


**Research Article**

## Ex vivo Microsphiltration Profile of Plasmodium Falciparum Infected Red Blood Cells in Patients with Malaria in Kéniéroba, Mali: Exploring the Spleen Retention Function

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### Abstract

*P. falciparum*- Malaria pathophysiology is still not fully understood. The main mechanisms of the malaria physiopathology involve interactions between host and parasite. Although, the role of the spleen has been mentioned in various clinical forms, supportive clinical evidence is still needed. We conducted a pilot study to determine the impact of the spleen functional status in different clinical forms of malaria. Ex vivo microsphiltration was utilized to assess the splenic function in patients received during routine consultation with mild malaria at the Kéniéroba health center, a high malaria endemic area in Mali. A total of 25 patients were enrolled for ex vivo microsphiltration. The spleen was non-palpable (Hackett stage 0) in two patients, palpable with deep inspiration (Hackett stage 1) in 22 patients and without deep inspiration (Hackett stage 2) in one patient. The parasitaemia ranged from 5360 -to 342720 trophozoites/ $\mu$ l with a mean parasitemia of 50774 trophozoites/ $\mu$ l  $\pm$  65540 trophozoites/ $\mu$ l. The mean hemoglobin level was 11.2g/dl  $\pm$  1.2 [8.7-13.4]. The retention rate of the infected red blood cell ranged from 11.11% to 94.44% with an average of 65.4%  $\pm$  23.7%. A higher ex vivo retention rate of infected red blood cells was observed in patients with palpable spleen ( $p= 0.03$ ). This pilot study proved the feasibility of exploring the spleen filtering function in malaria patients using the ex vivo microsphiltration explore of the venous collected red blood cell.

**Keywords:** Microsphiltration; Malaria; Plasmodium Falciparum; Splenic retention

**Abbreviations:** MRTC: Malaria Research and Training Center; PBS: Phosphate Buffer Saline; Pf: Plasmodium falciparum; RBCs: Red Blood Cells; RPMI: Roswell Park Memorial Institute Medium; RR: Retention Rate; USTTB: University of Sciences, Technics and Technologies of Bamako; WACCBIP: West African Centre of Cell Biology of Infectious Pathogens

### Introduction

Malaria is the most important tropical parasitic disease with 241 million cases and 627,000 deaths in 2020 worldwide [1]. Africa bore in 2020 a disproportionate share of the global malaria burden with 95% morbidity and 96% mortality rates [1]. Despite of the ongoing basic and experimental research on malaria, the malaria pathophysiology is not yet fully understood due to its complex and multifactorial mechanisms. The main mechanisms

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involve synergistic host-parasite interactions [2]. The high frequency of splenomegaly in malaria endemic areas, the occurrence of splenic ruptures during or immediately after malaria disease, the more frequently marked severity of the first episodes of malaria in splenectomized patients are suggestive of a central role of the spleen in the malaria pathogenesis [3]. Pathogenic stages of *P. falciparum* develop in red blood cells (RBCs) and modify their physical properties [4, 5]. Understandably, the spleen, responsible of controlling the RBCs deformability, influences the fate of infected RBCs. In fact, clinical manifestations of *P. falciparum* infection result from the development of asexual stages within the RBCs [6]. Splenic microcirculatory beds filter altered RBCs, so the spleen can naturally eliminate subpopulations of infected or uninfected RBCs modified during *P. falciparum* malaria [7]. The spleen seems to be more protective against severe manifestations of malaria in naive patients [8]. It is involved in parasite clearance after certain antimalarial treatments, including artemisinin derivatives (artesunate and dihydroartemisinin) [9]. The loss of RBCs during malaria contributes to malaria anemia, a clinical form associated with a subacute progression, frequent splenomegaly and a chronic subclinical parasitaemia. This loss of RBCs results from the splenic clearance of either newly infected RBCs or uninfected, but parasite-modified RBCs [10].

However, the strengthen filtering function of the spleen seems to be associated with a reduced risk of serious complications with and high parasitic loads, such as in cerebral malaria [11]. These hypotheses remain speculative despite their relevance. Exploring the role of the spleen in the pathophysiology of malaria remains overly complex due to the *in vivo* experimental challenges. Many technics have been described to explore the filtering function of the spleen. Microsphiltration is an experimental technic, which mimics the mechanical retention of particles with little deformation in the human spleen [12, 13]. This technic can be used either *in vitro* (plasmodium in culture) or, *ex vivo* (parasites harvested directly from patients). The *ex vivo* microsphiltration of RBCs from patients with malaria could be utilized to explore the splenic retention capability. Indeed, RBCs from malaria patients with have previously undergone splenic filtration *in vivo* could therefore provide informations on the splenic filtering functionality of the patient. Thus, the patient RBCs deformability, depending on the spleen filtering function will determine their *ex vivo*, microsphiltration retention rate. Such the *ex vivo* microsphiltration retention rate may reflect the ability of the spleen *in vivo* to filter out the least deformed RBCs. Here, we explored the *ex vivo* microsphiltration as tools to assess the splenic filtering function in patients with different clinical forms of malaria (supplementary Figure 1).

## Methods

### Study site

The patients were enrolled at the health center in the village of Kenioroba located along the Niger river in the Guinean-type forest savannah in Mali. In Kenioroba, malaria is endemic all year long with a stable and intense malaria transmission season (May to January).

### Study type and period

We conducted a prospective cross-sectional study during the 2018 transmission season.

### Study population and sampling

The study population consisted of all patients aged six (6) months or older and who presented to health centers for malaria or signs of malaria. The sampling was exhaustive involving all the patients who presented themselves to the health centers during the study period, who agreed to participate in the study and from whom we could collect a venous blood sample for laboratory tests. Blood collection was performed in patients those had more than 8 dg/l hemoglobin rate, more than 5000 *P.f* trophozoites/ $\mu$ l parasitemia only when the laboratory was ready to perform microsphiltration. Data from samples with 5% parasitemia were excluded from the analysis to avoid the confounding effects due to the filter saturation.

### Microsphiltration

Splenic retention of *P. falciparum* infected-RBCs in malaria patients has been studied using previous described tip microsphiltration method [14]. Briefely, calibrated metal microbeads composed of 96.5% tin, 3.0% silver and 0.5% copper of different diameters were used to make layers in tips of very narrow spaces to imitate the inter-endothelial clefts of the micro-veins of the red pulp of the human spleen (sinus). An equal-weight mixture (1 g) of microsphere powder (5-15  $\mu$ m in diameter and 15-25  $\mu$ m in diameter) was suspended in 5 mL 1% PBS/ AlbuMAX I solution (life technologies Cat#11020-021). A total of 800  $\mu$ L of this suspension of microspheres were poured into an inverted anti-aerosol pipette tip of 1000  $\mu$ L (Neptune, BarrierTips) and left to stand, leading to the formation of a layer of microspheres 5 mm thick at - above the aerosol filter. The microspheres were obtained from the Spherical Powder industry (24A, rue de la Résistance-BP 438, Annemasse 74108, France). *P. falciparum*-infected RBCs were collected from malaria patient and immediately kept at 4°C in a cooler until the sample are ready for microsphiltration (4 -5 hours). This procedure stop the parasite growth between the blood collection and the microsphiltration. A total of 600  $\mu$ L of RBCs suspension (patientsRBCs washed with RPMI and suspended at 1% hematocrit in PBS/AlbuMAX II 1%) were introduced instantly upstream of the layer of microspheres

and entrained through the layer of microspheres with 6 ml of PBS / AlbuMAX II 1% using an electric pump (Syramed  $\mu$ sp6000, Arcomed'Ag). The filtration rate was 60 ml / H. A downstream sample (6.6 mL) and an aliquot of the upstream sample were collected to determine parasitemia. Each sample was filtered in duplicate. The mean parasitaemias (% of infected RBCs in the RBC suspension) in the upstream (PAm) and downstream (PAv) samples were determined. The RBC retention rate (RR) for each sample was calculated using the following formula:  $TR = [(PAm - PAv) / PAm] \times 100$  [14]. The cells were counted using an Accuri C6 flow cytometer (Becton - Dickinson) after labeling with Syto 61.

### Statistical analysis

We used descriptive statistics to summarize the data. Quantitative variables were represented as means and standard deviations and categorical variables as frequencies. We used t-tests to assess statistical differences between the means. The box and whisker plot were used to visualize the distribution of data by quartiles. Simple linear regression was applied to determine the correlation between rates of spleen retention and parasitaemia, hemoglobin levels and age. For each variable, missing data was defined as no record of cases and unknown data, in this case the variable was not tested. A difference was considered significant at  $p < 0.05$ . All reported p values were bilateral. Statistical analyzes were performed on the complete data using statat version 14 software, the figures using prism version 8 software.

### Results

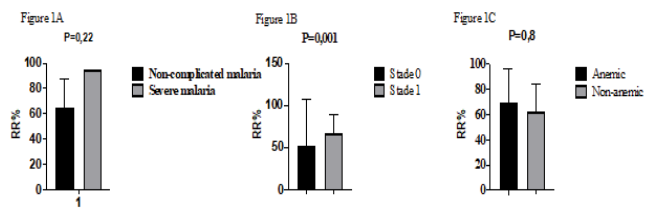
A total 25 blood samples from *P. f*-malaria patients were analyzed by microsphiltration. The sex ratio was 1.08. Only 4% (1/25) presented a severe malaria with prostration and vomiting. Two patients (8%) of patients had a no palpable spleen (hackette stage 0), 22 patients (88%) had a palpable deep inspiration spleen (stage 1 of Hackett) and one patient (4%) had a palpable spleen (Hackett stage 2) (Table 1). Patients were  $9.68 \pm 3.881$  years old on average with the extremes of 4 and 18 years old. Parasitemia ranged from 5,360 to 342,720 trophozoites/ $\mu$ l (mean:  $50,774.40 \pm 65,540,854$ ), the mean hemoglobin level was  $11.23 \pm 1.20$  g/dl with the extremes of 8.70 and 13.40 g/dl. Retention rates ranged from 11.11% to 94.44% with a mean of  $65.40 \pm 23.77\%$  (Table 2). We did not find significant difference between the average retention rates according to the clinical phenotype of malaria ( $p = 0.2$ ) (Figure 1A) and the anemia status ( $p = 0.8$ ) (Figure 1C). No correlation was observed between retention rates and parasitemia ( $p = 0.23$ ) (Figure 2A), the age ( $p = 0.6$ ) (Figure 2B) or hemoglobin levels ( $p = 0.21$ ) (Figure 2C). However, we observed a statistically significant difference between the average rate of splenic retention of RBCs infected and the Hackett stages ( $P = 0.001$ ) (Figure 1B).

**Table 1:** Clinical characteristics of the study participants.

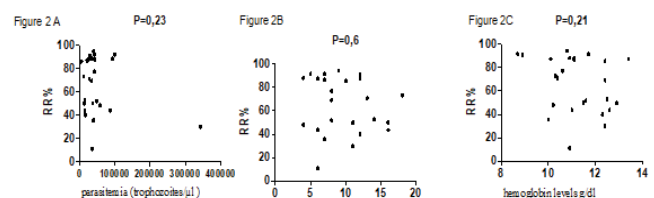
Hackett stages	Frequency	Percentage
Stade 0	2	8.0
Stade 1	22	88.0
Stade 2	1	4.0
Total	25	100.0
<b>Clinical phenotype</b>		
Non-complicated malaria	24	96.0
Severe malaria	1	4.0
Total	25	100.0
<b>Malaria severity criteria</b>		
Hyperparasitemia	0	0
Prostration et hyperparasitemia	0	0.0
Vomiting and Prostration	1	100.0
Convulsions	0	0.0
Total	1	100.0
<b>Statut anemia</b>		
Anemia	11	44.0
Non-anemia	14	56.0
Total	25	100.0

**Table 2:** biological characteristics of study participants.

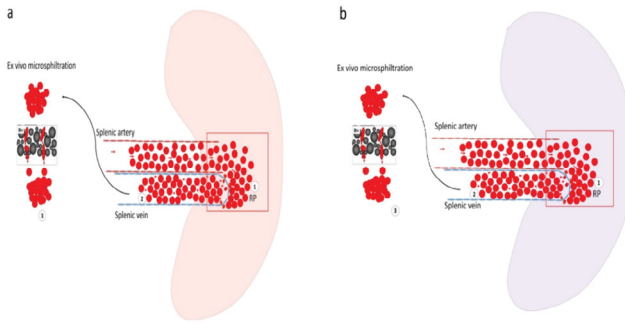
Characteristics	Average	Standar deviation	Minimum	Maximum
Parasitemia	50774.40	65540.85	5360	342720
Hemoglobin levels	11.22	1.20	8.7	13.4
Retention rate	65.40	23.77	11.11	94.44



**Figure 1:** Comparison of the mean spleen retention rates regarding the clinical parameters. 1A) Mean spleen retention rates regarding the clinical phenotype. 1B) Mean spleen retention rates regarding the clinical Hackett



**Figure2:** Correlations between the spleen retention rates and parasitemia, host ages and hemoglobin level. 2A) correlation between spleen retention rates and the parasitemia. 2B) correlation between spleen retention rates and the ages. 2C) correlation between spleen retention rates and the hemoglobin levels.



**Figure 3:** Illustration of the hypothetical retention behaviors of *Plasmodium falciparum* infected-RBCs in functional and altered spleen and the implication on *ex-vivo* microfiltration retention rate. Experimental hypothesis to predict the splenic filtration function using *ex-vivo* microfiltration of parasite-infected red blood cells from patients with mild malaria. a) In a fully functional spleen, the splenic artery brings infected RBCs into the splenic red pulp. Deformable infected RBCs arrive - through the inter- endothelial slit of the wall of the splenic veins in the lumen of the splenic vein while stiff infected RBC are retained in the splenic red pulp (1) resulting in a low venous parasitemia (2). The *ex vivo* microfiltration of venous deformable infected RBCs may yield a lower retention rate (3). b) In an abnormally functional spleen, stiff infected RBC are not maximally retained in the splenic red pulp. Therefore, infected RBCs accumulate less in the splenic red pulp (1) and venous parasitemia is higher (2). The *ex vivo* microfiltration of venous stiff infected RBC may result in a higher retention rate (3).

## Discussion

The role of the spleen in clinical malaria symptoms occurrence has long time been discussed [3]. However, direct evidence for these claims has still not been reported due to difficulties to explore *in vivo* the splenic function. In this study, we proposed to explore the spleen function remotely in patients with malaria using *ex vivo* microfiltration of RBCs infected. We carried out a cross-sectional study from May to December 2018 in Kéniéroba village. In this pilot study, clinical and biological data as well as *ex vivo* microfiltration retention rates were collected from 25 malaria patients. Microfiltration is an experimental exploratory technique of RBCs deformability. It mimics the splenic retention of less deformable RBCs. This technique has been validated on suspensions of parasitized RBCs at different stages in the development of *P. falciparum in vitro* [13]. In this study, we assessed the deformability of naturally infected RBCs using *ex vivo* microfiltration. We hypothesized that the deformability of venous *Pf*-infected RBCs may be informative of the spleen filtering function status. Thus, high *ex vivo* microfiltration retention rates of *P.falciparum*-infected RBCs would be associated with a dysfunction of the splenic filtering function in patients and vice versa (supplementary figure1). The mean splenic retention rate of the infected-RBCs was much higher ( $65.40 \pm 23.77\%$ , as compared to that reported by Diakite et al. 2016 ( $54.5 \pm 4.7\%$  in Hb AA subjects and  $44.5 \pm 3.3\%$  in Hb AS subjects) [15]. This high rate could be explained by the experimental conditions, in particular the time elapsed between the sampling and the microfiltration. Although anemia is associated with high

splenic retention rate of infected-RBCs *in vivo* [11, 16], which may result into low *ex vivo* microfiltration retention rates, we did not find a statistically significant correlation between the *ex vivo* retention rates of parasitized RBCs and the hemoglobin level ( $p = 0.21$ ). Our hypothesis assumed that low *ex vivo* retention rates would reflect high splenic retention *in vivo* that would be associated with a low parasitemia.

We found no correlation between ages and *ex vivo* microfiltration retention rate of infected-RBCs ( $p = 0.7$ ). Age could also influence the filtering function of the spleen in patients living in malaria endemic areas. In fact, in malaria endemic areas, patients are more likely to develop splenic insufficiency due to multiple challenges the spleen face during successive infections [3, 17]. Analysis of parasitemia according to the *ex vivo* microfiltration retention rate of infected-RBCs did not reveal any correlation between these two parameters ( $p = 0.23$ ). High parasite density has been associated with serious manifestations of the disease such as cerebral malaria. This clinical phenotype is in favors of low splenic retention *in vivo* [7]. No correlation between the parasitemia and the *ex vivo* retention rates was found in this study. Ended due to technical challenges we only worked on parasitemia more than 5000 parasites per microliter, also only data from samples with real parasitemia (flow cytometry) less than 5% to avoid saturation of the filter. As expected, we observed high *ex vivo* retention of RBCs infected from patients with clinical Hackett stages greater than or equal to 1 ( $p = 0.03$ ). In fact, the Hackett clinical stage defines the degrees of splenomegaly that may be associated spleen function impairment.

## Conclusion

This pilot study allowed to explore *ex vivo* filtering

function of the spleen in patients with malaria. Infected-RBCs from patients with higher clinical Hackett stages showed greater retention rates. Methodological corrections, especially in terms of planning and sample size, would help generating data to draw reliable conclusions.

## What is Known about this Topic

Functional spleen retains stiff red blood cells. Splenomegaly is associated with stiff red blood cells circulation. Microbeads retains stiff red blood cells

## What this Study Adds

Infected-RBCs from patients with higher clinical Hackett stages showed greater retention rates in microfiltration

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### Authors' Contributions

Study setup: SD. Sample collection: BK, MD. Data collection: BK, AG, SD. Data analysis: BK, SD. Manuscript writing: SD, BK. Manuscript review: SD, BK. All authors read and approved the final manuscript.

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### Ethics Approval and Consent to Participate

Before starting this study, we obtained a community consent from traditional and customary chiefs prior to the study. The study was approved by the ethics committee of the faculty of medicine and Pharmacy of the University of Sciences, Technics and Technologies of Bamako (USTTB), Mali. Written informed consent was obtained from a parent or guardian of each enrolled child. All methods were performed in accordance with the good laboratory and clinical practices guidelines and were respectful of the Helsinki declaration.

### Consent for Publications

All authors read and approved the final manuscript.

### Competing Interests

The authors declare that they have no competing interests.

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