Fine specificity of natural acquired antibodies for micro-heterogeneous sequences of the Pf3D7-MSP2 dimorphic domain in malaria-endemic African countries

Spécificité des anticorps naturels pour les séquences microhétérogènes du domaine dimorphique de Pf3D7-MSP2 dans des pays africains endémiques pour le paludisme

Balam S[1], Incandela NC[2], Konaté D[1], Agak GW[3], Diakité M[1], Moret R[4], Nebie I[5], Corradin G[5]
1 International Center for Excellence in Research of Mali (ICER-Mali), Faculty de medicine and Odontostomatology (FMOS), University of Sciences, Techniques and Technologies of Bamako (USTTB), Mali
2 University of California, Los Angeles (UCLA), Department of Chemistry and Biochemistry, USA
3 Division of Dermatology, David Geffen School of Medicine, UCLA, Los Angeles, CA, USA
4 ASAREN 01BP3916, Ouagadougou 01, Burkina Faso
5 Biomedical Science Department, Groupe de Recherche Action en Santé (GRAS), Ouagadougou - Burkina Faso
5 Immunobiology Department, University of Lausanne, Lausanne, Switzerland
***Auteur correspondant :** Saidou Balam, MD, PhD. balamsira@yahoo.fr / Saidou.balam@yahoo.fr / Saidou.balam@icermali.org

Abstract
Background. Micro-heterogenicity of 25-mer segments within the dimorphic domain of the MSP2 defines various genotypes of the 3D7 allelic family of *Plasmodium falciparum* (Pf3D7). This study investigates the specificity of African malaria-endemic antibodies (IgG) for this micro-heterogenicity. **Methods**. We tested, using standard and competitive ELISA, plasmas from Mali (N=75), Burkina Faso (N=49) and Tanzania (N=37) against 12 synthetic 25-mer peptides representing each micro-heterogenous segment. **Findings**. Overall, Burkina Faso antibody samples had significantly higher antibody levels ($p<0.0001$), and the largest proportion (45%-65%) of positive responders for all the 25 mer-peptides. In contrast, Mali samples had slightly lower antibody levels and a smaller proportion of responders (15%-57%), whereas Tanzania samples demonstrated the lowest antibody levels and fewest proportions of responders (3%-46%). Children 6-14 years-old exhibited higher antibody levels and a larger proportion of responders (20-80%) compared to ≤ 5 years-old (08-54%, $p = 0.001$) and ≥ 15 years-old (05-37%, $p < 0.0001$). Furthermore, a large number of responders recognized at least one, if not all, of the 25-mer epitopes. The 25-mer peptides showed cross-inhibition with each other. **Conclusion**. This work broadened our understanding of specific antibody response profiles against epitopes in the Pf3D7-MSP2 allelic dimorphic domain. This study also demonstrates the presence of multi-genotypic infections due to this allele in the African population. **Keys word**: African plasmas, Antibody specificity, Dimorphic domain, Microheterogeneous epitopes, Pf3D7-MSP2.

Résumé
Contexte. Une microhétérogénéité portant sur une séquence de 25 acides aminés (aa) située dans le domaine dimorphique de la protéine MSP2 distingue différents génotypes de la famille allélique 3D7 de *Plasmodium falciparum* (Pf3D7). Cette étude explore la spécifique des anticorps (immunoglobulines de type G) contre cette microhétérogénéité dans les sérums de trois pays africains endémiques au paludisme. **Méthodes**. Douze peptides d'une séquence de 25 acides aminés chacun représentant un des segments microhétérogènes ont été testés en ELISA sur des plasmas du Mali (N=75), du Burkina Faso (N=49) et de la Tanzanie (N=37). **Résultats**. Globalement, les échantillons du Burkina Faso avaient un niveau d'anticorps ($p<0,0001$) et une proportion de répondeurs (45%-65%) les plus élevés pour tous les peptides de 25 aa. Comparés au Burkina Faso, les échantillons du Mali avaient en revanche un niveau d'anticorps et une proportion de répondeurs légèrement inférieurs (15 %-57 %), mais plus grands que les échantillons de Tanzanie, lesquels avaient le taux d'anticorps et la proportion des répondeurs les plus faibles (3 %-46 %). Les enfants de 6-14 ans avaient un plus grand niveau d'anticorps et de proportion de répondeurs (20-80 %) que ceux de ≤ 5 ans (08-54 %, $p = 0,001$) et de ≥ 15 ans (05-37 %, $p < 0,0001$). Un grand nombre de répondeurs reconnaissait au moins un voire tous les 25-mer peptides. En outre, ces peptides ont montré un effet d'inhibition croisée les uns avec les autres. **Conclusion**. Cette étude contribue à une meilleure compréhension de la spécificité des anticorps naturels et de leurs épitopes caractérisant le domaines dimorphique de la protéine MSP2 de la famille allélique Pf3D7. Elle suggère aussi l'existence d'infections multigénotypiques dues á cet allèle au sein des populations africaines. **Mots clés** : Plasmas africains, Anticorps spécifiques, Domaine dimorphique, Epitopes microhétérogènes, Pf3D7-MSP2.

INTRODUCTION

Merozoite surface protein 2 (MSP2) of *Plasmodium falciparum* (*Pf*) (PfMSP2), is a blood-stage protein and a promising vaccine candidate that is characterized by unique domains [1-3]. The dimorphic (D) domain of MSP2 led to the definition of two allelic families of Pf, the 3D7-type (Pf3D7) and FC27-type (PfFC27) alleles [3-5]. Immune responses against the corresponding proteins and synthetic peptide-mimics have demonstrated that the 3D7-D domain of Pf3D7 (Pf3D7-D) was better correlated with protection from clinical malaria in endemic areas, suggesting a predominance of Pf3D7 parasite infections in endemic areas [3, 6, 7]. Mapping the B cell epitopes characterizing the D domains of the MSP2 allelic families (both Pf3D7-MSP2 and PfFC27-MSP2) in endemic blood samples has confirmed that Pf3D7 infections are predominant [6]. In addition, both proteins and synthetic peptides based on 3D7-D were antigenic and immunogenic for mice and humans [8, 9].

On the other hand, while the 3D7-D fragments demonstrate variety in their length and sequence, they also retain a high level of similarity [10-12]. Indeed, in a phase 2b of blood-stage vaccine study, it has been suggested that the conserved epitopes in the D domain were the most important determinants of vaccine-effectiveness against new Pf3D7-type infections [12]. Furthermore, distinct serogroups of Pf3D7-type alleles have already been identified based on sequence similarities and antibody cross-reactivity [13]. All these findings supported the notion that there is a multiplicity of infections and a diversity of 3D7-type msp2 allele genotypes in endemic regions [12-14]. How these regions of micro-heterogeneity in the Pf3D7-D domain "epitopes" shape immune responses in the naturally exposed population in African endemic areas has not yet been characterized. Here, we sought to explore how 25-mer peptides with sequences corresponding to these microheterogeneities shape antibody responses to the Pf3D7-MSP2 dimorphic domain in three African malaria endemic areas, including Mali (ML) and Burkina Faso (BF) in West Africa, and Tanzania (TZ) in East Africa. We hypothesize that characterizing variations in the fine specificity of antibody responses for the 3D7-MSP2 allele types, as a result of sequence micro-heterogeneities, will strengthen future multiepitope, vaccine-candidate design strategies. For this purpose, twelve micro-heterogenous 25-mer motifs characterizing each Pf3D7-msp2 genotype [3, 6, 8, 12], were selected and compared with each other in the GenBank database. The 25-mer microheterogeneous peptides were then tested in ELISA immunoassays to determine their antibody recognition by plasmas from the three African countries where P. falciparum is most widespread.

MATERIALS AND METHODS

2.1. Ethic statement, and permission

This study was conducted using plasma samples previously collected in different studies [6, 15-18]. All plasma used is this study are thus residual samples previously collected, which were anonymized and stored at -80°C. In Mali (ML), the approval was obtained from the Ethical Committee (EC) of the Faculty of Medicine, Pharmacology and Odonto-Stomatology (FMPOS), University of Bamako, Mali (N°0840/FMPOS). For Burkina Faso (BF), no ethical approval was required for a research study in 1998. However, verbal approval (permission) was obtained from the local health authorities and community leaders prior to the sample collection. For Tanzania (TZ), the approval was obtained from the Commission for Science and Technology (UTAFITI NSR/RCA 90). Furthermore, written informed consent (IC) was obtained from each adult, and an informed assent (IA) or IC from a parent or legal guardian was obtained for each minor. Anonymized plasmas from healthy Swiss adults with no exposure to malaria and no malaria history, and who gave their ICs to participate in a previous malaria vaccine study (2012, study NCT01605786) were used as negative controls.

2.2. Blood samples collection

A whole blood was collected in EDTA tubes, and then the plasma was extracted by centrifugation and stored at -80°C before the different tests. Blood was obtained via venipuncture in accordance with the ethical clearances or prevailing standards and guidelines of each respective country. For this study, samples selected were from three malaria-endemic African countries: Mali, ML (adults and children, N=75), Burkina Faso, BF (adults, N=49) and Tanzania, TZ (adults, N=37). The criteria for selecting these plasma

included ensuring all plasma samples be of African origin – from a country where malaria is endemic – and in sufficient quantity to perform ELISA assays. Samples from Burkina Faso (BF) were collected in June 1998 in the Goundry locality, situated 30km from Ouagadougou, the capital city. Samples from Mali were collected in December from 2009 to 2011 in Kenieroba, a village located in the Bancoumana district, 73 km from Bamako (the capital city), and from Dangassa village, 80 km from Bamako. In Tanzania (TZ), samples were remaining from those collected from 1982 to 1984 during a large-scale community-based study undertaken in Ifakara village in the Kilombero District in Morogoro. Qualified personnel, in accordance with the prevailing standards and guidelines, conduct plasma collections in each country. Furthermore, good laboratory practices were observed during sample gathering and handling. There was a minimal risk for participants, as only a small amount of blood was drawn from each participant. Risk of infection at the puncture site was also minimal, with the site having been disinfected prior to blood collection. The discomfort due to the puncture was also minor.

2.3. Synthetic peptides

The synthesis of long peptides was performed as previously described [3, 6, 16] at the Department of Immunobiology, University of Lausanne, Switzerland, using an Applied Biosystem 431A instrument (Foster City. CA. USA). Fmoc-protected amino acids were used in conjunction with acetyl capping of unreacted peptide chains after each coupling step. The 25-mer peptides were synthesized with the use of the MultiRespep Synthesizer (Bioanalytical Instrument, Intavis AG). The long synthetic peptides (LSP) spanned the entire sequence of the 3D7-D domain (3D7-D LSP); the twelve 25-mer peptides from MIP1 to MIP12 covered the micro-heterogeneity within the 3D7-D domain (Table 1). Peptide purity (>80%) was determined using analytical C18 HPLC and mass spectroscopy. After Lyophilization, the peptides were dissolved in phosphate buffered saline, PBS (Gibco® Invitrogen™) at a concentration of 1 mg/mL and stored at -20°C.

2.4. ELISA assays

- Indirect ELISA

Enzyme-linked Immunosorbent Assays (ELISA) were carried out in triplicate for each sample with the use of Maxisorp 96-well microtiter plates (Thermo scientific. Ref. 442404). Plates were coated with 50 µL/well using a 1µg/mL solution of 3D7-D LSP and a 5µg/mL solution for the 25-mer peptides, and stored overnight at 4°C. After blocking for 1 hour at room temperature (RT) with PBSx1-3% milk, the plates were incubated for 2 hours at RT with plasmas diluted to 1:200 in PBSx1-1.5% milk. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG was used as secondary antibody at a dilution of 1:2000 (Life technologies, Ref H10307) and incubated in each well for 1 hour at room temperature. The reaction was developed using TMB substrate (BD OptEIA. cat 555214) for 30 minutes in the dark at RT. The reaction was then quenched using 1M sulphuric acid (Merck. 1.00731.1000). Optical density (OD) was measured at both 450 nm and 630 nm wavelength, with the latter used for background correction, using a TECAN NanoQuant Infinit M200 PRO spectrophotometer. ELISA results were considered positive if the sample mean OD ≥ mean OD + 3SD of the negative control sample (Swiss naïve human plasmas, NHP).

- Competition ELISA

ELISA competition assays were performed by incubating competitor peptides with plasmas at a dilution of 1:300, corresponding to 50% of the maximum signal of the competitor peptide, for 1 hour at RT. The mixture was then added to wells coated with the peptides of interest. The plates were then incubated for 30 minutes at RT, and the reactivity was determined as previously described in indirect ELISA. Each test was performed in duplicate. The percentage of inhibition in the presence of competitor peptide was calculated as 100 − [(mean antibody OD with competitor peptide (inhibited well)) x100/mean antibody OD without competitor peptide (no inhibited well)].

2.5. Affinity purification of 3D7-D-LSP specific antibody

Burkina Faso adult donor N°2 (D2), a plasma that showed high antibody levels for the LSP of the 3D7-D and for the 25-mer micro-heterogenous peptide was used for IgG purification as previously described [16, 18]. Briefly, CNBr-sepharose 4B (Amersham Bioscience AB, Uppsala, Sweden) was activated with 1 mM HCl to prepare the antigen-Sepharose conjugate. Then, 5 mg of the 3D7-D-LSP antigen was dissolved in 1 mL of

coupling buffer (0.1 M NaHCO3 containing 0.5 M NaCl, pH 8.0). D2 plasma was diluted 5-fold with PBS (1x) containing 0.5 M sodium chloride. The mixed plasma with the antigen-sepharose conjugate was stirred O/N gently at 4°C. The bound antibody was eluted with a glycine solution (0.1 M, pH 2.5). The different fractions (F1, F2, F3) were then collected in TRIS solution (1 M, pH 8.0) to instantly neutralize the solutions before dialyzing them against phosphate buffer (0.1M, pH 7.0). Each fraction's antibody (IgG) concentration was determined by the absorbance of the solution at 280 nm. An indirect ELISA test was then performed to assess the recognition of 3D7-D-LSP and to determine the best-purified antibody fraction.

2.6. Statistics

All ELISA data are presented as an average optical density (OD) value from duplicate or triplicate experiments. GraphPad Prism software, version 8.0 was used for the data analysis. The Mann-Whitney test was utilized for comparing two groups, and a Kruskal-Wallis test followed by Dunn's multiple comparison test was used when more than two groups were considered. Fischer's exact and Chi-square tests were used to compare the proportion of responding plasmas between the groups. The statistical significance level was set at $p \leq 0.05$.

RESULTS

3.1. Regions of sequence micro-heterogeneity covering the 3D7-dimorphic domain

The 25-mer micro-heterogeneous peptide regions were identified and compared to the equivalent region (in blue) in the full-length sequence (88-residues, aa95-183) of the Pf3D7-D domain (D-LSP sequence) of MSP2, used here as reference [3, 6, 8]. This comparison with the reference led to the identification of genotypic differences across the 11 PfMSP2-3D7 variants previously isolated from Papua New Guinea (PNG) samples [12] (Table 1). In GenBank online data resources [19], we then matched each 25-mer with the reference 25-mer peptide, MIP1, and synthetized 12 peptides (MIP1 to MIP12) as representatives of each Pf3D7-MSP2 genotype variant. Furthermore, performing matching analysis of the twelve 25-mer peptides with each other resulted in a 1-4 aa discrepancy with an identity ≥ 83% (Table I).

Table I : Sequence of the full-length 3D7-D peptide and sequence alignment of the twelve of 25-mer micro-heterogenous peptides spanning the 3D7-type MSP2 alleles

Full-length sequence of 3D7-MSP2 dimorphic (D) domain:
AEASTSTSSENPNHKNAETNPKGKGEVQE PNQANKETQNNSNVQQDSQTKSNVPPTQ DADTKSPTAQPEQAENSAPTAEQTESPEL QS (95-183. 88 aa). *Genbank accesion number: UDB86418*

Sequence of 25-mer microheterogenous peptides	Peptide name	Number (%) of identical aa	Genbank accession number
PNHKNAETN PKGKGEVQE PNQANKE	MIP1(aa106 -131)	*Reference*	*XM_001 349542*
PNHNNAKTN PKGNGGVQE PNQANKE	MIP2	21 (83%)	*DQ1665 34*
PNHNNAKTN PKGNGGVQK PNQANKE	MIP3	20 (80%)	*DQ1744 42*
PNHKNAETN PKGKGEVQK PNQANKE	MIP4	24 (96%)	*DQ1626 22*
PNHKNAETN PKGKGEVQK TNQANKE	MIP5	23 (92%)	*U07001*
PNHNNAETN PKGKGEVQK SNQANKE	MIP6	22 (88%)	*M73810*
PNHNNAETN PKGNGEVQE PNQANKE	MIP7	23 (92%)	*AJ3187 55*
SNHNNAETN PKGKGEVQE PNQANKE	MIP8	23 (92%)	*DQ1665 45*
TNHNNAETN PKGNGKVQE PNQANKE	MIP9	21 (83%)	*U07009*
PNHNNAETN PKGKGQVQE PNQANKE	MIP1 0	23 (92%)	*U16842*
PNHNNAKTN PKGKGEVQK PNQANKE	MIP1 1	22 (88%)	*AY5345 07*
PNHNNAKTN PKGK-EVQEPNQAN	MIP1 2	22 (88%)	*DQ1685 71*

Sequence alignment of the full-length 3D7-dimorphic (3D7-D) and 25-mer micro-heterogeneous peptides covering the 12 3D7-types MSP2 allelic genotypes. Sequence names refer to GenBank accession numbers. Number and % of identical amino acids (aa)/25 aa shared among each of the 25-mer peptides (MIP2 to MIP 12)

compared with the reference peptide MIP1. The micro-heterogenous sequence is identified (in blue) in the reference 3D7-D sequence. The difference pertaining to aa is highlighted in red and underlined.

3.2. Fine specificity of antibody responses

against the 3D7-D micro-heterogenous 25-mer peptides in plamas from three malaria-endemic African countries

All 12 micro-heterogeneous peptides (MIP1 to MIP12) were tested in ELISA using African plasmas from Mali (ML), Tanzania (TZ), Burkina Faso (BF). All 12 micro-heterogeneous 25-mer peptides were recognized to varying degrees across the three countries. Notably, BF plasmas exhibited significantly higher antibody levels (Figure 1A, $p<0.0001$) and a higher prevalence of responders ranging from 45% to 65% as compared to plasmas from ML donors, which showed marginally higher antibody levels and a slightly broader response range of 15% to 57%, relative to TZ samples, which had the lowest proportion of responders (03% to 46%) (Table 2).

	Mali (N=75) a		**Tanzania (N=37) b**		**Burkina Faso (N=49) c**		**Aggregate (N=161)**		***p value***		
Peptides	n	(%)	n	(%)	N	(%)	n	%	a vs b	a vs c	b vs c
3D7-D LSP	50	67	14	38	49	100	38	68	**	****	****
MIP1	29	39	11	30	32	65	24	45	*ns*	**	**
MIP2	36	48	6	16	23	47	22	37	***	ns	**
MIP3	43	57	8	22	22	45	24	41	***	ns	*
MIP4	36	48	13	35	28	57	26	47	*ns*	*ns*	*Ns*
MIP5	29	39	9	24	32	65	23	43	ns	**	***
MIP6	31	41	17	46	27	55	25	47	ns	ns	*Ns*
MIP7	11	15	17	46	29	59	19	40	***	****	Ns
MIP8	18	24	8	22	26	53	17	33	ns	***	**
MIP9	13	17	2	5	32	65	16	29	*ns*	****	****
MIP10	21	28	3	8	29	59	18	32	*	***	****
MIP11	21	28	4	11	30	60	18	33	*	***	****
MIP12	16	21	1	3	25	51	14	25	**	***	****

The recognition profile for the 25-mer peptides was more consistent between ML and TZ samples, with peptides ranging from MIP1 toMIP6 inducing higher antibody levels for samples from both countries, except MIP7, which unlike Mali, showed higher antibody level in TZ samples (Figure 1A).

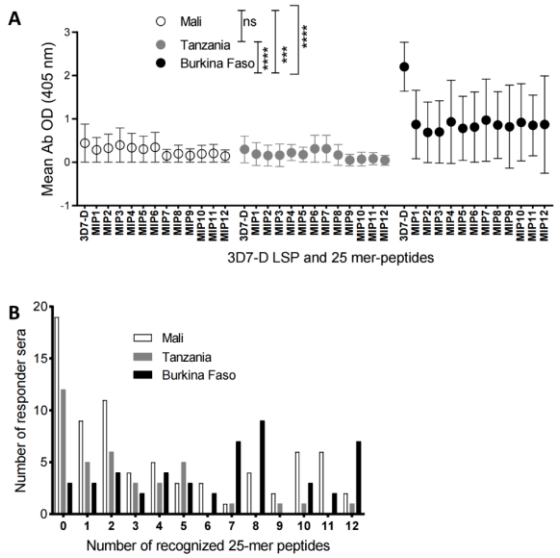

Figure 1: Antibody responses against the micro-heterogeneous regions of 3D7 across different endemic countries

ELISA was performed using the 3D7-D LSP and the 12 micro-heterogenous 25-mer peptides (MIP1 to MIP12) with plasmas from Mali, Tanzania and Burkina Faso. (A) Overall antibody Levels (mean OD value) against the micro-heterogeneous peptides are significantly disparate between countries, with higher levels in BF samples. Kruskal-Wallis test followed by Dunn's multiple comparison test was applied to compare OD values between the three country samples. (B) Responder proportions based on the number of recognized peptides regarding the country samples, Mali, Tz and BF. Ab, antibody; OD, optical density; ns, not significant; ***p<0.001; ****p<0.0001.

However, certain peptides – specifically MIP4 and MIP6 – were similarly well-recognized in all three countries with the responders proportion ranging between 35-57% (Table 2).

Furthermore, aggregated data from positive responder samples in all three countries highlighted a broader recognition of peptides MIP1-MIP6 (Table 2). In some instances, some samples recognized several or even all twelve 25-mer micro-heterogenous peptides, with a higher prevalence in BF and ML donors (Figure1B).

Further analysis of five BF samples (donors D1-D5) which showed high response levels against 3D7-D LSP revealed variations in antibody responses against the 25-mer peptides within the same endemic zone. Notably, donor N°2 (D2) showed higher antibody levels for all 25-mer peptides (Figure 2A). Using purified antibodies (pAb) from the D2 sample against the 3D7-D LSP identified peptides MIP7 and MIP8 as being better recognized 25-mer micro-heterogenous peptides (Figure 2B). A competition ELISA with the pAb from D2 showed strong cross-reactivity between the micro-heterogeneous 25-mer peptides (Figure 3A&B).

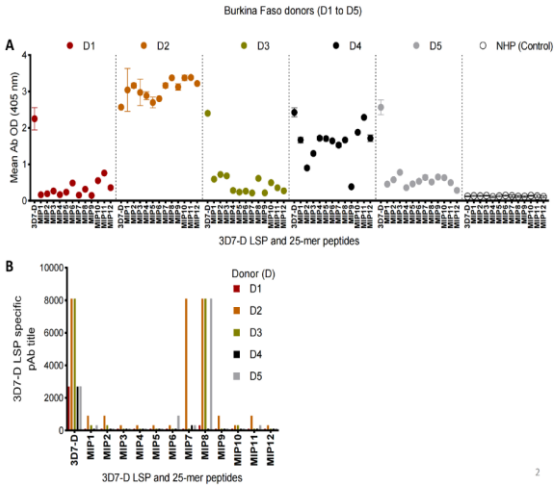

Figure 2: Purified antibody against the 3D7-D-LSP recognize the 25-mer micro-heterogenous peptides.

Five BF donor plasmas (D1 to D5) from which reactive purified antibodies (pAbs) against 3D7-D-LSP were raised were used to perform ELISAs using the 25-mer peptides (MIP1 to 12). (A) Recognition (Ab OD mean) of the 25-mer peptides by five-screened plasmas. The controls plasmas were obtained from Swiss naive adults. (B) Recognition of the 25-mer micro-heterogeneous peptides (antibody title) by the five-screened pAb against 3D7-D-LSP . The antibody title was determined at the starting dilution of 1/100.

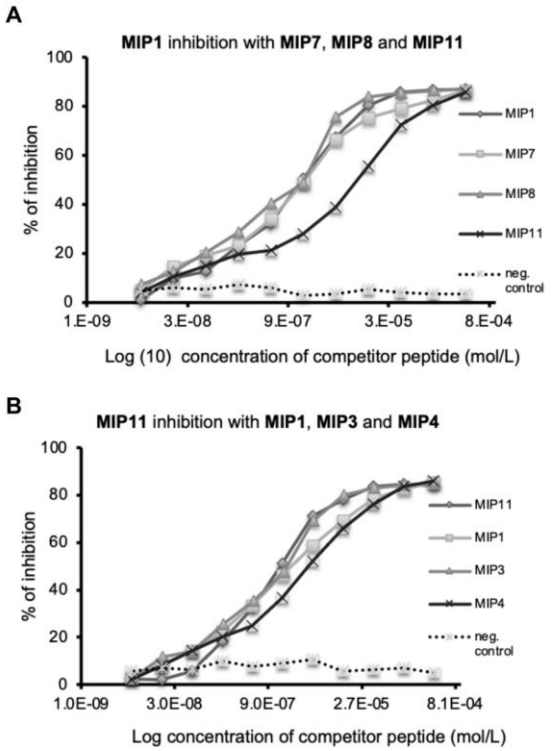

Figure 3 : Cross-reactive antibodies against the 25-mer micro-heterogenous peptides.

The highest recognizing plasma donor N°2 (D2) as shown in Figure 2 was used to perform an inhibitory ELISA for some of the 25 mer-peptides. (A) Inhibition of the MIP1 peptide (the reference sequence) was achieved by using MIP7 and MIP8, the most recognized epitopes. MIP11 is the least recognized by pAb as shown in Figure 2. (B) MIP11, one of the least recognized peptides by pAb was also inhibited by other poorly-recognized peptides, such as MIP1, MIP3 and MIP4. Naive Swiss plasma (NHP) was used as negative controls.

3.3. Fine specificity of antibody responses against the 3D7-D micro-heterogenous 25-mer peptides with regards to age in plasmas from Mali

We then examined antibody recognition of the 25-mer micro-heterogeneous peptides as a function of age. We designated three age groups (≤ 5, 6-14, and ≥ 15-years-old) for samples from Mali (ML) in order to assess the relevance of age in the fine specificity of antibody responses. Remarkably, the 6–14 year-old group showed significantly higher antibody levels (Figure 4A, $p<0.001$) – with the proportion of positive responders ranging from 20%-80% - than ≤ 5-year-old (8%-54% of responders) and ≥ 15 years old (5%-37% of responders) (Table 3). However, the antibody levels were comparable between the ≤ 5 and ≥ 15-year-old age groups (Table 3, Figure 4A). Recognition profiles for MIP1-MIP6 peptides tended to be consistent, with a higher proportion of responders to MIP1-MIP6 peptides than to MIP7-MIP12 peptides across each age group. This trend is consistent with the general trends observed across samples from the three countries (Table 3). In addition, 6–14-year-old groups showed a higher proportion of responders regarding the number of recognized 25-mer peptides. (Figure 4B).

Table III : Proportion of positive responders against the 25-mer long and 3D7-D peptides with regards to age-group in Mali

	≤ 5 y (N =26)a		***6-14 y (N= 30)b***		***≥ 15 y (N =19)c***		***p value***		
Peptides	*n*	*%*	*n*	*%*	*n*	*%*	*a vs b*	*a vs c*	*b vs c*
3D7-D LSP	13	50	25	83	12	63	**	*ns*	*Ns*
MIP1	6	23	16	53	7	37	*	*ns*	*ns*
MIP2	12	43	20	67	4	21	*ns*	*ns*	**
MIP3	14	54	24	80	5	25	*	*ns*	****
MIP4	8	31	21	70	7	37	**	*ns*	*
MIP5	7	27	19	63	3	16	**	*ns*	** *
MIP6	6	23	20	67	5	26	***	*ns*	**
MIP7	2	8	6	20	3	16	*ns*	*ns*	*ns*
MIP8	3	12	12	40	3	16	*	*ns*	*ns*
MIP9	4	15	8	27	1	5	*ns*	*ns*	*ns*
MIP10	6	23	13	43	2	11	*ns*	*ns*	*
MIP11	4	15	15	50	2	11	**	*ns*	**
MIP12	4	15	10	33	2	11	*ns*	*ns*	*ns*

Proportion of responder samples for each age group in Mali was determined. Fisher's exact test was applied to compare responder proportions between two age groups. N, total donor samples; n, number of responders; %, percentage of responders; LSP, long synthetic peptide; a, b and c correspond to the different age group (respectively ≤ 5, 6–14 and ≥ 15 years old); ns, not significant; *p≤0.05; **p<0.01; ***p<0.001; ****p<0.0001.

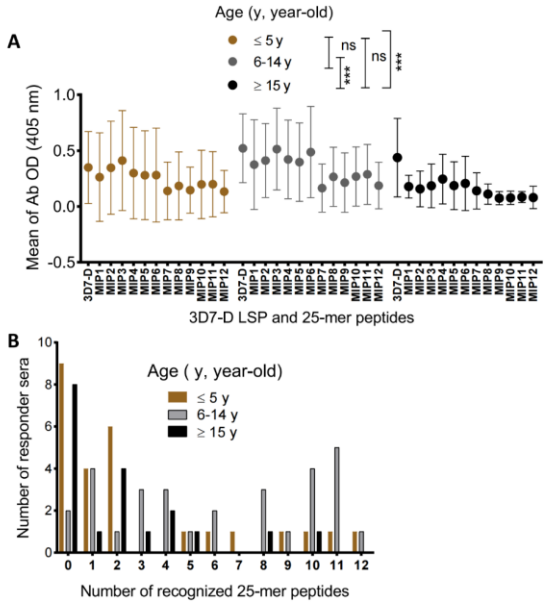

Figure 4 : Mali antibody responses against the micro-heterogeneities of 3D7 according to age group

The same plasmas from Mali shown in Figure 1 were further analysed according to age groups: ≤ 5-years-old; 6–14-years-old and ≥ 15 years-old. (A) Antibody levels against the micro-heterogeneous region were significantly higher in the 6–14 age group as compared to the two other age groups. (B) Distribution of the number of responders regarding the number of the recognized 25-mer peptides for the three age groups. Ab, antibody; OD, optical density; LSP, long synthetic peptide; ns, not significant; ***p<0.001.

DISCUSSION

This study aimed to characterize the fine specificity of natural-acquired malaria antibodies against micro-heterogeneous epitopes within the Pf3D7-D domain of MSP2. This led to the identification of different immune responses against all 12 Pf3D7 genotypes studied herein, with variations across all three African malaria-endemic areas and among different age groups. The results obtained may contribute to the characterization and selection of immunodominant epitopes characterizing the Pf3D7-D domain of MSP2. In previous studies, we and others have characterized the full-length sequence of the Pf3D7-MSP2 dimorphic domain and described the major epitopes for a single 3D7 genotype of MSP2, using samples from malaria-endemic areas and in mice [3, 6, 8]. Here, we refine the characterization of the MSP2-3D7 dimorphic domain antibody recognition by using micro-heterogenous 25-mer peptide fragments. These represent MSP2-3D7 and 11 other Pf3D7-MSP2 genotypes previously identified [12].

We thus tested all 12 micro-heterogeneous 25-mer peptides with samples from all three countries and found that they were recognized to varying extents. Despite significant differences among the three countries, antibody responses in samples from Burkina Faso and Mali were significantly greater for most of the 25-mer peptides compared to Tanzania. These finding raise the question of whether the selected Pf3D7 allelic family variants described in this study are more prevalent in West African countries (Mali and Burkina Faso) which may share the same climate and malaria burden, than in East African countries (Tanzania). [20, 21]. In addition, the trends in the recognition of these 25-mer epitopes (in antibody levels and responder rates) among the three countries support the hypothesis that Pf infection may be caused by a common 3D7 genotype in each endemic area.

Analysis of participant antibodies that recognized multiple 25-mer peptides revealed significant differences in how the Pf3D7-MSP2 genotype variants were identified across each malaria endemic area. In addition, the use of pAb further enhanced the detection accuracy of the 25-mer peptides.

Age is known to influence the outcome of malaria infection and naturally acquired immunity [22-24] since immunity levels typically increase with age throughout childhood before reaching a plateau in adulthood [25-28]. Here, we found significantly higher antibody levels against 25-mer peptides for 6–14 year-olds, followed by children under 5 years, and the lowest levels observed were in individuals 15 years and older. This pattern warrants further exploration. The lower antibody responses in children (≤ 5 years) are consistent with the loss of maternal immunity and the absence of infection-specific immunity that has not yet developed at that age [29]. On the other hand, this does not fully account for the lower immunity observed in those over 15 year, suggesting that factors such as immune-senescence and/or aging may impact host immunity to parasitic infections [30-32]. However, data from this age group must be interpreted with caution, due to the small number of participants (only 19 samples). Further research is needed to determine whether these results highlight a risk for adults or are the product of limited data. Altogether, our findings

further strengthen the notion that immunity levels against malaria are not only dependent on age and exposure, but also on the cumulative number of episodes of clinical malaria experienced by an individual [33-35].This micro-heterogeneity study within the 3D7-D domain enhanced characterization of natural immune responses against the Pf3D7-MSP2 genotype. A multi-epitope recognition observed in plasmas from African populations may suggest the existence of distinct serogroups within the Pf3D7-MSP2 allelic family. These findings imply that recognition of micro-heterogeneities may be dependent on the genetic background of the population and/or their exposure to the same allelic variants of Pf, highlighting the need to further investigation of these micro-heterogeneities in the context of infections with multiple genotypic and localized epidemics. This is of particular importance because it has been suggested that antibodies targeting these micro-heterogeneous repeats fail to protect children against new infections [12]. Furthermore, competing inhibition assays for the 25-mer micro-heterogenous peptides with each other further support the fine specificity of antibodies. Thus, antibody recognition was not only driven by sequence similarity between the 25-mer peptides (which was ≥ 83%), but also by competitive interactions during multi-genotypic exposures to *P. falciparum*, which may result in cross-immune reactions among populations. Indeed, some studies have postulated that pre-existing acquired immunity against either the 3D7 genotype or an ongoing infection can prevent novel infection from the same allelic family [12, 36]. Therefore, longitudinal investigations to understand the dynamics of specific antibodies to these micro-heterogenous epitopes and their correlation with protection from clinical malaria in endemic population settings are warranted.

CONCLUDING CONSIDERATION

By profiling specific antibody responses against micro-heterogeneous fragments of the Pf3D7-MSP2 allelic family – corresponding to the most common parasite – we have created an opportunity to determine the optimal immunodominant epitopes for eliciting pan-reactive and specific antibodies against the Pf allelic family. Furthermore, this study advances the approach of selecting multi-epitopes, paving the way for the development of multivalent malaria vaccines.

Author contributions

SB and GP designed the experiment. SB, NCI, DK and GP performed most experiments, tests, and analyses. SB and GP wrote the manuscript. NCI, DK, GAW, MD, RM and IN contributed to antigen and sample processing, and manuscript revisions. All authors read and approved the submitted version.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

REFERENCE

1. Taylor RR, Smith DB, Robinson VJ, McBride JS, Riley EM. Human antibody response to Plasmodium falciparum merozoite surface protein 2 is serogroup specific and predominantly of the immunoglobulin G3 subclass. Infection and immunity. 1995;63(11):4382-8. doi: 10.1128/iai.63.11.4382-4388.1995. PubMed PMID: 7591074.

2. Metzger WG, Okenu DM, Cavanagh DR, Robinson JV, Bojang KA, Weiss HA, et al. Serum IgG3 to the Plasmodium falciparum merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria. Parasite immunology. 2003;25(6):307-12. Epub 2003/09/26. doi: 10.1046/j.1365-3024.2003.00636.x. PubMed PMID: 14507328.

3. Flueck C, Frank G, Smith T, Jafarshad A, Nebie I, Sirima SB, et al. Evaluation of two long synthetic merozoite surface protein 2 peptides as malaria vaccine candidates. Vaccine. 2009;27(20):2653-61. Epub 2009/05/12. doi: 10.1016/j.vaccine.2009.02.081. PubMed PMID: 19428875.

4. Felger I, Marshal VM, Reeder JC, Hunt JA, Mgone CS, Beck HP. Sequence diversity

and molecular evolution of the merozoite surface antigen 2 of Plasmodium falciparum. Journal of molecular evolution. 1997;45(2):154-60. Epub 1997/08/01. doi: 10.1007/pl00006215. PubMed PMID: 9236275.

5. Thomas AW, Carr DA, Carter JM, Lyon JA. Sequence comparison of allelic forms of the Plasmodium falciparum merozoite surface antigen MSA2. Molecular and biochemical parasitology. 1990;43(2):211-20. Epub 1990/12/01. doi: 10.1016/0166-6851(90)90146-d. PubMed PMID: 2090943.

6. Balam S, Olugbile S, Servis C, Diakité M, D'Alessandro A, Frank G, et al. Plasmodium falciparum merozoite surface protein 2: epitope mapping and fine specificity of human antibody response against non-polymorphic domains. Malar J. 2014;13:510-. doi: 10.1186/1475-2875-13-510. PubMed PMID: 25526742.

7. Genton B, Al-Yaman F, Anders R, Saul A, Brown G, Pye D, et al. Safety and immunogenicity of a three-component blood-stage malaria vaccine in adults living in an endemic area of Papua New Guinea. Vaccine. 2000;18(23):2504-11. Epub 2000/04/25. doi: 10.1016/s0264-410x(00)00036-0. PubMed PMID: 10775784.

8. Balam S, Jafarshad A, Servis C, Frank G, Reed S, Pink R, et al. Immunogenicity of dimorphic and C-terminal fragments of Plasmodium falciparum MSP2 formulated with different adjuvants in mice. Vaccine. 2016;34(13):1566-74. Epub 2016/02/14. doi: 10.1016/j.vaccine.2016.02.013. PubMed PMID: 26874325.

9. Eacret JS, Gonzales DM, Franks RG, Burns JM, Jr. Immunization with merozoite surface protein 2 fused to a Plasmodium-specific carrier protein elicits strain-specific and strain-transcending, opsonizing antibody. Scientific reports. 2019;9(1):9022. Epub 2019/06/23. doi: 10.1038/s41598-019-45440-4. PubMed PMID: 31227760; PubMed Central PMCID: PMCPMC6588637.

10. Felger I, Irion A, Steiger S, HP. B. Genotypes of merozoite surface protein 2 of Plasmodium falciparum in Tanzania. Trans R Soc Trop Med Hyg. 1999;93(Suppl 1):3-9.

11. Felger I, Steiger S, Hatz C, Smith T, Beck HP. Antigenic cross-reactivity between different alleles of the Plasmodium falciparum merozoite surface protein 2. Parasite immunology. 2003;25(11-12):531-43. Epub 2004/04/01. doi: 10.1111/j.0141-9838.2004.00664.x. PubMed PMID: 15053774.

12. Flück C, Schöpflin S, Smith T, Genton B, Alpers MP, Beck HP, et al. Effect of the malaria vaccine Combination B on merozoite surface antigen 2 diversity. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2007;7(1):44-51. Epub 2006/05/02. doi: 10.1016/j.meegid.2006.03.006. PubMed PMID: 16647307.

13. Franks S, Baton L, Tetteh K, Tongren E, Dewin D, Akanmori BD, et al. Genetic diversity and antigenic polymorphism in Plasmodium falciparum: extensive serological cross-reactivity between allelic variants of merozoite surface protein 2. Infection and immunity. 2003;71(6):3485-95. Epub 2003/05/23. doi: 10.1128/iai.71.6.3485-3495.2003. PubMed PMID: 12761133; PubMed Central PMCID: PMCPMC155717.

14. Cortés A, Mellombo M, Benet A, Lorry K, Rare L, Reeder JC. Plasmodium falciparum: distribution of msp2 genotypes among symptomatic and asymptomatic individuals from the Wosera region of Papua New Guinea. Experimental parasitology. 2004;106(1-2):22-9. Epub 2004/03/12. doi: 10.1016/j.exppara.2004.01.010. PubMed PMID: 15013785.

15. Ayadi I, Balam S, Audran R, Bikorimana J-P, Nebie I, Diakité M, et al. P. falciparum and P. vivax Orthologous Coiled-Coil Candidates for a Potential Cross-Protective Vaccine. Frontiers in immunology. 2020;11. doi: 10.3389/fimmu.2020.574330.

16. Villard V, Agak GW, Frank G, Jafarshad A, Servis C, Nébié I, et al. Rapid identification of malaria vaccine candidates based on alpha-helical coiled coil protein motif. PLoS One. 2007;2(7):e645-e. doi: 10.1371/journal.pone.0000645. PubMed PMID: 17653272.

17. Olugbile S, Villard V, Bertholet S, Jafarshad A, Kulangara C, Roussilhon C, et al. Malaria vaccine candidate: design of a multivalent subunit α-helical coiled coil poly-epitope. Vaccine. 2011;29(40):7090-9. Epub 2011/08/02. doi: 10.1016/j.vaccine.2011.06.122. PubMed PMID: 21803099; PubMed Central PMCID: PMCPMC4165486.

18. Olugbile S, Kulangara C, Bang G, Bertholet S, Suzarte E, Villard V, et al. Vaccine

potentials of an intrinsically unstructured fragment derived from the blood stage-associated Plasmodium falciparum protein PFF0165c. Infection and immunity. 2009;77(12):5701-9. Epub 2009/09/30. doi: 10.1128/iai.00652-09. PubMed PMID: 19786562; PubMed Central PMCID: PMCPMC2786454.

19. https://rb.gy/hzofst. Accession Number prefixes: Where did the data originate? https://rbgy/hzofst.

20. Mafwele J, JW L. Relationships between transmission of malaria in Africa and climate factors. Scientific reports. 2022;12(1):14392. doi: 10.1038/s41598-022-18782-9.

21. https://rb.gy/7sd1za. Malaria: Monitoring the situation of children and women. https://rbgy/7sd1za.

22. Dassé R, Lefranc D, Dubucquoi S, Dussart P, Dutoit-Lefevre V, Sendid B, et al. Changes Related to Age in Natural and Acquired Systemic Self-IgG Responses in Malaria. Interdisciplinary perspectives on infectious diseases. 2011;2011:462767. Epub 2012/01/19. doi: 10.1155/2011/462767. PubMed PMID: 22253622; PubMed Central PMCID: PMCPMC3255176.

23. Dassé R, Lefranc D, Dubucquoi S, Dussart P, Dutoit-Lefèvre V, Sendid B, et al. [Singular, systemic, self-reactive IgG patterns related to age: relationship with cerebral malaria susceptibility in exposed subjects residing in an endemic area in Abidjan, Côte-d'Ivoire]. Bulletin de la Societe de pathologie exotique (1990). 2012;105(4):276-83. Epub 2012/08/14. doi: 10.1007/s13149-012-0252-y. PubMed PMID: 22886432.

24. Yazdani SS, Mukherjee P, Chauhan VS, Chitnis CE. Immune responses to asexual blood-stages of malaria parasites. Current molecular medicine. 2006;6(2):187-203. Epub 2006/03/07. doi: 10.2174/156652406776055212. PubMed PMID: 16515510.

25. Wilson S, Booth M, Jones FM, Mwatha JK, Kimani G, Kariuki HC, et al. Age-adjusted Plasmodium falciparum antibody levels in school-aged children are a stable marker of microgeographical variations in exposure to Plasmodiuminfection. BMC Infectious Diseases. 2007;7(1):67. doi: 10.1186/1471-2334-7-67.

26. Baird JK, Jones TR, Danudirgo EW, Annis BA, Bangs MJ, Basri H, et al. Age-dependent acquired protection against Plasmodium falciparum in people having two years exposure to hyperendemic malaria. The American journal of tropical medicine and hygiene. 1991;45(1):65-76. Epub 1991/07/01. doi: 10.4269/ajtmh.1991.45.65. PubMed PMID: 1867349.

27. Baird JK. Age-dependent characteristics of protection v. susceptibility to Plasmodium falciparum. Annals of tropical medicine and parasitology. 1998;92(4):367-90. Epub 1998/07/31. doi: 10.1080/00034989859366. PubMed PMID: 9683890.

28. Doolan DL, Dobaño C, Baird JK. Acquired immunity to malaria. Clinical microbiology reviews. 2009;22(1):13-36, Table of Contents. Epub 2009/01/13. doi: 10.1128/cmr.00025-08. PubMed PMID: 19136431; PubMed Central PMCID: PMCPMC2620631.

29. Schumacher RF, Spinelli E. Malaria in children. Mediterranean journal of hematology and infectious diseases. 2012;4(1):e2012073. Epub 2012/12/04. doi: 10.4084/mjhid.2012.073. PubMed PMID: 23205261; PubMed Central PMCID: PMCPMC3507524.

30. Xu W, Wong G, Hwang YY, Larbi A. The untwining of immunosenescence and aging. Seminars in immunopathology. 2020;42(5):559-72. Epub 2020/11/10. doi: 10.1007/s00281-020-00824-x. PubMed PMID: 33165716; PubMed Central PMCID: PMCPMC7665974.

31. Frimpong A, Kusi KA, Adu-Gyasi D, Amponsah J, Ofori MF, Ndifon W. Phenotypic Evidence of T Cell Exhaustion and Senescence During Symptomatic Plasmodium falciparum Malaria. Frontiers in immunology. 2019;10:1345. Epub 2019/07/19. doi: 10.3389/fimmu.2019.01345. PubMed PMID: 31316497; PubMed Central PMCID: PMCPMC6611412.

32. Felizardo AA, Marques DVB, Caldas IS, Gonçalves RV, Novaes RD. Could age and aging change the host response to systemic parasitic infections? A systematic review of preclinical evidence. Experimental gerontology. 2018;104:17-27. Epub 2018/01/26. doi: 10.1016/j.exger.2018.01.022. PubMed PMID: 29366738.

33. Rodriguez-Barraquer I, Arinaitwe E, Jagannathan P, Boyle MJ, Tappero J, Muhindo M, et al. Quantifying Heterogeneous Malaria

Exposure and Clinical Protection in a Cohort of Ugandan Children. The Journal of infectious diseases. 2016;214(7):1072-80. Epub 2016/08/03. doi: 10.1093/infdis/jiw301. PubMed PMID: 27481862; PubMed Central PMCID: PMCPMC5021229.

34. White M, Watson J. Age, exposure and immunity. eLife. 2018;7. Epub 2018/08/22. doi: 10.7554/eLife.40150. PubMed PMID: 30129437; PubMed Central PMCID: PMCPMC6103766.

35. Rodriguez-Barraquer I, Arinaitwe E, Jagannathan P, Kamya MR, Rosenthal PJ, Rek J, et al. Quantification of anti-parasite and anti-disease immunity to malaria as a function of age and exposure. eLife. 2018;7. Epub 2018/07/26. doi: 10.7554/eLife.35832. PubMed PMID: 30044224; PubMed Central PMCID: PMCPMC6103767.

36. de Roode JC, Culleton R, Bell AS, Read AF. Competitive release of drug resistance following drug treatment of mixed Plasmodium chabaudi infections. Malar J. 2004;3:33. Epub 2004/09/16. doi: 10.1186/1475-2875-3-33. PubMed PMID: 15367331; PubMed Central PMCID: PMCPMC517944.