

# Paucity of *Plasmodium vivax* Mature Schizonts in Peripheral Blood Is Associated With Their Increased Cytoadhesive Potential

Stefanie C. P. Lopes,<sup>1,2</sup> Letusa Albrecht,<sup>1</sup> Bruna O. Carvalho,<sup>1,a</sup> André M. Siqueira,<sup>2,3</sup> Richard Thomson-Luque,<sup>2,4</sup> Paulo A. Nogueira,<sup>5</sup> Carmen Fernandez-Becerra,<sup>4</sup> Hernando A. del Portillo,<sup>4,6</sup> Bruce M. Russell,<sup>7,8</sup> Laurent Rénia,<sup>7</sup> Marcus V. G. Lacerda,<sup>2,3</sup> and Fabio T. M. Costa<sup>1</sup>

<sup>1</sup>Departamento de Genética, Evolução e Bioagentes, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil; <sup>2</sup>Fundação de Medicina Tropical Dr Heitor Vieira Dourado, Manaus, Amazonas, Brazil; <sup>3</sup>Universidade do Estado do Amazonas, Manaus, Amazonas, Brazil; <sup>4</sup>Barcelona Centre for International Health Research, CRESIB, Barcelona, Spain; <sup>5</sup>Instituto Leônidas e Maria Deane, Fiocruz, Manaus, Amazonas, Brazil; <sup>6</sup>Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain; <sup>7</sup>Singapore Immunology Network, Biopolis, Agency for Science Technology and Research, Singapore and <sup>8</sup>Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

**There is now a growing body of evidence that challenges the current view that *Plasmodium vivax*-infected erythrocyte (Pv-iE) are unable to sequester. Here we used ex vivo adhesion assays with Pv-iE before and after maturation to demonstrate a higher binding potential of schizonts compared to other asexual stages. These experimental results are correlated with our observations in a panel of 50 *vivax* malaria patients where schizonts were completely absent in 27 isolates, and few schizonts were observed in the remaining patients. These observations prompt a paradigm shift in *P. vivax* biology and open avenues to investigate the role of Pv-iE sequestration.**

**Keywords.** malaria; *Plasmodium vivax*; schizonts; disproportionality; cytoadhesion; sequestration.

Malaria in humans is caused by 5 species of parasites of the genus *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*). Malaria caused by *P. falciparum* is the most lethal form of disease and predominates in Africa. Although *P. vivax* infections are rarely fatal, this parasite is the most geographically widespread, with 2.48 billion people worldwide at risk [1].

The severity of *P. falciparum* is partially attributed to the ability of the mature asexual stages to cytoadhere to the microvasculature of several organs [2]. The absence of mature *P. falciparum* parasites in the peripheral blood circulation of patients is an unequivocal evidence of cytoadherence and parasite sequestration. Adhesion of *P. falciparum*-infected erythrocytes (Pf-iEs) is mediated by electron-dense protuberances on the surface Pf-iEs, named knobs, which contain several antigens, including *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is expressed at the cell surface [2]. PfEMP1 is directly involved in the adhesion of Pf-iEs to various host receptors [2]. Because *P. vivax*-infected erythrocytes (Pv-iEs) lack “sticky” knob structures and because all parasite forms can be observed in the peripheral blood of patients, it has become dogma that *P. vivax* lacks the ability to cytoadhere and therefore sequester.

Recently, we demonstrated that Pv-iEs are able to cytoadhere to endothelial cells expressing receptors known to mediate *P. falciparum* cytoadhesion, such as ICAM-1 and CSA [3]. The adhesive capacity of *P. vivax* was further confirmed by Chotivanich and collaborators, who showed that Pv-iEs adhere to CSA-coated plates and placenta sections via hyaluronic acid [4]. Moreover, although no PfEMP1 homolog has been found in *P. vivax*, variable protein members (VIR proteins) have been shown to play a role in adhesion. First, computational analysis using a *P. falciparum*-based algorithm revealed putative adhesive motifs in VIR proteins [5]. Second, antibodies toward VIR proteins partially prevented adhesion [3]. Finally, specific *vir* genes transfected into *P. falciparum* mediated specific adhesion to the ICAM-1 protein [6]. Here, we evaluated the potential differences in cytoadherence capability between the *P. vivax* mature forms, trophozoites and schizonts. In parallel, we determined the distribution of the different blood-stage forms (ring, trophozoite, and schizont) in freshly drawn peripheral blood from *P. vivax*-infected patients.

## METHODS

### Ethics Statement

All protocols and consent forms were approved by the Ethics Review Board of the *Fundação de Medicina Tropical*

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<sup>a</sup>Present address: Instituto Oswaldo Cruz, Fiocruz Rio de Janeiro-RJ, Brazil.

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Correspondence: Stefanie C. P. Lopes, PhD, Dep. Genetics, Evolution and Bioagents, University of Campinas (UNICAMP), Institute of Biology, Cidade Universitária Zeferino Vaz, Rua Monteiro Lobato, 255, PO Box: 6109, 13083-862, Campinas, SP, Brazil (stefaniecplopes@gmail.com).

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### Study Area and Subjects

Patients were recruited and examined at FMT-HVD, a tertiary care center for infectious diseases in Manaus, Amazonas State, Brazil. Up to 7 mL of peripheral blood was collected immediately after confirmation of *P. vivax* infection by a thick blood smear ( $n = 50$ ). Patients were then treated with chloroquine and primaquine according to the standard protocol recommended by the Brazilian Malaria Control Program. *P. vivax* monoinfection was subsequently confirmed by polymerase chain reaction (PCR) analysis as described elsewhere [7].

### Parasitemia Determination

Blood samples from *P. vivax* patients were collected in sodium citrate tubes. Two thin blood smears were prepared and stained with Giemsa stain. The parasitemia was determined by calculating the number of parasites per 500 leukocytes (Supplementary Figure 1A). The parasite stages were determined for a minimum of 200 parasites and were separated into sexual (gametocytes) (Supplementary Figure 1B) and asexual forms (Supplementary Figure 1C). The asexual forms were further divided into the following 3 groups: (i) ring (<22 hours post-invasion); (ii) trophozoite, including late forms (23–40 hours post-invasion); and (iii) schizont (>40 hours post-invasion) (Supplementary Figure 1C).

### Parasite Purification and Maturation

Samples with >50% asexual forms at the trophozoite stage were selected for the cytoadhesion assays ( $n = 8$ ). First, the selected blood samples were filtered using a CF-11 column to remove white blood cells, as described elsewhere (Supplementary Figure 1D) [8]. Second, we performed parasite enrichment by Percoll 45% gradient as described by our group previously [3] (Supplementary Figure 1E). Half of the parasites obtained after Percoll enrichment were used immediately in cytoadhesion assays, the other half were cultured as described previously by Russell et al [8] for 18–22 hours for parasite maturation (Supplementary Figure 1F).

### Cytoadhesion Assays

The cytoadhesion assays were performed as described elsewhere [3]. Briefly, human lung endothelial cells (HLECs) were grown to confluence on 8-well culture slides (BD), and then,  $5 \times 10^4$  Pv-iEs, either in vitro matured or not matured, were added to each well in cytoadhesion medium (RPMI 1640 pH 6.8). The culture slides were incubated for 1 hour at 37°C and then extensively washed to remove any unbound parasites. The slides were then fixed in methanol and stained with Giemsa stain (Supplementary Figure 1G). The number of Pv-iEs adherent to the endothelial cell monolayer was

determined, and cytoadhesion was expressed as the mean  $\pm$  standard deviation of the number of erythrocytes per  $\text{mm}^2$  observed in 3 wells.

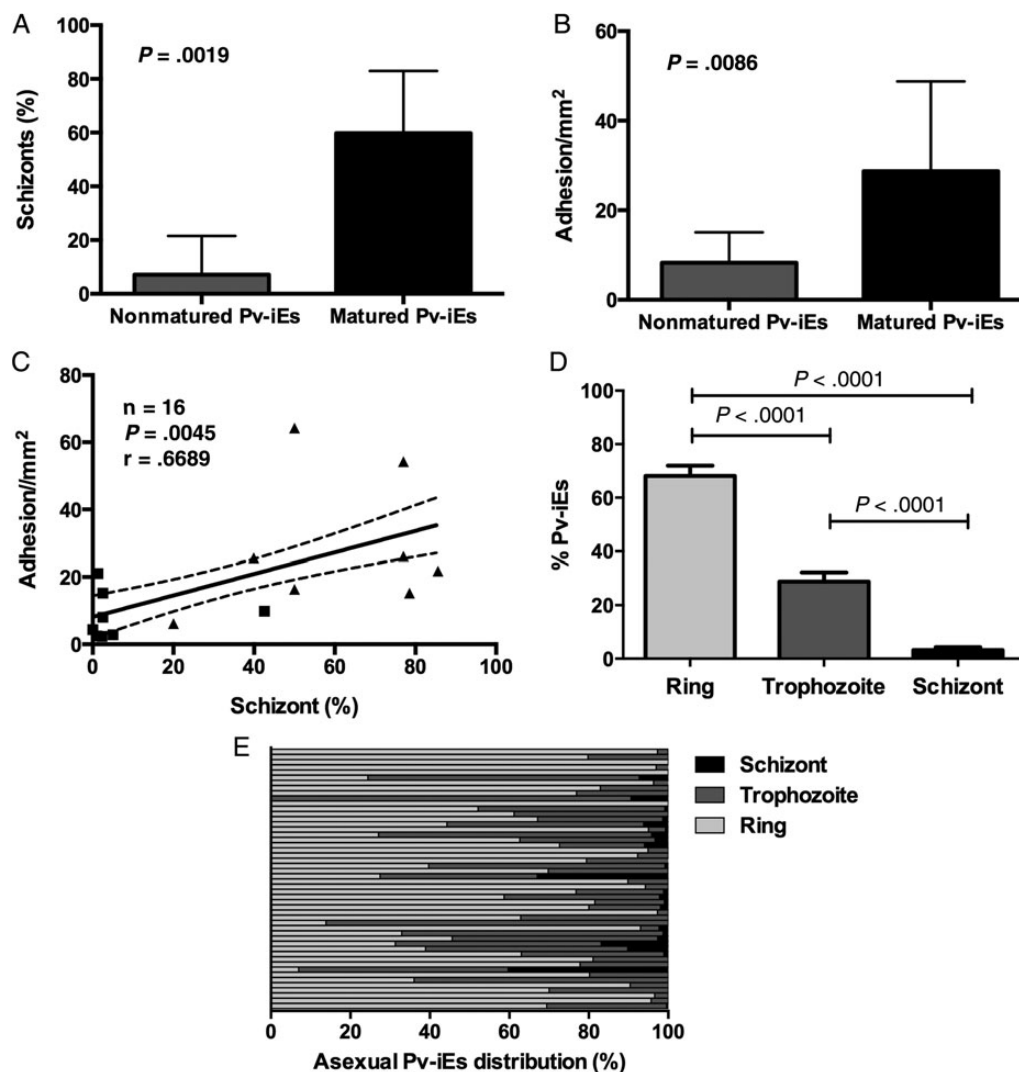
### Statistical Analysis

The Mann-Whitney test was used to compare 2 groups, and the Kruskal-Wallis test was used for comparisons between >2 groups. Correlations were analyzed using the Spearman correlation. Differences were considered statistically significant when  $P < .05$ . Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, CA).

## RESULTS

To evaluate the capacity of *P. vivax* blood stages to cytoadhere, we performed cytoadhesion assays at 2 time-points for each of the 8 isolates: (i) immediately after blood withdrawal; parasites from these samples were Percoll-enriched purified and were primarily trophozoites; and (ii) after 18–22 hours of cultivation (in vitro maturation), in which the majority of parasites were schizonts. Pv-iE maturation increased the amount of schizonts from 7% to 59% (Figure 1A). Moreover, the adhesion assays performed with matured and nonmatured Pv-iEs showed that the matured parasites displayed a higher (3.5-fold) binding ability to HLECs than the nonmatured forms (Figure 1B). Additionally, we also performed adhesion assays using 3 other isolates containing a majority of parasites in ring stage and no adhesion was observed (data not shown). Importantly, we found a positive correlation between the schizont percentage and the cytoadhesion index (Figure 1C).

After demonstrating that the matured parasites have higher ability to cytoadhere and this higher ability is correlated to an increased proportion of schizonts in matching patient isolates, we wished to see if this result was reflected in a wider clinical survey of vivax malaria patients. We hypothesized that if the schizonts are more cytoadhesive they may “sequester”, resulting in a lower than expected proportion of schizonts (relative to other asexual stages) in the peripheral circulation of patients. For this purpose, 50 patients were included in this study after being diagnosed with clinical *P. vivax* malaria by microscopy. *P. vivax* monoinfection was further confirmed with a sensitive PCR method [7]. No mixed infection was detected. Of these 50 patients, the majority ( $n = 27$ ) had no schizonts in circulation at the time of blood withdrawal. Notably, in the remaining individuals who presented with schizonts in the peripheral circulation ( $n = 23$ ), only a small proportion of this form was observed (Figure 1D). A predominance of parasite ring forms ( $68.09\% \pm 22.22\%$ ) compared to the other forms was observed (trophozoites,  $28.82\% \pm 23.62\%$ , and schizonts,  $3.09\% \pm 7.87\%$ ; Figure 1D and 1E), indicating biased distribution of the asexual forms in the thin blood smears of all *P. vivax* patients analyzed.



**Figure 1.** Distribution of *Plasmodium vivax* asexual stages in patients' peripheral blood, schizont percentage, and Pv-iE cytoadhesion rate in HLECs before and after short-term in vitro maturation. The percentage of schizonts before and after short-term in vitro maturation (A). The number of Pv-iEs adhered per mm<sup>2</sup> in HLECs before and after short-term in vitro maturation (B). The correlation between the schizont percentage and the Pv-iE cytoadhesion rate to HLECs (C). The percentage of asexual Pv-iEs stages in the peripheral blood of each patient (D). The mean percentage of asexual Pv-iE stages in 50 patients (E). In panel C, the squares represent Pv-iEs before maturation, whereas the triangles represent Pv-iEs after maturation. The statistical analyses were performed using the Mann-Whitney test or Kruskal-Wallis test for comparisons between 2 or more groups, respectively. The correlational analyses were performed using the Spearman test. Abbreviations: HLEC, human lung endothelial cell; Pv-iE, *P. vivax*-infected erythrocyte.

## DISCUSSION

In *P. falciparum*, the absence of mature trophozoites, schizonts, and developing gametocytes in the peripheral blood circulation of humans is clear evidence for the sequestration of these stages [2]. It has long been postulated that Pv-iEs do not sequester because all stages of this parasite can be observed in the peripheral blood of patients.

We have previously shown that Pv-iEs purified from patients' blood cytoadhere ex vivo to endothelial cells and placental cryosections, most likely by binding to ICAM-1 and CSA host

receptors [3]. This ability was further confirmed by an independent group that showed that Pv-iEs collected in Thailand can cytoadhere ex vivo to CSA and HA soluble receptors and placental cryosections [4]. Moreover, transfection of the nonadherent *P. falciparum* strain 3D7 with a *P. vivax* specific *vir* gene allowed this strain of *P. falciparum* to adhere to ICAM-1 under flow conditions [6]. Therefore, there is now a growing body of evidence supporting that *P. vivax* may have an adhesive phenotype, although this parasite does not express PfEMP-1 or generate knob structures at the surface of the iEs. Other *Plasmodium* species, including *P. knowlesi* and

*P. chabaudi*, which do not have knobs, also cytoadhere. These parasite species and other knobless parasites, such as *P. berghei* and *P. falciparum* gametocytes, have previously been shown to sequester. Collectively, these findings suggest that cytoadhesion is not a feature exclusive to *P. falciparum*; therefore, cytoadhesion is not a phenomenon restricted to PfEMP1 and knob-positive parasites.

Here, we demonstrated a higher adhesive capacity of in vitro-matured Pv-iEs compared to nonmatured isolates. Moreover, we found a positive correlation between the schizont percentage and the index of cytoadhesion. We also examined the blood thin smears of 50 patients and showed that although all parasite forms are known to be detectable in the peripheral blood of *P. vivax*-infected patients, there is a disproportion of circulating parasite forms because the mature stages, including the trophozoites and schizonts, were less prevalent than the immature stages. Consistent with our findings, elegant studies from the middle of the 20th century have previously shown a low proportion of mature schizonts compared to the other asexual stages [9]. These findings indicate that the low proportion of schizonts observed in patients' peripheral blood circulation may be associated with a more adhesive phenotype. This also suggests that, in contrast to the situation for *P. falciparum*, not all Pv-iEs are sequestered; therefore, some Pv-iEs can be detected in the circulation. Another possibility is that sequestration of *P. vivax* may be saturated at a much lower parasitemia than that observed for *P. falciparum*. Indeed, *P. falciparum* schizonts have been observed in the circulation of patients with high parasitemia. This finding deserves further study.

An additional explanation that we could not completely exclude in our observations is the fact that *P. vivax* is a synchronous parasite, and infected people seek treatment usually soon after the malarial paroxysm, a time that coincides with the release of merozoites and therefore with the appearance of the young trophozoite in the peripheral blood. However, because 70% of malaria patients in Brazil seek medical attention within 48 hours of onset of symptoms and according to the old literature, synchrony in malaria infections is observed only at the end of the first week of the disease [10], we do not expect to observe synchronous infections in our patients, which give support to our hypothesis that circulating mature form paucity is related to sequestration.

Finally, the recent findings of a few Pv-iEs inside the lung microvasculature after flushing the tissue obtained by autopsy of an aparasitemic patient at the time of death [11] and a study showing the presence of large numbers of intact Pv-iEs in the spleen [12] support the hypothesis that *P. vivax* undergo sequestration in vivo. The sequestration of *P. vivax* was first suggested by Bass et al [13], who suggested that it would be unlikely that merozoites released by circulating erythrocytes are able to attach to and invade new erythrocytes. Importantly, Bass published this hypothesis prior to the finding that *P. vivax* selectively targets

reticulocytes for invasion [14]. The encounter between these *P. vivax* circulating merozoites and the parasite's target cells would be even more difficult because reticulocytes are rare in the periphery. Collectively, our findings suggest that this bias in parasite stage distribution in the peripheral blood may be related to a cytoadhesive phenotype of *P. vivax* matured stages. Finally, although the role of *P. vivax* sequestration remains unknown and is unlikely to be directly associated with clinical severity, we hypothesize that sequestration at sites enriched within this parasite's target cells, such as the spleen and bone marrow, contribute to *P. vivax* biology and chronicity. Nevertheless, further studies are necessary to identify the receptors that *P. vivax* schizonts cytoadhere to and to identify the parasite proteins involved in this phenomenon. These studies should include additional ex vivo studies using human target organs, which may allow a better understanding of *P. vivax* pathogenesis.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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**Potential conflicts of interest.** All authors: No potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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