partly distinguished on the basis of the merozoite proteins in parasite isolates. The differences in MOI as well as the genetic and antigenic differences in the clinically defined groups are consistent with a role for parasite antigenic variability in malaria pathogenesis.

Background

The wide spectrum of disease manifestations in *Plasmodium falciparum* malaria infections is most likely determined by a combination of host, pathogen and environmental factors. Parasite heterogeneity could play a role in the severity of disease, with some "strains" being more virulent than others. It has been hypothesized that a majority of *P. falciparum* strains cause uncomplicated malaria (UM), whereas the most severe form, cerebral malaria (CM), could be caused by a few distinct highly virulent strains [1, 2], and that severe malarial anaemia (SMA) could be a complication occurring in a

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certain proportion of infections with "mild" strains [3]. Although a number of studies have addressed whether particular parasite strains, defined as genotypes, are differentially distributed by malaria disease manifestations [4–12], more evidence suggests a role in disease severity for *var* gene-encoded variant surface antigens expressed on infected erythrocytes that mediate cytoadhesion. For example, among polymorphic *var* gene families, upregulation of group A and DC8 *var* has been associated with severe malaria [13] and a more restricted subset with CM [14], but associations are less clear for genotypes of antigens involved in other parasite functions like invasion [15–17].

Due to their extensive polymorphism, *P. falciparum* merozoite surface proteins (MSP) 1 and 2 are common genotyping markers [18–21] using polymerase chain reaction (PCR) [22] or other methods like restriction fragment length polymorphism (RFLPs) [23, 24] or sequencing [23, 25–28]. MSP-1 has a dimorphic structure spanning most of the molecule represented by alleles of the MAD20 and K1 *P. falciparum* isolates [29]. The most polymorphic region of MSP-1 is the N-terminal block 2, but all allelic sequences fall into one of three main types represented by the K1, MAD20 and RO33 isolates, with hybrids generated by intragenic recombination. K1-like and MAD20-like types vary in the sequence, length and number of tandem repeats, whereas RO33 is a non-repetitive sequence with little variation between isolates. MSP-2 also has an essentially dimorphic structure [25, 30], represented by the IC1 and FC27 *P. falciparum* isolates, which correspond to serogroups A and B identified by reactivity with monoclonal antibodies (mAbs) and immunofluorescence microscopy (IFA) [26, 31]; hybrids also exist [27, 32]. A central variable region, composed of non-repetitive sequences surrounding two domains of polymorphic tandem repeats, differs substantially between the two families. The FC27 MSP-2 family (serogroup B) has 32- and 12-amino acid residue motifs, which can be tandemly repeated in different isolates [33]. Members of the IC1/3D7 (serogroup A) allelic family have shorter repeats of 4–8 amino acid residues [34], forming so-called R1 and R2 regions. Some IC1/3D7 alleles are also characterized by the presence or absence of short sequences within the 3’ non-repetitive variable blocks (D1 and D2 deletions). The extent to which these polymorphisms translate into antigenic diversity defined by antibody binding, and on the specificity of human immune responses, has been less characterized.

The number of parasite genotypes infecting a host, known as multiplicity of infection (MOI), can also impact naturally acquired immunity [35] and the severity of clinical symptoms [4, 5, 9, 12, 15, 17, 36–43]. Higher MOI has been associated with asymptomatic rather than symptomatic individuals in some studies [36–41] but not in others [11, 31, 44–47]. It has been proposed that in older children (> 2 years), high MOI may be a feature of low-level chronic parasitaemia that could confer cross-protection against incoming parasites via partially type-specific immune responses [48]. However, very few studies have examined whether MOI differs between patients presenting with CM and SMA [8, 17].

Here the association between MSP-1 and MSP-2 parasite genotypes, MOI and severe malaria clinical presentations was investigated in Malawian paediatric patients diagnosed with SMA, CM, CM + SMA, or UM. As part of this analysis, the relationship between *P. falciparum* allelic polymorphisms and antigenic diversity of MSP-1 and MSP-2 was also assessed by comparing the data obtained by PCR and IFA typing methods.

Methods

Patients and samples

Plasmodium falciparum isolates were obtained from whole blood samples collected from children < 12 years of age presenting with a malaria illness, in Blantyre, Malawi, during 1996 and 1997 transmission seasons. Children with severe malaria were admitted to the Blantyre Malaria Project and Wellcome Research Programme, Department of Pediatrics, Queen Elizabeth Central Hospital. Clinical data, including state of consciousness, history of convulsions and prior drug treatment, and basic demography, were recorded for all patients. A finger prick blood sample was taken to assess *P. falciparum* parasitaemia and haematocrit. Microscopic examination of reverse Fields’-stained thick and thin blood films was performed, counting parasites against at least 200 white blood cells or 500 red blood cells (RBCs), respectively. Children were defined as having CM if they had Blantyre Coma Score (BCS) < 3 [49] with asexual parasitaemia of any density and no other obvious cause of the clinical syndrome. SMA was defined as haematocrit < 16% or haemoglobin (Hb) < 5 g/dl. Children with CM or SMA were all admitted to hospital; the patient samples represented all children admitted during these time periods. Children with UM were symptomatic but had no recent history of coma or convulsions, and were fully conscious (BCS of 5). They were part of a convenience sampling recruitment, either (i) ambulant children screened for enrolment in studies of novel antimalarial therapy at Ndirande Health Centre, Blantyre, (ii) patients admitted to the hospital with a final diagnosis of UM, or (iii) ambulant controls attending the hospital for blood examination for malaria parasites, with confirmed infection. The CM and SMA patients received intravenous quinine until two consecutive smears were negative, and the UM patients were prescribed sulfadoxine-pyrimethamine treatment.

All participants were asked to return after a month for a follow up visit or if they were sick after discharge, resulting in some children coming for multiple convalescent visits. Informed consent was obtained from parents or guardians, and the study obtained ethical approval from the Malawi National Health Sciences Research Committee (Study Approval Number P.11/07/593).

Five ml of venous blood were taken in lithium heparin or EDTA at study enrolment and follow up visits, centrifuged, and the buffy coat and plasma removed. Packed red cells including parasitized RBCs (PRBCs) were washed 3×in sterile phosphate buffered saline (PBS). When parasitaemia was at least 0.05%,≅0.3 ml RBCs were used to set up short-term *P. falciparum* in vitro cultures [44, 50]. The residual RBCs were stored at − 70 °C for analysis of parasite DNA.

Genotyping of *P. falciparum* by PCR

Plasmodium falciparum DNA was extracted from 20 to 100 µl PRBCs by the quick boiling method [51]. PRBCs were washed 5×in 0.5 ml of ice-cold 5 mM sodium phosphate pH 8.0 and centrifuged for 10 min. The supernatant was removed, 50 µl of sterile ddH$_2$O were added to the pellet and boiled for 10 min. After a 10 min spin (g=10,000), the pellet was discarded and the supernatant was used as DNA template for PCR. Block 2 of MSP-1 was amplified by a hot-start nested PCR method. Primers O1 and O2 were used first in the outer reaction, as described [52] (Fig. S1). The inner amplification was carried out using 1–2 µl of the previous reaction and 3 sets of block 2 type-specific primers in separate reactions to distinguish the known types of block 2 of MSP-1 (K1, MAD20 and RO33) and to detect size differences among alleles of each type due to repeat sequences [53]. Dimorphic regions of MSP-1 and MSP-2 were typed by the dimorphic-form specific (DIFS) PCR method [54]. A combination of 3 primers consisting of two 5’ type-specific primers and a 3′ common primer could distinguish between MAD20 and K1 alleles of MSP-1 (primers difs 1, 2 and 3) and between IC1 and FC27 alleles of MSP-2 (primers difs A, B and C) by differences in the size of bands in a hot-start single PCR (Fig. S1). The DIFS typing methods do not detect size differences between PCR bands within an allelic type, and thus the maximum number of different clones that DIFS alone can resolve is 2 in mixtures of the MSP-1 MAD20 and K1 dimorphic types, or MSP-2 IC1 and FC27 types. In all cases, a 50 µl reaction mixture was prepared, containing 1–10 µl of extracted DNA solution, 5 µl of 2 mM dNTP mix, 0.5 µl of each primer (25–100 pmol), 5 µl of 10x*Taq* polymerase buffer and 2 U *Taq* polymerase (Boehringer Mannheim). The PCR reactions were run in a Hybaid Omnigene Temperature Cycler. A 10 µl aliquot from each amplification was electrophoresed at 90 V on 2% agarose gels in 1xTBE buffer, stained with 0.5 µg/ml ethidium bromide and visualized by UV transilluminator. The minimum number of parasite genotypes per infection was estimated by combining the number of distinct PCR bands detected for each locus in the 3 reactions. The prevalence of a given genotype was measured as the percentage (%) of parasite isolates which were positive for that genotype.

Parasite DNA sequencing

Plasmodium falciparum isolates from 1996 that appeared to be single clone infections by initial PCR amplification were selected to carry out sequencing of the block 2 region of MSP-1, and almost full-length MSP-2 gene. PCR products were purified using spin UF 100 minicolumns (Costar). Direct automated sequencing was performed with the corresponding PCR primers with ABI PRISM™ Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer). Sequences were analysed by the GCG package.

In vitro culture of *P. falciparum* isolates and IFA serotyping

Parasites were incubated at 37 °C in RPMI-HEPES medium (Life Technologies) supplemented with gentamicin 10 µg/ml (Life Technologies), NaHCO$_3$ 2 mg/ml (Sigma) and 10% human AB serum from non-malaria exposed Australian blood donors, in a gas mixture of 1% O$_2$, 5% CO$_2$ and 94% N$_2$. Parasites were grown for≅48 h until they matured to schizonts, as judged by thin smear examination. When cultures contained schizonts, the PRBCs were harvested and washed 2 in 10 ml PBS to remove all traces of human serum. Cells were resuspended to≅3–5% haematocrit in PBS and 20–25 µl aliquots were placed onto wells of 12-well multispot microbiological slides (Hendley-Essex). The slides were dried in a culture hood, packed and stored at − 20 °C in self-sealed plastic bags containing silica gel as desiccant.

Type-specific Ab probes can identify distinct allelic forms of MSP-1, MSP-2 and of exported protein EXP-1, and thus the presence of mixed infections in individuals. Serological reagents included mAbs and polyvalent mouse sera specific for *P. falciparum*, MSP-1, MSP-2 and EXP-1 (Table S1). Three known MSP-1 block 2 types, represented by the RO33, MAD20 and K1 isolates, (Fig. S1) were identified by specific mAbs [44] and/or polyclonal mouse sera [53]. In addition, mAbs were used that detect MSP-1 sequence polymorphisms in blocks 3 and 4b (the C-terminal half of block 4), in dimorphic regions (block 6 to 16) of the MAD20-type or Well/K1-type, and conserved epitopes (block 17) [44]. Polymorphisms within and between the two major serogroups of MSP-2, A and B (Fig. S1), were similarly identified by mAbs [26] or mouse sera against recombinant MSP-2.

Working dilutions of Abs were prepared in 1% bovine serum albumin (BSA) in PBS containing 0.01% sodium azide and kept at 4 °C, and IFA serotyping was carried out as described [44]. Briefly, slides with schizont PRBCs were fixed in acetone for 5 min and 25 µl of working dilutions of Abs were placed on separate wells and incubated at room temperature in a wet box. After 30 min, Abs were removed and slides were washed 3 × in PBS. After the slides were dried on a warm plate, 15 µl of FITC (fluorescein isothiocyanate)-conjugated anti-mouse immunoglobulin (1/80) were placed in each well, incubated and washed as above. Parasite DNA was stained in 4′,6′-diamino-2-phenylindole (DAPI) (1:100,000) for 5 min, and the blood films counterstained in 0.1% Evans Blue. After 5 min, the slides were rinsed in PBS and mounted under coverslip with Citifluor (City University, London) or 50% glycerol in PBS. Reactions were read at magnification of 315 × or 630 × and incident light of 450–490 nm for FITC-fluorescence (green) and 390–440 nm for DAPI (blue). In each isolate, the percentage of schizonts giving mAb-specific positive fluorescence was recorded for each mAb. Details on IFA data processing are provided in Supplementary Materials.

Statistical analysis

MOI was calculated for MSP-1 and MSP-2 separately and combined by selecting the maximum number of different clones resolved by each marker in each isolate. Associations between the presence of particular parasite genotypes or MOI and malaria disease status and outcome of the disease (full recovery, neurological sequelae or death) were first analysed by Chi-square tests (χ^2). Mean MOI were compared among the different groups of patients by *t*-tests. Data were further analysed by multiple regression models simultaneously accounting for confounding factors such as age and sex in the analysis [55]. The outcome variable was distributed as a binary trait. Additional analysis compared the SMA and CM forms of clinical malaria to determine whether those disease presentations were affected differently. Using the PROC GENMOD procedure [55] for analysis of binomial data, a linear regression model was fitted to the binomial disease-type data with variables including the minimum MOI, the presence or absence (1 or 0) of *MSP-1* or *MSP-2* genotype, parasitaemia (fitted as a continuous covariate on the logarithmic scale), age (classified as < 1, 1, 2, 3, 4, 5, ≥ 6), sex, area of residence (urban, peri-urban or rural), duration of symptoms (in hours), history of prior drug treatment within 1 week (yes or no), and date of admission (fitted as month within year). A similar analysis was done for disease recovery with or without neurological sequelae, or death as outcomes. Parasitaemia was analysed as a dependent variable of factors such as the presence or absence of a genotype, and the MOI, using the PROC GLM procedure of SAS for continuous data. In all models, two-way interactions among factors were tested for significance, and all non-significant factors were excluded from the models in the final analysis. Significance is reported at the 5% level.

Results

MSP-1 and MSP-2 *P. falciparum* genotypes and malarial disease severity

A summary of clinical and parasitological data on the children is given in Table 1. PCR genotyping was performed in 466 cases to identify distinct allelic forms of MSP-1 and MSP-2 and to resolve MOI, with overall efficiencies of DNA amplification of 89.2% (MSP-1 DIFS), 95.5% (MSP-1 block 2 nested PCR), and 76.6% (MSP-2 DIFS).

The dimorphic MAD20 type of MSP-1 strongly predominated over the alternative K1 type (Table 2), but children with SMA (with or without CM) were more commonly infected with K1 parasites than children without SMA (20.3% *vs.* 8.9%, $\chi^2 = 6.0$, $p < 0.025$) (Fig. 1). The prevalence of MSP-1 dimorphic K1-type parasites was significantly higher in SMA than CM cases ($p = 0.03$) after controlling for the possible confounding factors in multivariable regression models. Among single clone infections in children with CM, none were of the dimorphic K1 genotype (Table 2).

Regarding MSP-2, although the dimorphic IC1 type was more prevalent overall, in children with SMA it was less prevalent (Table 2), and a significantly higher proportion of children with SMA had mixed IC1 + FC27 infections (36%) compared to those without SMA (17%) ($\chi^2 = 6.74$, $p < 0.01$) (Fig. 2). Overall, children with SMA were more commonly infected with MSP-2 FC27-type parasites than children without SMA (66% *vs.* 42%, $\chi^2 = 6.45$, $p < 0.025$). By contrast, children with CM carried a significantly lower proportion of mixed MSP-2 IC1 + FC27 genotypes (11%) than did children without CM ($n = 136$, 27%) ($\chi^2 = 8.73$, $p < 0.005$). In CM children with single clone infections there was a significantly higher prevalence of IC1-type infections (61.5%) than in children with SMA (34%) ($\chi^2 = 8.96$, $p < 0.005$) (Table 2).

Children with CM also had significantly lower prevalence of MSP-1 MAD20 block 2 type parasites than children with UM after adjustment for parasitaemia, age, sex, MOI and presence of the K1 and RO33 block 2 types ($p < 0.05$). This difference might be partially explained by a higher MOI in UM than CM, as the significance was lost when this was included in the model ($p = 0.07$). Nevertheless, the MAD20 type itself seemed to have an independent effect on disease in the model that was not observed when the presence of either K1 or RO33 type parasites was analysed in like manner.

Table 1 Clinical and parasitological data of the patients

Diagnosis [a]	n [b] patients	Age ± SD Arithmetic mean	% area of [c] residence					Parasitaemia [d] Geometric mean	Duration [e] symptoms	% prior [f] treatment	% with neurological sequelae	% deaths	n [g] convalescent
			A	B	C	D	E	(95% CI)					
CM	93	3.62 ± 2.10	39	40	11	8	2	28,219 (16,025–49,691)	56 ± 40.9	36	19	11	78
SMA	50	2.35 ± 1.41	28	26	25	11	10	11,814 (4,693–29,738)	83 ± 50.5	33	2	9	41
CM + SMA	26	3.09 ± 1.69	29	50	15	4	2	56,485 (27,079–117,820)	71 ± 38.6	42	17	17	22
UM	92	2.79 ± 1.54	84	15	0	0	1	26,738 (15,545–45,990)	52 ± 38.1	8	0	0	23

[a] CM = cerebral malaria; SMA = severe malarial anaemia; CM + SMA = both CM and SMA; UM = uncomplicated malaria

[b] Number of patients from whom a RBC sample was obtained for parasite typing on admission. Data on clinical diagnosis, age, area of residence, parasitaemia, duration of symptoms, prior treatment and number of neurological sequelae and deaths refers to these patients

[c] Percentage of patients whose area of residence was: A. Blantyre town; B. periurban areas; C. villages; D. towns other than Blantyre; E. unknown

[d] Parasitaemia calculated as parasites (ring forms) per microliter of blood

[e] Longest duration of symptoms (any of: fever, vomiting, diarrhoea, convulsions, unconsciousness, stop eating, stop drinking/sucking) in hours, arithmetic mean ± SD, as recorded by interviewed parents

[f] Percentage of patients with a history of anti-malarial treatment (sulfadoxine-pyrimethamine and/or quinine) in the past week

[g] Number of patients who came back to hospital for a follow up visit a month after admission. A RBC sample was also collected for parasite typing

Table 2 Prevalence (%) of MSP-1 and MSP-2 genotypes detected by PCR

Diagnosis [a]	[b]	**MSP-1**												**MSP-2**			
	No. of	**Dimorphic**				**Block 2**								**Dimorphic**			
	PCR+	**n typed**	**%M** [c]	**%K**	**%M+K**	**n typed**	**%K**	**%R**	**%M**	**%K+R**	**%K+M**	**%R+M**	**%K+R+M**	**n typed**	**%IC1**	**%FC27**	**%IC1+FC27**
SM	169	163	86.5	2.5	11.0	168	20.8	5.9	7.7	11.9	22.0	**1.8**	26.8	160	52.5	27.5	20.0
CM	93	89	92.1	0	7.9	92	25.0	5.4	8.7	13.0	22.8	1.09	22.8	91	**61.5**	27.5	**11.0**
SMA	50	49	77.6	6.1	**16.3**	50	14.0	8.0	6.0	8.0	18.0	0	38.0	44	**34.1**	29.5	**36.4**
CM+SMA	26	25	84.0	4.0	12.0	26	19.2	3.8	7.7	15.4	26.9	7.7	19.2	25	52.0	24.0	24.0
UM	92	88	88.6	5.7	5.7	90	22.2	1.1	4.4	5.6	15.6	**7.8**	42.2	84	53.6	21.4	**25.0**
Total	397	354	90.4	2.5	7.1	379	24.0	6.3	6.1	9.2	19.3	4.5	27.7	304	52.0	23.7	21.3

[a] SM = severe malaria; CM = cerebral malaria; SMA = severe malarial anaemia; CM + SMA = both CM and SMA; UM = uncomplicated malaria. Total includes all acute and convalescent samples tested. The prevalence in each disease group refer only to acute stages

[b] Number (No.) of isolates that were PCR positive for any of the typed loci

[c] M = MAD20, K = K1, R = RO33

Percentages of isolates which either contained only one detectable parasite clone, possessing the indicated genotype, or mixed isolates that contained more than one parasite genotype. Statistically significant differences in prevalence between patient groups are bolded; p values are detailed in the results text

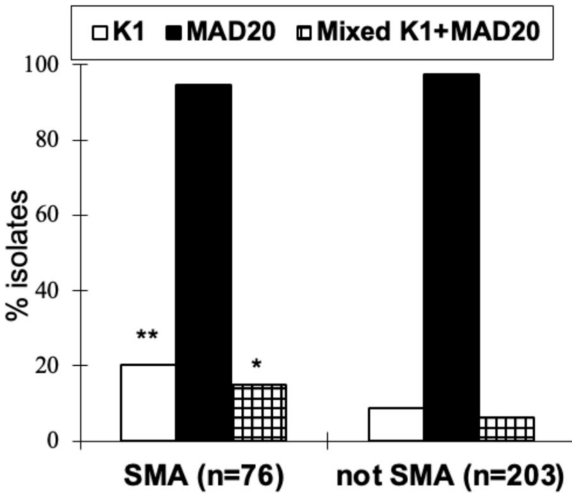

Fig. 1 Prevalence of dimorphic genotypes of MSP-1 in patients with or without severe malarial anaemia. The percentage of isolates containing parasites of the MSP-1 dimorphic K1 type was significantly higher in children presenting with severe malarial anaemia (SMA) compared to children without SMA, regardless of other comorbidities including cerebral malaria.** SMA *vs.* not SMA, K1, $p<0.025$ * SMA *vs.* not SMA, K1 + MAD20, $p<0.05$

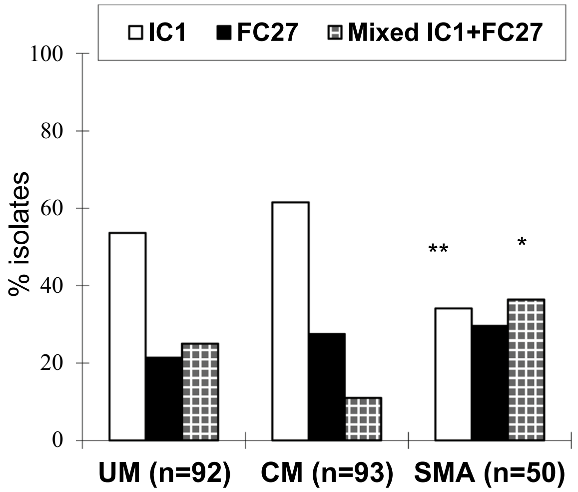

Fig. 2 Prevalence of dimorphic genotypes of MSP-2 by malaria clinical presentation. The percentage of isolates containing parasites of the MSP-2 dimorphic IC1 type (serogroup A) was significantly lower in children presenting with severe malarial anaemia (SMA) compared to children without SMA, regardless of other comorbidities including cerebral malaria (CM).** SMA *vs.* not SMA (CM + UM), IC1, $p<0.025$ * SMA *vs.* not SMA (CM + UM), IC1 + FC27, $p<0.01$

Children infected with MSP-2 FC27-type parasites had higher geometric mean parasite densities (18,841/µl, 95% CI: 42,702–8,313) than patients infected with IC1-type parasites (10,270/µl, 95% CI: 18,518–5,696). The difference was significantly higher in mixed IC1 + FC27 (24,495/µl, 95% CI 43,822–13,692) compared to single IC1 infections ($p<0.05$). This effect was independent of disease status, since IC1 + FC27 mixed infections predominated in SMA patients, whose parasite densities were lower than those of CM or UM patients (Table 1). No association was found between MSP-1 genotypes and parasite density.

Considering disease outcome (survivors, sequelae or death), there was an increased prevalence of mixed MSP-1 block 2 K1 and RO33 type parasites among children who died (7/32 = 22%) than among survivors (28/347 = 8%) ($\chi^2 = 6.66$, $p<0.025$); nevertheless, the overall frequencies of these genotype combinations were low.

MOI and malarial disease severity

Combination of MSP-1 block 2 and DIFS genotyping increased the level of resolution of MOI. The mean MOI per isolate was significantly lower in CM (mean ± SD 2.25 ± 0.89) than in UM (2.64 ± 0.98) or in CM than in SMA (2.70 ± 1.20) (*t* tests, $p<0.01$) (Table 3). This was because single-clone infections were significantly more common in CM (20.4%) than in UM (8.7%) cases ($\chi^2 = 5.11$, $p<0.025$). The UM and SMA groups had similar MOI. Conversely, MOI ≥ 4 was less frequent in CM (7.5%) than in combined non-cerebral cases (n = 160, 20%) ($\chi^2 = 7.02$, $p<0.01$). MOI ≥ 3 was also less common in CM (36.5%) than in UM and SMA cases pooled together (n = 142, 50.7%) ($\chi^2 = 4.54$ $p<0.05$). All results remained statistically significant when parasitaemia, age, sex, date of admission, area of residence, prior drug intake or duration of symptoms were accounted for in multiple regression models.

Polymorphisms detected by IFA serotyping versus PCR genotyping and sequencing

After evaluating the diversity of the MSP-1 and MSP-2 genetic loci, the MSP-1 and MSP-2 protein diversity was investigated at the serological level through reactivity with well characterized type-specific Abs in the subset of *P. falciparum* isolates that could be grown in vitro. Out of 206 blood samples collected, 200 were selected for culturing on the basis of parasitaemia, and 146 PRBCs isolates matured to schizonts (73%).

IFA typing identified 58.6% mixed *P. falciparum* infections overall, with a mean ± SD MOI per isolate of 2.0 ± 1.0 (Table S2). MSP-1 typing had a higher power to detect multiple infections than MSP-2 typing, probably due to the larger panel of Abs used. When both markers were combined, almost equivalent results to MSP-1 alone were obtained.

Combining all clinical isolates, the MSP-1 MAD20 dimorphic type predominated (96.3%) over the

Table 3 Analysis of mixed infections detected by MSP-1 and MSP-2 PCR genotyping

Number			**%**			
isolates n		**clones/isolate mean ± SD**	**1 clone**	**2 clones**	**3 clones**	**≥ 4 clones**
Total	279	2.46 ± 1.00	15.8	40.5	29.0	14.7
CM	93	**2.25 ± 0.89**	**20.4**	43.0	29.0	**7.5**
SMA	50	**2.70 ± 1.20**	16.0	32.0	28.0	**24.0**
CM + SMA	26	2.35 ± 0.80	11.5	50.0	30.8	7.7
UM controls	92	**2.64 ± 0.98**	**8.7**	41.3	30.4	**19.6**

The number of clones was determined as the maximum number of distinct PCR bands combining the MSP-1 and MSP-2 loci in each isolate (see Methods)

Significant differences in multiplicity of infections between patient's groups are highlighted in bold and comparisons explained in the results text

alternative K1/Well-type (13.4%), consistent with the PCR results (97% MAD20 *vs.* 9.6% K1/Well). Regarding the polymorphic MSP-1 block 2, K1 predominated (78.4%) over RO33 (47.8%) and MAD20 (40.3%) by IFA, similarly to PCR (81% K1 *vs.* 59% MAD20 and 49% RO33). MSP-2 results were more variable by year and typing method. In 1996, 9/12 (75%) isolates had MSP-2 serotype A (IC1/3D7) and 7/12 (38%) had serotype B (FC27) by IFA; 9 of these could be sequenced, yielding 5 IC1/3D7genotypes, 3 FC27 genotypes, and 1 hybrid allele. In 1997, the proportion of MSP-2 A or B serogroups was the same (68% each) by IFA. However, among single clone infections, serogroup B (56.9%) predominated over A (43.1%), while in mixed infections, serogroup A (58.2%) predominated over B (47%) in majority clones (> 50%). By PCR, IC1/3D7 alleles were more prevalent (73%) than group FC27 alleles (48%).

Although the sensitivity of IFA to detect mixed infections was lower than that of PCR (overall mean MOI of 2.46 ± 1.00, maximum MOI of 6), some typing Abs bind to other variable fragments of the MSP antigens that were not amplified in the PCR assay, and to another protein (EXP-1) Thus, IFA analyses could provide additional insights on the extent of MSP epitope diversity and MOI in Malawi, and in comparison to other endemic areas (see Supplementary results).

Relationship between genetic and antigenic polymorphisms

PCR and IFA paired data were available from 146 isolates that were successfully cultured in vitro and typed for MSP-1 and MSP-2. Agreement between techniques was high, with 544/611 (89%) concordance. The best correlation between PCR and IFA typing was for the dimorphic region of MSP-1, and there was a very good correlation for MSP-1 polymorphic block 2 types. Concordance for MSP-2 types was also reasonably good, particularly for the dimorphic type A. In 8% of paired comparisons, a genotype identified by PCR was not detected by IFA, most probably because it was a minority population in a mixed infection, detected with a higher sensitivity by PCR. Only in 2.9% of all comparisons was a positive IFA result not reproduced by PCR, as detailed for each antigen in Supplementary results. Therefore, the data indicate that differences in gene sequences captured by PCR typing translated into distinct antigenic epitopes differentially recognized by type-specific Abs.

Longitudinal genotyping in recurrent infections

Sixty participants returned for follow up visits during the same malaria season (once, n = 38; twice, n = 14; 3 times, n = 6; 4 times, n = 2), mostly within 1-month intervals (range 0.5–3 months). Of these, 41 were CM survivors (including 9 with SMA + CM), 16 were hospitalized initially with SMA, and 3 had UM. At convalescence, most children had mild or no symptoms. However, 11 children had CM sequelae and 3 returned with SMA. The genotyping data were sequentially compared to assess whether there was evidence of new infection or recrudescence in subsequent visits, and if this was associated with particular syndromes. Parasite isolates from 37 participants could be typed more than once and, of these, 27 were infected with genotypes that were present at the index infection, suggesting recrudescence; of these, the MOI was reduced in 12. Five participants had different parasite genotypes in the follow-up samples, suggesting reinfection, and 6 had a mixture of previous and new parasites. There was no specific clustering by disease diagnosis.

Discussion

An unbalanced distribution of *P. falciparum* genotypes was found associated with disease manifestations, even after adjusting for MOI, in participants with carefully-defined severe malaria or UM; this was a large sample size compared to prior studies and with the novelty of also resolving the diversity of the antigens using type-specific

Ab reagents in addition to PCR. Divergences in genotype prevalences were most noticeable between CM and SMA. Specifically, the MSP-1 dimorphic K1-type was more frequent in parasites from children with SMA, whereas it was rarely detected in children with CM. In addition, parasites from children with CM were less likely to be of the MSP-1 block 2 MAD20-type compared to parasites from UM. SMA was associated with MSP-2 FC27-type parasites, whereas children with CM were more commonly infected with MSP-2 IC1-type parasites. Analysing the MOI, diversity was lower in CM than either UM or SMA, and did not differ between UM and SMA. About half of CM patients still had PCR detectable parasites in post-discharge visits, most of them with genotypes detected in the acute episode, suggesting recrudescences presumably associated with suboptimal therapeutic efficacy of the drugs administered at the time. However, after the introduction of artemisinin derivatives to Malawi, such recrudescences would be much less expected. Furthermore, the genotypes defined at the DNA sequence level for the selected MSP-1 and MSP-2 polymorphic and dimorphic loci targeted were distinctly recognized by mouse Abs and, therefore, largely reflected at the amino acid level as different serotypes.

Data were consistent with the concept that in malaria, as in other infectious diseases, the heterogeneity of parasite populations contributes to differential pathogenesis that may be manifested as specific disease syndromes, also within severe malaria. Indeed, historical data collected during experimental malaria infections indicated that *P. falciparum* strains could vary in the severity of the disease induced [56]. Even though the antigens used to define genotypes are expressed on the merozoite surface and do not mediate key pathogenic functions such as cytoadhesion, MSP-1 and MSP-2 are involved in invasion of host cells that is essential for the blood stage life cycle responsible for symptoms. Thus, MSPs having diverse protein structures might differentially interact with the host cell and result in dissimilar invasion efficiencies, which could be tested in in vitro cultures using well-characterized single-clone *P. falciparum* isolates. This would translate into varying growth and multiplication rates that could impact disease outcomes. In fact, an association between MSP-2 FC27-type parasites and higher parasite density was found, like another study in PNG [24], and the FC27-type has also been associated with symptomatic malaria in children [57, 58] and with higher pro-inflammatory IL-6 [10]. On the other hand, the presence of the MSP-1 K1-type may signal an incomplete, slower or less efficient parasite clearance, perhaps chronic low-grade infection, and prolonged RBC destruction, eventually resulting in SMA. Another possibility is that the near absence of the MSP-1 K1-type among CM patients (and, conversely, the predominance of the alternative MAD20-type) indicates a requirement for the MAD20 genotype in some step of CM pathogenesis, or that an unknown gene for pathogenicity is closely linked to the MAD20-type alleles in the Malawian isolates. That is, certain MSP gene loci might be linked to other genes that more directly mediate immune evasion and severity functions, thus being just markers but not mechanistically involved. CM patients have shown broadly increased expression of *var* genes (group A and DC8 var) that encode PfEMP1 proteins involved in parasite sequestration, while children with SMA had high transcription of DC8 *var* only [13]. The presence of common *var* profiles in severe malaria patients of different ages across distant geographical sites, as well as syndrome-specific disease signatures, have been demonstrated for those variant gene families. Furthermore, two studies reported an association between a specific K1 MSP-1 allele with a specific *var* gene (var-D) in patients with severe malaria versus UM, both being in strong linkage disequilibrium [6, 59].

Some studies have found a higher prevalence of the MSP-1 block 2 MAD20-type, alone or in combination with RO33-type, in UM compared to severe malaria (SMA or hyperparasitaemia) [5, 38] but others have not [4, 10], and others have failed to find any associations between genotypes and disease [45, 60]. Conflicting results may reflect distinct genetic characteristics in parasites and/or hosts in different countries, as with contrasting reports on correlations between rosetting and severe malaria [61–64]. In addition, the prevalence of parasite strains associated with the clinical categories studied here could also change over time in a given geographic location.

Relatively few studies have examined the relationship between MOI and malaria severity. The findings agree with reports from Senegal [4], Gabon [5], and Cameroon [9] where high MOI was positively associated with SMA. It was speculated that CM could be associated with a limited number of clones multiplying to high parasitaemias, while UM cases could be caused by a larger number of lower density clones [4]. Alternatively, CM could be characterized by the expansion of one or a few clones, such that the ability of the PCR to detect minor clonal populations could be compromised [65]. Moreover, fever and TNF responses associated with CM pathogenesis may reduce low-density clones below the PCR detection threshold [66]. MOI has also been associated with parasite density and host age in Senegal [22]. The number of genotypes carried by asymptomatic individuals decreased with age, from a mean of 4, in children under 15 years, to 2 in adults, in parallel with decreases in parasite prevalence and density. The findings further support the idea that the more coexisting clones, the broader the repertoire of immunity protecting against an uncontrolled expansion of a superinfecting parasite clone (concomitant immunity or premunition). The observation that premunition is acquired at a rate which is dependent upon degree of exposure [67] may explain why the positive association between high MOI and clinical disease was found in Sudan [46], where transmission is unstable and immunity against previously experienced genotypes has time to wane.

SMA might occur if genetically diverse infections were less rapidly cleared by hosts. In the experimental *Plasmodium chabaudi* model, multiple-clone infections in mice caused more anaemia and body weight loss than single-clone infections [68]. It was suggested that this could be because diverse genotypes could select a larger number of specific T- or B-cells, and thus stimulate greater immune response causing either destruction of RBCs, or higher production of cytokines (e.g. TNF) involved in the pathogenesis of SMA [69, 70].

This study has some limitations, including the cross-sectional design -associating certain parasite characteristics with certain clinical categories at one point in time- and the use of classical PCR technology rather than sequencing for detection of distinct clones. Given that some malaria manifestations can occur as part of a continuum (e.g. UM becoming SMA over time), there may be some loss of precision explaining the crossover for some categories. However, the typing by Abs is unique, and the good overall agreement between the two techniques strengthens further the validity of the PCR data, notwithstanding emergence of new technologies. This study was indeed the first to systematically compare PCR and IFA typing techniques in a large number of samples, and both are easily amenable to field studies in endemic countries. However, both techniques have limitations when used on their own, and are more valuable in combination. A major advantage of IFA over PCR is that it allows the quantification of each parasite clone in a mixed infection, and the resolution of the phenotypes of MSP-1 and MSP-2 proteins [44]. However, PCR is more sensitive than IFA and thus able to detect parasite clones at low densities. The few discrepancies between both methods may be attributable to the presence of recombinant alleles. With hybrids, PCR alone may give misleading results which could be resolved by DNA hybridization with allele-specific probes, RFLP analysis or, ideally, by quantitative PCR or sequencing.

In conclusion, CM and SMA appeared to represent different expressions of malaria disease in relation to *P. falciparum* genotype distributions and MOI. Together with the literature, the findings indicate a trend from the highest MOI in asymptomatic infections, through decreasing MOI in the milder forms of malaria. The lowest MOI in CM may suggest dominant fast-growing clones, and the high multiplicity in SMA could be related to persistent multiple clones each contributing to RBC destruction and associated pathogenesis. Finally, parasite genetic polymorphisms correlated well with antigenic polymorphisms of encoded proteins as recognized by type-specific Abs. Such data are needed to better understand the effect of *P. falciparum* antigen diversity on the specificity of human immune responses (addressed in the accompanying manuscript [71]) and the acquisition of immunity to malaria. This information is also relevant for malaria vaccine development as most target candidates are products of polymorphic genes.

Abbreviations

Ab	Antibody
BSA	Bovine serum albumin
CM	Cerebral malaria
DAPI	4′,6′-Diamino-2-phenylindole
DIFS	Dimorphic form-specific
Hb	Hemoglobin
IFA	Immunofluorescence microscopy
mAb	Monoclonal antibody
MOI	Multiplicity of infection
MSP	Merozoite surface protein
PCR	Polymerase chain reaction
PRBC	Parasitized red blood cells
RBC	Red blood cells
RFLPs	Restriction fragment length polymorphism
SMA	Severe malaria anaemia
TNF	Tumour necrosis factor
UM	Uncomplicated malaria

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-025-05662-6.

Supplementary material 1

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Author contributions

T.E.T. recruited the patients and defined their clinical presentation. J.S.M designed the laboratory studies and generated key reagents. C.D. and S.J.R collected and processed the samples and parasites towards antigen typing. C.D. performed the experiments and analyses, and wrote the initial draft of the manuscript under the guidance of J.S.M. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

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