1 Plasmodium falciparum increased time in circulation underlies persistent asymptomatic infection in the dry season

2 Infection in the dry season


4 The dry season is a major challenge for Plasmodium falciparum parasites in many malaria endemic regions, where water availability limits mosquitoes to only part of the year. How P. falciparum bridges two transmission seasons months apart, without being cleared by the host or compromising host survival is poorly understood. Here we show that low levels of P. falciparum parasites persist in the blood of asymptomatic Malian individuals during the 5- to 6-month dry season, rarely causing symptoms and minimally affecting the host immune response. Parasites isolated during the dry season are transcriptionally distinct from those of subjects with febrile malaria in the transmission season, reflecting longer circulation within each replicative cycle, of parasitized erythrocytes without adhering to the vascular endothelium. Low parasite levels during the dry season are not due to impaired replication, but rather increased splenic clearance of longer-circulating infected erythrocytes. We propose that P. falciparum virulence in areas of seasonal malaria transmission is regulated so that the parasite decreases its endothelial binding capacity, allowing increased splenic clearance and enabling several months of subclinical parasite persistence.
Introduction

The mosquito-borne Plasmodium falciparum parasite is responsible for over 200 million malaria cases yearly, and killed nearly 400,000 African children in 2018. P. falciparum causes disease while multiplying asexually within red blood cells (RBCs) and exporting its variant surface antigens to the RBC surface. Variant surface antigens mediate adhesion to vascular endothelium, thereby helping the parasite avoid splenic clearance. During each ~48h replicative cycle in RBCs, P. falciparum follows a regulated transcriptional pattern from the invading merozoite, through the ring- and trophozoite-stages, and to the multinucleated schizont, which yields 16-32 new merozoites. In parallel with a predictable transcriptional pattern, the parasite develops a network of membrane structures in the infected RBC (iRBC) and at the trophozoite stage the host cell membrane presents knobs exposing parasite-derived P. falciparum erythrocyte membrane protein 1 (PfEMP1). The multigene family var, is expressed in a monoallelic fashion, coding for PfEMP1s that bind host endothelial cell receptors, with different binding phenotypes associating with varying virulence and pathological outcomes. In Mali and many African regions, malaria cases are restricted to the rainy season when the mosquitoes transmitting P. falciparum are present, while subclinical P. falciparum infections can persist throughout the dry season, enabling the parasite to bridge transmission seasons several months apart. We have recently shown that although P. falciparum-specific humoral immunity is higher in subclinical P. falciparum carriers in the dry season, it decreases similarly during this time in carriers and non-carriers, suggesting that chronic low parasitaemia in endemic settings may not maintain nor boost malaria immunity. While much is known about immune responses to clinical malaria, and to some extent to subclinical infections during the transmission season, the impact on immunity of subclinical P. falciparum persistence in the dry season has not been extensively studied. Host survival during the dry months is essential for resuming P. falciparum transmission in the ensuing rainy season, thus the parasite has likely evolved strategies to prevent potentially fatal host pathology, and assure persistence during mosquito-free periods. In this study we address the host and parasite features that associate with parasite persistence between two transmission seasons, and provide insights into the complex interaction between P. falciparum, its human hosts and the surrounding environment. By comparing parasites from the dry season to malaria-causing parasites in the transmission season, we show that despite inducing a minimal immune response and conserving its replication ability, P. falciparum dry season parasitaemias can be maintained low by splenic clearance of a large proportion of iRBCs that circulate longer than observed in malaria cases.
RESULTS

P. falciparum persists during the dry season. In a cohort study in the Malian village of Kalifabougou, we followed ~600 individuals from 3 months to 45 years of age during 2017 and 2018. As reported earlier for this site, clinical episodes of malaria (temperature ≥37.5°C, ≥2500 asexual parasites/μL, and no other clinically discernible cause of fever) were largely restricted to the transmission seasons (June - December), whereas nearly all subjects remained free of symptomatic malaria during the intervening dry seasons (January - May). Specifically, there were 3 and 3 febrile malaria cases diagnosed during the 2017 and 2018 rainy seasons, respectively, while only 12 and 5 febrile malaria episodes occurred during the dry seasons (Fig. 1a and Table 1).

Table 1  | Characteristics of study participants, clinical cases, and dry season asymptomatic infections stratified by year

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<th>MAL</th>
<th>May</th>
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<tr>
<td>n</td>
<td>female %</td>
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Despite the very low incidence of clinical malaria in the dry season, we consistently observed 10-20% prevalence of subclinical P. falciparum infections during this time. Analysing over 400 individuals with paired data at the beginning (January) and end (May) of each dry season, we found that 20% of individuals were P. falciparum PCR+ in January 2017 and January 2018, and 15% and 12% of individuals were P. falciparum PCR+ at the end of the 2017 and 2018 dry seasons, respectively (Fig. 1b). Older children and young adults carried subclinical P. falciparum more frequently than young children at the end of the dry season (Fig. 1c and Table 1), as reported earlier for this cohort. Also, as previously reported, we observed that subclinical P. falciparum carriers at the end of the dry season were very likely to have been infected since its beginning (January 2017 or January 2018), while uninfected subjects at the beginning of dry season remained uninfected until its end (Fig. 1b). The odds ratios (OR) of maintaining the same infection status through the entire dry seasons were OR 90.9 (95% CI 108 (38.6, 213.8) P < 0.0001) in 2017 and OR Odds ratio 43.5 95% CI (17.5, 107.5) P < 0.0001) in 2018 (Supplementary Table 1). Consistent with the continued absence of clinical malaria during the dry season, parasitaemias of subclinical carriers determined by RT-qPCR and flow-cytometry were found to remain low, or in some individuals decline as the dry season progressed, while clinical cases of malaria in the wet season presented with high parasite burdens (Fig.1d).
**Figure 1**

*P. falciparum* persists during the dry season.  

**a.** Frequency of clinical malaria episodes every 2 days over 2 years in a cohort of ~600 subjects aged 3-45 years. Clinical malaria defined as axillary temperature ≥37.5°C, ≥2500 asexual parasites/μL of blood, and no other discernible cause of fever.  

**b.** Prevalence of subclinical *P. falciparum* detected by PCR in paired individuals (rows) at the beginning (Jan), middle (Mar), and end (May) of two consecutive dry seasons (2017 left, 2018 right). Columns are sorted such that the same individual is represented by each row at the three timepoints in each dry season.  

**c.** Age-stratified point prevalence of subclinical *P. falciparum* infection detected by PCR at the end of the dry season in May 2017 (left) and May 2018 (right).  

**d.** Parasite load detected by qRT-PCR (left) and flow cytometry (right) of RDT+ subclinical children at the beginning (Jan), mid (Mar) and end (May) of the dry season and children with their first clinical malaria episode (MAL) in the wet season. Parasitaemia data represented as median ± IQR. *P* values determined using Kruskal-Wallis test with multiple comparisons.

**P. falciparum** induces a minimal immune response during the dry season. To test the hypothesis that host immunity may contribute to the suppression of parasitaemia during the dry season, we compared the immune responses of subclinical carriers of *P. falciparum* (May+) versus non-infected children (May-). We profiled serological markers of inflammation and cytokines, circulating immune cells, and humoral responses to *P. falciparum* variant surface antigens (VSAs) of age- and gender-matched children who did or did not carry *P. falciparum* during the dry season, as detected retrospectively by PCR. Inflammation markers that had been reported to be elevated in clinical cases of malaria, such as C-reactive protein (CRP), von Willebrand factor (vWF), and hepcidin were quantified in plasma samples obtained at the beginning (Jan) and end (May) of the dry season. None of the three markers were significantly different in subclinical carriers compared to uninfected children at either timepoint (Fig. 2a). We complemented these serological analyses with a multiplex bead array to detect 32 cytokines...
and chemokines and observed no differences between children with or without *P. falciparum* at the end of the dry season (May) in all but one of the quantified analytes (Supplementary Table 2). Only CXCL1, a pro-inflammatory chemokine known to recruit neutrophils, that has thus far not been associated with malaria in the clinical setting, was significantly increased in children with *P. falciparum* persistent parasitaemias at the end of the dry season (Fig. 2b). In contrast, CCL3, IL-10, IL-6 and IL-1β, previously associated with clinical malaria were comparable in the plasma of infected versus uninfected children at the end of the dry season (Fig. 2b and Supplementary Table 2). We next quantified the proportions of major leucocyte populations from thawed PBMCs collected at the end of the dry season from children with or without subclinical *P. falciparum* (gating strategy in Supplementary Fig. 1). We observed that monocytes, T cells, B cells and NK cell subpopulations were not significantly different between children who carried (May-), or not (May-) *P. falciparum* (Fig. 2c and Supplementary Fig. 2). To interrogate differences in cell function, we quantified intracellular cytokines, activation or cytotoxicity markers, transcription factors or exhaustion markers of freshly collected PBMCs from *P. falciparum* subclinical infected- and non-infected children at the end of the dry season. The levels of the activation marker CD25, transcription factor T-bet or cytokine IL-2 of CD4 T cells; Granzyme B of CD8 T and NK cells, and exhaustion marker FCRL5 of atypical memory B cells were comparable between children who carried or not *P. falciparum* (Fig. 2d and Supplementary Table 3). We further questioned whether memory B cells (MBCs defined as CD19+, CD10-, CD21- and CD27+ or -, gating strategy in Supplementary Fig. 1) specific for *P. falciparum* were affected in subclinical carriers compared to non-infected individuals at the end of the dry season. Using biotinylated AMA-1 and MSP1, we quantified AMA-1- or MSP1-specific MBCs in both children who carried *P. falciparum* parasites, and non-infected children at the end of the dry season. We found that the proportion of class-switched *P. falciparum*-specific MBCs (AMA1+ or MSP1+ IgG, IgM- MBCs) was significantly increased in subclinical carriers at the end of the dry season, while no such difference was found in non-class-switched MBC population (AMA1+ or MSP1+ IgG, IgM- MBCs) (Fig. 2e). Within the IgG- MBC subpopulations (classical, activated or atypical) we failed to observe differences between *P. falciparum* carriers and uninfected individuals at the end of the dry season in *P. falciparum*-specific classical and atypical MBCs, but detected increased *P. falciparum*-specific activated MBCs in subclinical carriers (Supplementary Fig. 2). Using another multiplex bead array, we quantified humoral responses of *P. falciparum* subclinical carriers and uninfected individuals at the beginning and end of the dry season, to 35 different domain types of the VSA multigene family *var*, which were grouped according to their endothelial-receptor affinity (CD36, EPCR or unknown receptor) and PfEMP1...
We observed that more subclinical carriers (May+) than non-infected individuals (May-) were reactive against PfEMP1 domains binding to CD36, EPCR or to unknown receptors at both time-points, and also that the proportion of individuals reactive to the different PfEMP1 domains decreased over the dry season independently of individual infectious status (Fig. 2f and Supplementary Fig. 2), these differences parallel our previously published data on *P. falciparum*-specific humoral responses to non-VSA, suggesting comparable humoral dynamics for PfEMP1s and non-VSA antigens. Additionally, we observed that the magnitude of IgG reactivity to A, B or B/A types of PfEMP1 declined similarly from the beginning to end of the dry season in children who carried subclinical infection (May+) or were uninfected (May-) during the dry season (Fig. 2g and Supplementary Fig. 2). Antibodies against PfEMP1 domains (Fig. 2f-g), against a large set of *P. falciparum* non-VSA, and also particularly against RBC invasion-related proteins were consistently higher in subclinical carriers compared to non-infected children at the end of the dry season, so we questioned if the difference in humoral response at the end of the dry season could impose variance in inhibition of merozoite invasion in vitro. We tested merozoite invasion of a laboratory-adapted *P. falciparum* strain in the presence of plasma from Malian children who carried parasites or not during the dry season, and evaluated the antibodies’ ability to block RBC invasion. Testing complete and antibody-depleted plasma, we observed that antibody-depleted Malian plasma allowed for ~5-fold increase in invasion of merozoites compared to complete Malian plasma, while antibody depletion had no differential effect on the control German plasma used (Fig. 2h and Supplementary Fig. 2). Notably, however, plasma from Malian children carrying subclinical infections (May+) or not carrying parasites (May-), had similar ability to inhibit merozoite invasion, suggesting that the antibodies remaining elevated at the end of the dry season have no significant effect on inhibiting merozoites invasion of RBCs, and are unlikely to contribute to the maintenance of low parasitaemias through this mechanism.
Fig. 2 | *P. falciparum* induces a minimal immune response during the dry season. a, Plasma concentration of C-reactive protein (CRP) (May− n=71; May+ n=117), von Willebrand factor (vWF) (May− n=33; May+ n=51) and Hepcidin (May− n=41; May+ n=37) of paired samples at the beginning (Jan) and end of the dry season (May) carrying *P. falciparum* (May+) or not (May−). Boxplots indicate median, 25th and 75th percentiles, *P* values determined using one-way ANOVA with multiple comparisons correction. b, Plasma concentration of cytokines from children carrying (May+ n=21) or not (May− n=12) *P. falciparum* infection at the end of the dry season. ANOVA with Sidak multiple comparisons test. c, Expression of surface markers of frozen PBMCs collected from children that carried (May+ n=23) or not (May− n=28) *P. falciparum* at the end of the dry season. Data represented as median ± IQR. *P* values determined using Kruskal-Wallis test with multiple comparisons. d, Expression of intracellular markers of freshly collected PBMCs from children that carried (May+ n=29) or not (May− n=24) *P. falciparum* subclinical infections during the dry season. Data represented as median ± IQR. *P* values determined using Kruskal-Wallis test with multiple comparisons. (Supplementary Table 3 for details). e, *P. falciparum*-specific memory B cells (MBCs), defined as AMA1+ or MSP1+, in *P. falciparum* carriers (May+, n=23) or non-carriers (May− n=28) at the end of the dry season for class-switched (IgG+ IGM− MBCs) or non-class-switched (IgG− IGM+ MBCs). Data represented as median ± IQR. *P* values determined by Mann-Whitney test. f, Proportion of children with antibodies specific to PfEMP1 domains with different binding activity at the beginning (Jan) and end (May) of the dry season (Jan and May− n=106; Jan 260 and May+ n=112). Boxplots indicate median, 25th and 75th percentiles, *P* values determined with 261 RM one-way ANOVA (with Greenhouse-Geisser correction) for each of the binding receptor affinity. g, Magnitude of anti-PfEMP1 domains of A and B subtypes between the beginning and end of the dry season in children carrying or not subclinical *P. falciparum* (Jan and May+, n=112, Jan and May− n=106). h, Parasitaemia after invasion in the presence of complete plasma or antibody-depleted plasma from children who carried (May+ n=29) or not (May− n=24) *P. falciparum* subclinical infections during the dry season. Data represented as median ± IQR. *P* values determined using Kruskal-Wallis test with multiple comparisons.
269 *P. falciparum* genetic diversity is maintained throughout the year. Next, we asked whether 270 *P. falciparum* parasites that persist through the dry season are genetically distinct from those 271 that cause acute malaria during the transmission season. To that end, we measured the size of 272 the merozoite surface protein 2 gene (*msp2*), which is highly polymorphic and discriminates 273 different *P. falciparum* genotypes. Through nested PCR followed by fragment analysis using 274 capillary electrophoresis, we compared paired samples from 93 subclinical carriers at the 275 beginning (Jan) and end (May) of the dry season, with 136 samples from clinical cases of 276 malaria in the ensuing transmission season (MAL). The number of clones detected per 277 individual did not significantly differ between parasites isolated during the dry season or 278 transmission season, nor did the percentage of individuals with different numbers of clones 279 (Fig. 3a-b). Furthermore, the size and distribution of *msp2* clones identified during the dry 280 season were similar to those isolated from clinical malaria cases during the transmission season 281 (Fig. 3c-d), with the most frequent clone sizes being the same at any of the time-points analysed.

282

**Figure 3**

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284 **Fig. 3 | P. falciparum genetic diversity is maintained throughout the year.** a, Number of *P. falciparum* clones in subclinical PCR+ children in beginning (Jan) and end (May) of the dry season, and first clinical malaria case (MAL) during the transmission season, determined by size differences in the polymorphic region of 1 *msp2*. b, Proportion of individuals with different number of *P. falciparum* clones in the beginning (Jan) and end (May) of the dry season and clinical malaria cases (MAL). c, Size of the 5 *msp2* alleles FC27 (left) and IC/3D7 (right) in 6 beginning (Jan) and end (May) of the dry season, and clinical malaria cases (MAL). d, 8 Prevalence of different *msp2* clone sizes of 9 FC27 (left) and IC/3D7 (right) allelic families in 9 Jan (n= 93), May (n= 93) and clinical malaria 1 (MAL, n= 136). Comparisons were performed with Mood’s median test.

304 Transcriptome of circulating subclinical *P. falciparum* at the end of the dry season differs from that of *P. falciparum* during clinical malaria. We performed RNA sequencing of 306 leucocyte-depleted blood from 12 children with persistent subclinical *P. falciparum* at the end of 307 the dry season (May), and from 12 age- and gender- matched children presenting with their first 308 clinical malaria case in the ensuing transmission season (MAL). RNA-Seq libraries from these 309 samples were run on an Illumina HiSeq, producing 0.5 to 3 million reads mapping uniquely to
the *P. falciparum* genome (Supplementary Table 5). Principal component (Fig. 4a) and unsupervised clustering (Fig. 4b) analyses showed segregation of transcription profiles based on seasonality. Differentially expressed genes (DEGs) determined at a false discovery rate threshold of 5% resulted in 1607 DEGs, 1131 transcripts up-regulated, and 476 transcripts down-regulated in the dry season compared to clinical malaria samples (Fig. 4c and Supplementary Table 6). Validation of the RNA-Seq data was performed by RT-qPCR of eight DEGs and the correlation between the two methods resulted in highly significant $r^2=0.929$ (Fig. 4d and Supplementary Table 7). Furthermore, samples from additional children (6 end of the dry season and 12 malaria cases during the transmission season) were used to quantify expression of three DEGs in parallel with the initial 24 samples, and revealed similar fold changes by RT-qPCR (Fig. 4e). We investigated similarities in the DEGs obtained in this study with those of previous reports comparing parasite physiological states and transcriptomes from a range of clinical malaria severities, or parasites causing malaria in high versus low transmission areas, but no enrichment was found (Supplementary Fig. 3), suggesting that singular mechanisms may be at play during the dry season. Functional and Gene Ontology analysis of the dry season DEGs using DAVID revealed a significant enrichment (Benjamini-Hochberg adjusted $P < 0.05$) of transcripts involved in cellular processes related with several metabolic pathways and also *Phagosome, DNA replication* or *Homologous recombination* (Fig. 4f).

Indeed, DEGs involved in metabolic pathways suggest that *Glycolysis, Glycerophospholipid, Purine and Pyrimidine* pathways were increased in parasites from the end of the dry season (May), while *Fatty acid biosynthesis*, appeared downregulated comparing to parasites from clinical malaria (MAL) in the wet season (Fig. 4g). To follow-up on possible metabolic differences between parasites persisting through the dry season and parasites causing malaria in the transmission season we used liquid chromatography–mass spectrometry to profile both hydrophilic and hydrophobic metabolites from the plasma of 12 subclinical children with *P. falciparum* infections at the end of the dry season (May), and of 12 children presenting with their first clinical malaria case (MAL) in the rainy season. We found significant separation between metabolites present in the two groups of samples (Fig. 4h and Supplementary Table 8), however the difficulty of normalization of measured metabolite levels to parasite burden, plus the strong parasitaemia differences at the time of the blood draw, hinder conclusive interpretation of what may be seasonal or parasite induced metabolic alterations (Supplementary Table 9).
Fig. 4 | Transcriptome of circulating *P. falciparum* at the end of the dry season differs from malaria-causing *P. falciparum* during the transmission season. a, Principal components analysis and b, unsupervised clustering analyses of RNA-Seq data of *P. falciparum* parasites collected at the end of the dry season (May, n=12) and from clinical malaria cases (MAL, n=12). c, Heatmap showing normalized reads of differentially expressed genes (DEGs) (rows) for each subject (columns) from *P. falciparum* collected at the end of the dry season (May, n=12) and at the first clinical malaria case (MAL, n=12) in the ensuing transmission season. Analysis performed using Bioconductor package DESeq2, with $P_{adj} < 0.05$ considered significant. d, RT-qPCR validation of RNA-Seq data for 8 labelled DEGs (n=24). $P$ and $r^2$ values were determined using Pearson correlation. e, RT-qPCR validation of initial 24 samples used in RNA-Seq analysis (left) and 18 additional samples (right) for three DEGs. Data represented as mean ± SD $P$ values determined with non-parametric Mann-Whitney test. f, Summary of KEGG pathways significantly enriched with DEGs in dry season obtained using the DAVID tool. Blue bars indicate $P_{adj}$ for the enrichment of each pathway in the gene list. The grey dashed line indicates the threshold $P = 0.05$. The orange line indicates the percentage of genes in each pathway that was present in the DEG list. g, Heatmap showing normalized reads of differentially expressed genes (DEGs) involved in different metabolic pathways (rows) for each subject (columns) from *P. falciparum* collected at the end of the dry season (May, n=12) and at the first clinical malaria case (MAL, n=12) in the ensuing transmission season. h, Principal components analysis of target metabolites’ data of plasma samples from subclinical *P. falciparum* carriers at the end of the dry season (May, n=12) and from clinical malaria cases (MAL, n=12).
393 **P. falciparum replication is not impaired in the dry season.** We then tested the hypothesis that dry season parasitaemias were maintained low and subclinical due to decreased parasite replication capacity during this period. We cultured *P. falciparum* in vitro directly after blood draw for 36 to 48 h, from rapid diagnostic test positive (RDT,) samples of asymptomatic individuals at three time points of the dry season (January, March and May), and from samples of children presenting with their first clinical malaria episode of the ensuing transmission season (MAL). By flow cytometry, we measured the increase in parasitaemia and parasite development at 0, 16, 24, 30, 36 and 48 h after in vitro culture. Parasite in vitro growth rates of samples from >40 subclinical donors during the dry season, and ~30 malaria cases in the transmission season indicated that the highest growth determined between any two time-points of the short-term culture was similar throughout the year. Parasitaemias increase in vitro between 2 and 5-404 fold at any point in the year, 2.8-fold in January, 95% CI (2.3 , 3.3), 4.1-fold in March, 95% CI (3.4 , 4.7), 3.6-fold in May, 95% CI (2.6 , 4.6), and 2.8-fold in malaria cases, 95% CI (2.1 , 3.5). However, the number of hours in culture needed to increase parasitaemia was shorter in the dry season samples than in samples from malaria-causing parasites in the transmission season (Fig. 5a). In accordance with an earlier increase in parasitaemia in vitro during the dry season, we could frequently identify on Giemsa smears mature schizonts after 16 and 24 h of culture, and young ring-stages after 30 or 36 h in the dry season samples; while mature schizonts of malaria-causing parasite samples were mostly observed after 36 h in culture and young ring-stages were largely found after 48 h in vitro (Fig. 5b). When we calculated the number of hours in culture at which the highest increase of parasitaemia could be detected for each sample, we observed that it decreased from the beginning to the end of the dry season, Jan 26.4 h, 95% CI (24.5 , 28.3), Mar 24.9 h, 95% CI (23.9 , 25.9), May 22.7 h, 95% CI (20.8 , 24.6), and was maximum during malaria cases in the transmission season, MAL 44.0 h, 95% CI (41.5 , 46.6) (Fig. 5c). Nevertheless, when we measured the number of merozoites per multinucleated schizont prior to or at the time of the highest increase in parasitaemia in vitro, we obtained comparable values at the end of dry season and in clinical malaria cases in the wet season (Fig. 5d). Finding later developmental parasite stages at earlier times in this short-term in vitro experiment during the dry season could indicate a faster than 48 h intraerythrocytic replicative cycle, or alternatively, that dry season parasites circulate longer without adhering to the host vascular endothelium, and are more developed than circulating parasites in clinical malaria cases at the time of the blood draw. To test the latter, we used the RNA-seq data described in Fig. 4 to estimate, with a likelihood-based statistical method previously described, the age in hours post-invasion (hpi) of circulating parasites from subclinical children at the end of the dry
season (May) and from clinical cases during the wet season (MAL). We determined that parasites circulating in the dry season had a transcriptional signature of ~17 hpi, 95%CI (14.05, 20.8), while parasites circulating in malaria cases during the wet season had a transcription profile comparable to parasites with ~7 hpi, 95% CI (6.5, 7.7) (Fig. 5e). Accordingly, by imaging the thick blood films made in the field at the time of the blood draw, we confirmed that trophozoites were present on the end dry season samples, while clinical malaria samples in the transmission season had only ring-stages, with much smaller areas (Fig. 5f-g).

**Figure 5**

*Fig. 5. | Replication of persistent dry season P. falciparum is not impaired.*

a, Parasitaemia fold change at 0, 16, 24, 30 and 36 h post-culture of parasites collected from children at different times during the dry season (Jan, Mar and May) and clinical malaria case (MAL). Fold change is defined as %iRBC t(n)/ %iRBC t(n-1). Data is represented as median ± IQR.

b, Giemsa-stained thin blood smears 16, 24, 36 or 48 h post-culture of P. falciparum parasites collected from children during the dry season (Jan, Mar and May) and clinical malaria case (MAL). Scale bars, 2 μm.

c, Time of highest increase in parasitaemia detected during in vitro culture of P. falciparum parasites collected from children in Jan, Mar and May during the dry and clinical malaria case (MAL). Data represented as mean ± SD P values determined using Kruskal-Wallis multiple comparisons test.

d, Number of merozoites inside multinucleated schizonts determined by flow cytometry at the end of the dry season (May) and clinical malaria (MAL) samples.

e, Maximum likelihood estimation (MLE) of the hours post-invasion of dry season (May) and clinical malaria (MAL) parasites. Data is represented as mean ± SD P values determined using Mann-Whitney test.

f, Giemsa-stained thick blood films of P. falciparum parasites collected straight from the arm of children, at the end of the dry season (May) and at their first clinical malaria (MAL). Scale bars, 5 μm.

g, P. falciparum area measured from Giemsa-stained thick smears in subclinical infections at the end of the dry season (May) and in malaria cases (MAL) in the wet season. Data is represented as median ± IQR.
Infected erythrocytes in circulation at the end of the dry season are at higher risk of splenic clearance. To investigate if longer circulation of iRBCs in the dry season would impact host RBC deformability and potentiate splenic clearance, we used a microsphiltration system that mimics the narrow and short inter-endothelial slits of the human spleen with different sized microspheres. Using freshly collected blood samples from asymptomatically infected children at the end of the dry season and from children presenting with febrile malaria during the transmission season, we assessed retention in the microspheres and flow-through of circulating iRBCs at time zero, and after 6, 18 and 30 h in vitro. We observed that iRBCs collected from malaria (MAL) cases were not significantly retained in the spleen-like system at 0, 6 or 18 h post-culture, and that only after 30 h was the percentage of iRBCs in the flow-through reduced, indicating splenic retention of iRBCs (Fig. 6a). Conversely, iRBCs in RDT+ blood collected at the end of the dry season (May) had significantly reduced flow-through immediately after the blood draw (~25% retention of 0 h iRBCs in the microsphere-system), and over 50% retention of iRBCs after 6 h or 18 h in culture (Fig. 6a). Accordingly, we observed that trophozoites or schizonts which fail to flow through the microsphere system were circulating (at 0 h) only in the dry season samples (Fig. 6b).

We then investigated whether differences in cytoadhesion, affecting the length of time that parasitized cells remain in circulation, could explain the observed age distributions and microsphiltration results. For this we used a mathematical model to describe the within-host growth and removal of infected red blood cells (iRBC) from circulation through cytoadhesion in the deep vasculature and through splenic retention (see Methods). Both processes were assumed to be dependent on the parasite’s developmental stage, increasing as the parasite starts to express adherence-mediating surface antigens and RBC modification leads to higher cell rigidity. Whereas cytoadhering parasites still replicate, those filtered out by the spleen were assumed to be completely removed. As shown in Fig. 6c, effective growth rates and population sizes of low-cytoadhering parasites are significantly lower than those of high-cytoadhering parasites, which can avoid splenic clearance before parasitized cells become too rigid to pass through the spleen. We then obtained estimated parasite age distributions for both scenarios (Fig. 6d) by sampling from the modelled parasite population at random points over the simulated infection time course, akin to blood sampling from a population. As low-adhesion parasites are predominantly removed by the spleen towards the end of their life-cycle, they show a much broader age-range than high-cytoadhesion parasites, which are removed from circulation earlier through cytoadhesion and therefore show a narrower and younger age range, in agreement with the observed age distribution from thick blood smears (Fig. 5e-f). Next, we simulated a
microfiltration experiment by “growing” our sampled model parasites older and evaluating their projected average flow-through based on our assumed, age-dependent splenic retention function (see Methods). As illustrated in Fig. 6e, the throughput of high-cyoadhering parasites is very high for the first 6-10h before dropping off gradually as parasites grow older. In contrast, samples from low-cyoadhering parasites with their more uniformly distributed age range already have a much-reduced flow-through at 0h, which, however, remained more stable as parasites mature over the next 30h; this is again in line with empirical observations (Fig. 6a).

These mathematical results suggest that cyoadhesion alone can explain the differences between parasites sampled during the dry season (low-adhesion) and parasites sampled from malaria cases (high-adhesion).

To investigate if the expression of cyoadhesion-mediating PIEMP1 proteins differed in abundance or quality in parasites found subclinical in the dry season and parasites found in malaria cases during the wet season, we assembled the var genes from the RNA-Seq reads of the 24 samples from end of the dry season and malaria cases (see methods). Expression of several var genes has been shown to remain fairly stable between ~10 and 20h post invasion which should be close to the average ages estimated for parasites in malaria cases’ and dry season samples respectively. Using a recently developed analytical pipeline, we could detect LARSFADIG motifs identifying PIEMP1 coding genes in 8 out of 12 samples from the dry season, and in 10 out of 12 malaria cases (Table 2).

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Table 2 | LARSFADIG motifs that identify PIEMP1 coding genes of 12 subclinical individuals at the end of the dry season (May) and 12 first clinical malaria episodes in the ensuing wet season (MAL).
We were able to annotate some full length var genes, including both the start N-terminal sequence (NTS) and the acidic terminal sequence (ATS) domains, and also many isolated fragments, and generally we observed more contigs with LARSFADIG motifs and var gene fragments in the wet season samples (Supplementary Table 10). We used different methods to access an enrichment of higher expressed var genes in the wet versus the dry season samples. Although we did not see statistically significant enrichment, we observe a trend that the top expressed var genes in each individual in the wet season are higher expressed (Fig. 6f). Furthermore, we identified known var gene domains such as Duffy Binding Like (DBL) and Cysteine Rich Interdomain Regions (CIDR) as well as the NTS and ATS, and searched for typical 5’ upstream (UPS) sequences associating with different pathological outcomes in the dry season and malaria cases’ assembled vars. Although we were unable to determine the UPS type of expressed vars due to the short assembly of the 5’ UTR region, we did find more var genes with a DBLz domains in the malaria samples (13 out of 61 in var fragments longer than 3.5 kb) compared to the dry season (1 out of 11 in var fragments longer than 3.5 kb) (Supplementary Table 10).
Infected erythrocytes in circulation at the end of the dry season are at higher risk of splenic clearance. a, *P. falciparum* iRBCs filtration through 2 beads mimicking the human spleen at 0, 18 and 30 h post culture at the end of the dry season (May) and during clinical malaria (MAL) relative to the non-filtered same-time control. Flow-through % is defined as downstream %iRBCs / upstream %iRBCs) x 100 (n= 8, mean ± SD, *P* values determined by Dunn's multiple comparison test of the mean rank of each condition to 0h MAL, * shows *P* < 0.001) b, Percentage of circulating non-ring stage iRBCs at the end of the dry season (May, n= 50) and during malaria cases (MAL, n= 39) determined by flow cytometry. Mean ± SD *P* values determined with Mann-Whitney test. c, Within-host dynamics simulation of growth rates and population sizes over five replication cycles of low-cytoadhering (left), and high-cytoadhering (right) parasites, stratified as circulating (red lines), cytoadhering (orange dashed lines) and total biomass (black lines). d, Simulation of circulating parasites’ age distribution over two replication cycles after repeated sampling of low- and high-cytoadhering parasites (Low), and high-cytoadhering parasites (High). e, Simulation of circulation and passage through the spleen of parasites aging over time, with low-cytoadhering (Low), and high-cytoadhering (High) parasites. f, Expression level of the highest expressed *var* gene at the end of the dry season (May, n= 8) and during a clinical malaria case (MAL, n= 10).

Discussion

Asymptomatic individuals carrying *P. falciparum* at the end of the dry season in areas of seasonal malaria have been broadly described, but how the parasite bridges two rainy seasons without promoting malaria symptoms or being cleared remained elusive. In this study, with samples from Malians exposed to alternating six-month dry and transmission seasons, we show that within each 48h replicative cycle, *P. falciparum* iRBCs circulate longer in the bloodstream during the dry season, allowing increased clearance in the spleen, and thus preventing high parasitaemias which could lead to immune activation or malaria symptoms. Although asymptomatic parasitaemia at the end of the dry season is associated with a lower risk of clinical malaria in the ensuing wet season, clearance of parasitaemia with anti-malarials prior to the transmission season does not increase subsequent malaria risk, and the persistence
of infection during the dry season does not prevent nor slow the decline of *P. falciparum*-specific antibodies\(^{13}\) (Fig. 2 f-g). Consistent with these earlier observations, low parasitaemia during the dry season did not elicit detectable inflammation, nor affect immune cell function (Fig. 2), possibly indicating that chronic low parasitaemia in seasonal endemic settings might differ from Controlled Human Malaria Infections in naïve individuals, where low-level parasitaemias appear to induce immunity (reviewed in\(^{40}\)); and also pointing that slow and continuous stimulation of the immune system is less effective than sudden changes in antigenic stimulation\(^{41}\). Nevertheless, cumulative immunity may be determinant to sustain the dry season reservoir of *P. falciparum*. Dry season subclinical carriers have higher anti-*P. falciparum* humoral immunity (Ref. 13 and Fig. 2f-g), and higher *P. falciparum*-specific MBCs (Fig. 2e) than non-infected individuals, suggesting that a certain cumulative exposure is necessary to carry subclinical infections through the dry season. Additionally, we and others have shown that end of dry season parasitaemias are more frequent in older than younger children\(^{13,42}\), which is consistent with an age-dependent decrease in parasitaemia and increase in anti-parasitic immunity\(^{43,44}\). It is possible that within each *P. falciparum* infection, sequential presentation on the surface of iRBCs of the different variants of multi-gene families, and its corresponding ordered acquisition of antibodies\(^{45,46}\) favours progressively less virulent parasites. Accordingly, a recent study of Controlled Human Malaria Infections including malaria-naïve and semi-immune individuals observed clinical cases in malaria-naïve individuals with parasites expressing VSAs; while chronic infections appeared in semi-immune with intermediate antibody levels\(^{47}\).

Reports from the transmission season show that increasing malaria severity associates with different parasite transcriptional profiles\(^{26,48-50}\), but the persisting dry season reservoir had not been investigated. Our data shows that, while *P. falciparum* causing malaria in the transmission season has a ring-stage transcriptional signature, parasites persisting at the end of the dry season resemble more developed intraerythrocytic stages, which we confirmed both visually and through differential growth kinetics in vitro (Fig. 5). From the bulk RNA-Seq analysis, it is unclear if the differences in parasite gene-expression between the dry season and malaria cases are solely imposed by the hpi of the parasites collected, but single-cell transcript analysis of iRBCs\(^s\) will allow comparing stage-matched pools of parasites to better understand how *P. falciparum* achieves low cytoadhesion in the dry season. Also of interest, will be to revisit earlier reports of transcriptional differences between parasites inducing varying degrees of malaria severity\(^{26,48,49}\), and question if these could be partially imposed by the number of hpi of circulating parasites. In fact, Tonkin-Hill and colleagues found a bias towards early trophozoite transcription in the non-severe malaria samples compared to the ring-stage transcriptional
profile of the severe malaria cases, which could be due to differing adhesion efficiencies in vivo. Interestingly, in vitro replication rates of severe and uncomplicated malaria causing parasites has not consistently explained the higher parasitaemias observed in severe malaria cases, suggesting that adhesion efficiency differences may also contribute. Continued asexual replication (independently or coupled with immunity) may lead to progressively less adhesive iRBCs as observed in parasites collected during the dry season. In a rodent-malaria model, uninterrupted asexual-stage growth led to bias in gene expression of VSA and parasite virulence; and the transition between acute and chronic phases is suggested to be independent of adaptive immunity. Also, in humans it has been suggested that continued asexual replication can skew the PfEMP1 expression profile, which is consistent with our var genes RNA-Seq data (Fig. 6f and Table 2). The mechanisms by which the parasite adapts to the dry season, and how transmission is assured in the rainy season ensues remain to be investigated. In a varying or unpredictable environment, organisms can overcome unfavourable conditions by sensing environmental changes and adapting their individual developmental program to increase survival; or alternatively, stochastic population heterogeneity increases the probability of survival under changing conditions. P. falciparum may sense and respond to environmental cues of transmissibility opportunity, as has been described for detection of nutrient availability, sexual commitments, or appropriate environment for gametogenesis. Such a mechanism could act through epigenetic modulation of VSAs, or be seasonally imposed by different metabolic states of the host, driving a shift on the parasite from a fast- to a slow-growing program as the transmission season ends and persistence is required, and return to fast growth as transmission resumes. In an avian-malaria model, chronic P. relictum has been shown to respond to bites from uninfected mosquitoes and increase its replication promoting transmission. Parasite survival during the dry season is imperial, but will only be efficient in resuming transmission if these retain the ability to produce gametocytes that mosquitoes can be uptake, thus investigating potential adaptive changes in the sexual stages of P. falciparum during the dry season will likely also reveal seasonal adjustments. The survival of P. falciparum-infected individuals during the dry season is advantageous for the parasite, and low adhesion of infected erythrocytes is likely a central feature to the subclinical carriage of P. falciparum demonstrating the adaptability of P. falciparum parasites to the vector-free period.

Data availability
RNA-Seq data (normalized counts data and raw sequencing reads) have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE148125 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148125](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148125). All processed metabolomics spectral data and analytical metadata from this study have been deposited into the NIH Metabolomics Workbench (project ID no. ###). The data file of assembled var gene fragments of all isolates are available at: [https://github.com/ThomasDOtto/varDB/tree/master/Otherdatasets/Andrade_DryWet2020](https://github.com/ThomasDOtto/varDB/tree/master/Otherdatasets/Andrade_DryWet2020).

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**Author contributions**

CMA, HF, RTL, NFL, CA, JH, CSH, SL, MN, HC, DS, CM, SR, KM, MVH, ELA and SP performed experiments and analysed data; SD, DD, KK, AO, BB and PDC designed, conducted and supervised field work generating the clinical data and samples; JM, NOS and TDO performed bioinformatic analysis; MEN, CL, TL, AM, and AF, ML provided technical expertise and TL and LT provided essential reagents; MR performed mathematical models; TMT, JS and VW provided statistics expertise; CMA prepared figures and CMA, HF, RTL, TMT, NSO and TDO and helped prepare the manuscript; TMT, AF, ML, TL, TDO, MR and PDC provided insightful comments to the manuscript. PDC discussed initial field and study designs. SP designed the study and wrote the manuscript. All authors read and approved the final manuscript.

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Advances, and Challenges.


