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The Use of Crude *Plasmodium falciparum* Antigens for Comparison of Antibody Responses in Patients with Mild Malaria vs. Cerebral Malaria

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ABSTRACT

Background: Cerebral malaria (CM) is one of the major causes of death in African populations infected with *Plasmodium falciparum*. Only 1% of infected subjects develop CM. The reasons for these differences are not fully understood, but it is likely that the host humoral response against blood-stage antigens plays a role in protection from malaria, although the precise targets and mechanisms mediating immunity remain unclear. **Objective**: The purpose of this study was to distinguish between defined *P. falciparum*-specific Ab response patterns in patients presenting with mild malaria (MM) vs. CM. **Methods**: We used a panel of *P. falciparum* conserved antigens including crude blood-stage extracts schizont, merozoite and parasitised erythrocyte membranes and MSP-1p19, *Pf*EB200, R23 and GST-5 recombinant antigens in a retrospective case-control study of symptomatic adults, one group presenting confirmed CM without fatal outcome and another group with MM. We further matched *P. falciparum*-specific Ab responses with those from individuals living in an endemic setting known to have protective immunity and considered them as "immune control" subjects (IC). Total IgG, IgM and IgG subclass Ab responses were determined using ELISA method. **Results**: Substantial Ab responses were found in symptomatic patients, significantly lower than the "immune control" subjects, and with a limited quantitative difference between MM versus CM. Interestingly, asynchronous IgM response was evidenced in CM contrary to MM. **Conclusion:** Our results suggest that the contribution of an efficient IgG response against parasite multiplication is of importance in the evolution towards CM manifestation without fatal outcome and deserves further analysis for vaccine candidates.

Keywords: Antibody Response, Cerebral Malaria, Non-Complicated Malaria, *Plasmodium falciparum*

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INTRODUCTION

Plasmodium falciparum malaria is one of the leading lethal infections in sub-Saharan Africa populations. With approximately 1–2 million deaths annually, the situation seems far from future improvement as it is linked with increasing poverty-related problems, growing resistance of anopheline vectors to insecticides and of parasites to drug treatment (1). In non-immune individuals, early diagnosis and appropriate treatment most frequently lead to recovery within a few days but a late diagnosis or inadequate therapy may lead to the development of clinical complications such as anaemia, cerebral malaria, and pulmonary and renal failure, increasing the risk of death (2). In populations living in high malaria endemic areas, a tolerance of persistent parasitaemia is described and explained by the acquisition of antiparasitic immunity. This effective immunity against *P. falciparum* malaria develops slowly over time after repeated exposure and protects against the development of symptomatic and severe illness (3). Nevertheless, protection is critically dependent on the presence of certain types of antibodies (Abs) as clearly demonstrated by passive transfer in experimental models (4) and humans (5).
Insufficient and/or inadequate Ab responses could take place upstream amongst the multiple factors contributing to the development of severe manifestations such as cerebral malaria (CM). We hypothesize that an insufficient level of Ab responses could be possibly evidenced at the "earliest" state of infection and explains the occurrence of severe complications. In this line, we examined and compared the profiles of Abs to a panel of *P. falciparum* Ags in urban adults presenting symptoms of malaria leading to hospitalisation or consultation. We performed a retrospective case-control study of well-categorised patients with acute malaria symptoms: one group of patients were hospitalised for confirmed CM without fatal outcome and another group of individuals were admitted to the hospital with mild malaria (MM). Additionally, we matched *P. falciparum*-specific Abs responses in acute malaria patients with those from individuals living in an endemic setting and known to have protective immunity, which are referred here as immune controls (IC) and are hypothesised to display a large panel of naturally acquired protection-associated Ab responses before infection (3). The Ags tested were: (i) crude blood-stage of *P. falciparum* extracts (schizont, merozoite and parasitised erythrocyte membranes (IRBCm); (ii) MSP1p19 and *Pf*EB200, R23, GST-5 recombinant antigens. This work is an extention of some previously published results (6) but provides novel data and interpretations related to the group of adults with CM.

MATERIALS AND METHODS

Sample Collection and Study Population. Three categories of subjects, namely cerebral malaria (CM) patients, mild malaria (MM) patients and immune control (IC) subjects were enrolled in the study. Informed consent was obtained from the participants and/or their relatives prior to inclusion, after giving them verbal information in their native language. The protocols were approved by the National Ethic Committee and the Ministry of Health of Senegal. Clinical background and information were collected for each patient from physician's records and study questionnaires.

Inclusion Criteria. The following criteria were used to enrol the subjects into different clinical groups:

Cerebral Malaria (CM). CM was defined as unrousable coma (non-purposeful response or no response to a painful stimulus by Glasgow modified score <9) with microscopically diagnosed *P. falciparum* and haveing no other clinically evident cause of impaired consciousness such as hypoglycemia, meningitis, and encephalitis following WHO criteria (2). Patients with other infections in addition to malaria parasites were excluded.

Mild Malaria (MM). Patients who had fever with *P. falciparum* parasitaemia of < 25 000 parasites/µl of blood with no evidence of impaired consciousness or seizures at the time of enrollment and no other past background of mental illness, meningitis or accidental head injury were included.

Immune Control (IC). Individuals identified as immune controls (IC) were from the village of Ndiop, a rural meso-endemic area of transmission. IC subjects were positive for malaria parasite but had no clinical symptoms and were described as resistant to malaria infection as evidenced throughout the longitudinal follow-up carried out over several years (3). A set of 39 samples from subjects (14.2 - 62 years, mean 29.8 years) were enrolled after the 1998 transmission season year was used. CM and MM patients were treated at Hospital Principal in Dakar (Senegal)- the patients and were from the city of Dakar and suburbs. In this area, more than 2 million people are exposed to less than 0.5 infective bites per individual per year during the raining season with a high variable density of the vector (7).

A mean incidence of 2.4% of clinical access (26 cases out of 1067) was observed and there was no difference between adults and children (8). Samples were collected up on admission to the hospital and from mid-September to the end of December over the years 1998–1999. The group with symptomatic malaria was composed of 72 patients aged over 13 years; 36 patients had confirmed CM and recovered without after-effects (mean age 28 years), and 36 had MM (mean age 31.8 years) (Table 1). 70 Healthy controls from Dakar who did not have malaria infection (as determined by microscopy) or any other febrile illness were, selected at the same period of enrolment and were analysed as our previous studies (6,9). Data showed low IgG and IgM antibody levels against *P. falciparum* sexual blood antigens and were not significantly different from European nonexposed subjects used as negative controls in ELISA protocols.

For all subjects, parasitaemia was measured by examination of Giemsa stained blood smears using a magnification of 1000x and measuring the parasite count per 1000 leukocytes. The count per microliter was calculated according to WHO recommendations. There was no significant difference in the distribution of the age or in the parasite densities according to disease severity (Table 1). No correlation was found between age and parasitaemia levels in the three groups. CM or MM patients treated with antimalarial drugs were excluded from the study.

Blood samples were collected and plasma was separated from red blood cells by centrifugation, and stored at –20 °C until use.

Table 1. Characteristics of the study population

	Cerebral Malaria (CM)	Mild malaria (MM)	Immune control (IC)
Number of patients	36	36	39
Sex (M/F)	18/29	20/16	17/12
Mean age in years (Min - Max)	28.0 (14 – 73)	31.8 (15 - 62)	29.8 (14.2 – 62)
Parasite density (paras./µl)[a]	5435	5727	6125
Mean delay before consultation (days)	5.1	4.2	nc[b]
Patients consulting >4 days after onset of the symptoms (%)	51%	38%	nc[b]

[a]Parasitaemia was counted on Giemsa stained thick smears for 1000 leukocytes and expressed as parasites per microliter of blood, according to WHO standards. Geometric means were calculated

[b]nc = not concerned

Antigens and ELISA Procedure. Three crude antigenic extracts were prepared: (i) a lysate of in vitro matured schizont enriched *P. falciparum* IRBC; (ii) entire merozoites; (iii) erythrocyte membranes from red blood cells infected by mature parasites (IRBCm) and control ghosts (RBC) prepared according to our previous methods. The preparation of these Ag sources was done by using FCR3 strain of *P. falciparum* as described (9). The total protein concentration in the parasite preparations was estimated by a Biorad® precision assay. Ag preparations were kept at –80°C in working aliquots until use.

Three purified recombinant proteins fused to *Schistosoma japonicum* glutathione-*S*-transferase (GST) in the pGEXA vector were used: (i) R23 which contains 11 copies of a 6 amino acid repeat derived from the central domain of antigen R45; the consensus sequence is HKSDSN/S/H (10); (ii) *Pf*EB200, which contains 13 repeats with characteristic Glu-Glu dimmers and derived from *Pf*332, a conserved giant protein accessible on the infected red blood cell surface in late schizonts (11); (iii) GST-5 which is the recombinant product expressing the 1450 bp EcoRI fragment of the C-proximal region of *Pf*EMP3 (FUP/SP Palo Alto alias FCR3). *Pf*EMP3-cl5 (or GST-5) was identified as a target of variant immune response whose expression level of *Pf*EMP3 was shown to be modulated during antigenic variation (12). The major surface associated merozoite Ag MSP1p19 was tested in the present study; the C-terminal domain of the Palo Alto MSP1 allele, was produced in *Sodoptera frugiperda* infected with the recombinant baculovirus and purified by nickel agarose chromatography (13).

All Ags, diluted in sterile PBS, were coated in ELISA plates with 100 µl/well. Each crude antigen preparation was coated overnight at 10–15 µg/ml, GST-fused recombinant protein at 1 µg/ml, and MSP1p19 at 0.5 µg/ml. Plasma samples were diluted 1:100 in PBS with 1% BSA/0.05% Tween 20, and the ELISA procedure was then performed as previously described (9,14,15). Reagents were from Sigma Chemicals (St Louis, MO) unless stated otherwise. For the determination of IgG sub-classes, human sub-class specific mouse mAbs were used and assayed using peroxidase-labelled goat anti-mouse IgG (1:2000). Calibration was done beforehand, and optimal concentrations were: IgG1 (clone NL16), 1:2000; IgG2 (clone HP6002) and IgG3 (clone ZG4), 1:10 000; IgG4 (clone GB7B), 1:30 000 as described elsewhere (9,16,17).

Negative control (pool of sera from European citizens not exposed to *P. falciparum* or other infective species and positive control (pool of sera from clinically immune adults living in Dielmo) were included in the assay. Results were expressed as OD ratios ($OD_{sample}/OD_{negative\ control}$). The OD signal of the samples was individually corrected for GST or RBC signal. In this study, individuals with OD ratio>2 (corresponding to a mean OD±2SD of controls) were considered as positive responders (17).

Statistical Analysis. Comparisons of antibody levels between different groups were done by the MannWhitney rank test, the Kruskal-Wallis test, the Wilcoxon signed rank test and the Spearman rank correlation test for non-normally distributed data. The exact Fisher's test was used to compare between groups. P values <0.05 after correction of Bonferroni for multiple comparisons were considered significant. Statistical analyses were performed using Statview 5.0® software (SAS Institute, Cary, NJ).

RESULTS

Level and Incidence of IgG Responses Against Different Ags. High levels of IgG responses and high percentage of responders (percent of samples giving an OD ratio value greater than 2) were found against crude extracts Ag preparations in three groups of individuals: with symptoms (CM, MM) or without symptoms (IC).

Levels of IgG responses were not significantly different between CM and MM groups (Table 2).

Table 2. Levels and incidences of IgG and IgM responses against parasite extracts and recombinant antigens

	Cerebral Malaria (n=36)				Mild malaria (n=36)				Immune control (n=39)			
	mean OD	Ratio[a]		%	mean OD	Ratio[a]		%	mean OD	Ratio[a]		%
	Value±SD	mean±SD	med	resp[b]	Value±SD	mean±SD	med	resp[b]	Value±SD	mean±SD	med	Resp[b]
IgG to schizont	0.61 ± 0.38	2.5 ± 1.5	2.1	50%	0.69 ± 0.33	2.9 ± 1.2	2.6	69%	0.92 ± 0.36	6.0 ± 2.4	6.3	98%
IgM to schizont	0.22 ± 0.20	1.8 ± 1.1	1.3	31%	0.32 ± 0.35	2.1 ± 1.5	1.6	42%	nd[c]	-	-	-
IgG to merozoite	0.64 ± 0.40	2.4 ± 1.0	2.3	61%	0.69 ± 0.32	2.5 ± 0.9	2.7	69%	0.98 ± 0.44	3.4 ± 1.2	3.5	88%
IgM to merozoite	0.22 ± 0.18	2.0 ± 1.0	1.9	47%	0.27 ± 0.27	2.2 ± 1.5	1.9	50%	nd[c]	-	-	-
IgG to IRBCm	0.34 ± 0.32	3.0 ± 1.7	2.5	50%	0.41 ± 0.24	3.5 ± 1.4	3.3	88%	0.89 ± 0.52	4.4 ± 2.1	4.6	83%
IgM to IRBCm	0.06 ± 0.06	1.7 ± 0.6	1.8	08%	0.07 ± 0.06	2.3 ± 1.2	1.9	17%	nd[c]	-	-	-
IgG to *Pf*EB200	0.35 ± 0.20	1.3 ± 0.4	1.0	14%	0.34 ± 0.26	1.3 ± 0.7	1.0	11%	0.40 ± 0.74	3.2 ± 4.2	1.1	38%
IgG to *Pf*EMP3-cl5	0.23 ± 0.40	2.2 ± 2.4	1.0	22%	0.30 ± 0.48	2.8 ± 3.1	1.0	31%	0.90 ± 0.88	7.4 ± 6.4	5.2	73%
IgG to R23	0.43 ± 0.27	1.3 ± 0.6	1.0	08%	0.36 ± 0.29	1.2 ± 0.7	1.0	8%	0.12 ± 0.24	2.0 ± 1.9	1.1	23%
IgG to MSP1p19	1.51± 0.55	7.2 ± 2.5	8.0	94%	1.62 ± 0.47	7.8 ± 2.2	8.5	100%	0.71 ± 0.52	7.7 ± 4.8	6.2	85%

[a] Mean Ab levels are shown as mean OD ± SD, mean OD ratio ± SD and median OD ratio (med) of each group; the background signals for IRBC and GST fusion proteins were subtracted from the individual Ag-specific signal

[b] Incidence of positive responses i.e. individuals with OD ratio ≥ 2

[c] nd = not done

In contrast, levels of IgG response against schizont, merozoite and IRBCm antigens were significantly higher in IC subjects ($p<0.001$) compared to MM and CM groups. Additionally, the percentage of responders against schizont Ags was significantly higher in IC subjects than in CM and MM patients ($\chi^2>11$; $p<0.001$). Interestingly, there was a significantly lower incidence of IgG responses against IRBCm in CM patients compared to MM ($\chi^2>11$; $p<0.001$).

IgG antibody levels and prevalence of positive IgG responses against IRBCm-associated recombinant Ag (R23, *Pf*EB200 and *Pf*EMP-cl5) were not different between MM and CM patients. However, the levels of IgG against all the IRBCm-associated recombinant Ags appeared to be significantly higher ($p<0.001$) in IC subjects than in those with acute malaria symptoms (CM and MM).

We also found a lower percentage of IgG responders against *Pf*EB200 and *Pf*EMP-cl5 in MM and CM (11% and 31% of responders) compared to IC individuals (38% and 73%) ($\chi^2>7$; $p<0.01$). An overall high percentage of responses and IgG levels against MSP1p19 were observed, without significant variations among the different groups.

The majority of IgG responses showed a significant degree of colinearity, with similar characteristics for all three groups. This was found for IgG responses against different extracts where p values ranged from 0.7 to 0.9 ($p<10^{-4}$) and there was a significant degree of colinearity between IgG responses against *P. falciparum* extracts and MSP1p19 ($p=0.5–0.8$; $p<0.001$). However, the degree of colinearity was lower between IgG responses against IRBCm-associated recombinant Ags and *P. falciparum* extracts. There were limited differences between the groups of individuals, i.e. the colinearity of IgG responses to different Ags was mostly significant simultaneously in the three groups.

In addition, there was no correlation between IgG responses and the age of individuals in the MM and CM groups. In the IC group, only IgG responses against schizont and IRBCm were correlated with age, possibly due to the limited number of adults in this group.

Levels and Incidence of IgM Responses Against Somatic Ags. IgM responses against shizont, merozoite and IRBCm extracts were measured in the CM group vs. the MM group. As summarised in Table 2, we found similar levels and prevalences of IgM responses in MM and CM groups. Substantial levels of IgM against all three extracts were evidenced with decreasing incidence (50% to 15%) from merozoite to IRBCm.

As shown in Figure 1, there was a marked difference in the reciprocal relationship between IgG and IgM responses against *P. falciparum* extract in the MM group compared to the CM group. In MM group, IgG and IgM responses against merozoite (Figure 1a) and schizont extracts (Figure 1c) were significantly correlated ($p<0.01$, $p=0.45$ and 0.58, respectively), and become significant for IRBCm ($p=0.35$, $p=0.04$, but non-significant after Bonferoni correction), contrary to patients with CM for their responses against merozoite (Figure 1b) and schizont extracts (Figure 1d).

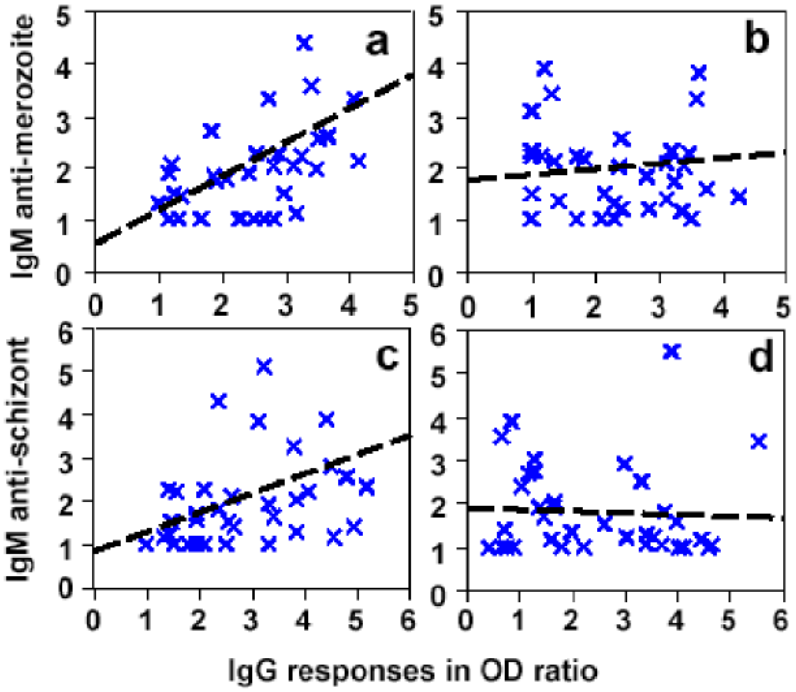

Figure 1. Scatter plot of the relationship between IgG and IgM responses against merozoite (a,b) and schizont (c,d) in clinical groups. Shown are the reciprocal relationship between IgG responses (*x* axis) vs IgM responses (*y* axis): on upper panel, against *P. falciparum* merozoite for individuals with mild malaria (MM) (a), and with cerebral malaria (CM) (b); lower panel, against schizont extract for the MM group (c), and for CM group (d). A significant relationship between IgG and IgM responses was found for Ndiop MM ($p<0.001$, Spearman rank test) but not for CM patients.

IgG Subclasses Against Schizont, Merozoite and MSP1p19. The IgG subclasses of MM, CM and IC groups against schizont and merozoite antigens extracts are shown in Figure 2. Despite similar levels and incidences of total IgG responses against schizont extract, a slightly different profile of IgG subclasses between MM and CM groups was found with schizont extract. In MM group, IgG responses contained mainly of IgG1 and IgG3 (approx. 70% and 30%, respectively), whereas in the CM group, it was mostly restricted to the IgG1 subclass (Figure 2a).

As indicated in Figure 2, when comparing IgG subclass levels between MM and CM groups, we found IgG2 and IgG3 responses against schizont Ag extract to be significantly lower (Figure 2a) and IgG2 level against merozoite Ag extract also significantly lower (Figure 2b) ($p<0.02$) in CM group.

In IC group, IgG Abs against merozoite extract contained predominantly IgG1 and IgG3. Interestingly, all sera of the control group were positive for IgG anti-merozoite extract (100% prevalence).

The mean levels of IgG1 and IgG3 from the IC subjects were significantly higher than in MM and CM patients ($p<0.01$ for IgG1; $p<0.001$ for IgG3) regardless of disease category (Figure 2b).

Comparing the levels of IgG1 and IgG3 against schizont extract in IC individuals versus CM and MM patients, we found that these responses were significantly higher ($p<0.001$) in clinically immune subjects from Ndiop than in symptomatic patients from Dakar. Similar results were found for IgG2 responses against schizont extract (Figure 2a).

IgG subclass Abs against MSP1p19 were mainly IgG1 and appeared similar to anti-merozoite IgG subclass distribution, without any significant difference between CM and MM (data not shown).

IgG Abs to MSP1p19 Ag were predominantly IgG1 in IC subjects. We found also a high level of IgG3 anti-MSP1p19 Abs but not like IgG1 level. Significantly lower prevalence of IgG1 and IgG3 Abs anti-MSP1p19 was observed in CM and MM patients compared to IC individuals ($p<0.01$) and regardless of disease category.

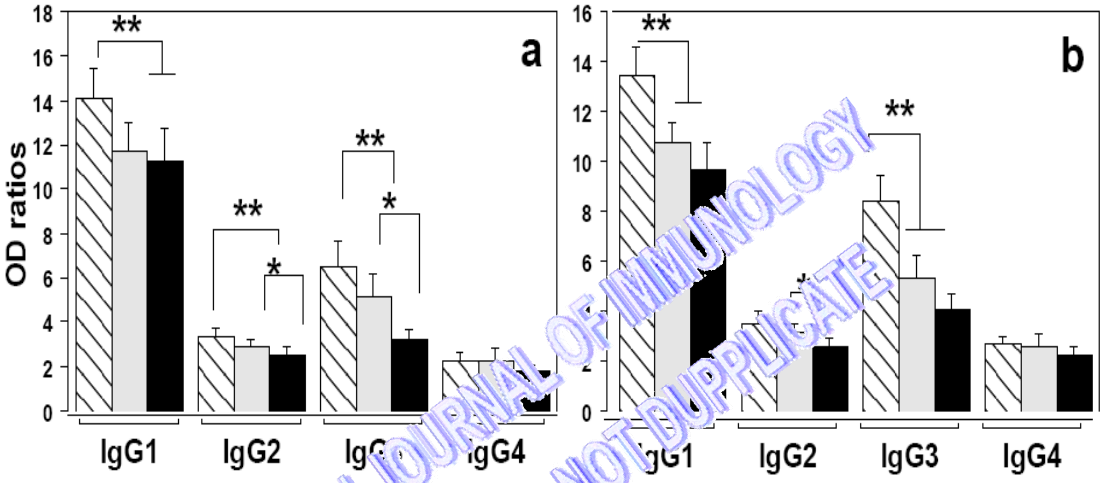

Figure 2. Pattern of IgG subclass Ab responses against schizont and merozoite antigens in MM, CM and IC groups. Shown are box plots of anti-shizont (a) and anti-merozoite (b) IgG subclass Ab responses in patients with symptoms of MM (light grey boxes), cerebral malaria (dathan in CM and MM, i.e. IgG1, IgG2 and IgG3 for schizont Ag, IgG1 and IgG3 for merozoite ($p<0.01$, Kruskal-Wallis Test).

DISCUSSION

This study aimed to evaluate *P. falciparum*-specific IgG and IgM antibodies responses in patients with mild malaria vs. cerebral malaria. We focused on Ab responses against a set of *P. falciparum* total and recombinant Ags in individuals living in an urban area of low endemicity (7) who developed acute symptoms of illness upon infection and required hospitalisation or consultation. It is commonly admitted that certain types of Abs, in terms of both Ag recognition and subclass are mediated or associated with protection against clinical symptoms and severe illness. Indeed, two groups of patients from the same urban locations, who were considered non-protected against clinical malaria, were selected and categorised on the basis of the clinical symptoms. These indi-

viduals, regardless of age, previous history of potential infection and variable conditions of exposure to infective bites, can be considered to be equally at risk for infection resulting in non-fatal cerebral malaria symptoms or not. Limitations to such a study include a lack of knowledge about the occurrence of malaria episode and the exact timing where a treatment was given, leading to a highly variable sequence of events before reaching the hospital. In addition, these individuals are likely to be partially immune, as they were adults who had been infected in the past. We do not know whether antibody levels at the admission in the hospital reflect pre-existing immune responses or whether they are a response to the current infection. Because these unknown factors could differ between the two disease groups, we have interpreted our results with caution, and compared them with a third group used as Immune control group. This control group consisted of non-urban individuals, who acquired natural immunity by repeated exposure in an age-dependent manner. Indeed, their history of infection and the circulating strains of parasite were substantially different. In these villagers, children are known to have a much higher degree of efficient anti-malarial immunity than the older urban adults living in Dakar (18,19). However, as we investigated Ab responses to "conserved" Ags, the use of such well-defined samples from non-urban individuals could be considered as relevant "immune" controls for comparison with parameters of "protection-associated Ab profiles" in this study, despite the different geographical settings.

The pathogenesis of malaria complications is not completely understood but is generally accepted to be multi-factorial. Cerebral malaria syndrome occurs preferentially in young individuals and in adults who have a low degree of immunity. It is possible that adaptive immune responses to key conserved Ag might provide sufficient additive capacity to innate immunity (20) in decreasing the parasite load and contributing to the prevention of cerebral syndromes. In this regard, our findings highlight lower levels of total IgG against most Ags (except MSP1p19) in clinical groups (CM and MM patients) than in Ndiop villagers used as Immune controls. However, substantial Ab responses were found in CM and MM patients with almost no quantitative difference between mild versus cerebral cases. The differences in antibody patterns between clinical groups and immune controls could reflect differences antedating the current infection or differences in response to it. Epidemiological studies indicated that in areas of high malaria transmission, antibody levels tend to increase with age and exposure as immunity is acquired (21). We cannot exclude the possibility that in the context of a low/non-immune background, infection by *P. falciparum* induces a high Ab response against numerous Ag determinants of the parasite, obscuring potential analysis to target key protection-associated Ags in the context of cross-sectional recruitment of symptomatic infected urban patients.

For several decades there has been very good *in vivo* evidence that Abs can mediate protection against the blood stage of *P. falciparum*. Total IgG to *P falciparum* crude extracts was only weakly associated with protection, emphasizing the value of examining subclasses and not just total IgG in studies of human immunity. More recent data suggest that anti-malarial Ab isotypes can be functionally sub-classified: protective Abs belong to a restricted panel of isotypes, specifically IgG1 and IgG3, whereas IgG4 Abs are considered non-protective (16,22,23). Our results suggest that the IgG subclasses responses in malaria patient could be used as indicators to differeniate mild malaria

from cerebral malaria. These data are in agreement with a previous study in the same location where the differential anti-schizont IgG3 Abs response was the most relevant prognostic factor of fatal outcome in severe malaria in hospitalised patients, rather than cytokine levels (24). We found that a high level of imbalanced malaria-specific IgG subclass Ab responses plays a central role for the efficacy of protection-associated mechanisms and for the design of vaccine candidates (22). In this regard, these data emphasise the asynchronous IgM response occurring in cerebral malaria, resulting from a possible disturbance of the anti-*P. falciparum* immune responses. Such skewed asynchronous IgM responses in CM compared to MM, suggest that individuals were not able to respond to the whole panel of Ags despite comparable cycles of parasite multiplication *in vivo*. Whether this observation results from a substantial limitation of the Ab repertoire to the IRBCm-associated variant (25) or from a lower concentration of Ab levels against cryptic Ags in CM resulting in a lower anti-parasite efficient immune response (26), has to be further analysed. An important finding of our study is that the MM patients showed a significantly reciprocal correlation between IgG and IgM responses to schizont and merozoite antigens extracts, contrary to CM patients. This result is related to asynchronous IgM responses in CM. Although, we cannot exclude the presence of extremely high concentrations of *P. falciparum* soluble antigens in severe malaria cases (27). It is evident that parasite soluble factors can contribute to the severity of disease from their ability to induce excessive production of cytokines and dysregulation of the immune system (27,28). Differences in antibody level may reflect differences in Th1/Th2 cytokine balance (29), which may be due to the disease (30) or the host genetics (31). However, additional evidence to confirm this observation is required and validation will depend on further studies. For example, it would be interesting to know whether the parasitaemia and the humoral responses against *P. falciparum* soluble antigens or toxins such as *Pf*GPI, have an impact on the clinical symptoms of malaria.

Humoral immune responses to MSP1p19 are known to be protective against *P. falciparum* infection and clinical malaria (32). In addition, the association of lower IgGs, IgG1 and IgG3 Abs titers to MSP1p19 with malaria severity (such as CM) has been demonstrated in previous studies (15,21,23,33). For example, in a recent study about the differences in IgG Abs responses to MSP complex proteins among CM patients, MM patients and healthy control subjects from a malaria-endemic part of India, significantly lower levels of Abs responses were observed for IgG1 to MSP1p19 (not IgG3) in CM patients compared to MM patients (34). In the current study, any significant variation of IgG subclasses against MSP1p19 was not found between CM and MM patients and these results are in concordance with the Indian study, except for IgG1 Abs. Interestingly, we observed that mean levels of IgG3 and IgG1 anti-MSP1p19 from immune control donors were significantly higher than MM and CM patients; these findings confirm that IgG1 and IgG3 Abs to MSP1p19 are correlated with clinical immunity to *P. falciparum*, and with reduced parasitaemia and fever (32). As demonstrated in previous studies, these antibodies have been shown to inhibit both erythrocyte invasion and parasite growing *in vitro* (33).

Results from this study suggest that the contribution of an efficient Ab response against parasite multiplication is important in the evolution towards cerebral manifestation. In urban areas with low unstable transmission, individuals at risk have a large heterogeneity of previous infections including previous trips in endemic areas. This pre-infection immune baseline is of importance for possible discovery of a preferential Ag target that discriminates non-fatal cerebral cases from uncomplicated malaria subjects (35).

Whether the qualitatively different Ab responses observed in these CM patients are responsible for, or a consequence of the harmful effect of the infection is an open question requiring further investigation with additional recruitment and the analysis of a larger panel of Ag targets and functional anti-parasite activity of IgG responses.
To elucidate the relationship between naturally acquired antibody responses to blood stages of *P. falciparum* infection and malaria disease, large protective cohort studies, severe malaria cases and uncomplicated malaria patients should be performed, including detailed immunological analyses of IgG subclasses, fine specificity, inhibitory activity and affinity/avidity of antibodies and assessment of cytokine responses, HIV and nutritional status, and host genetic factors.

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