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Host and parasite factors of *Plasmodium falciparum* asymptomatic persistence during the dry season

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Affidavit

I hereby declare that the experiments for the presented work are the result of my own work, unless otherwise stated. I have not used any other sources or aids than those indicated in my thesis. The thesis in its current or similar version has not been submitted anywhere else.

Heidelberg, 04.03.2021

Carolina Morgado de Andrade

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Summary

Malaria is a mosquito-borne disease caused by *Plasmodium* parasites and transmitted by female mosquitoes of the genus *Anopheles*. The majority of deaths occurs in the African continent and in 2019 *Plasmodium falciparum* killed nearly 400,000 people. In many areas of the globe, dry season periods limit *Anopheles* mosquito availability reducing malaria transmission to virtually zero. In Mali, during the 5- to 6-month dry season *P. falciparum* persists in a fraction of the human population in clinically silent infections that serve as reservoir to restart transmission when mosquitoes return in the ensuing wet season. However, the mechanism by which *P. falciparum* bridges rainy seasons without promoting malaria symptoms or being cleared by host immunity remained elusive.

In this work, we used samples from a village in Mali where individuals are exposed to an alternating six-month dry and rainy season. We show that *P. falciparum* persists in the blood of asymptomatic individuals during the dry season at low parasite densities and very seldom causing disease. By comparing immune system markers in individuals that carried or not infections during the dry season, we found that asymptomatic infections only minimally affect the host immune response during this period.

We compared the transcription profile in parasites collected at the end of the dry season or during the first clinical malaria case in the ensuing transmission season and we found that the parasites segregated by season, where parasites from the dry season have a transcription profile reflecting a longer circulation within each 48-hour intra-erythrocytic cycle when compared to malaria causing *P. falciparum.* In agreement with a different transcriptional profile that indicates a longer circulation of *P. falciparum* in the dry season, we found more developed parasites in the dry season by microscopy and flow cytometry in comparison with parasites isolated from malaria patients in the transmission season. And we further show that circulation of older parasites in the dry season increases splenic clearance, promoting the low parasite densities found in the dry season. All together we describe how seasonally altering the competence of adhesion to the endothelium by parasites, which is a hallmark of *P. falciparum* malaria, affects parasitaemia growth and disease presentation.

To understand how *P. falciparum* achieves this less adhesive phenotype during the dry season, we investigated if parasites could respond to seasonal serologic factors. We cultured *P. falciparum* parasites in the presence of plasmas from different times of the year and

determined parasite development, replication and host cell remodelling in vitro, which could seasonally favour asymptomatic infections. We did not observe any differences in parasite development, replication, or host cell remodelling when parasites were grown in different season plasmas. Although the in vitro experiments we were able to do could not disprove the existence of a sensing mechanism of the season by *P. falciparum*, because it may be that we were unable to provide the condition exactly replicating what occurs in vivo, it seems unlikely since we also observed older parasites in circulation in asymptomatic infections during transmission season. This all suggesting that the longer circulation phenotype observed is not unique to the dry season, but likely linked to low parasitaemias that remain below the clinical radar.

Finally, we questioned when the *P. falciparum* dry season asymptomatic infections were transmitted during the preceding transmission season. We followed retrospectively children that were infected at the end of one dry season until when they had first become parasite positive in the preceding transmission season, capturing fortnightly longitudinal data on parasite density fluctuations, and measuring the parasite genetic diversity by *ama*1 amplicon sequencing. We found that clonal infections that reached the end of the dry season had been mainly transmitted towards the end of the rainy season, while clones transmitted early in the transmission season were unlikely to persist until the end of the dry season. These data suggest that infection length is tailored to bridge the length of the dry season, and favours a model where early transmitted infections that are not treated will present a “dry season” phenotype still during the transmission season (as observed in asymptomatic infections during the transmission season), while later transmitted infections are more likely reach the end of the dry season and resume transmission as the wet season ensues.

The results from this work begin to elucidate on the mechanisms that allow *P. falciparum* to survive the dry season, bridging two transmission seasons. The knowledge acquired in this work about the complex interaction between *P. falciparum,* the human host and the environment, might inform strategies that will aid to the ultimate goal of malaria eradication by targeting the silent reservoirs of *P. falciparum.*

Zusammenfassung

Die Erreger der Malaria, Parasiten des Genus *Plasmodium*, werden durch weibliche Anopheles Moskitos übertragen. In 2019 verlief eine Infektion mit *Plasmodium falciparum* für nahezu 400 000 Menschen tödlich, die Mehrheit davon auf dem afrikanischen Kontinent. In weiten Teilen der Erde, wird die Verfügbarkeit des Anopheles Vektors stark durch jahreszeitliche Trockenzeiten eingeschränkt, während dieser Perioden fällt Malariatransmission auf beinahe Null. In Mali, während der 5-6-monatigen, jährlichen Trockenzeit, persistiert *P. falciparum* in einem Teil der Bevölkerung als eine klinisch stille, asymptomatische Infektion. Diese Infektionen dienen als Reservoir für einen erneuten Transmissionsstart, wenn mit Beginn der folgenden Regenzeit Moskitos zurückkehren. Die Mechanismen, die dem Parasiten erlauben, die Trockenzeit zu überbrücken, ohne Symptome auszulösen, und gleichzeitig Beseitigung durch die Wirtsimmunität zu vermeiden, sind jedoch nicht bekannt.

In dieser Arbeit haben wir Proben aus einem Dorf in Mali untersucht, in dem sich eine je sechsmonatige Regen- und Trockenzeit abwechseln. Wir zeigen, dass *P. falciparum* im Blut von asymptomatischen Individuen über die Trockenzeit persistiert, Infektionen in diesem Zeitraum wiesen eine geringe Parasitendichte auf und waren nur selten krankheitsauslösend. Durch den Vergleich von Immunmarkern in *P. falciparum*-infizierten und nicht infizierten Individuen während der Trockenzeit, ermittelten wir, dass asymptomatische Infektionen nur minimal die Immunantwort des Wirts über diese Zeitspanne beeinflussen.

Außerdem verglichen wir das Transkriptionsprofil von Malariaparasiten, die entweder am Ende der Trockenzeit oder während der ersten Malariaepisode der folgenden Regenzeit isoliert wurden, wir fanden eine Segregation der Parasiten nach Jahreszeit, wobei Parasiten aus der Trockenzeit ein Transkriptionsprofil aufwiesen, das eine längere Zirkulation innerhalb jedes 48-stündigen intra-erythrozytischen Zyklus widerspiegelt, verglichen mit Malaria verursachenden *P. falciparum*. Übereinstimmend mit einem Transkriptionsprofil, dass auf eine längere Zirkulation von *P. falciparum* während der Trockenzeit hindeutet, fanden wir mit Hilfe von Mikroskopie und FACS spätere Entwicklungsstadien des Parasiten in der Trockenzeit verglichen mit Parasiten, die von Malariapatienten in der Regenzeit isoliert wurden. Darüber hinaus zeigen wir, dass die Zirkulation älterer Parasiten in der Trockenzeit die erhöhte Entfernung infizierter Erythrozyten durch die Milz zur Folge hat, was zu den niedrigen Parasitendichten, wie sie in der Trockenzeit beobachtet werden, beitragen könnte. Alles in allem beschreiben wir, wie eine saisonale Veränderung der Kompetenz zur Endotheladhäsion des Parasiten – ein wichtiges

Merkmal der *P. falciparum* Malaria – eine Veränderung in Parasitendichte und Krankheitsbild herbeiführen kann.

Um zu verstehen, wie *P. falciparum* diesen weniger adhäsiven Phänotyp während der Trockenzeit annimmt, untersuchten wir, ob Parasiten auf saisonale serologische Faktoren reagieren können. Dafür setzten wir *P. falciparum* Blutkulturen mit Blutplasma von über das Jahr verteilten Zeitpunkten an und ermittelten Parasitenentwicklung, Replikation, sowie Wirtszellremodellierung *in vitro*; Veränderungen in diesen Bereichen könnten asymptomatischen Infektionen in der Trockenzeit erklären. Hinsichtlich Parasitenentwicklung, Replikation, sowie Wirtszellremodellierung wurde kein Effekt des Wachstums in verschiedenen Plasmen beobachtet.Wir können die Existenz einer Art jahreszeitspezifischen Sensors des Parasiten nicht endgültig widerlegen, es bleibt die Möglichkeit, dass unsere *in vitro* Konditionen nicht exakt die Situation *in vivo* wiederspiegelten, dennoch erscheint uns diese eher als unwahrscheinlich, da wir auch in asymptomatischen Infektionen in der Regenzeit ältere Parasiten in Zirkulation beobachteten. Dies weist darauf hin, dass der hier beschriebene Phänotyp der längeren Zirkulation der Parasiten möglicherweise nicht spezifisch für die Trockenzeit ist, sondern stattdessen allgemein mit niedrigen, subklinischen Parasitämien zusammenhängen könnte.

Schließlich fragten wir uns, wann in der vorangegangenen Regenzeit asymptomatische Infektionen der Trockenzeit übertragen wurden. Wir folgten retrospektiv Kindern, die am Ende der Trockenzeit eine Infektion aufwiesen, zurück bis zum Zeitpunkt ihres ersten positiven Tests in der Regenzeit. Wir erhoben zweiwöchentlich longitudinale Daten über Fluktuationen in der Parasitendichte und maßen die genetische Diversität durch *ama*1-Amplikonsequenzierung. Klone, die am Ende der Trockenzeit nachgewiesen wurden, waren vor allem gegen Ende der Regenzeit übertragen worden, während Klone, deren Übertragung früh in der Regenzeit stattfand, selten bis zum Ende der Trockenzeit persistierten. Diese Daten legen nahe, dass die Infektionslänge auf das Überbrücken der Trockenzeit zugeschnitten ist, und unterstützen ein Modell des Infektionsverlaufs, in dem unbehandelte, früh übertragene Infektionen einen „Trockenzeitphänotyp“ schon während der Regenzeit vorweisen (wie auch in asymptomatischen Infektionen in der Regenzeit beobachtet wird), während später übertragene Infektionen mit höherer Wahrscheinlichkeit das Ende der Trockenzeit erreichen und erneut übertragen werden können.

Die hier vorgestellten Ergebnisse, stellen einen Schritt zum besseren Verständnis der Mechanismen dar, die *P. falciparum* erlauben, die Zeit zwischen zwei Regenzeiten zu überbrücken. Das in dieser Arbeit gewonnene Wissen über die komplexe Wechselbeziehung zwischen *P. falciparum*, dem menschlichen Wirt und der Umwelt, kann Strategien informieren, die, indem sie auf die asymptomatischen Reservoire des Parasiten abzielen, zum Ziel der endgültigen Ausrottung von Malaria beitragen.

Abbreviations

*ama*1	apical membrane antigen 1
CD	Cluster of Differentiation
CI	Confidence Interval
CIDR	Cysteine-Rich Interdomain Regions
CRP	C Reactive Protein
CSA	Chondroitin Sulphate A
DBL	Duffy-binding-like
Dec	December
DEG	Differential Expressed Gene
DHA	Dihydroartemisinin
DNA	Deoxyribonucleic Acid
EBL	erythrocyte binding-like proteins
EEF	Exo-Erythrocytic Form
EPCR	Endothelial Protein C Receptor
GLUT1	Glucose Transporter 1
GWAS	Genome-Wide Association Studies
h	Hours
hpi	Hours post invasion
*hrp*2	histidine-rich protein 2
*hrp*3	histidine-rich protein 3
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IQR	Inter-Quartile Range
iRBC	infected Red Blood Cell
Jan	January
KEGG	Kyoto Encyclopedia of Genes and Genomes
LysoPC	Lysophosphatidylcholine
MAL	Malaria
Mar	March

MBC	Memory B Cells
MLE	Maximum Likelihood Estimation
msp	Merozoite Surface Protein
NK	Natural Killer
Oct	October
OR	Odds Ratio
pALD	*Plasmodium* aldolase
PBMC	Peripheral Blood Mononuclear Cell
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
Pf	*P. falciparum*
PfEMP1	*P. falciparum* Erythrocyte Membrane Protein 1
PfRhs	*P. falciparum* reticulocyte-binding protein homologs
pLDH	*Plasmodium* lactate dehydrogenase
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
RNA	Ribonucleic Acid
RPMI	Roswell Parks Memorial Institute
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
UPS	Upstream Promoter Sequence
varATS	*var* gene Acidic Terminal Sequence
vWF	von Willebrand Factor
WBCs	White Blood Cells
WHO	World Health Organization

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CHAPTER

INTRODUCTION 1

1.1 Malaria: an overview

Malaria is a mosquito-borne disease caused by Apicomplexa parasites of the genus *Plasmodium* and transmitted by female mosquitoes of the genus *Anopheles.* Five species of *Plasmodium* can cause the disease in humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi* and the sympatric *P. ovale* subspecies *P. ovale wallikeri* and *P. ovale curtisi* (Ashley, Pyae Phyo, & Woodrow, 2018; Cowman, Healer, Marapana, & Marsh, 2016). *P. falciparum* and *P. vivax* are the predominant species and *P. falciparum* is the most widespread in Africa and responsible for the majority of deaths.

Malaria is currently endemic in 86 countries. In 2019, the World Health Organization (WHO) estimated 229 million cases of malaria (95% confidence interval (CI), 211-252), 409 000 of which resulted in death (95% CI 387 000-460 000), and children under the age of five accounted for 67% of those. Five African countries alone reported 51% of all malaria cases and the African continent alone reported 94% of all deaths in 2019 (World Health Organization, 2020).

Malaria is closely associated with poverty, affecting the economy of endemic countries where it reinforces poverty, stalling economic growth; on the other hand, poverty may also promote malaria transmission (Sachs & Malaney, 2002). It is estimated that between years 2000 and 2015, malaria mortality in Africa decreased by 57%, corresponding to a decrease of 37% of all-cause mortality annual mortality from 1,007,000 deaths in 2000 to 631,000 deaths in 2015 (Gething et al., 2016). During the same period, *P. falciparum* prevalence in the African continent decreased by 40%, mainly due to the introduction of long-lasting insecticide-treated bed nets that is estimated to have averted 68% of malaria cases (Bhatt et al., 2015). Although malaria mortality declined, the last WHO malaria report showed that the decline has stalled in recent years (World Health Organization, 2020). An article examining of the economics of malaria in a

stylized model concludes that current funding is insufficient to successfully eliminate malaria (Maskin, Monga, Thuilliez, & Berthelemy, 2019).

1.2 *Plasmodium* (*falciparum*) life cycle

Human *Plasmodium spp.* infections start upon a blood meal by an infected female *Anopheles* mosquito, where the mosquito deposits sporozoites in the skin of the host. Through a mode of locomotion known as gliding motility, *Plasmodium* sporozoites – the mosquito salivary-gland and liver-infective stage – are deposited in the skin of the host. Through a mode of locomotion known as gliding motility, *Plasmodium* sporozoites reach a blood vessel and enter the bloodstream, reaching the liver within minutes (reviewed in Cowman et al., 2016 Lindner, Miller, & Kappe, 2012).

In the liver, sporozoites cross the liver sinusoids (fenestrated blood vessels lined with endothelium and Kupffer cells) and traverse several hepatocytes within transient vacuoles by a process known as cell traversal where they disrupt the hepatocytes plasma membrane without productive invasion (Mota et al., 2001; Risco-Castillo et al., 2015). Ultimately, sporozoites productively invade a hepatocyte through an invagination of the host cell plasma membrane forming the parasitophorous vacuole. During the entire liver stage development, the parasite will reside surrounded by the parasitophorous vacuole membrane (PVM) the main interface between the parasite and the host. This period lasts 7-10 days in humans, and during this time the parasite is called exo-erythrocytic form and differentiates and proliferates through schizogony, and undergoing rapid growth and DNA replication, where tens of thousands of merozoites are produced (reviewed in Lindner et al., 2012). The liver-stage of malaria infection is clinically silent, no symptoms are reported during this time. In two of the human malaria-causing species, *P. vivax* and *P. ovale,* cryptic liver-stage forms may occur – hypnozoites – that can stay dormant for months or years, before resuming development and causing disease relapses., constituting a major problem in malaria eradication. The liver-stage culminates with the release of merosomes – merozoite filled vesicles – into circulation that will burst in the microvasculature of the lungs releasing merozoites into the bloodstream (Baer, Klotz, Kappe, Schnieder, & Frevert, 2007).

In the blood, continuous asexual blood-stage replication cycles give rise to the periodic fevers typically associated with the disease and other malaria-associated symptoms may also appear. The cycle begins when merozoites invade erythrocytes, first attaching to their surface, then

entering and enclosing themselves inside. Invasion of the host erythrocyte is a fast process that takes ~2 minutes and involves pre-invasion, active invasion and echinocytosis (Weiss et al., 2015). Pre-invasion is the initial contact of the parasite with the host erythrocyte and involves a series of interactions between *P. falciparum* reticulocyte-binding protein homologs (PfRhs) and the erythrocyte binding-like proteins (EBL). Active invasion of the erythrocyte occurs by the parasite's actomyosin motor. The parasite is then enclosed in the erythrocyte, and echinocytosis occurs – abnormal form of the erythrocyte in which spiky protrusions are observed (reviewed in Cowman et al., 2016; Cowman, Tonkin, Tham, & Duraisingh, 2017). Inside the red blood cell (RBC), the parasite acquires a biconcave disc form known as ring stage. The parasite then rounds up and becomes a trophozoite, actively feeding, growing and remodelling the host erythrocyte. The next parasite form is the schizont, where the parasite nucleus will divide to form approximately 16-32 nuclei which will derive into new 16-32 merozoites (reviewed in Cowman et al., 2016). During its growth, *Plasmodium* parasites use host erythrocyte haemoglobin as a nutrient source. Heme that accumulates from hemoglobulin degradation is detoxified into hemozoin crystals in the parasite's digestive vacuole (Goldberg, Slater, Cerami, & Henderson, 1990). At the late schizont stage, merozoites become visible at the RBC periphery, each one containing a nucleus. Once the RBC breaks down and the merozoites are released into the bloodstream the cycle can start all over again. During the ~48 h replicative cycle in the host erythrocyte, *P. falciparum* follows a tightly regulated transcriptional pattern starting from the invading merozoite, through the ring- and trophozoite-stages, and to the multinucleated schizont (Bozdech, Llinas, et al., 2003). Close to 1% of parasites instead of continuing asexual development cycle, develop into gametocytes – the sexual blood stages of the parasite (Eichner et al., 2001). Sexual replication is epigenetically regulated by the transcription factor *ap2-g* (Kafsack et al., 2014). Maturation of *P. falciparum* gametocytes lasts 8-10 days and takes place in the bone marrow, and only mature gametocytes are seen in circulation (Joice et al., 2014). Sexual and asexual infected and non-infected erythrocytes are ingested by the mosquito upon a blood meal.

In the mosquito, the ingested blood will go to the mosquito midgut, where a drop in temperature, rise in pH, and the presence of xanthurenic acid will trigger parasite gametogenesis, where gametocytes transform into extracellular gametes (Billker et al., 1998). Male gametocytes undergo three mitotic replication rounds that produce eight male gametes (microgamete) in a process called exflagellation that was the first ever description of a malaria

parasite, in 1880 by Alphonse Laveran. The motile microgamete fertilizes the female gamete (macrogamete), forming a diploid zygote that initiates mitosis without nuclear or cellular division (Janse, Van der Klooster, Van der Kaay, Van der Ploeg, & Overdulve, 1986). The zygote will then transform into a motile form – the ookinete – that migrates and penetrates the midgut epithelium shifting from the lumen to the midgut basal lamina, where it transforms into a sessile spherical form – the oocyst. Inside the oocyst, mitotic replication begins and thousands of sporozoites are formed. These are then released into the mosquito haemolymph and through recognition of specific host receptors invade the mosquito salivary glands (reviewed in Frischknecht & Matuschewski, 2017). The life cycle can then be resumed in the human host during the mosquito's next blood meal.

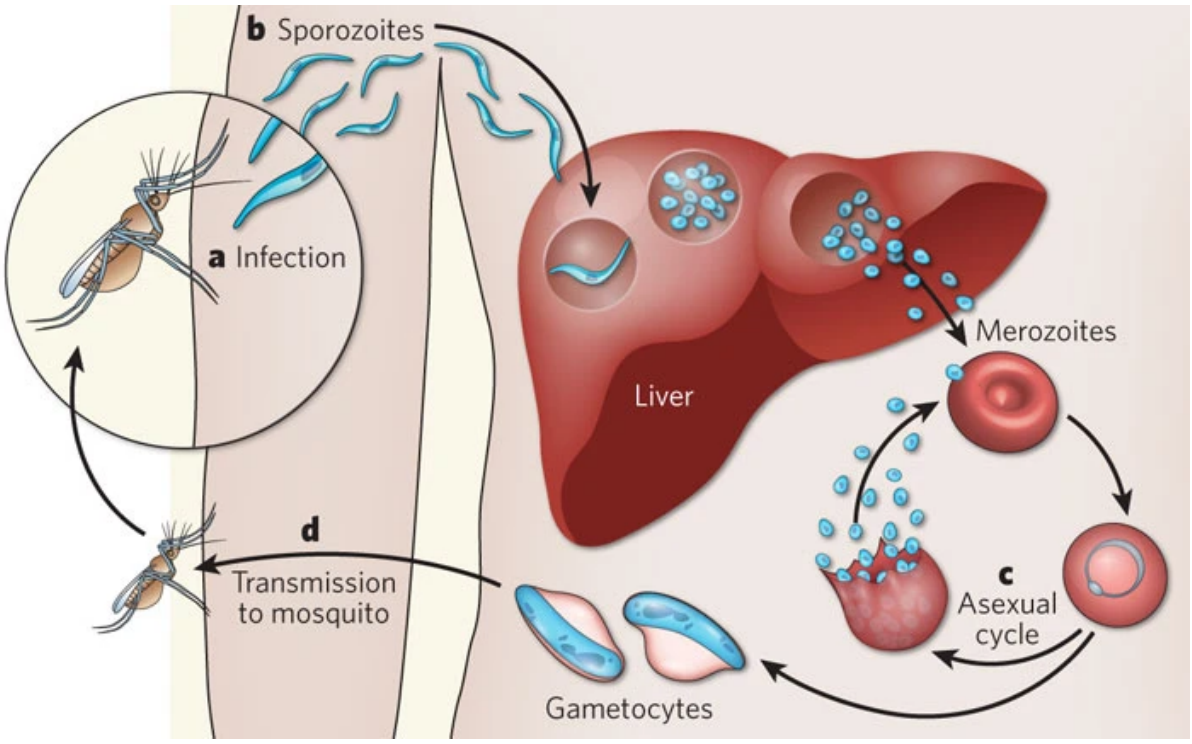


Figure 1 | *Plasmodium falciparum* life cycle

a, Parasite transmission occurs when an infected female *Anopheles* mosquito takes a blood meal on a susceptible host. The mosquito deposits sporozoites into the skin, which enter the blood stream eventually reaching the liver. **b,** In the liver, sporozoites traverse several hepatocytes until infecting one. The liver stage of infection ends with the release of merozoites that invade erythrocytes initiating the blood stage of infection. **c,** *P. falciparum* parasites will develop asexually for 48 hours and malaria associated symptoms might appear. **d,** *Plasmodium* differentiates into gametocytes, and the mosquito upon a blood meal ingests them. Fertilization of gametes will occur in the mosquito's midgut, and parasite development will continue. Upon the mosquito's next blood meal, sporozoites are deposited

into the host's skin and the cycle continues. Figure reproduced with authorization from Springer Nature as available in Michalakis & Renaud, 2009.

1.3 *P. falciparum*'s host cell remodelling and pathogenesis

During erythrocytic development, *P. falciparum* lives inside the parasitophorous vacuole and remodels its host cell extensively. Through a network of membrane structures (Marti, Good, Rug, Knuepfer, & Cowman, 2004), the parasite is able to export proteins, across the PVM and into the host erythrocyte. Maurer's clefts – closed cisternal compartments – are involved in the trafficking of proteins to the host erythrocyte (Lanzer, Wickert, Krohne, Vincensini, & Braun Breton, 2006). One of these proteins is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which localizes to the knobs – electron-dense protrusions present in the infected erythrocyte surface at around 20 hours post-invasion (Leech, Barnwell, Miller, & Howard, 1984; Oh et al., 2000; Udeinya, Schmidt, Aikawa, Miller, & Green, 1981). Adhesion is a hallmark of *P. falciparum* malaria that prevents clearance of infected erythrocytes in the spleen and is central to malaria pathogenesis.

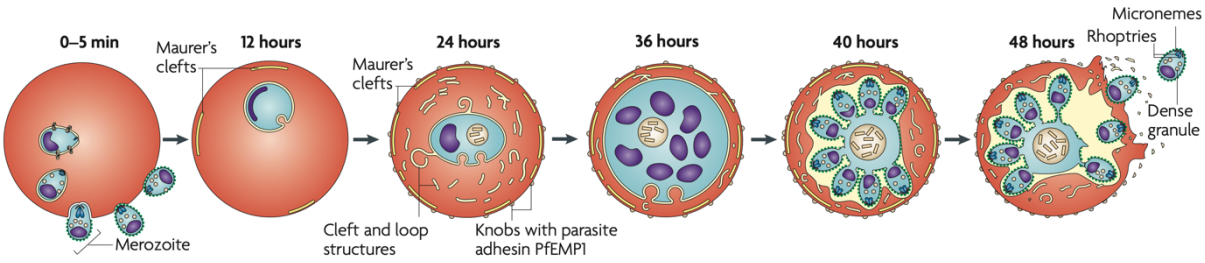
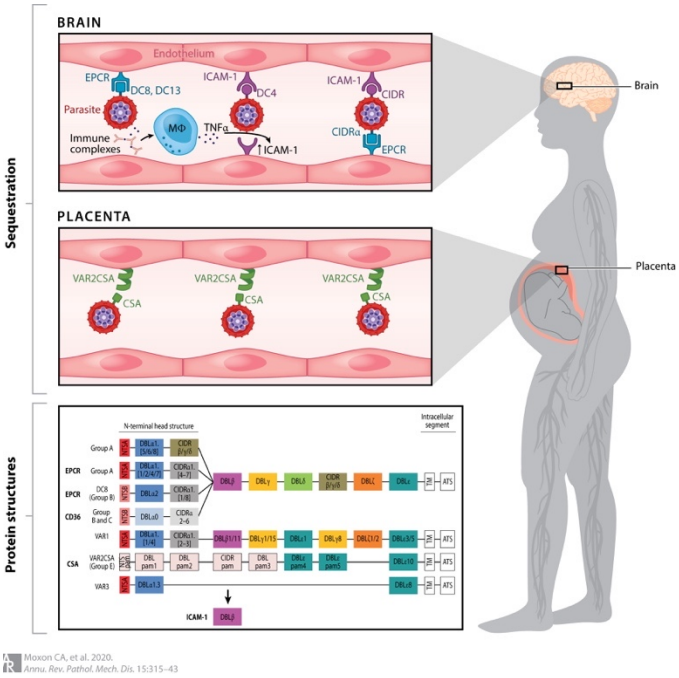


Figure 2 | Blood-stage development and host cell remodelling in *P. falciparum*

(from left to right) Blood stage infection starts with attachment and invasion of the red blood cell by *P. falciparum* merozoites. During blood-stage development, the parasite develops inside the parasitophorous vacuole. Development starts with ring stage (0-24 hours), continuous through the trophozoite stage (24-36 hours) and finalizes in the schizont stage (40-48 hours). At the final stage (48 hours), the red blood cell ruptures and daughter merozoite are released into circulation. PfEMP1, *P. falciparum* erythrocyte membrane protein 1. Figure reproduced with authorization from Springer Nature as available in Maier, Cooke, Cowman, & Tilley, 2009.

PfEMP1 is a transmembrane immunodominant protein that interacts with the receptors of host cells, mainly endothelial cells. It is encoded by ~60 different copies the multigene family *var* per genome. *Var* genes undergo monoallelic expression, such that exclusively only one gene is transcribed while all the others are silenced (Scherf et al., 1998). PfEMP1s can be divided into three major groups – A, B and C – based on their upstream promoter sequence (UPS) and chromosome location (J. D. Smith et al., 1995). Each PfEMP1 contains Duffy-binding-like (DBL) domains and cysteine-rich interdomain regions (CIDRs) in tandem in the extracellular sequence, and a shorter acidic terminal sequence in the cytoplasmic tail. DBL and CIDR domains bind to specific host receptors and this binding specificity associates with varying parasite virulence and disease outcomes. The thrombospondin receptor CD36 has been identified as a receptor for the most N-terminal DBL-CIDR domain cassettes (Baruch et al., 1997) and has been associated with uncomplicated malaria, whereas binding to ICAM-1 receptor has been associated with severe malaria (Ochola et al., 2011; J. D. Smith et al., 2000) mainly in infected erythrocytes that are able to bind both ICAM-1 and EPCR (Lennartz et al., 2017). Endothelial protein C receptor (EPCR) binds to DC8- and DC13-type PfEMP1 and has been associated with severe malaria (Lavstsen et al., 2012; Turner et al., 2013). In the placenta, VAR2CSA-expressing parasites bind to host receptor chondroitin sulphate A (CSA) and lead to placental malaria (Fried & Duffy, 1996). After one or more pregnancies, women are often found protected from placental malaria due to VAR2CSA-specific antibodies (reviewed in Hviid & Jensen, 2015).

In the spleen, infected erythrocytes that fail to adhere are removed from circulation. Remodelling of the infected erythrocyte membrane stiffens the red blood cell, making it less deformable and less likely to pass through the splenic sinusoidal filtration system (Paulitschke & Nash, 1993). Infected erythrocytes squeezed through the sinusoids of the spleen, may also release their parasite which is retained in the spleen and the free erythrocyte is released back into circulation in a process called splenic pitting (Angus, Chotivanich, Udomsangpetch, & White, 1997). Macrophages can also phagocyte infected erythrocytes in the spleen contributing to infected RBC clearance (Joice et al., 2016).



Moxon CA, et al. 2020. *Annu. Rev. Pathol. Mech. Dis.* 15:315–43

Figure 3 | Adhesion phenotypes of *P. falciparum* infected erythrocytes

Schematic representation of infected red blood cells in different organs and mediated by different receptor-ligand interactions. ATS, acidic terminal region; CIDR, cysteine-rich interdomain region; CSA, chondroitin sulfate A; DC, domain cassette; EPCR, endothelial protein C receptor; ICAM-1, intercellular adhesion molecule 1; Mϕ, macrophage; TM, transmembrane; TNF, tumor necrosis factor. Specific PfEMP1 structures scheme adapted from Storm et al., 2019. Figure reproduced with authorization from Annual Reviews as available in Moxon, Gibbins, McGuinness, Milner, & Marti, 2020.

1.4 Seasonal transmission of *P. falciparum*

During their lifecycle, mosquitos require water to lay their eggs after mating. In many areas of the globe, mainly tropical and subtropical regions, water availability is limited during a period known as the dry season. The duration of this period differs depending on the region (Figure 4), but during this time water and mosquitos are scarce. For long, it has been debated whether mosquitoes aestivate or migrate during this time (T. Lehmann et al., 2010), and although it still unclear what is the contribution of either possibility, a recent study has shown windborne migration of *Anopheles* mosquitos in the Sahel of Mali (Huestis et al., 2019). Concurrent with the absence of mosquitoes during the dry season, malaria transmission drops to virtually zero and virtually no malaria cases are observed during this time.

It has been consistently observed that during the dry season a portion of individuals remain infected without symptoms of clinical malaria disease (Babiker, Abdel-Muhsin, Ranford-

Cartwright, Satti, & Walliker, 1998; Obeng-Adjei et al., 2017; Ouedraogo et al., 2016, Portugal et al., 2017). In agreement with absence of transmission during the dry season, it has also been described that children that start the dry season uninfected are unlikely to become infected during the dry season. Moreover, the parasites in asymptomatic carriers at the end of the dry season are shown to be the same since the beginning in each individual (Hamad et al., 2000; Portugal et al., 2017). The parasites remaining in the host during the dry season are an important reservoir responsible for restarting transmission upon mosquitos' reappearance in the ensuing transmission season.

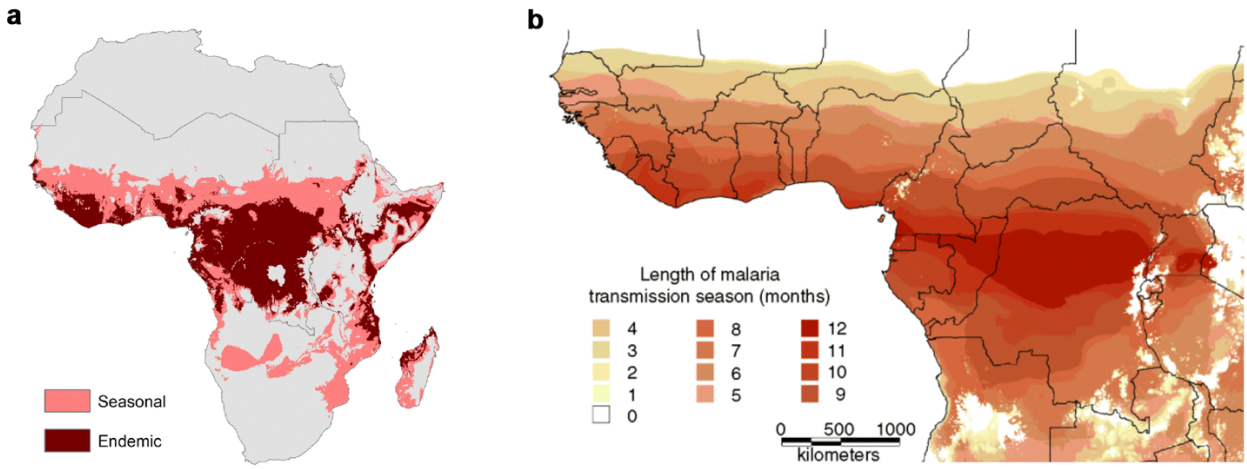


Figure 4 | Seasonal transmission of *P. falciparum* in Africa

a, Endemic (10-12 months) and seasonal transmission of *P. falciparum* in the African continent. Reproduced from Ryan, Lippi, & Zermoglio, 2020. **b,** Seasonal *P. falciparum* transmission represented with different lengths in West and Central Africa. Reproduced with authorization by John Wiley & Sons from Gemperli et al., 2006.

1.5 Asymptomatic *P. falciparum* infections

P. falciparum infection can cause debilitating disease and is responsible for high mortality. However, asymptomatic infections are common in endemic areas. Although a standard definition does not exist, an asymptomatic *P. falciparum* infection is usually defined as the presence of parasites in the host's peripheral blood and the absence of fever (axillary temperature < 37,5 °C) and of other malaria-associated symptoms (Laishram et al., 2012). In

fact, it is estimated that more than 75% of infections detected in community surveys are asymptomatic (B. M. Greenwood, 1987). However, longitudinal tracking of these infections is necessary to distinguish chronic asymptomatic infections from pre-symptomatic ones. Anecdotic reports from individuals that left malaria-endemic areas (thus eliminating the possibility of new infections) have shown that asymptomatic infections can last as long as 13 years, in blood-transfusion from an asymptomatic donor that left and endemic area 13 years prior, to an non-immune recipient or 9 years after leaving an endemic area in a splenectomised individual (Ashley & White, 2014).

Immunity is believed to be contribute to the establishment of asymptomatic infections, since these are more are commonly seen in high transmission endemic areas and occur mainly in older children and young adults. An early study with 3 non-immune individuals with sequential inoculations of *P. falciparum* has shown that although the first two inoculations were accompanied by a fast-growing parasitaemia and a clinical attack. The third inoculation, however, had a lower parasitaemia, no clinical attack and was maintained for the following 50 days. These results are in agreement that a certain level of acquired immunity necessary to establish an asymptomatic infection (Jeffery, 1966). For long, asymptomatic infections at the end of the dry season were thought to be responsible for partial protection of subsequent clinical malaria because it has consistently been shown that individuals carrying parasites in the end of the dry season have a decreased risk of developing clinical malaria in the ensuing transmission season (Crompton et al., 2008; Hamad et al., 2000; Males, Gaye, & Garcia, 2008; Portugal et al., 2017; Sonden et al., 2015). However, asymptomatic infections do not seem to *per se* protect against disease with a new *P. falciparum* infection (Buchwald, Sixpence, et al., 2019) and clearing parasites that remain asymptomatic during the dry season does not increase the subsequent risk of clinical malaria (Portugal et al., 2017). Immunity in *P. falciparum* asymptomatic infections is further detailed below.

Genetic factors such as immune system polymorphisms, hemoglobinopathies and other gene variants in erythrocytes have been associated with disease susceptibility in endemic areas by genome-wide association studies (GWAS) (Laishram et al., 2012; Taylor & Fairhurst, 2014).

Transmission to mosquitoes in asymptomatic infections is possible (J. T. Bousema et al., 2004). Studies from 1954 described that microscopy gametocyte-negative infections were still able to successfully infect *Anopheles* mosquitoes (Muirhead-Thomson, 1954a, 1954b). Sexual

conversion rates, measured by *ap2-g* expression, were higher in chronic infections than incident infections, suggesting that in an early infection, the parasite invests in asexual reproduction, as evidenced by the multiplication rate, whereas in a chronic infection, the investment shifts to sexual reproduction by gametocyte production. Moreover, gametocytes were more capable of mosquito transmission in asymptomatic infections, even if gametocytes were present in symptomatic infections (A. Barry et al., 2020). It is estimated that in low-transmission areas, asymptomatic sub-microscopy infections (below the limit of detection by microscopy) are responsible for 20-50% transmission to mosquitoes (Okell et al., 2012). A recent study genotyped *P. falciparum* in asymptomatic and symptomatic individuals and naturally fed mosquitoes in their households and estimated that asymptomatic infections were responsible for 94.6% of mosquito infections (Sumner et al., 2021). Different transmission intensities also have an impact in the association between asymptomatic infections and the risk of developing clinical malaria. In areas of high and moderate to high transmission, asymptomatic infections were associated with a reduced incidence of clinical malaria in children above 3 years, when compared to uninfected children. In areas of low transmission, however, asymptomatic children had a higher risk of developing clinical malaria than uninfected children, suggesting that transmission intensity contributes to acquisition of immunity to malaria disease (Wamae et al., 2019).

1.6 Detection of asymptomatic infections

Detection of asymptomatic infections is challenging since lower parasite densities fails to be detected by common diagnostic methods, such as microscopy and rapid diagnostic test (RDT).

Microscopy is the gold standard for the identification of a *Plasmodium* infection. It has a low cost and allows distinguishing infection by different parasite species. These involves the mechanical lysis of RBCs, and parasitaemia is measured counting the number of parasites against the number of white blood cells (WBCs), assuming a standard number of WBCs per microliter of blood (usually 8000 WBCs per µl). The sensitivity of thick blood smears is 50–500 parasites/µl. Besides the low sensitivity of blood smears, another downside is that it misses the diagnosis of parasites that might be sequestered at the time of blood draw (Moody, 2002).

Rapid diagnostic test (RDT) is an immunochromatographic assay that usually detects one of 3 abundant soluble antigens – histidine-rich protein 2 (HRP2), *Plasmodium* lactate

dehydrogenase (pLDH), or *Plasmodium* aldolase (pALD) – from a small amount of blood (5-20 μl) in a nitrocellulose strip that contains monoclonal antibodies directed against a specific target in a relatively short amount of time (5-20 min). RDTs are relatively cheap, fast and require little training to be performed and have a sensitivity of ~100 parasites/μl (Moody, 2002). HRP2 RDTs are the most commonly used, based on a higher sensitivity and heat-stability when compared with the other two. Based on their structural homology, RDTs for HRP2 also detected histidine-rich protein 3 (HRP3). HRP2 is a protein that is expressed during the erythrocytic development exclusively in *P. falciparum* and can remain in the human serum for weeks after parasite clearance, giving rise to false positives. Although RDTs detecting pLDH can usually aid to clarify a false positive result, gametocytes also produce pLDH and could also give a false-positive result if malaria treatment only cleared asexual stages from circulation (Bell, Wongsrichanalai, & Barnwell, 2006; Wongsrichanalai, Barcus, Muth, Sutamihardja, & Wernsdorfer, 2007). Recently, also false negatives have been reported based on parasites lacking the *hrp2* and/or *hrp3* genes. The deletion of these genes in *P. falciparum* leads to a negative result and a failure in diagnosing a *P. falciparum* infection. The failure to treat *hrp2/hrp3* negative parasites can potentially lead to the selection of these parasites and an increase of transmission of *P. falciparum*. This is in agreement with the recently observed high proportion of patients being treated for malaria with parasites lacking *hrp2* and *hrp3* (Alemayehu et al., 2021). Due to the implications these gene deletions have in public health and malaria elimination campaigns, WHO recommends that if an *hrp2/3* deletion is suspected in a symptomatic infection with a negative HRP2 RDT, pLDH RDT test or microscopy by two independent microscopists should be performed (World Health Organization, 2016) .

Polymerase chain reaction (PCR) is currently the most sensitive method for the detection of *Plasmodium* infections. It usually targets the human *Plasmodium* species 18S ribosomal RNA gene. Nested PCR was introduced in the early 90s and has a sensitivity of 0.5-1 parasites/μl (Snounou et al., 1993). Nowadays, quantitative PCR (qPCR) and nucleic acid sequence- based amplification (NASBA) are widely used in research settings for the detection of *Plasmodium* species. They have similar sensitivities of 0.02 parasites/μL (Schneider et al., 2005). It is an expensive method, more laborious and although not routinely used in the field, it is able to detect infections that other methods may have missed. An analysis of 106 surveys has found that microscopy detected, on average, only 54,1% of all PCR positive infections (Okell et al., 2012). Even though, a very sensitive method for the detection of a *P. falciparum* infection,

sequestration of parasites during their blood-stage development, removes these parasites from circulation, which can undermine successful parasite detection, giving rise to false negatives. Figure 5 summarizes the sensitivity of *P. falciparum* infection by microscopy, quantitative PCR (qPCR) and quantitative real-time PCR (qRT-PCR) in relation to parasite density and transmission intensity.

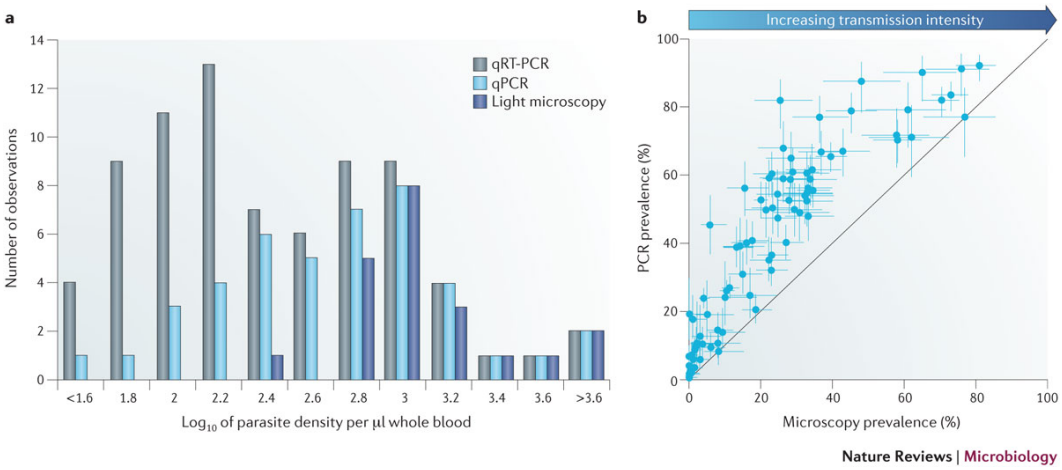


Figure 5 | Sensitivity of P. falciparum diagnostic methods in relation to parasite density and transmission intensity

a, Sensitivity of molecular detections methods such as quantitative real time PCR (qRT-PCR) and quantitative PCR (qPCR) and microscopy to detect *P. falciparum* infections. Methods were applied to the same blood samples of 315 asymptomatic individuals from Papua New Guinea. Detection by qRT-PCR is the most sensitive method, detecting infections at low parasite densities and the least sensitive method is light microscopy (Wampfler et al., 2013). **b**, Prevalence of *P. falciparum* infections detected by microscopy (x axis) compared with those determined by PCR (y axis). Data from 86 population surveys, with 95% confidence interval. Each data point represents data from one survey. Line represents expected correlation between both detection methods, should prevalence of infections be equally detected in both methods. Arrow represents malaria transmission, which is usually ranges from low (prevalence <10% by microscopy) to high (prevalence <75% by microscopy). Asymptomatic sub microscopic infections are more prevalent in areas of low transmission intensity (Okell et al., 2012). Figure reproduced with authorization from Springer Nature as available in T. Bousema, Okell, Felger, & Drakeley, 2014.

1.7 Genotyping *P. falciparum* infections

P. falciparum genome was sequenced in the beginning of the century and since then genomic data has rapidly evolved (Gardner et al., 2002). Although PCR can identify low parasitaemia infections, in order to understand parasite dynamics in the host, and between hosts, it is necessary genotype at the clonal level. Genotyping of *P. falciparum* to identify circulating clones has since aided in studying transmission, determine the success of intervention campaigns, vaccine candidates and drug treatment, and track natural selection of specific alleles at the population level (reviewed in Neafsey & Volkman, 2017). There are several methods to determine the genotype of infecting clones such as, microsatellites, genotyping of polymorphic genes, single nucleotide polymorphism (SNP) and amplicon sequencing. Vaccine candidates such as merozoite surface protein 1 and 2 (*msp*1 and *msp*2), glutamate-rich protein (*glurp*) and apical membrane antigen 1 (*ama*1) have been extensively studied and are highly polymorphic making these ideal targets for genotyping *P. falciparum* infections (Farnert, 2008). *msp*2 gene shows a higher degree of polymorphism than *msp*1 or *glurp* making *msp*2 the most sensitive to distinguish polyclonal infections (Snounou et al., 1999). Moreover, *P. falciparum* genome is haploid during the entire blood-stage since recombination only occurs in the mosquito vector, thus these genes are stable in the human host (Walliker et al., 1987).

Complexity of infection, defined by the number of parasite clones per host, is often higher in areas of intense transmission, due to the higher entomological inoculation rate and therefore more frequent recombination in the mosquito (Arnot, 1998). A recent study from a region of high transmission in Malawi has described the genetic diversity between genotypes in 15 malaria samples based on their transmission. Using single-cell sequencing, they characterized infection according to their genomes' relatedness: a monoinfection, an infection with only one parasite clone; a super infection, characterized by parasites transmitted by different mosquitos and a co-transmission infection, where different parasites are circulating in the host, but their relatedness suggests they were co-transmitted by the same mosquito, where recombination occurred giving rise to related parasite clones. This study showed that super infection is rare, having only occurred in 1 patient, whereas all other had monoinfections or co-transmission infections (Nkhoma et al., 2020).

Although genotyping is very powerful to detect asymptomatic infections, *P. falciparum* sequestration from peripheral circulation undermines complete detection of the full representation of circulating genotypes. A study of 25 adults with blood collected for 7

consecutive days and SNP genotyping has shown that a single blood sample is very unlikely to ever capture the full repertoire of parasite clones circulating in an individual. A single blood collection fails to detect all genotype diversity in an infection and should be considered as only snapshots in time (Nkhoma, Banda, Khoswe, Dzoole-Mwale, & Ward, 2018). A study performed in a high endemic area in Tanzania which genotyped *P. falciparum msp1* and *msp*2 genes in blood samples every 6 hours during 5 days in 1 asymptomatic child. The authors found that the circulating genotypes of samples within 6 hours apart could differ up to six genotypes, showing how fast this transition of circulation can occur (Farnert, Lebbad, Faraja, & Rooth, 2008).

1.8 Immunity to *P. falciparum*

To develop immunity to malaria years of exposure to *P. falciparum* are required in endemic areas. In fact, sterile immunity – protection from blood-stage parasitaemia – is unlikely or never achieved. However, intense exposure in endemic areas renders young children protected from severe malaria at an early age of ~5 years, having uncomplicated malaria through childhood and adolescence, and protection from clinical disease is often achieved at late adolescence (reviewed in Crompton et al., 2014). In agreement with acquired immunity to clinical malaria in parallel with exposure, repeated *P. falciparum* infections elicit protective variant-specific anti- PfEMP1s antibodies (Bull et al., 1998).

Several experimental malaria studies have described sterilizing immunity in animal models and humans (reviewed in Crompton et al., 2014). However, this sterilizing immunity was achieved when individuals were challenged with homologous parasites, which is unlikely to occur in an endemic area due to the broad genetic diversity of *P. falciparum* (A. E. Barry, Schultz, Buckee, & Reeder, 2009). In fact, a study showed that sterile acquired immunity is not achieved in endemic areas, since both children and adults that start the transmission season PCR negative, become PCR positive in similar number of days, having the same risk of becoming infected with *P. falciparum* (Tran et al., 2013). Moreover, studies have shown that children succumb to clinical malaria when infected with a new parasite clone (Babiker et al., 1998; Bull, Lowe, Kortok, & Marsh, 1999).

Humoral response has been shown to me a major player in malaria immunity for more than 60 years, since the transfer of purified IgG from malaria-immune adults to children with acute malaria led to the resolution of fever and a decrease in the number of asexual blood-stage parasites (Cohen, Mc, & Carrington, 1961). Studies with Malian children have shown that the

levels of antibodies to *P. falciparum* antigens are generated after each transmission season. These antibody levels peak during the transmission season, whereas decrease significantly during the dry season. The authors suggest that the short-lived immunoglobulin G (IgG), are antibodies mounted during the transmission season by short-lived plasma cells that disappear during the dry season of malaria. And that the only the antibodies specific for *P. falciparum* which are still present at the end of the dry season are generated by long-lived plasma cells. With cumulative exposure to *P. falciparum*, the levels of *P. falciparum* specific antibodies that remain in the dry season increase until adulthood, when acquired immunity is achieved (Crompton et al., 2010).

Humoral immunity in *P. falciparum* asymptomatic infections

In a low transmission area, protein microarray serological analyses of IgG and IgM found no significant differences in antibody breadth and magnitude between asymptomatic and symptomatic individuals (J. S. Lehmann et al., 2017). However, a different study in a high transmission area has shown, with the same method, significant differences between asymptomatic and uninfected individuals in breadth and magnitude of anti- *P. falciparum* antibodies, although the antibody levels decrease similarly during the dry season regardless of the infection status (Portugal et al., 2017). Previous studies have also shown that the risk of symptomatic malaria is inversely proportional to antibody breadth (Rono et al., 2013). However, it has recently been shown that treating parasitaemias in asymptomatic individuals at the end of the dry season does not increase the risk of developing malaria symptoms in the ensuing transmission season (Portugal et al., 2017), challenging the previous notion that silent infections decrease the risk of clinical malaria (T. Smith, Felger, Tanner, & Beck, 1999), but also suggesting that asymptomatic infection is a marker for *P. falciparum* exposure that leads to a higher antibody breadth and protection (Portugal et al., 2017).

A study with asymptomatic Kenyan children has shown that antibody levels against *P. falciparum* merozoite antigens (*ama*1, *msp*1 and *msp*3) were significantly higher when compared with uninfected children. However, the strength to which the antibody binds to its ligand (avidity) was negatively associated with asymptomatic parasitaemia for *msp*1, which might suggest that asymptomatic infections interfere with maturation of antibody avidity (Ibison et al., 2012). Also in Kenya, a different study has shown that asymptomatic infections

associate with higher level of IgG antibodies against *P. falciparum* variant surface antigens (VSAs) in children when compared to uninfected ones (Kinyanjui et al., 2004).

A study from a low transmission area in Peru links clinical immunity with antibody responses against *P. falciparum* invasion ligands. This study determined antibody responses against merozoite proteins that take part in attachment and invasion of red blood cells – Reticulocyte binding-like protein (PfRH) and erythrocyte binding-like protein (EBL) families – showing that IgG responses are higher in individuals with asymptomatic infection, when compared to uninfected individuals (Villasis et al., 2012).

Cellular immunity in *P. falciparum* asymptomatic infections

A study with Malian adult samples compared γδ T cells and Vδ2+and Vδ2- subsets between asymptomatic and clinical cases and observed no differences in their levels at the start of transmission season, and that this levels also did not discriminate between the risk of developing clinical malaria during follow-up. Moreover, although activation (measured by CD38 co-expression) of γδ T cells increased from the beginning until the end of the study (22 weeks later), it showed no differences between asymptomatic and symptomatic individuals (Diallo et al., 2019). In a study from the low transmission country Haiti, authors report differences between the immune response in PBMCs collected from asymptomatic and symptomatic individuals. Culturing PBMCs with *P. falciparum* infected erythrocytes, they saw an increase in CD8+ T cells of PBMCs from asymptomatic carriers' and in natural killer T (NKT) like cells. Regulatory T cells (Treg) (defined as $CD4^{+}CD25^{+}CD127^{low}$), responsible for suppression of immune response were also increased in asymptomatic individuals when stimulated with infected erythrocytes. Furthermore, authors also show that 11 cytokines and chemokines were upregulated in asymptomatic individuals, when compared to symptomatic ones, that have been previously associated with activation and persistence of cells from the monocyte lineage or markers of differentiation (J. S. Lehmann et al., 2017). However, the role of monocytes in an asymptomatic infection remains unclear.

Tregs were also assessed in a different study from Indonesia, an area with unstable malaria transmission. Tregs in asymptomatic infections there were divided as activated or resting according to their CD45RA expression. Treg cell numbers were lower in children with asymptomatic *P. falciparum* infection, compared with symptomatic and non-infected controls. In both asymptomatic children and adults , activated Tregs ($CD25^{high}$ $CD45RA^{-}$) proportion was

significantly smaller when compared to uninfected controls. The authors hypothesize that a reduction in proportion of activated Tregs associates in clinical immunity in asymptomatic individuals (Kho et al., 2015).

In children from Mali, anti-inflammatory cytokine IL-10 is upregulated after an episode of symptomatic malaria in children predominantly produced by $CD4^+CD25^+$Foxp3 T cells, and to a lower extent can remain upregulated in asymptomatic children during the 6-month dry season when compared to uninfected children (Portugal et al., 2014). A study in Indonesia assessed the levels of γδ T cells in asymptomatic children with *P. falciparum* and/or *P. vivax* infections. The authors observed that asymptomatic children had a higher proportion of γδ T cells when compared with uninfected children, and that levels of these cells decrease after 9 months of clearing *Plasmodium* infection since children that cleared infection more recently had higher γδ Tcell values. The marker of exhaustion PD-1 was also increased in γδ $CD4^+$ T cells and this increase remained after clearance of infection. T-bet^+ (Th1) and ROR $γt^+$(Th17) in $CD4^+$ T cells were also increased in asymptomatic children and showed a long-lasting presence, since these values did not decrease after clearance of infection. PBMCs from asymptomatic children cultured and stimulated for 96-hours *with P. falciparum* infected RBCs shown an increase in anti-inflammatory cytokine IL-10 production, and this increase remained after clearance. Expression of Tregs ($CD4^+CD25^+FOXp3^+$) was decreased in asymptomatic children, but TNF receptor 2 (TNF-RII) in these cells was increased suggesting that asymptomatic children have more Tregs with higher suppressive capacity as compared to uninfected children (de Jong et al., 2017). TNF-RII has also been associated with parasite elimination and studies from Indonesia that compare asymptomatic children with uninfected ones have showed that TNF-RII expression is increased in asymptomatic children (Wammes et al., 2013; de Jong et al., 2017).

Individuals exposed to malaria, develop a subset of memory B cells (MBCs) known as atypical MBCs. This B cell subset characterized by lack of CD27 and CD21 expression, is similar to exhausted MBCs observed in HIV patients and was first described in a longitudinal study in Mali at the end of the dry season. The authors have shown that this subset is increased in malaria exposed individuals when compared to naïve individuals, but also that asymptomatic infected children had an increase in atypical MBCs when compared to uninfected children. However, the percentage of atypical MBCs was not correlated with risk of clinical malaria in the ensuing transmission season (Weiss et al., 2009). It has also been shown, comparing PBMCs from Mali,

a high transmission area, and Peru, a low transmission area, that this MBC compartment increases with transmission intensity, being higher in Malians individuals compared to Peruvian (Weiss et al., 2011). A study from 2010 hypothesized that the memory B cell compartment responsible for producing long-lived plasma cells would expand with age, or that this expansion was caused by repeated antigen exposure. The authors showed, by comparing memory B cells frequency against two *P. falciparum* antigens versus Tetanus Toxoid antigens, that memory B cells specifics for *P. falciparum* antigens would expand with *P. falciparum* exposure, but would contract during the six-month dry season. Contrarily, the Tetanus-Toxoid memory B cell compartment suffered no changes. Continuous exposure to new infections is required to maintain the *P. falciparum* atypical MBCs (Weiss et al., 2010).

The majority of the studies mentioned here compared symptomatic children with asymptomatic, while few compared asymptomatic infections with uninfected individuals. Some of the studies that compared the immune status in asymptomatic infections with uninfected individuals, found that these differ in their immunophenotype. Although asymptomatic infections are defined by the absence clinical symptoms, they may not be completely undetected by the immune system. Immunity is also correlated with transmission and intensity of exposure to *P. falciparum*. However, during the dry season, transmission is interrupted and no studies, to our knowledge, have described the impact of asymptomatic infections in the host immune system.

1.9 *Plasmodium* ability to detect the environment

Plasmodium spp. have been shown to sense and respond to either a change between human and mosquito hosts, and also nutritional changes inside a single host. *Plasmodium* has no ability to synthetize many nutrients *de novo* and is auxotrophic for most (reviewed in Kumar, Skillman, & Duraisingh, 2020). *Plasmodium* depends on its host for acquisition of nutrients that assure its survival. It is therefore important that it is able to sense, adapt and respond to a changing environment in the host to assure survival and transmission.

In the mosquito, *Plasmodium* gametocytes undergo gametogenesis to start sexual reproduction. When transitioning from the human host to the mosquito, *Plasmodium* gametocytes sense the drop in temperature, an increase in pH and the presence of the mosquito-specific factor xanthurenic acid in the mosquito midgut, and respond to it by

differentiating into gametes that will then fertilize and continue the life-cycle (Billker et al., 1998). A recent study has also shown that sporozoite and ookinete mosquito stages can adapt their motility according to different substrates (Ripp et al., 2020).

In the liver, after sporozoite invasion, *Plasmodium* parasites transform into a round exo-erythrocytic form (EEF). *Plasmodium* parasites have been observed to develop into EEFs in vitro without the host hepatocytes, in both human and mouse -infecting *Plasmodium* species with an increase in temperature to 37 °C, the addition of serum (Kaiser, Camargo, & Kappe, 2003) as well as sodium bicarbonate (Hegge, Kudryashev, Barniol, & Frischknecht, 2010). Pumilio-2 (Puf2), member of the RNA binding protein family PUF is a key regulator in the transformation of sporozoites into EEFs. Sporozoites that are absent of Puf2, fail to detect the environment and initiate EEF development in the mosquito salivary glands (Gomes-Santos et al., 2011).

During liver stage infection, limiting amounts of glucose can hinder parasite development. To counteract low glucose levels and to promote an increase in glucose uptake, *P. berghei* translocates glucose transporter 1 (GLUT1) to the membrane of the infected hepatocyte (Meireles et al., 2017). Dietary supplementation with a combination of arginine, lysine and valine amino acids has shown to have an detrimental effect on the development of *P. berghei* parasites in the mouse liver (Meireles et al., 2020). Administration of a high fat diet in mice, impaired *P. berghei* liver infection by 90% mediated by oxidative stress, leading to a reduction in severe malaria (Zuzarte-Luis et al., 2017). Moreover, an ongoing blood-stage infection has been shown to impair a subsequent liver-stage infection by the host iron regulatory hormone hepcidin, by arresting parasites in the liver and progression to a new blood-stage infection (Portugal et al., 2011).

In the blood, *Plasmodium* parasites can sense and adapt to their environment. When in a host subjected to caloric restriction, *P. berghei* has been shown to reduce its replication, reducing the number of merozoites produced per schizont, leading to an overall increase in survival and protection from experimental cerebral malaria. In a host with limiting nutrient availability, this strategy increases the survival of both host and parasite. Moreover, *P. falciparum* grown in vitro supplemented with serum from a caloric restricted mouse, also shows a decreased number of merozoites per schizont. This phenotype could be reversed by the knockout of the putative serine/threonine kinase KIN (Mancio-Silva et al., 2017). *P. falciparum* can respond to the absence of isoleucine in culture by entering a dormant stage. This dormant stage can be

reversed upon re supplementation with isoleucine, provided it is done within 72 hours, before parasite survival is compromised (Babbitt et al., 2012). A recent study has shown histone acetyltransferase PfGCN5 is associated with genes important for maintenance of cellular homeostasis and to be upregulated in nutrient stress conditions (Rawat, Malhotra, Shintre, Pani, & Karmodiya, 2020). In response to a high parasite density in vitro, *P. falciparum* undergoes cell death. Addition of nutrients to culture may delay but not reverse cell death, meaning that this death is independent of the depletion of nutrients. Transcriptome of parasites grown at high parasite densities indicates stress and associate with mechanisms of epigenetic control (Chou et al., 2018). The sexual conversion of *P. falciparum* also has been shown to be induced in vitro in limiting concentrations of the host-derived lipid lysophosphatidylcholine (LysoPC), and high LysoPC concentrations repress sexual differentiation (Brancucci et al., 2017). Limiting levels of lysoPC in the human has also been associated with a higher sexual conversion in human *P. falciparum* infection (Usui et al., 2019). Drug induced stress by the addition of dihydroartemisinin (DHA) to trophozoite in vitro culture resulted in ~4-fold increase of sexual conversion in *P. falciparum* (Portugaliza et al., 2020).

Although only one study clearly reported *P. falciparum* ability to sense its environment (Mancio-Silva et al., 2017), *Plasmodium spp.* and *P. falciparum* in particular has been described to be able to detect and respond to host environmental conditions. Depending on successful transmission to the mosquito for sexual reproduction, evolutive strategies might play a role to ensure the parasite survival and persistence during the dry season in the human host.

1.10 Duration of *P. falciparum* infections

As previously mentioned, in situations where individuals leave an endemic area, asymptomatic *P. falciparum* infections have been reported to have lasted a maximum of 13 years (Ashley & White, 2014). This is, however, an extreme situation. In high endemic areas, it has been shown that during transmission season there is fast acquisition of new clones with replacement of the previous clones (Daubersies et al., 1996).

Knowledge of the duration of *P. falciparum* infections comes often from malariotherapy studies, when prior to the discovery of appropriate antibiotics to treat neurosyphilis, patients were infected with malaria parasites to induce fever that could kill *Treponema pallidum* and thus treat neurosyphilis. Although this experimental malaria studies occurred in non-immune

individuals in non-endemic areas, they provided important information about the duration of infections. An analysis on previous studies report that the patients were infected for an average of 211.6 days, where the minimum number of days a patient was infected with *P. falciparum* was 14 days, and the maximum was 417 days (Sama, Dietz, & Smith, 2006).

Studies from Sudan have shown by *msp*1, *msp*2 and *glurp* genotyping, that individuals may carry the same clone for an entire year (Babiker et al., 1998; Hamad et al., 2000). However, these infections occur in areas of very low transmission and were reported to be mainly monoclonal infections. There is an age dependence on the multiplicity of infection (MOI, the number of clones per person), where it peaks in children between 3-7 years of age (T. Smith, Beck, et al., 1999). A study in high transmission area in Malawi has reported that school-aged children had longer persistent asymptomatic infections when compared to children younger than 5 years of age. Compared to adults, school aged children had similar duration of asymptomatic infections. The number of new infections per person, per year was lower in adults when compared to school-aged and young children. The median duration of one genotype was estimated to be around 40 days (Buchwald, Sorkin, et al., 2019).

A recent study looked at the duration of *P. falciparum* infections in Uganda by *ama*1 amplicon sequencing based on the gender of the host and has observed that although both genders have similar force of infection (new blood-stage infection per period of time), females cleared asymptomatic infections naturally (without the use of antimalarials) much faster than males. Moreover, they also observed that new infections were cleared faster than infections that were in the host since the beginning of the study, and that monoclonal infections were cleared faster than polyclonal infections. Authors also report slower clearance by clone and infection event when infections have higher parasite densities. They estimated that infection persisted from 103 days to 447 days, by clone, and from 87 to 536 days by infection event (Briggs et al., 2020).

Duration of *P. falciparum* infections differ greatly between study sites, transmission intensities, age of study participants or genotyping protocols that can have different sensitivities. Moreover, during the dry season, transmission of *P. falciparum* is interrupted due to the absence of mosquitoes. *P. falciparum* genotypes are maintained in each person during this time, without the acquisition of new clones (Portugal et al., 2017).

1.11 Aim of the study

During the dry 6-month season in Mali, a fraction of the population remains infected with *P. falciparum* constituting a reservoir for resuming transmission in the following wet season. Throughout the dry season, malaria cases are very rare and anti- *P. falciparum* antibodies drop similarly in individual who carried or not *P. falciparum* (Portugal et al., 2017). During this time, *P. falciparum* faces the challenge of surviving in the human host without the possibility of transmission for several months. Increasing parasite burden to levels potentially causing disease during this time, could risk killing the host and losing the possibility of transmission. Increasing parasitaemias could, on the other hand, induce an effective immune response clearing *P. falciparum* from the host, and thus eliminating the possibility of transmission upon mosquitos' return in the ensuing transmission season.

The work presented here intends to clarify host and parasite factors that allow asymptomatic *P. falciparum* persistence during the dry season.

We questioned if the host immunity would be controlling asymptomatic infections during the dry season. To this end, we profiled and compared the immune response during the dry season in infected and non-infected age- and gender-matched individuals.

On the other hand, the absence of disease during this time could the product of a parasite mechanism that allows persistence below the immunological radar in the dry season, so we compared *P. falciparum* parasites persisting through dry season with parasites causing clinical-malaria in the ensuing transmission season in age- and gender-matched children. We characterized their genetic diversity and transcriptome, and measured their development and replication ability in vitro freshly after blood draw.

Furthermore, we searched for a parasite encoded mechanism detecting the season, which could benefit *P. falciparum*. We cultured *P. falciparum* collected at different times of the year with different season plasmas and measured parasite replication and host cell remodelling. And we investigated if the dry season silent parasitaemia phenotype could also be observed during transmission season.

Finally, in addition or alternatively to the sensing mechanism hypothesis, we determined length of *P. falciparum* clones persisting asymptomatically through the dry season searching for

patterns of when clones bridging two transmission seasons transmission would more likely be transmitted.

This study addressed 5 major questions:

1. Do *P. falciparum* infections during the dry season elicit an immune response?
2. Are parasites that survive the dry season different from parasites causing first clinical malaria episodes in the transmission season?
3. Can parasites sense a change in seasonal environment in vitro and respond by altering their replication, development and/or host cell remodelling?
4. How similar are asymptomatic infections in the dry season comparing to asymptomatic infections during transmission season?
5. When were the *P. falciparum* clones that survive until the end of the dry season transmitted in the preceding transmission season?

This study aimed to fill an important gap in the knowledge regarding how asymptomatic infections persist during the dry season, which may inform the design of public health strategies to control and eventually eliminate malaria at its hidden reservoirs.

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MATERIALS & METHODS 2

2.1 Materials

2.1.1 Equipment

Equipment	Brand/ Company
Aqualine waterbath	Lauda
Axio Lab.A1 Ligth microscope	Zeiss
Bio-Plex 200 System	BioRad
Bioanalyzer	Agilent
Biometra Thermocycler	Analytik Jena
Cytation3 Plate Reader	BioTek
CO2 Incubator Heracell 150i	Thermo Scientific™
CryoCube F570 Freezer	Eppendorf
Digital Microscope Camera Axiocam 305	Zeiss
DNA 1000 chips	Agilent
DNA sequencer 3730	Applied Biosystems
Exactive Plus Orbitrap	Thermo Scientific™
FACS Canto II	BD Biosciences
FACS Fortessa	BD Biosciences
FACS LSR II	BD Biosciences
Fluid aspiration system BVC control	VACUUBRAND GMBH
Heraeus™ Fresco™ 21 microcentrifuge	Thermo Scientific™
Heraeus™ Multifuge™ X3 Centrifuge	Thermo Scientific™
Herasafe KS 15 Safety cabinet	Thermo Scientific™
HiSeq 2500 Rapid flow cell	Illumina
LabChip GX Touch HT Nucleic Acid Analyzer	PerkinElmer GmbH
MiniSpin® centrifuge	Eppendorf

Pipetus® Pipetboy	Hirschmann
Prominence 20 UFLCXR System	Shimadzu
qTower³ Thermocycler	Analytik Jena
QuadroMACS™ separator	Miltenyi Biotec
RNA Pico chip	Agilent
SU1550 centrifuge	Sunlab
ThermoMixer® F1.5	Eppendorf
Titramax 100 Shaker	Heidolph Instruments GmbH & CO
TripleTOF 5600	SCIEX
UV2 Sterilizing PCR Workstation	UVP

2.1.2 Disposables

Disposables	Brand/Company
0.22 µm filters	Whatman
24-well plates, sterile	Greiner Bio-One
48-well plates, sterile	Greiner Bio-One
96-well PCR Plate	Analytik Jena
96-well plates for ELISA (Immunoplates, Maxisorp F)	Fischer Scientific
96-well plates, flat bottom with lid	Corning
96-well plates, round bottom	Greiner Bio-One
Aspiration pipettes, 2ml unplugged	Carl Roth
Cell strainers Easy Strainer, 70um	Greiner Bio-One
Cryotubes	Greiner Bio-One
DNA LoBind tubes	Eppendorf
EASYseal™ sealing film	Greiner Bio-One
Eppendorfs, 1.5ml	Sarstedt
FACS tubes,	Sarstedt
Falcon tubes, 15ml	Greiner Bio-One
Falcon tubes, 50ml	Sarstedt
Filter tips	Greiner Bio-One
Glass slides	A.Hartenstein

Harris cutting mat	Whatman
Harris Uni-Core 3mm punches	Whatman
Latex gloves	Semperguard
LS/LD collumns	Miltenyi Biotec
Malaria Ag P.f test of histidine-rich protein II	SD BIOLINE
MicroAmp Optical Adhesive Film	Thermo Scientific™
Needles	Becton Dickinson
Nunc™ EasYFlask™ Cell Culture Flasks	Thermo Scientific™
Optical sealing foil	Analytik Jena
Pipetting reservoir	Bio-Pure
Pipetting reservoir, sterile	VWR
Plasmodipur	EuroProxima
Plate sealers, adhesive	Sigma
Protein Saver 903 Filter paper	Whatman
Puradisc 13 syringe filter 1.2 µm	Whatman
Serological pipettes	Greiner Bio-One
Serological pipettes	Sarstedt
Steri Cups, 0.22µm	Millipore
Steri-Flip vacuum filtration system	Millipore
Syringes, Luer-Lock	Becton Dickinson
Tips	Sarstedt
Tips	Greiner Bio-One
TouchNTuff Nitril gloves	Ansell
Vacutainer CPT Tubes	Becton Dickinson
Vacutainer K3EDTA Tubes	Becton Dickinson

2.1.3 Chemicals and Reagents

Chemicals/ Reagents	Brand/ Company
10 x PBS	Carl Roth
10x PBS for molecular biology	Invitrogen™
96% Ethanol	Zentrallager Universiät Heidelberg
Agencourt RNAClean SPRI beads	Beckman Coulter

Albumax II	Gibco
Aqua Dead Cell Stain	Thermo Fisher Scientific
BSA	Carl Roth
Chelex 100 for molecular biologu	BioRad
D-sorbitol	Sigma-Aldrich
DMSO	Sigma-Aldrich
E64	Sigma-Aldrich
FBS, South American, 500ml	Gibco
Gentamycin, 20ml (50mg/ml)	Carl Roth
Giemsa	Merck
Glycerol	Honeywell
HEPES	Gibco
Hypoxanthine, 100x	CC Pro
Lymphoprep solution	Fresenius Kabi
Methanol	Sigma-Aldrich
MitoTracker Deep Red	Applied Biosystems
Nuclease-Free Water	Invitrogen
Penicillin-Streptomycin	GIbco
Pierce IgG Elution Buffe	Thermo Fisher Scientific
Pierce Protein G Plus Agarose beads	Thermo Fisher Scientific
Pierce Protein L Plus Agarose beads	Thermo Fisher Scientific
Poly-L-lysine	Merck Millipore
RAL 555 Kit	BioRepair GmbH
RPMI 1640 (+ L-Glutamine)	Gibco
RPMI 1640 w/25mM HEPES	Gibco
Sodium bicarbonate, 7.5%	Gibco
SYBR Green I	Invitrogen
TRIzol LS	Ambion
Trypan Blue Stain	Gibco
TURBO DNase	Invitrogen
Tween 20	AppliChem
TWEEN® 20 for molecular biology	Sigma-Aldrich

β-mercaptoethanol	Gibco

2.1.4 Kits

Kit	Brand/ Company
CRP Human ELISA Kit	Invitrogen
DNeasy Blood & Tissue Kit	Qiagen
Express qPCR SuperMix	Invitrogen
FastStart™ High Fidelity PCR-System	Roche
FoxP3 Staining Buffer Set	eBioscience
GoTaq DNA Polymerase	Promega
Hepcidin 25 ELISA Kit	DRG Diagnostics
Human vWF A2 Domain Sandwich ELISA	R&D Systems
Human/Mouse/Rat Ribo-Zero Magnetic Kit	Epicentre
KAPA Hyper Prep Kit	KAPA Biosystems
KAPA Quant Kit for Illumina Sequencing	KAPA Biosystems
Milliplex Human Cytokine/Chemokine Magnetic Bead Panel 41-Plex Kit	Merck
NEBNext MasterMix	NEB
Phusion Blood Direct PCR Kit	Thermo Fisher Scientific
Power SYBR Green PCR Master Mix	Applied Biosystems
QIAquick 96 PCR Purification Kit	Qiagen
Ready-SET-Go! ELISA Kit for human IgG	eBioscience
Ready-SET-Go! ELISA Kit for human IgM	eBioscience
SuperScript VILO cDNA Synthesis	Invitrogen

2.1.5 Antibodies

Antibody	Brand/Company
CD14-BV71 (clone M5E2)	BD Biosciences
CD14-PE (clone 61D3)	eBioscience
CD16 -BV605 (clone 3G8)	BioLegend
CD16-BV421(clone 3G8)	BD Biosciences
CD16-BV711 (clone 3G8)	BD Biosciences
CD19 -BV421 (clone HIB19)	BioLegend
CD19- PerCPcy5.5 (clone SJ25-C1)	eBioscience
CD19-PE Texas Red (clone HIB19)	BD Biosciences
CD21-APC (clone HB5)	eBioscience
CD21-BV421 (clone B-ly4)	BD Biosciences
CD21-FITC (clone BL13)	Beckman Coulter
CD21PE TexasRed (clone B-ly4)	BD Biosciences
CD25-PEcy7 (clone BC96)	eBioscience
CD27-AlexaFluor 700 (clone O323)	BioLegend
CD27-APCcy7 (clone O323)	BioLegend
CD27-BV421 (clone M-T271)	BioLegend
CD27-BV650 (clone O323)	BioLegend
CD27-FITC (clone M-T271)	BioLegend
CD27-PEcy7 (clone M-T271)	BD Biosciences
CD3-BV510 (clone OKT3)	BioLegend
CD3-BV711 (clone UCHT1)	BD Biosciences
CD3-PerCPcy5.5 (clone OKT3)	BioLegend
CD4-APC (clone RPA-T4)	BD Biosciences
CD4-FITC (clone OKT4)	eBioscience
CD4-PEcy5 (clone OKT4)	BioLegend
CD56- BV510 (clone HCD56)	BioLegend
CD56-BV605 (clone HCD56)	BioLegend
CD8 -APCcy7 (clone SK1)	BD Biosciences
CD8-PEcy7 (clone RPA-T8)	BD Biosciences
FCRL5-PE (clone 509f6)	BioLegend

Granzyme A-AF647 (clone CB9)	BioLegend
Granzyme B-PE Texas Red (clone GB11)	BD Biosciences
	Jackson
Human secondary F(ab′)2 Goat Anti-Human IgG	ImmunoResearch
IL-2-FITC (clone MQ1-17H12)	eBioscience
T-bet-PE Texas Red (clone O4-46)	BD Biosciences
TCRγδ-PE (clone B1.1)	eBioscience

2.1.6 Primers

Gene		Sequence
	Forward	5′ CCT GTT GTT GCC TTA AAC TTC 3′
18s rRNA (rPLU)	Reverse	5′ TTA AAA TTG TTG CAG TTA AAA CG 3′
	Forward	5′-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3′
18s rRNA (rFAL)	Reverse	5′-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3′
tRNA ligase	Forward	5′-TGAGTGATATGGATAATA TAAAGGAACAAA-3′
(PF14_ 0198)	Reverse	5′-GGATGATATTTCACAA ACGTATCTTTCT-3′
human GAPDH	Forward	5′-ACAACTTTGGTATCGTGGAAGG-3′
	Reverse	5′-GCCATCACGCCA CAGTTTC-3′
msp2	Forward	5′-ATGAAGGTAATTAAAACATTGTCTATTATA -3′
(outer domain)	Reverse	5′-CTTGTACACGGTACATTCTT-3′
IC/3D7 (msp2	Forward	5′-AGAAGATGCAGAAAGAAKCCTYCTACT-3′
allelic family)	Reverse	5′-GATGTAATCGGGGGATCAGTTTGTTC G-3′ VIC
FC27 (msp2	Forward	5′-AATACAAGAGGTGGGCRATGCTCCA-3′
allelic family)	Reverse	5′-TTTTATTTGGTGCATTGCCAGAACTTGAAC-3′ 6-FAM
	Forward	5' AAGAGATATATCATCCGATTATGAAATAGAAAT 3'
sir2	Reverse	5' AATCCATCTACATTTTGTGTTACTACTGATT 3'
	Probe	6-FAM 5' CCAAACTTTCTAAAGTTGATAAAGCTACATGACCATTGTT 3' BHQ
	Forward	5' TTTCCTTTGCTTATATCTTAATACGTTCAACT 3'
rex3	Reverse	5' CTTGTGAAGTTGTTGCTTCTATATGTGAT 3'
	Probe	6-FAM5' ACCACCTCATACGAAGGGAGCAGTTTC 3' BHQ
KARHP	Forward	5' GTGAGAACCATCGTGTGCACTT 3'

	Reverse	5' TGTGGACCTGCCGCTATAGATT 3'
	Probe	6-FAM 5' TGTTCCAGCAGATGCACCAAATGGCT 3' BHQ
PHISTc GEX20 (PFB0900c)	Forward	5' TTTGGTTATGGTTATACGAACAAGG 3'
	Reverse	5' TTTTCTACATTATTCAAGATTTTCTTTCCT 3'
	Probe	6-FAM 5' ATCCTCATACAGATTAATTTCAAGTTGAATCAAAACGT 3' BHQ
STARP antigen (PF07_0006)	Forward	5' CCGTGGTTGTTGTAATATTATTATTGTCT 3'
	Reverse	5' TAAATACAAAACCCATTGATAATAATAACACA 3'
	Probe	6-FAM 5' TGTCTGTGTTCTTATTATCTGTTTCCTTTGTGCCTG 3' BHQ
UBE4B (PF08_0020)	Forward	5' TATGCCTGTAAATACGACCGATTC 3'
	Reverse	5' TTGCATTATTATTATTAGAGTTGGTGGTTG 3'
	Probe	6-FAM 5' CCTCTTGTGGAACATCATTATTACCATTGCTGT 3' BHQ
MIF	Forward	5' GGTAGCAACGAAGCTTATTGTTTTGTA 3'
	Reverse	5' CAAGTTGCTTACAAGGAGTTTCGTTAT 3'
	Probe	6-FAM 5' ATCAGCAAGAGCAGAATTATTTGACCTATTAATTCCTCCA 3' BHQ
GlyRS	Forward	5' TGAGTGATATGGATAATATAAAGGAACAAA 3'
	Reverse	5' GGATGATATTTCACAAACGTATCTTTCT 3'
	Probe	CalFluorGold540 5' TAAGCTCTTCCAAACTACCACATCCATCATTCTCT 3' BHQ
varATS	Forward	5'- CCCATACACAACCAAYTGGA-3'
	Reverse	5'-TTCGCACATATCTCTATGTCTATCT-3'
	Probe	6-FAM-TRTTCCATAAATGGT-NFQ-MGB
AMA1	Forward	5'-GCTGAAGTAGCTGGAACTCAA-3'
	Reverse	5'-TTTCCTGCATGTCTTGAACA-3'
AMA1 + adapter	Forward	5'- acactctttccctacacgacgctcttccgatct-CCATCAGGGAAATGTCCAGT-3'
	Reverse	5'-gtgactggagttcagacgtgtgctcttccgatct-TTTCCTGCATGTCTTGAACA-3'

2.1.7 Software

Software	Company /Developer
2x2 contigency table	Richard Lowry http://vassarstats.net/odds2x2.html
7900HT Fast Real-Time PCR	Applied Biosystems
Beacon Designer software	Premier Biosoft
EM-Menu	TVIPS GmbH

EndNote X9	Thomson Reuters
FACS Diva	BD Biosciences
FlowJo	Tree Star
GeneMapper	ThermoFisher
GraphPad	Prism
Illustrator	Adobe
ImageJ	NIH
Perseus	Max Planck Institute of Biochemistry
Photoshop	Adobe
Rstudio	
SmartSEM	Zeiss
Zen2 core v2.4	Zeiss

2.2 Methods

2.2.1 Study individuals and ethical approval

Samples and clinical data were acquired from 2011 to 2019 in a cohort study with ~600 individuals conducted in the village of Kalifabougou, Mali. The study is registered at ClinicalTrials.gov (identifier NCT01322581), was approved by the Ethics Committee of Heidelberg University Hospital; the Faculty of Medicine, Pharmacy and Odontostomatology at the University of Bamako; and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health Institutional Review Board. Written informed consent was obtained from all study participants or their parents/guardians when underaged. Exclusion criteria at enrolment were: haemoglobin <7 g dl−1, axillary temperature ≥37.5 °C, acute systemic illness or use of antimalarial or immunosuppressive medications in the 30 days preceding enrolment. Clinical malaria cases were defined by axillary temperature ≥37.5 °C, ≥2,500 asexual parasites per µl of blood and no other apparent cause of fever. Clinical malaria cases were treated with a 3-day course of artemether and lumefantrine. Study participants included individuals aged 3 months to 45 years. Cross-sectional time-points were performed at the beginning (January), middle (March) and end (May) of the dry season and at peak of transmission season (October).

2.2.2 Sample collection

Dried blood spots on filter paper (Protein Saver 903, Whatman), thick blood smear and venous blood (4 or 8 ml depending on whether donor age was below or above 4 years) were collected on dry season cross-sectional visits and at their first malaria episode of the transmission season. Blood samples were drawn by venipuncture and collected in sodium citrate-containing cell preparation tubes (Vacutainer CPT Tubes, BD). PBMCs, plasma and RBC pellets were separated by centrifugation and used freshly or stored at -80 °C within the next 3 h. In May 2012, an additional 2 ml of venous blood from RDT+ individuals was collected into EDTA tubes (Vacutainer K3EDTA Tubes, BD) and processed directly at the field site. Plasma (used for metabolomic analysis) was separated by centrifugation and immediately frozen in liquid N_2. The buffy coat was discarded, and leucocytes were removed from the RBC pellet, first by density gradient on Lymphoprep solution (Fresenius Kabi), and then by Plasmodipur (EuroProxima) filtration, all according to the manufacturer instructions and as previously described Auburn et al., 2011. RBC pellets were then frozen in liquid N_2 and were later used for the RNA-seq and RT–qPCR validation.

2.2.3 *P. falciparum* detection

P. falciparum parasites were detected by light microscopy of Giemsa-stained thick blood smears, counted against 300 leukocytes of patients presenting with clinical symptoms of malaria. Leukocytes have an average count of 7,500 cells per µl. Each smear was evaluated independently by two expert microscopists, and a third resolved discrepancies. *P. falciparum* infections during the dry season at cross-sectional time-points were detected by Rapid Diagnostic Test (SD BIOLINE Malaria Ag P.f test of histidine-rich protein II) with a sensitivity of ~100 parasites per µl (Ratsimbasoa et al., 2008). Retrospectively, nested PCR was performed in all samples collected during the cross-sectional time-points from filter papers (2017) or frozen RBC pellets (2018). 18S ribosomal RNA gene (Snounou et al., 1993) of *P. falciparum* was amplified following a previously described protocol (Tran et al., 2013). Briefly, DNA extraction or direct PCR was performed from blood samples from 2 mm circular punch of a dried blood spot or 2 µl of frozen RBC pellet, respectively and was performed with Phusion Blood Direct PCR Kit (Thermo Fisher Scientific) and the following cycling conditions 98 °C, 5 minutes, 30 cycles of amplification (98 °C, 1 second, 61 °C, 5 seconds, and 72 °C, 30 seconds) and 72 °C, 1 minute. A second nested PCR was performed with 1 µl of PCR product from the first PCR, with

the following cycling conditions: 95 °C, 2 minutes, 30 cycles of amplification (95 °C, 1 minute, 58 °C, 1 minute, 72 °C, 1 minute) and 72 °C, 5 minutes. The sensitivity of the nested PCR is around 0.5– 1 parasite per µl (Tran et al., 2013).

2.2.4 *P. falciparum* quantification

By flow cytometry, RBC pellets were washed and stained with 5× SYBR Green I (Invitrogen) and 7.5 µM MitoTracker (Applied Biosystems) for 30 min at 37 °C. Cells were acquired using an LSR II and analyzed using FlowJo software 10.2 or higher versions (both BD). By RT–qPCR, RBC pellets were thawed, and RNA was extracted immediately TRIzol LS (Ambion) according to manufacturer instructions, and complementary DNA (cDNA) was synthesized using SuperScript IV VILO Master Mix with ezDNase (Invitrogen). RT–qPCR was run on qTower^3(Analytik Jena) using Power SYBR Green PCR Master Mix (Applied Biosystems) with primers for the *P. falciparum* reference gene glycine-tRNA ligase and human GAPDH.

For *ama*1 amplicon sequencing, DNA was extracted from 4 x 3mm punches of dried blood spots (DBS) following the Chelex-Tween previously described protocol (Teyssier et al., 2019). Briefly, DBS were incubated overnight with 0.5% Tween in nuclease-free water overnight at 4 °C, washed for 30min at 4 °C in 1xPBS and extracted by incubating at 95 °C for 10 min with 10% Chelex-100 (BioRad) in nuclease-free water. DNA was stored at -20 °C until further use.

After DNA extraction, qRT-PCR was performed with TaqMan™ Multiplex Master Mix (Applied Biosystems), varATS primers and varATS TaqMan probe and 5 µl of genomic DNA, following a previously described protocol (Hofmann et al., 2015). The PCR data was acquired with qTower3 (Analytik Jena) with the following cycling conditions: 2 min at 50 °C, 10 min at 95 °C, 15 sec at 95 °C and 1 min at 55 °C for 45 cycles.

To allow for determination of parasite density, a standard curve was performed. PfMali2k1 parasites were synchronized with 2 sorbitol synchronizations and ring-stage parasitaemia was determined by Giemsa-stained thin blood smears. Parasite density was calculated based on the RBC count in a Neubauer-chamber. Serial dilution from 10^6 to 10^{-2} parasites per µl was performed by diluting the blood in uninfected RBCs. The blood was then diluted to a 50% haematocrit in 1x PBS, blotted on a filter paper and DNA was extracted as previously described.

2.2.5 Serologic analyses

CRP, vWF and hepcidin in human plasmas were detected through the CRP Human ELISA Kit (Invitrogen), Human vWF A2 Domain Sandwich ELISA (R&D Systems) and Hepcidin 25 ELISA Kit (DRG Diagnostics), respectively, all according to manufacturer instructions. Results were measured using the Cytation3 plate reader (BioTek). To measure circulating analytes in human plasma we used Milliplex Human Cytokine/Chemokine Magnetic Bead Panel 41-Plex Kit (Merck) according to manufacturer instructions and results were read on a Bio-Plex 200 System (Bio- Rad). Antibody reactivity to PfEMP1 domains was measured using PfEMP1 domains covalently coupled beads following the method previously described by Cham et al., 2010. Briefly, PfEMP1 coupled beads were incubated for 30 min with diluted plasma samples, followed by incubation with F(ab')2 Goat Anti-Human IgG (Jackson ImmunoResearch) human secondary detection antibody for 30 min. Results were read on a Bio-Plex 200 System. The threshold for reactivity of each PfEMP1 domain was calculated with the average value of 21 German controls plus three standard deviations. Reactivity to PfEMP1 A, B and B/A subtypes was determined by the fluorescence intensity of reactive individuals in the beginning (January) and end (May) of the dry season of subclinical carriers and non-infected individuals. Slopes of subclinical carriers and non-infected individuals were compared using a linear non-interaction model.

2.2.6 Processing of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from the Vacutainer CPT Tubes and separated by centrifugation. If not used immediately, PBMCs were frozen within 3h of the blood collection in FBS (Gibco) containing 7.5% DMSO (Sigma-Aldrich). PBMCs were first frozen at -80 °C for 24 h and then transferred to liquid N2 for long-term storage. For experiments where frozen PBMCs were used, cells were quickly thawed in a 37 C water bath, washed with 10% FBS in PBS and incubated with complete RPMI supplemented with 10% FBS, 1% penicillin-streptomycin and 0.5% β-mercaptoethanol. In all experiments, individuals from both groups analysed were thawed and assayed simultaneously. Trypan blue exclusion dye was used to determine viability of PBMCs after thawing. Immediately after thawing, or washing in the case of fresh PBMCs, $1–2 \times 10^6$ PBMCs were incubated for 30 min at 4 °C with different combinations of the following fluorescently labelled surface antibodies diluted 1:25 and stained in 50 µl of 4% FBS in PBS: CD14-PE/BV71, CD16-BV421/ BV605/BV711, CD19-PETexasRed/

BV421/PerCPcy5.5, CD21-FITC/PE Texas Red/BV421/APC, CD27- FITC/PEcy7/AlexaFluor 700/BV421/BV650/APCcy7, CD3-BV510/BV711/PerCPcy5.5, CD4-FITC/PEcy5/APC, CD8-PEcy7/APCcy7, TCRγδ-PE and CD56-BV510/BV605 (see 2.1.5 Antibodies). After fixing and permeabilizing the cells with FoxP3 Staining Buffer Set (eBioscience) according to manufacturer instructions, PBMCs were stained for 30 min with 1:25 diluted fluorescently labelled intracellular antibodies: CD25-PEcy7, T-bet-PE Texas Red, IL-2-FITC, Granzyme B-PE Texas Red, FCRL5-PE and Granzyme A-AF647. Data were acquired using BD LSR II or LSR Fortessa flow cytometers (BD) and analysed using FlowJo 10.2 or higher versions. Gating strategy is described in Supplementary figure 1.

2.2.7 Flow cytometry with *P. falciparum*–specific B cell probes

Immediately after thawing, PBMCs were stained with biotinylated recombinant *Pf*MSP1 and *Pf*AMA1 coupled to fluorescently labelled streptavidin (Hopp et al., 2020), simultaneously with the following labelled monoclonal antibodies: CD3-BV510 (clone UCHT1), CD4-BV510 (clone SK3), CD8-BV510 (RPA-T8), CD14-BV510 (clone M5E2), CD16-BV510 (clone 3G8), CD56-BV510 (clone HCD56), CD10-BUV737 (clone Hi10a), IgD-BUV737 (clone IA-2), CD19-ECD (clone J3-119), CD21-PE-Cy7 (clone B-ly4), CD27-BV605 (clone M-T271), IgM-PerCP-Cy5.5 (clone G20-127) and IgG-AlexaFluor700 (clone G18-145) (see 2.1.5 Antibodies). Aqua Dead Cell Stain was added for live/ dead discrimination (Thermo Fisher Scientific). Data was acquired on LSR Fortessa X20 (BD), and analysed using FlowJo 10.2 or higher versions. *Pf*MSP1- or *Pf*AMA1-specific MBCs were identified after exclusion of $CD3^+CD4^+CD8^+CD14^+CD16^+CD56^+$ non-B cells and $CD10^+$ immature and IgD^- B cells. To increase the frequency of specific B cells detected in any given sample, PBMCs were stained simultaneously with *Pf*AMA1 and *Pf*MSP1 probes. Therefore, *Pf*AMA1 and *Pf*MSP1 probe-binding cells are indistinguishable by flow cytometry analysis and are referred to together as 'Pf-specific' cells. Influenza hemagglutinin (HA) antigen was used as a non-Pf-specific cells control. A complete gating strategy is available in Supplementary figure 2.

2.2.7 *P. falciparum* culture

P. falciparum parasites were maintained in fresh human O^{Rh+} erythrocytes at 5% haematocrit in RPMI 1640 (Gibco) complete medium (with L-glutamine and HEPES), 7.4% sodium

bicarbonate (Gibco), 100 µM hypoxanthine (C.C.Pro) and 25 mg ml^{-1} gentamycin (Gibco)) added with 0.25% Albumax II (Gibco), at 37 °C either in the presence of a gas mixture containing 5% O_2, 5% CO_2 and 90% N_2 or using the candle jar system method described by Trager & Jensen, 1976.

Infected red blood cell pellets were frozen at ring-stage in Sorbitol-glycerolite solution – 28% Glycerol (Honeywell), 3% Sorbitol (Applichem) and 0.65% NaCl (Sigma-Aldrich). Briefly, after centrifugation of RBCs, for 250 ul of pellet, 300 ul of heat-inactivated plasma was added, followed by dropwise addition of 500 ul of Sorbitol-Glycerolyte solution. Cells were first frozen at -80 °C for 24 h and then transferred to liquid N_2 for long-term storage.

Frozen infected RBC pellets were rapidly thawed at 37 °C, and 0.1x 12% NaCl solution was added dropwise. After 5 min incubation, 10x 1.6% NaCl solution was added dropwise. After centrifugation, RBC pellet was washed with 10x complete RPMI. After washing, *P. falciparum* parasites were put in culture with fresh human O^{Rh+} erythrocytes at 5% haematocrit in RPMI 1640 complete medium, supplemented with 0.25% Albumax II as previously described.

P. falciparum parasites at a ring-stage estimated by Giemsa-stained thin smears, were synchronized with sorbitol by the previously described protocol Radfar et al., 2009. Briefly, ring-stage parasite culture was washed, and incubated with 5% sorbitol for 8 min at 37 °C, followed by two more washes with complete medium, and put in culture with fresh O^{Rh+} erythrocytes at 5% haematocrit in complete RPMI 1640 medium as previously described. Successful synchronization was assessed by Giemsa-stained thin smears.

2.2.8 *P. falciparum* invasion assay

3D7 *P. falciparum* parasites were cultured and regularly synchronized by the use of 5% sorbitol Radfar et al., 2009 and heparin to prevent re-invasion. At the early schizont stage, E64 compound (Sigma-Aldrich) was used to prevent merozoite egress. 5-8 h later, segmented schizonts were assessed by Giemsa-stained thin smears. Merozoites were then purified through filtration as previously described Boyle et al., 2010. Merozoites were put in culture in the presence of non-infected RBCs and RPMI 1640 medium supplemented with 25% human plasma, complete or antibody-depleted, from different donors. After allowing invasion to occur for 30 min, medium was removed, and fresh complete RPMI 1640 supplemented with Albumax

was added to the culture. After 30 h in culture, iRBCs were fixed as previously described Tonkin et al., 2004 and stained with 5% SYBR Green for 30 min. Data was acquired on FACS Canto II (BD) and analysed using FlowJo software 10.2 or higher versions.

2.2.9 Antibody depletion from human plasma

In order to deplete antibodies from human plasmas, we used Pierce Protein G and Protein L Plus Agarose beads (Thermo Fisher Scientific). Briefly, plasmas were incubated with the same volume of both Protein G and Protein L plus agarose beads for 30 min. After, plasma was collected, and the antibodies were eluted from the beads using Elution Buffer (Thermo Fisher Scientific). Plasma was incubated with beads for an additional two times to achieve successful depletion. Depletion efficiency was measured by quantifying the levels of IgG and IgM in the plasma before and after depletion with Ready-SET-Go! ELISA Kit (eBioscience). Results were acquired on Cytation3 (BioTek) plate reader.

2.2.10 *msp*2 fragment analysis by capillary electrophoresis

DNA was extracted from two 3-mm circular punches of blood spots on filter papers (Whatman 3MM), using the DNeasy Blood & Tissue Kit (Qiagen). Genotyping of *msp2* was performed using a previously described nested PCR reaction (Liljander et al., 2009). The first PCR amplified the outer *msp2* domain with the following cycling conditions: 95°C for 5 min, 58°C for 1 min, extension at 72°C for 1 min followed by 94°C for 30 sec, for 23 cycles, 58°C for 1 min and 5 min at 72°C. The second PCR used fluorescently labelled primers to identify two *msp2* allelic families: IC/3D7 and FC27 with the following cycling conditions: The cycle conditions for the primary PCR: 5 min at 95°C, 1 min at 61°C, 1 min at 72°C, 1 min at 94°C, steps 2-4 for 22 cycles, 30 sec at 61°C and 5 min at 72°C. 1 µl of nested PCR product was added per well with 9 µl of Hi-Di formamide (Applied Biosystems) and 0.5 µl size standard (GSTM-LIZ® 1200, Applied Biosystems). The size standard contains 73 single- stranded DNA fragments ranging in size from 20 bp to 1200 bp. Fragment analysis was performed by capillary electrophoresis using a 3730 DNA sequencer (Applied Biosystems) and analysed using GeneMapper 5 software (Thermo Fisher Scientific), where a cut off of 300 relative fluorescent units was set. Fragments with a

size difference less than 3 base pairs were considered to be the same allele within each allelic type.

2.2.11 Transcriptome analysis

Total RNA was isolated from frozen RBC pellets using TRIzol as reported previously (Bozdech, Llinas, et al., 2003). RBC samples were frozen in liquid N_2 immediately after a blow draw. RNA preparation quality was measured using a Bioanalyzer (Agilent) obtaining an average RNA integrity number value of 5.4 and 5.3 for dry season and clinical malaria cases samples respectively. Average RNA yields tested with the same method were 27.4 ng and 25.0 ng for dry season and clinical malaria samples respectively. To perform next-generation sequencing (NGS), twenty-four samples were first selected based on parasitemia and the highest 12 titers for the wet and dry seasons. The total RNA input used in NGS sample preparation ranged from 100 pg up to 100 ng. Samples were prepared for transcriptome analyses essentially as described previously (Broadbent et al., 2015) with the following modifications: to adapt the protocol to a low-input ribosomal RNA depletion, Agencourt RNAClean SPRI beads (Beckman Coulter) were resuspended in 19 µl after DNase treatment. To deplete ribosomal RNA (rRNA), the Human/Mouse/Rat Ribo-Zero Magnetic Kit (Epicentre) was used following the Clontech modified protocol for removal of rRNA from small amounts of total RNA (100 ng). Fragmentation and cDNA synthesis were performed as previously described (Broadbent et al., 2015, Andrade et al., 2020). Processed fragmented samples and purified cDNA were analysed with a Bioanalyzer using Pico RNA chips (Agilent). cDNA samples were prepared for NGS using the KAPA Hyper Prep Kit (KAPA Biosystems) following manufacturer's instructions. The quality of the amplified libraries was tested using Bioanalyzer with DNA 1000 chips (Agilent). Quantification of nucleic acid content from libraries was performed using a KAPA Quant Kit for Illumina Sequencing (KAPA Biosystems). Samples were pooled based on parasitemias and were subject to cluster generation and sequencing using a HiSeq 2500 Rapid flow cell (Illumina). Raw sequences were trimmed from adapter sequences and low-quality bases using the FASTX-Toolkit v0.0.14. Finally, sequences were aligned to a reference *P. falciparum* genome, build ASM276v1, using HISAT2 v2.0.4 (Kim, Langmead, & Salzberg, 2015) and genes were mapped using htseq-count: v0.6.1 (Anders, Pyl, & Huber, 2015). To calculate parasite age (in hours post invasion) an estimation based on genes expression was used as detailed in Andrade et al., 2020;

Lemieux et al., 2009). To identify differential expression, data was processed using the Bioconductor package DESeq2 v1.26.0 (Love, Huber, & Anders, 2014) as described in Andrade et al., 2020. Reads were validated by RT-qPCR on the twenty-four previously mentioned samples and on an extra set of eight-teen samples: six dry season and 12 clinical cases during the wet season. Reverse transcripts were first generated using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) and purified using the QIAquick 96-well protocol (Qiagen). Relative gene expression was determined on eight transcripts by RT-PCR using Invitrogen Express qPCR SuperMix with premixed ROX reference dye (Invitrogen) as detailed in Andrade et al., 2020. Data was processed using the 7900HT version 2.4 sequence detection system software.

2.2.12 Metabolite profiling

Plasma metabolomics was conducted employing both a targeted and untargeted profiling using several LC-MS platforms to analyse small molecules and lipids and obtain a broad metabolite coverage. Briefly, plasma samples were split into two independent samples and prepared for hydrophilic and hydrophobic metabolic profiling as detailed in Andrade et al., 2020. Detection of hydrophilic metabolites was performed using reverse-phase high-performance LC–MS in an AB SCIEX 5600 (QTOF) TripleTOF and Thermo Exactive Plus Orbitrap and separated on the AB SCIEX 5600 by reverse-phase HPLC. Eluates were further analysed with a 5600 TripleTOF using a Duospray ion source (AB SCIEX) and metabolites were separated using a Thermo Exactive Plus Orbitrap. Detection of hydrophobic metabolites was performed using reverse-phase high-performance LC–MS using an AB SCIEX 5600 TripleTOF. Metabolites were separated using a Waters Acquity UPLC CSH C18 column. Finally, all data was removed from backround noise by normalization and blank subtraction before statistical analysis.

2.2.13 *P. falciparum* field isolates short-term culture and analysis

RBC pellets either from RDT+ samples in cross-sectional time-points or malaria cases samples in the transmission season, were cultured at 7% haematocrit in complete RPMI supplemented with 0.25% Albumax II at 37 °C in a candle jar for 36 or 48 hours. To allow *P. falciparum* parasites to grow to their maximum potential, malaria case samples RBCs were diluted 1:10, 1:25 and 1:50 in fresh human O^{Rh+} erythrocytes.

2.2.14 *P. falciparum* analysis by flow cytometry

Parasitaemia and parasite development were assessed flow cytometry. RBC pellet was stained with Sybr Green I (Invitrogen) and MitoTracker (Applied Biosystems) for 30 min at 37 °C. Samples were acquired on LSR II flow cytometer (BD) and analysed using FlowJo software (BD) 10.2 or higher versions. Parasitaemia fold change was determined for each sample (ratio of %iRBCs at each time point over its preceding one). The time of highest increase of parasitaemia was the time point at which the ratio of %iRBCs at a given time point over its preceding one was the highest for each sample. Progeny number was determined by dividing SYBR Green fluorescence of multinucleated schizonts before or at the time of the highest increase in parasitaemia in vitro by the fluorescence of the smallest ring stage population that sample presented. Gating strategy available in Supplementary Figure 2.

2.2.15 *P. falciparum* size measurement by light microscopy

Giemsa-stained thin blood smears were analysed by light microscopy with Axio Lab.A1 (Zeiss) microscope. Images were acquired on 100x objective using the Zen2 Core v2.4 software. Area of each parasite was measured using ImageJ software (NIH).

2.2.16 *P. falciparum* in vitro culture with human plasma

P. falciparum parasites were synchronized either with sequent sorbitol synchronizations, or by sequent magnetic activated cell sorting (MACS) –Multinucleated schizonts were purified, allowed to invade for 3-4 h, and selected for recently-invaded rings with a second MACS. Following synchronization, young rings were put in culture in fresh human O^{Rh+} erythrocytes at 2% haematocrit in RPMI 1640 medium without glucose, supplemented with L-glutamine, 25mM HEPES, 7.4% sodium bicarbonate, 25 mg ml^{-1} gentamycin (all Gibco) and 5.5mM D-glucose (Sigma-Aldrich) at 37 °C either in the presence of a gas mixture containing 5% O_2, 5% CO_2 and 90% N_2 or using the candle jar system method described by Trager and Jensen Trager & Jensen, 1976. Depending on the experiments, *P. falciparum* parasites were supplemented with different concentrations of human plasmas, either total or antibody-depleted (with Pierce protein G and L plus agarose beads (Thermo Scientific)). *P. falciparum* parasites were grown in 96-well round-bottom plates with a total volume of 70 µl at 2% haematocrit, supplemented

with human plasma. After a determined number of hours in culture, parasites were stained with SYBR green I and MitoTracker. Parasitaemia and parasite development were acquired using FACS Canto II or LSR II (BD) and analysed using FlowJo software 10.2 or higher versions.

2.2.17 FCR3 *P. falciparum* culture in different plasmas

P. falciparum parasites were supplemented with human plasma as described previously. Multinucleated schizonts were purified by incubation with gelafundin for 15 min at 37 °C. After allowing for invasion to occur for 3-4 h, sorbitol synchronization was performed to select for recently invaded rings stage parasites. After 30-38 h post invasion, *P. falciparum* parasites were put in culture with 25% antibody-depleted human plasma in fresh human O^{Rh+} erythrocytes at 2% haematocrit in RPMI 1640 medium without glucose, supplemented with L-glutamine, 25mM HEPES, 7.4% sodium bicarbonate, 25 mg ml^{-1} gentamycin (all Gibco) and 5.5mM D-glucose (Sigma-Aldrich) at 37 °C in the presence of a gas mixture containing 5% O2, 5% CO2 and 90% N_2. 48 h later, RBC pellet was washed and fixed overnight at 4 °C with 2% PFA and 0.016% Glutheraldehyde in 0.1 M Cacodylate buffer.

2.2.18 Transmission electron microscopy of *P. falciparum* infected erythrocytes

After fixation, RBCs pellet were embedded in 3% low-melt agarose in 0.1M Cacodylate buffer. Briefly, after dissolving agarose solution at 80 °C, pellet was infiltrated for 1min in agarose solution at 37 °C, hardened on ice for 15 min, and then placed again in agarose solution, infiltrated for 1 min at 37 °C, hardened on ice for 15 min, and cut into cubes with 1-2 mm edges. RBC pellet was washed in 0.1 M Cacodylate buffer and stained with 1% OsO_4 in 0.1 M Cacodylate buffer for 1h at RT. Pellet was washed in 0.1 M Cacodylate buffer and then in water, and then stained with 1% Uranyl Acetate overnight at 4 °C. The following day, the pellet was washed in water and then dehydrated by incubation with different concentrations of acetone (30%, 50%, 70%, 90%), each concentration incubated for 10min at RT and incubated at 4 °C overnight in 100% acetone. After, we proceeded with resin infiltration in Spurr's resin by incubating with different concentrations (25% 50% 75%) of Spurr's resin in acetone for 45min each, followed by incubation with 100% Spurr's resin incubation overnight at 4 °C. Lastly, RBC pellet was polymerized with 100% Spurr's resin by incubation at 60 °C overnight. RBC pellet

was sectioned into 70 nm sections using a diamond knife (Diatome ultra 35°) on Ultramicrotome EM UC6 (Leica) and placed on a copper grid coated with pioloform film. Samples were acquired using JEM-1400 (Jeol Ltd.) transmission electron microscope with EM-Menu (TVIPS GmbH) software. Images were analysed using ImageJ (NIH) software.

2.2.19 Scanning electron microscopy of *P. falciparum* infected erythrocytes

12 mm coverslips were cleaned with two sonication steps in Milli Q water, followed by 1h incubation in 0.1 N HCl for 1h, washed with water and incubated for 1h in 96% EtOH. After washing, coverslips were stored in 96% EtOH until further use. Coverslips were then coated with 0.01% Poly-L-lysine (Merck Millipore) 20 min at RT. After coating, RBC pellet was gently added to the coverslips, and allowed to settle for 15 min at RT. After washing with 0.1 M Cacodylate buffer, the samples were stained with 1% OsO_4 solution for 1h at RT, washed with 0.1 M Cacodylate buffer and then with water and was dehydrated by incubation with different concentrations of acetone (30%, 50%, 70%, 90%), each concentration incubated for 10min at RT and incubated twice in 100% acetone. Critical point drying of the coverslips was performed using Leica EM CPD300, with coverslips mounted on pins with silver glue and sputtered with a 10 nm film of palladium gold using Leica EM ACE200. Samples were acquired using Zeiss Leo 1530 (Zeiss) scanning electron microscope at 2 kV accelerating voltage at 4-6 mm working distance at 12,000 x magnification using the SE2 detector at 3000x2000 image setting in scanning speed 3, using SmartSEM (Zeiss) software. Images were analysed using ImageJ (NIH) software.

2.2.20 *ama*1 heminested PCR

To determine haplotypes circulating in PCR+ samples, we performed hemi-nested PCR of the 236 base-pair segment of apical membrane antigen *ama*1) gene of *P. falciparum* following previously published protocols (Briggs et al., 2020; Miller et al., 2017).

Briefly, after DNA extraction and parasite density quantification, positive samples were amplified for *ama*1 gene. PCR was performed with 5 µl of DNA, *ama*1 primers and Fast Start High Fidelity PCR kit (Roche) with the following cycling conditions: 2 min at 95 °C, 30 sec at 95 °C, 30 sec at 55 °C, 1 min at 72 °C for a total of 20 cycles, and then 10 min at 72 °C.

A second nested PCR was done with PCR products from the first PCR where a 5′ linker sequence was added. The cycling conditions were the ones previously used, with a different number of cycles based on the parasite density (10, 20 or 25 cycles for samples with $10^3 – 10^4$, $10 – 10^3$, $1^{-1} – 10$ parasites per µl, respectively).

Finally, a third nested PCR was done with the 15 µl PCR product from the second PCR with NEBNext Master Mix (NEB) with primers binding to the linker sequences and carrying Illumina sequence adapters.

Samples were then pooled, purified by bead cleaning and sequenced using Miseq platform (Illumina) with 250 bp paired-end. Data was analysed using SeekDeep software (Hathaway, Parobek, Juliano, & Bailey, 2018) as described in Briggs et al., 2020. Data analysis was performed using R software.

2.2.21 Haplotype length analysis

To determine the duration of each haplotype infection, dried blood spots (DBS) were collected every 2 weeks, and the number of days each clone stayed in the host was determined. Due to the imperfect detection of *P. falciparum* clones, either because of parasite sequestration or as a result of low parasite densities that lead to the absence of detection of minor haplotypes, we performed our analysis to allow for a miss of 2 “skips”, the equivalent to one month. We identified a haplotype as a new infection if it was missing for more than 2 “skips” (one month). Since clones are not transmitted exactly when the DBS were collected, we determined the calendar day in the middle of the two collection time-points in which the clone appears. Individuals that start the dry season without parasites are likely to be negative at the end of the dry season, in agreement with absence of transmission (Portugal et al., 2017; Andrade et al., 2020). Due to the lack of sample collection during the dry season, haplotypes that were only detected at the end of the dry season (May 2013) were assumed to have been transmitted 14 days after the last time-point during transmission season (December 2012). Haplotypes present in May 2012 that were carried during transmission season in 2012 were excluded from the analysis. Data analysis was performed in R. Graphs and statistical analysis were performed in GraphPad Prism 9.

2.2.22 Statistical analysis

Statistical testes used are described for each experiment in the corresponding figure legend. Statistical significance was defined as a two-tailed *P* value of ≤ 0.05. All analyses were performed with GraphPad Prism versions 6.0, 8.0 or 9.0, JMP 14.0.1 or R.

CHAPTER

RESULTS 3

3.1 *Plasmodium falciparum* infection persists during the dry season in 2017 and 2018

During the years 2017 and 2018, we followed ~600 individuals aged 3 months to 45 years, in a cohort study in Kalifabougou, Mali. As observed before (Portugal et al., 2017), clinical malaria cases were restricted to the transmission seasons (from June to December), whereas during the dry season (from January to May) nearly all individuals remained malaria free. In fact, during transmission season in 2017 and 2018, 386 and 347 malaria cases were reported, respectively, whereas only 12 and 5 malaria episodes occurred during the dry season (Table 1 and Figure 6 a). Although malaria cases barely occurred during the dry season, we consistently observed 10-20% prevalence of *P. falciparum* asymptomatic infections during this time. We analysed *P. falciparum* infection status by PCR in over 400 individuals paired in the beginning (January) and end (May) of the dry season and detected that 20% of individuals were *P. falciparum* PCR^+ both 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 (Figure 6 b). In agreement with our previous report on the same cohort Portugal et al., 2017, we observed that *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 non-infected individuals at the beginning of a dry season remained non-infected until its end (Figure 6 b). The odds ratios (OR) of maintaining the same infection status through each entire dry season were OR 90.9 (95% confidence interval (CI) (38.6 - 213.8) $P < 0.0001$) in 2017, and OR 43.5 (95% CI (17.5 - 107.5) $P < 0.0001$) in 2018 (Table 2). As previously reported for this cohort (Portugal et al., 2017), we observed that asymptomatic infections were more frequent in older children and young adults (Table 1 and Figure 6 c). To evaluate how dynamic these persisting parasitaemias were, we quantified *P. falciparum* parasitaemias in asymptomatic individuals during the dry season or clinical malaria

cases during transmission season by RT-qPCR and flow cytometry. We found that during the dry season parasitaemias remained low or even decreased in some individuals, whereas clinical malaria cases in the transmission season had very high parasitaemias (Figure 6 d).

Table 1 | Characteristics of study participants, stratified by year.

	all			MAL		May	
	n	female %	Age, y, mean (95% CI)	Malaria cases n	Age malaria case (y, 95% CI)	% Pf+ in May n	Age %Pf+ in May (y, 95% CI)
2017	604	46,7	11.2 (10.5 - 11.8)	398	9.4 (8.8 - 10.1)	13,4	13.3 (11.8 - 14.9)
2018	603	46,3	10.8 (10.1 - 11.5)	352	9.2 (8.5 - 10.0)	10,9	11.8 (10.0 - 13.6)

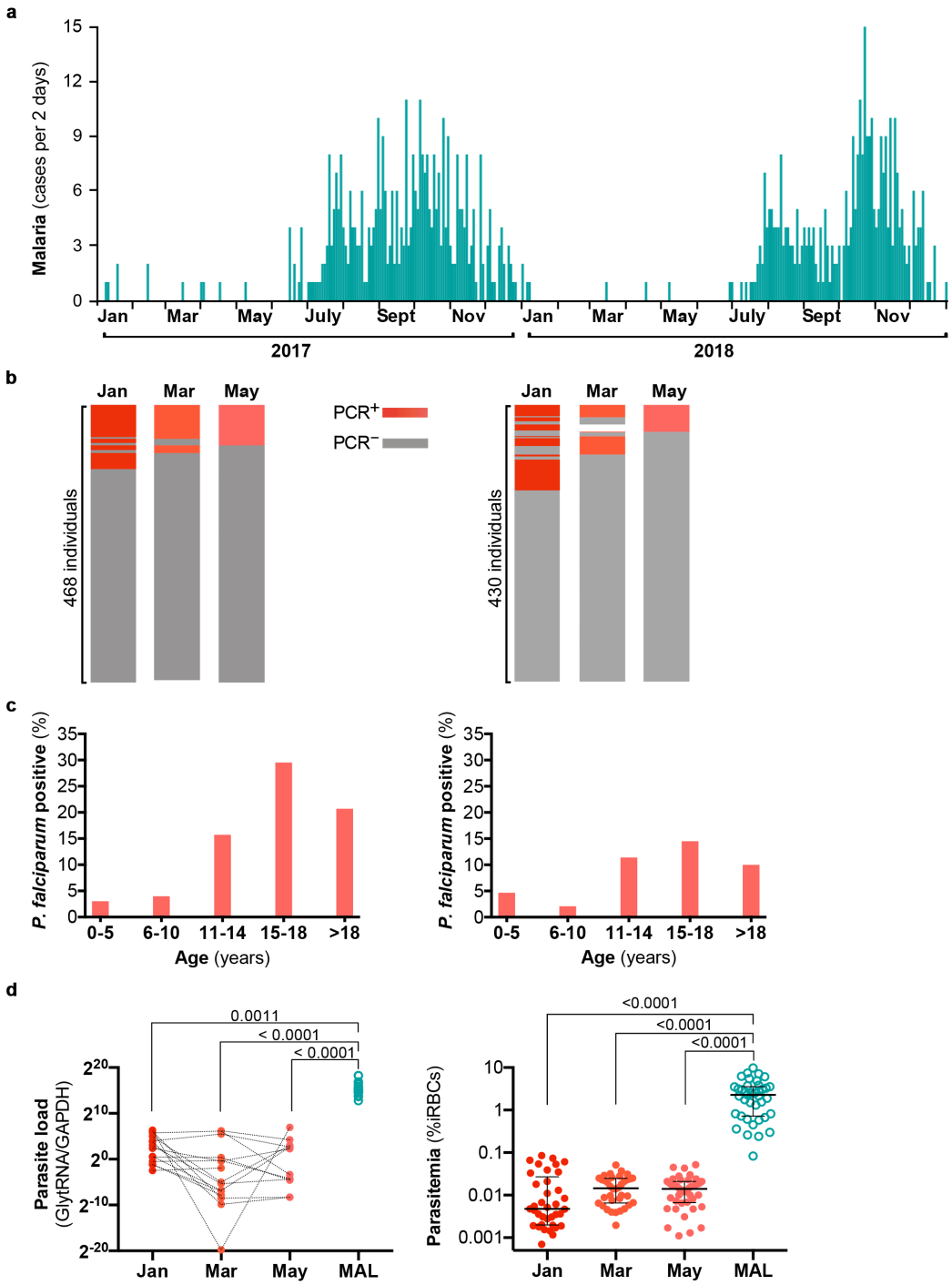

Figure 6 | *P. falciparum* reservoir during the dry season.

a, Clinical malaria frequency in a cohort of ~600 individuals aged 3 months to 45 years measured every 2 days for 2 years. Clinical malaria diagnosed by axillary temperature ≥37.5 °C and ≥2,500 asexual parasites per µl of blood and no other apparent cause of fever. **b,** Prevalence of asymptomatic *P. falciparum* determined by PCR in paired individuals (rows) at the beginning (January), middle (March) and end (May) of two consecutive dry seasons (2017, left; 2018, right). Columns are sorted to have the same individual represented in a single row at the three time points in each dry season. **c,** Prevalence of asymptomatic *P. falciparum* infection determined by PCR at the end of the dry season in May 2017

(left) and May 2018 (right) stratified by age. **d,** Parasite load detected by RT–qPCR (left) and flow cytometry (right) of RDT$^+$ asymptomatic children at the beginning (January), mid (March) and end (May) of the dry season and children with their first clinical malaria episode (MAL) in the transmission season. Parasitaemia data represented as median ± IQR; Kruskal–Wallis test with multiple comparisons. Figure reproduced from Andrade et al., 2020.

Table 2 | Contingency tables with PCR data from beginning and end of the dry season of 2017

	Neg May17	Pos May17	
Neg Jan17	365	7	372
Pos Jan17	35	61	96
	400	68	468

Odds ratio 2017: 90.88 (CI: 38.63;213.82)

	Neg May18	Pos May18	
Neg Jan18	331	6	337
Pos Jan18	52	41	93
	383	47	430

Odds ratio 2018: 43.5 (CI: 17.46 , 107.54)

3.2 Host immune responses are minimal at the end of the dry season and do not predict clinical malaria in the ensuing transmission season

Knowing that *P. falciparum* infections do not cause clinical malaria in the dry season, we inquired if the immune system could prevent the increase of parasitaemias during this time. To address this, we compared immune responses of asymptomatic carriers of *P. falciparum* (May$^+$) versus non-carriers (May$^-$) during the dry season. We determined levels of 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 (May$^+$) or did not (May$^-$) carry *P. falciparum* during the dry season, detected retrospectively by PCR. Serological inflammation markers C- reactive protein (CRP), von Willebrand factor (vWF) and hepcidin were previously reported to be increased during clinical malaria (Burte et al., 2013; O'Donnell et al., 2009; Park, Ireland, Opoka, & John, 2012) and were quantified in plasma samples during the dry season. We observed no differences in the levels of these inflammation markers in plasma samples collected from asymptomatic carries (May$^+$) or non-carriers (May$^-$) either at the beginning nor at end of the dry season (Figure 7 a). We then profiled the levels of cytokines and chemokines circulating in the plasma of *P. falciparum* infected or non-infected individuals at the end of the dry season, using a multiplex bead array, and observed no differences in all except one chemokine – CXCL1 (Table 3 and Figure 7 b). We then quantified

the levels of major leukocyte populations by flow cytometry in fresh and frozen peripheral blood mononuclear cells (PBMCs) collected from children at the end of the dry season, carrying or not *P. falciparum* infection. We observed that the percentage of monocytes, T cells, B cells and NK cells subpopulations were not different between these children (Figure 7 c). To determine potential differences in cell function, we quantified intracellular cytokines, activation or cytotoxicity markers, transcription factors or exhaustion markers of freshly collected PBMCs. We observed that 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 similar between children who carried or not *P. falciparum* (Figure 7 d and Table 4). Additionally, we inquired if *P. falciparum* memory B cells (MBCs, defined as $CD19^{+}$, $CD10^{-}$, $CD21^{-}$ and $CD27^{+}$or^{-}) were differently affected during the dry season in asymptomatic carriers compared to non-infected individuals. We quantified *P. falciparum* specific MBCs by using biotinylated *P. falciparum* antigens to apical membrane antigen 1 (*ama*1) and merozoite surface protein 1 (*msp*1) (Hopp et al., 2020). We observed that that the proportion of class-switched *P. falciparum*-specific MBCs (*ama*1^{+} or *msp*1^{+} IgG^{+} IGM^{-} MBCs) was significantly increased in asymptomatic carriers at the end of the dry season, albeit no difference was found in the non-class-switched MBC population (*ama*1^{+} or *msp*1^{+} IgG^{-} IGM^{+} MBCs). Within the IgG^{+} MBC subpopulations, we did not observe differences between asymptomatic carriers and non-carriers at the end of the dry season in *P. falciparum*-specific classical and atypical MBCs, but detected increased *P. falciparum*-specific activated MBCs in asymptomatic carriers (Figure 7 e and f). A previous study from our laboratory identified that children that carry *P. falciparum* infections during the dry season have higher *P. falciparum*-specific humoral responses than children that do not carry parasites during the same period (Portugal et al., 2017). However, responses to variant surface antigens (VSA) multigene family *var* were missing from this protein array analysis. We used a multiplex bead array to 35 domain types of the VSA multigene family *var*, which were grouped according to their endothelial-receptor affinity (CD36, EPCR or unknown receptor) and PfEMP1 upstream promotor sequence (UPS) type (A, B or B/A types) (Lavstsen, Salanti, Jensen, Arnot, & Theander, 2003). Comparable with our previous study in non-VSA *P. falciparum* specific humoral responses, we observed that the percentage of individuals reactive against PfEMP1 domains binding to CD36, EPCR or to unknown receptors was higher in asymptomatic carriers when compared to non-carriers, both at the beginning and end of the dry season. Moreover, we observed that the proportion of

individuals reactive to the different PfEMP1 domains decreased over the dry season independently of individual infection status (Figure 8 a). We further 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 asymptomatic infection (May^{+}) or were uninfected (May^{-}) during the dry season (Figure 8 b). Antibodies against PfEMP1 domains (Figure 8 a and b), against a large set of *P. falciparum* non- VSA (Portugal et al., 2017), and also particularly against red blood cell (RBC) invasion-related proteins (Figure 8 c) were higher in asymptomatic carriers compared to non-infected children at the end of the dry season. We questioned if the difference in humoral response at the end of the dry season could differently inhibit *P. falciparum* merozoite invasion in vitro. We allowed 3D7 *P. falciparum* merozoites to invade RBCs in the presence of total or antibody-depleted plasma collected at the end of the dry season from infected or non-infected children, and observed that in the presence of complete Malian plasma there was ~5-fold more merozoite invasion inhibition than in the presence of antibody- depleted Malian plasma, while antibody depletion had no differential effect on the control German plasma used (Figure 8 d and e). However, we observed no differences in inhibition of merozoite invasion when invasion occurred in plasma from infected or non-infected children, suggesting that the differences in antibodies present at the end of the dry season have no significant effect on inhibition of merozoite invasion.

Lastly, we inquired if the immune status at the end of the dry season correlated with clinical malaria episode in the ensuing dry season. We stratified individuals by the occurrence of a clinical malaria episode in the transmission season and questioned if their responses in the end of the preceding dry season, in May, could explain the difference in clinical presentation. We did not observe any differences in the levels of inflammation markers (Table 5), cytokines (Table 6), PBMCs subpopulations and cell functions (Table 7) or *P. falciparum* specific MBCs (Figure 8 f and g) at the end of the dry season in children that would have (MAL) or would not have (no MAL) a clinical malaria case in the ensuing transmission season. Notably, when we re-analysed the previously published protein array against 862 *P. falciparum* proteins (Portugal et al., 2017) segregating children by the occurrence or not of a clinical malaria episode in the transmission season we observed that *P. falciparum*-specific IgG breadth was reduced in children that would present with clinical malaria in the following transmission season (Figure 8 h). Moreover, we observed that children that experienced malaria in the ensuing transmission season had a higher loss of *P. falciparum* specific breadth of antibodies during the dry season (Figure 8 i).

We observed that *P. falciparum* asymptomatic dry season infections induce only minimal response on the host, and that the host immune response at the end of the dry season does not predict the risk of clinical malaria in the ensuing transmission season.

Table 3 | Multiplex bead array quantification of cytokines and chemokines in the plasma children with (May+) or without (May-) persisting *P. falciparum* at the end of the dry season. (n=12 May- and n=21 May+).

Cytokine	May- Median (Interquartile range)	May+ Median (Interquartile range)	Adjusted P Value
CXCL1 (GRO)	421.4 (102.2 - 455.8)	656.3 (374.8 - 1095)	<0,0001
CXCL10 (IP10)	202.4 (71.52 - 245.2)	137.3 (107.4 - 383.8)	0,2705
CCL2 (MCP1)	134.1 (103.1 - 280.8)	252.4 (154.1 - 356.8)	0,9973
CCL22 (MDC)	465.6 (220.9 - 817.7)	400.1 (288.5 - 624.5)	0,9991
sCD40L	166.9 (91.06 - 439.7)	181.3 (77.97 - 514.1)	0,9997
CCL3 (MIPa)	15.41 (9.91 - 28)	6.33 (4.35 - 18.69)	>0.9999
CCL4 (MIP1b)	14.37 (11.49 - 25.77)	16.47 (14.08 - 25.65)	>0.9999
CCL7 (MCP3)	3.86 (2.45 - 6.95)	2.65 (1.98 - 3.91)	>0.9999
EGF	7.72 (3.26 - 18.96)	11.35 (3.99 - 21.3)	>0.9999
Eotaxin	12.59 (11.63 - 15.99)	15.45 (11.9 - 26.76)	>0.9999
FGFa	7.82 (4.53 - 10.26)	2.74 (1.99 - 4.24)	>0.9999
Fractaline	5.09 (4.10 - 7.78)	5.58 (4.75 - 12.16)	>0.9999
GCFS	178.9 (42.32 - 755.2)	185.9 (65.12 - 542.7)	>0.9999
GMCSF	0.53 (0.34 - 1.26)	1.5 (0.73 - 2.65)	>0.9999
IFNa2	2.72 (0.91 - 6.64)	5.73 (3.80 - 14.1)	>0.9999
IFNy	0.84 (0.51 - 2.84)	2.07 (0.64 - 3.21)	>0.9999
IL1a	10.26 (6.18 - 30.8)	18.16 (5.39 - 34.62)	>0.9999
IL1b	0.43 (0.13 - 0.68)	0.69 (0.36 - 1.43)	>0.9999
IL-2	1.86 (1.32 - 2.60)	1.92 (1.53 - 2.59)	>0.9999
IL-3	0.36 (0.23 - 0.45)	0.41 (0.25 - 0.69)	>0.9999
IL-6	1.66 (0.81 - 15.63)	2.68 (1.67 - 6.24)	>0.9999
IL-7	2.42 (1.18 - 49.67)	3.47 (1.25 - 8.30)	>0.9999
IL-8	18.7 (14.5 - 76.06)	44.99 (22.13 - 122.6)	>0.9999
IL-9	2.64 (0.73 - 7.38)	2.80 (0.97 - 11.84)	>0.9999
IL12P40	5.32 (0.40 - 15.76)	9.972 (3.62 - 17.51)	>0.9999
IL13	1.11 (0.17 - 9.57)	0.57 (0.23 - 97)	>0.9999
IL15	1.61 (1.33 - 4.87)	1.76 (1.47 - 2.57)	>0.9999
IL-10	10.23 (7.23 - 24.33)	21.53 (13.28 - 25.77)	>0.9999
IL-17	1.59 (1.57 - 1.62)	1.61 (1.58 - 1.76)	>0.9999
IL1RA	1.02 (0.58 - 6.65)	2.52 (1.05 - 8.07)	>0.9999
TGFb	1.18 (0.86 - 1.66)	1.58 (1.23 - 3.09)	>0.9999
VEGF	3.46 (1.29 - 8.54)	3.46 (1.20 - 13.14)	>0.9999

(shown are Adjusted P values calculated through one-sided Sidak's multiple comparisons test)

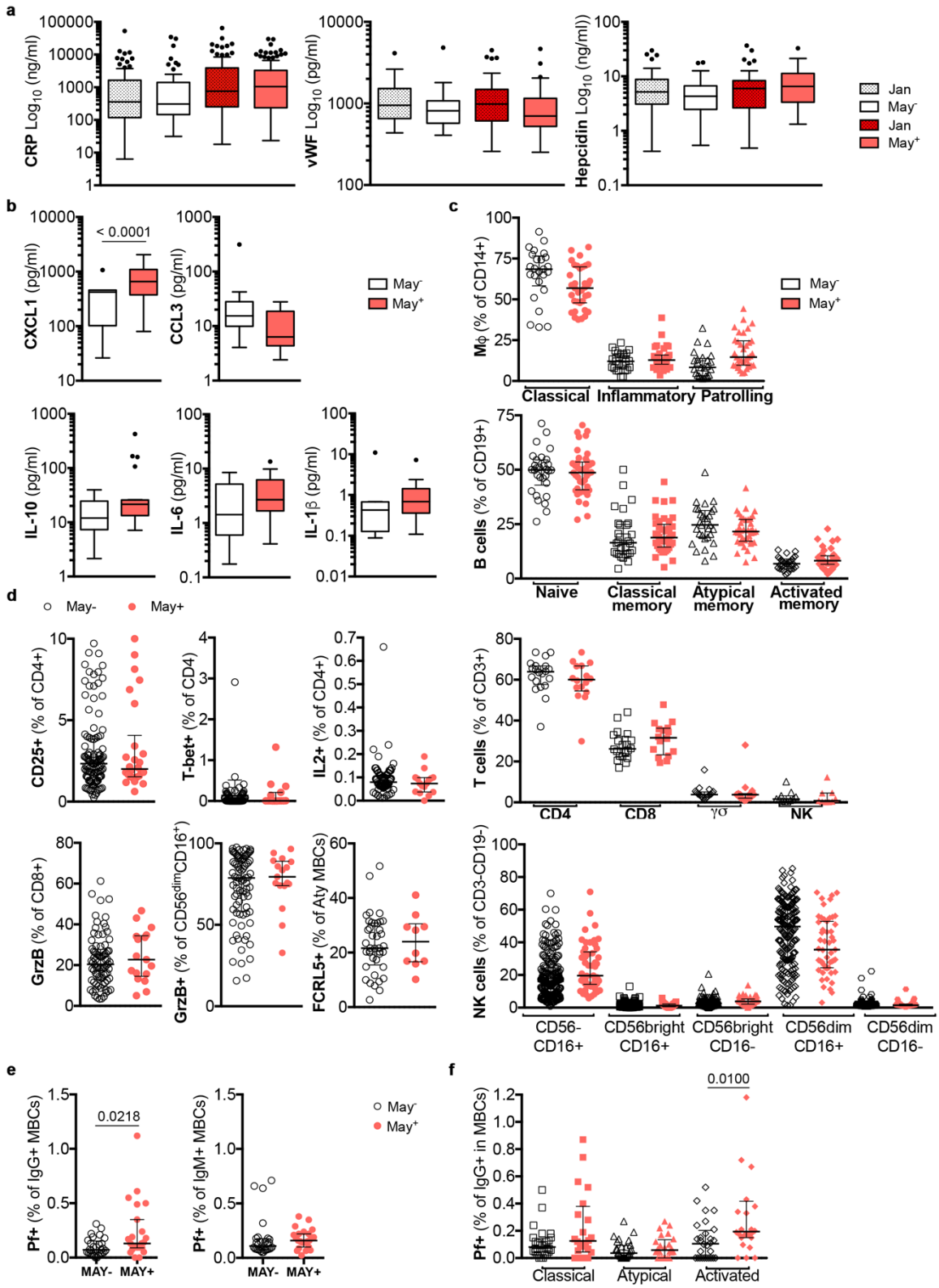

Figure 7 | Immune responses to *P. falciparum* during the dry season.

a, CRP (n = 71May−, 117 May+), vWF (n = 33 May−, 51 May+) and hepcidin (n = 41 May^{-}, 37 May^{+}) of paired plasma samples at the beginning (January) and end (May) of the dry season from individuals carrying *P. falciparum* (May^{+}) or not (May^{-}). One-way ANOVA with Tukey's multiple comparisons test.

b, Plasma cytokines from children carrying (n = 21 May$^+$) or not (n=12 May$^-$) *P. falciparum* at the end of the dry season. One-way ANOVA with Sidak's multiple comparisons test. **c,** Surface markers of PBMCs from children who carried (May$^+$) or did not carry (May$^-$) *P. falciparum* at the end of the dry season. One-sided Dunn's Kruskal-Wallis multiple comparisons test. **d,** Intracellular markers of fresh PBMCs from children carrying (May$^+$) or not carrying (May$^-$) *P. falciparum* at the end of the dry season (all data in Table 4). One-sided Dunn's Kruskal-Wallis multiple comparisons test. **e**, *P. falciparum*– specific AMA1$^+$ or MSP1$^+$ MBCs 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$^-$) or non-class-switched (IgG$^-$IgM$^+$) MBCs (Table 4). Mann–Whitney test. **f,** Proportion of *P. falciparum*-specific IgG$^+$ MBCs in classical, activated or atypical MBCs of *P. falciparum* carriers (May$^+$, n = 23) or uninfected individuals (May$^-$, n=28) at the end of the dry season. One-sided Dunn's Kruskal-Wallis multiple comparisons test. Adapted from Andrade et al., 2020.

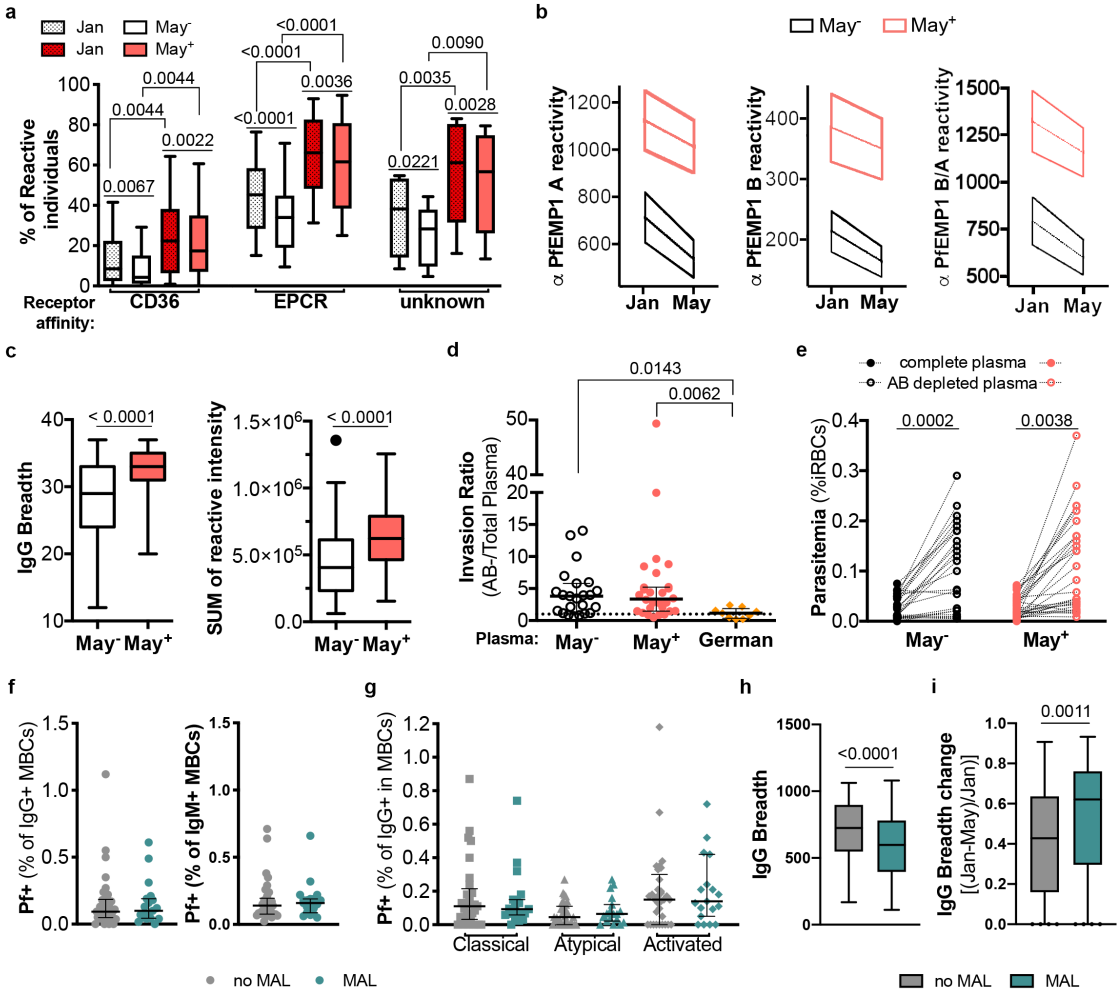

Figure 8 | Immune responses to *P. falciparum* during the dry season (continuation of Fig. 7).

a, Proportion of children with antibodies specific to PfEMP1 domains at the beginning and end of the dry season (n = 106 January and May^{-}, 112 January and May^{+}). Repeated-measures one-way ANOVA (with Greenhouse–Geisser correction) for each binding receptor affinity. Boxplots indicate median ± IQR. Values >1.5 times the IQR are plotted as individual points (Tukey method). **b,** Magnitude of anti-PfEMP1 domains antibody response between the beginning and end of the dry season in children carrying or not carrying asymptomatic *P. falciparum* (n = 112 January and May^{+}, 106 January and May^{-}). Slopes compared with a linear non-interaction model. **c,** Breadth (left) and magnitude (right) of *P. falciparum* IgG reactivity to invasion-related antigens at the end of the dry season in non-infected and asymptomatic children carrying *P. falciparum* (n=143 May^{-}, 139 May^{+}). Individual antigen reactivity (detailed in Portugal et al., 2017) of invasion-related antigens based on Cowman, Berry, & Baum, 2012 **d,** *P. falciparum* invasion ratio between merozoite invasion in antibody (AB) depleted plasma and merozoite invasion in paired complete plasma from asymptomatic carriers and non-infected children (n=28 May^{+}, 23 May^{-}), and malaria-naïve control (German adults' plasma, n=9). One sided Dunn's Kruskal-Wallis Multiple Comparisons test. Dashed line at invasion ratio of 1. **e,** Parasitaemia after invasion in complete or antibody-depleted plasma from children who carried or did not carry (n = 29 May^{+}, 24 May^{-}) *P. falciparum* asymptomatic infections during the dry season **f,** *P. falciparum*- specific AMA1^{+} or MSP1^{+} MBCs at the end of the dry season, in individuals that had clinical malaria (MAL, n= 19) or not (no MAL, n= 33) in the ensuing transmission season for class-switched (IgG^{+} IgM^{-}) or non-class-switched (IgG^{-} IgM^{+}) MBCs. **g,** Proportion of *P. falciparum*-specific IgG^{+} MBCs in classical, activated or atypical MBCs at the end of the dry season in individuals that had (MAL, n=19) or not (no MAL, n=33) clinical malaria in the ensuing transmission season. **h**, Breadth of *P. falciparum* specific IgG reactivity at the end of the dry season, in children that had (MAL, n= 134) or not (no MAL, n=148) a clinical malaria episode in the ensuing transmission season. **i,** Change in the breadth of *P. falciparum* specific IgG reactivity during the dry season, defined as (January-May)/January, in children that had (MAL, n= 101) or not (no MAL, n=137) a clinical malaria episode in the ensuing transmission season (**d, f, g**) Data indicate median ± IQR. (**e, h, i**) Breadth is the number of antigens to which the level of IgG reactivity exceeds 2 SDs above the no DNA control. Magnitude is the sum of log2 -IgG intensity values for all antigens per sample. Boxplots indicate median ± IQR, Tukey method, two-tailed Mann-Whitney test. Adapted from Andrade et al., 2020.

Table 4 | Expression of surface and intracellular markers of PBMCs from children that carried *P. falciparum* parasites (May^+) or remained uninfected (May^-) during the dry season.

Parameter	n	n May-	May- Mean (SD)	n May+	May+ Mean (SD)	p value	Significant?	test
CD3 (% of Lymphocytes)	252	201	71.72 (8.54)	51	73.75 (7.61)	0,074	No	Mann-Whitney
CD4 (% of CD3 T cells)	235	188	61.9 (6.54)	47	63.63 (5.62)	> 0,9999	No	Kruskal-Wallis
CD8 (% of CD3 T cells)	252	201	29.52 (5.61)	51	28.68 (5.4)	> 0,9999	No	Kruskal-Wallis
NKT cells (% of CD3 T cells)	252	201	2.659 (1.81)	51	2.15 (1.21)	> 0,9999	No	Kruskal-Wallis
Granzyme B (% of CD8 T cells)	97	80	22.2 (12.66)	17	24.51 (12.69)	> 0,9999	No	Kruskal-Wallis
Granzyme B (% of NKT cells)	97	80	43.02 (21.75)	17	44.92 (22.59)	> 0,9999	No	Kruskal-Wallis
Granzyme B (Geo Mean of CD8 T cells)	97	80	3399 (3169)	17	3294 (2149)	> 0,9999	No	Kruskal-Wallis
Granzyme B (Geo Mean of NKT cells)	97	80	18733 (19005)	17	13471 (14620)	> 0,9999	No	Kruskal-Wallis
T-bet (% of CD4 T cells)	76	61	0.13 (0.38)	15	0.1656 (0.3477)	> 0,9999	No	Kruskal-Wallis
T-bet (% of CD8 T cells)	80	62	3.13 (4.64)	18	2.31 (3.795)	> 0,9999	No	Kruskal-Wallis
T-bet (% of NKT cells)	80	62	4.86 (7.85)	18	3.65 (6.19)	> 0,9999	No	Kruskal-Wallis
T-bet (Geo Mean of CD4 T cells)	74	59	2577 (6708)	15	1879 (1293)	> 0,9999	No	Kruskal-Wallis
T-bet (Geo Mean of CD8 T cells)	77	59	1327 (212.6)	18	1571 (677.4)	0,141	No	Kruskal-Wallis
T-bet (Geo Mean of NKT cells)	74	57	1424 (327.4)	17	1533 (411.2)	0,453	No	Kruskal-Wallis
IL2 (% of CD4 T cells)	84	68	0.094(0.084)	16	0.076 (0.047)	> 0,9999	No	Kruskal-Wallis
IL2 (% of CD8 T cells)	92	72	0.081(0.067)	20	0.082 (0.061)	> 0,9999	No	Kruskal-Wallis
IL2 (% of NKT cells)	92	72	3.01(3.58)	20	3.03 (5.40)	> 0,9999	No	Kruskal-Wallis
CD25 (% of CD4 T cells)	133	107	3.0 (2.30)	26	3.27 (2.74)	> 0,9999	No	Kruskal-Wallis
CD25 (% of CD8 T cells)	144	114	0.03 (0.034)	30	0.026 (0.020)	> 0,9999	No	Kruskal-Wallis
CD25 (% of NKT cells)	144	114	0.52 (0.51)	30	0.48 (0.44)	> 0,9999	No	Kruskal-Wallis
CD25 (Geo Mean of CD4 T cells)	133	107	2846 (424.2)	26	2769 (438.6)	> 0,9999	No	Kruskal-Wallis
CD25 (Geo Mean of CD8 T cells)	119	94	3615 (6454)	25	2981 (1753)	0,604	No	Kruskal-Wallis
CD25 (Geo Mean of NKT cells)	137	109	4710 (4167)	28	4378 (2489)	> 0,9999	No	Kruskal-Wallis
IL2 (Geo Mean of CD4 T cells)	82	67	5534 (1913)	15	5374 (1551)	> 0,9999	No	Kruskal-Wallis
IL2 (Geo Mean of CD8 T cells)	91	71	5262 (1705)	20	5118 (1355)	> 0,9999	No	Kruskal-Wallis
IL2 (Geo Mean of NKT cells)	91	72	6113 (5387)	19	5407 (2237)	> 0,9999	No	Kruskal-Wallis
CD19 (% of Lymphocytes)	249	198	15.85 (3.98)	51	16.84 (4.45)	0,241	No	Mann-Whitney
Activated MBCs (% of CD19 B cells)	225	177	3.52 (2.0)	48	4.86 (2.87)	0,704	No	Kruskal-Wallis
Classical MBCs (% of CD19 B cells)	225	177	14.47 (6.09)	48	12.92 (5.11)	> 0,9999	No	Kruskal-Wallis
Naive (% of CD19 B cells)	225	177	70.7 (9.2)	48	68.1 (9.8)	> 0,9999	No	Kruskal-Wallis
Atypical MBCs (% of CD19 B cells)	225	177	11.34 (5.51)	48	14.1 (6.41)	0,423	No	Kruskal-Wallis
FCRL5 (% of Activated MBCs)	50	40	19.32 (8.31)	10	22.36 (10.62)	> 0,9999	No	Kruskal-Wallis
FCRL5 (% of Classical MBCs)	50	40	2.06 (1.45)	10	2.61 (2.29)	> 0,9999	No	Kruskal-Wallis
FCRL5 (% of Naive MBCs)	50	40	0.46 (0.61)	10	0.63 (0.57)	> 0,9999	No	Kruskal-Wallis
FCRL5 (% of Atypical MBCs)	50	40	22.94 (10.59)	10	24.17(9.66)	> 0,9999	No	Kruskal-Wallis
Granzyme B (% of Activated MBCs)	97	80	24.05 (20.02)	17	22.79 (19.61)	> 0,9999	No	Kruskal-Wallis
Granzyme B (% of Classical MBCs)	97	80	6.13 (10.71)	17	9.4 (14.57)	> 0,9999	No	Kruskal-Wallis
Granzyme B (% of Naive MBCs)	97	80	2.43 (5.52)	17	3.73 (8.18)	> 0,9999	No	Kruskal-Wallis
Granzyme B (% of Atypical MBCs)	97	80	6.84 (11.36)	17	7.62 (11.67)	> 0,9999	No	Kruskal-Wallis
IL2 (Geo Mean ofActivated MBCs)	91	72	841.4 (281)	19	954 (281.6)	0,649	No	Kruskal-Wallis
IL2 (Geo Mean ofClassical MBCs)	91	72	754.6 (249.7)	19	864 (243.3)	0,348	No	Kruskal-Wallis
IL2 (Geo Mean ofNaive MBCs)	91	72	557.8 (175.5)	19	632.5 (174.4)	0,580	No	Kruskal-Wallis
IL2 (Geo Mean ofAtypical MBCs)	91	72	648.7 (202.6)	19	775.2 (230.9)	0,148	No	Kruskal-Wallis
Granzyme B (Geo Mean of Activated MBCs)	96	80	4121 (3791)	16	3773 (3121)	> 0,9999	No	Kruskal-Wallis
Granzyme B (Geo Mean of Classical MBCs)	97	80	2359 (2015)	17	2564 (2019)	> 0,9999	No	Kruskal-Wallis
Granzyme B (Geo Mean of Naive MBCs)	97	80	1851 (1500)	17	1899 (1565)	> 0,9999	No	Kruskal-Wallis
Granzyme B (Geo Mean of Atypical MBCs)	97	80	2231 (2087)	17	2207 (1771)	> 0,9999	No	Kruskal-Wallis
CD25 (Geo Mean of Activated MBCs)	91	72	88.82 (34.49)	19	100.6 (25.79)	> 0,9999	No	Kruskal-Wallis
CD25 (Geo Mean of Classical MBCs)	91	72	163.4 (29.63)	19	168.5 (21.83)	> 0,9999	No	Kruskal-Wallis
CD25 (Geo Mean of Naive MBCs)	91	72	57.71 (16.91)	19	50.82 (10.1)	> 0,9999	No	Kruskal-Wallis
CD25 (Geo Mean of Atypical MBCs)	91	72	38.51 (10.56)	19	42.23 (10.08)	> 0,9999	No	Kruskal-Wallis
CD56-CD16+ (% of CD3-CD19-	249	198	18.83 (13.22)	51	24.77 (14.38)	> 0,9999	No	Kruskal-Wallis
CD56dimCD16- (% of CD3-CD19-)	249	198	1.95 (2.24)	51	1.99 (1.72)	> 0,9999	No	Kruskal-Wallis
CD56dimCD16+ (% of CD3-CD19-	249	198	45.73 (19.98)	51	37.86 (17.53)	> 0,9999	No	Kruskal-Wallis
CD56brightCD16- (% of CD3-CD19-)	249	198	3.03 (2.26)	51	4.00 (2.47)	0,766	No	Kruskal-Wallis
CD56brightCD16+ (% of CD3-CD19-	249	198	1.4 (1.55)	51	1.52 (1.14)	> 0,9999	No	Kruskal-Wallis
Granzyme A (% of CD56-CD16+)	50	40	32.05 (28.37)	10	12.63 (12.04)	0,256	No	Kruskal-Wallis
Granzyme A (% of CD56dimCD16-)	50	40	17.02 (15.51)	10	8.92 (5.98)	> 0,9999	No	Kruskal-Wallis
Granzyme A (% of CD56dimCD16+)	50	40	35.02 (25.13)	10	18.71 (17.21)	0,780	No	Kruskal-Wallis
Granzyme A (% of CD56brightCD16-)	50	40	7.71 (9.69)	10	3.61 (5.98)	> 0,9999	No	Kruskal-Wallis
Granzyme A (% of CD56brightCD16+)	50	40	19.05 (18.66)	10	10.6 (15.33)	0,444	No	Kruskal-Wallis
Granzyme B (% of CD56-CD16+)	97	80	54.99 (21.82)	17	54.35 (21.6)	> 0,9999	No	Kruskal-Wallis
Granzyme B (% of CD56dimCD16-)	97	80	16.45 (11.94)	17	15.72 (11.77)	> 0,9999	No	Kruskal-Wallis
Granzyme B (% of CD56dimCD16+)	97	80	72.86 (21.37)	17	77.09 (16.52)	> 0,9999	No	Kruskal-Wallis
Granzyme B (% of CD56brightCD16-)	97	80	6.038 (7.042)	17	7.82 (7.589)	> 0,9999	No	Kruskal-Wallis
Granzyme B (% of CD56brightCD16+)	97	80	24.72 (25.01)	17	20.92 (16.2)	> 0,9999	No	Kruskal-Wallis
MΦ(CD14 +) (% of PBMCs)	234	187	6.356(4.591)	47	5.68 (3.97)	0,464	No	Mann-Whitney
Classical MΦ (% CD14 +)	234	187	73.92(13.3)	47	69 (19.78)	> 0,9999	No	Kruskal-Wallis
Inflammatory MΦ (% of CD14 +)	234	187	12.83 (7.75)	47	13.15 (9.171)	> 0,9999	No	Kruskal-Wallis
Patrolling MΦ (% of (CD14 +)	234	187	12.21 (8.95)	47	17.01 (14.87)	0,763	No	Kruskal-Wallis
Activated (% of B cells)	52	28	3.827 (2.126)	24	8.807 (6.443)	0,1209	No	Kruskal-Wallis
Classical (% of B cells)	52	28	19.5 (6.028)	24	17.75 (4.915)	> 0,9999	No	Kruskal-Wallis
Atypical (% of B cells)	52	28	9.866 (3.702)	24	14.4 (6.849)	0,5455	No	Kruskal-Wallis
Naive (% of B cells)	52	28	66.8 (7.496)	24	59.05 (14.19)	> 0,9999	No	Kruskal-Wallis
Pf specific (IgM+ of non-naive mature B cells)	52	28	0.1765 (0.1833)	24	0.1753 (0.09002)	0,1046	No	Mann-Whitney
HA specific (IgM+ of non-naive mature B cells)	52	28	0.05646 (0.05032)	24	0.04124 (0.02893)	0,5074	No	Mann-Whitney
Pf specific (IgG+ of non-naive mature B cells)	52	28	0.1005 (0.08167)	24	0.237 (0.2624)	0,0218	Yes	Mann-Whitney
HA specific (IgG+ of non-naive mature B cells)	52	28	0.163 (0.1773)	24	0.09068 (0.06453)	0,1151	No	Mann-Whitney
Pf specific (% IgG+ of Classical)	52	28	0.1098 (0.1107)	24	0.2724 (0.3818)	0,6448	No	Kruskal-Wallis
Pf specific (% IgG+ of Atypical)	52	28	0.0615 (0.06772)	24	0.08229 (0.08235)	> 0,9999	No	Kruskal-Wallis
Pf specific (% IgG+ of Activated)	52	28	0.1297 (0.1439)	24	0.3593 (0.3929)	0,01	Yes	Kruskal-Wallis
HA specific (% IgG+ of Classical)	52	28	0.1306 (0.1008)	24	0.0773 (0.0575)	0,3174	No	Kruskal-Wallis
HA specific (% IgG+ of Activated)	52	28	0.313 (0.779)	24	0.08079 (0.09381)	> 0,9999	No	Kruskal-Wallis
HA specific (% IgG+ of Atypical)	52	28	0.1906 (0.1914)	24	0.1117 (0.12)	0,3054	No	Kruskal-Wallis

Table 5 | Inflammatory markers in the children's plasma collected at the end of the dry season, segregated by clinical malaria case (MAL) or not (no MAL) in the ensuing transmission season.

Inflammation marker	n no MAL	n MAL	no MAL Median (Interquartile range)	MAL Median (Interquartile range)	p value
Hepcidin	49	29	5.1 (2.9 - 8.8)	4.3 (2.6 - 9.9)	0.5458
vWF	58	26	689.9 (510.4 - 1116)	888.9 (654.7 - 1202)	0.1878
CRP	126	62	682.1 (203.3 - 2344)	423.4 (174.2 - 2422)	0.5269

Two-tailed Mann– Whitney test.

Table 6 | Multiplex bead array quantification of cytokines and chemokines in the plasma of children collected in May, segregated by clinical malaria case (MAL) or not (no MAL) in the ensuing transmission season.

Cytokine	n no MAL	n MAL	no MAL Median (Interquartile range)	MAL Median (Interquartile range)	Adjusted P value
CXCL1 (GRO)	23	5	656.3 (432.6 - 1065.0)	293.0 (203.1 - 438.6)	<0.0001
CXCL10 (IP10)	26	5	148.2 (98.5 - 279.4)	137.3 (86.1 - 427.9)	0.9996
CCL2 (MCP1)	24	5	233.7 (135.8 - 318)	215.8 (159.6 - 362.7)	>0,9999
CCL22 (MDC)	26	6	422 (290.4 - 732.3)	369 (207.1 - 632)	0.5234
CCL3 (MIPa)	26	5	10.2 (4.6 - 19.1)	14.81 (8.2 - 19.8)	>0.9999
CCL4 (MIP1b)	27	4	15.3 (13.1 - 26.7)	19.1 (14.9 - 24.0)	>0.9999
CCL7 (MCP3)	25	6	2.8 (2.1 - 4.3)	3.2 (1.9 - 4.9)	>0.9999
EGF	27	6	9.1 (3.6 - 20.9)	8.4 (2.6 - 41.8)	>0.9999
Eotaxin	24	4	15.0 (11.7 - 27.0)	14.3 (10.8 - 19.2)	>0.9999
FGFa	22	3	3.5 (2.1 - 7.3)	3.2 (2.6 - 8.4)	>0.9999
Fractaline	26	6	5.9 (4.7 - 11.2)	4.8 (3.1 - 7.3)	>0.9999
GCFS	10	3	258.1 (37.2 - 677.7)	185.9 (64.3 - 293.6)	0.9972
GMCSF	21	5	1.3 (0.5 - 2.6)	0.9 (0.4 - 1.8)	>0.9999
IFNa2	27	6	4.7 (0.8 - 12.3)	1.5 (0.8 - 2.7)	>0.9999
IFNy	25	5	2.1(0.6-3.7)	0.7 (0.4 - 0.9)	>0.9999
IL-10	27	5	19.7 (8.5 - 25.5)	21.0 (9.2 - 26.3)	>0.9999
IL-17	25	5	1.6 (1.6-1.7)	1.6 (1.6-1.8)	>0.9999
IL-2	24	5	1.8 (1.5 - 2.6)	2.5 (1.6 - 4.4)	>0.9999
IL-3	22	3	0.4 (0.2 - 0.7)	0.5 (0.3 - 0.5)	>0.9999
IL-6	23	4	2.7 (1.6 - 6.6)	1.9 (1.1 - 6.6)	>0.9999
IL-7	17	4	2.3 (1.2 - 6.5)	4.4 (1.1 - 140.1)	>0.9999
IL-8	23	5	48.4 (25.2 - 122.5)	17.8 (8.6 - 20.2)	>0.9999
IL-9	7	1	2.8 (1.8 - 9.0)	0.2 (0.2 - 0.2)	>0.9999
IL12P40	23	4	9.6 (5.2 - 17.7)	2.6 (0.3 - 9.9)	>0.9999
IL13	11	4	0.5 (0.19 - 1.1)	7.3 (2.2 - 9.9)	>0.9999
IL15	24	6	1.7 (1.5 - 2.5)	3.1 (1.4 - 5.5)	>0.9999
IL1a	14	1	18.2 (5.9 - 33.5)	14.59(14.59-14.59)	>0.9999
IL1b	24	6	0.7 (0.3 - 1.2)	0.4 (0.3 - 2.3)	>0.9999
IL1RA	22	3	2.2 (1.0 - 9.1)	0.6 (0 - 1.144)	>0.9999
sCD40L	24	5	189.6 (92.2 - 454.9)	122.9 (50.5 - 166.9)	0.229
TGFb	27	4	1.4 (1.1 - 2.1)	1.4 (1.0 - 4.0)	>0.9999
VEGF	24	5	3.5 (1.3 - 13.9)	3.2 (1.2 - 6.6)	>0.9999

One-way ANOVA with Sidak's multiple comparisons test.

Table 7 | Expression of surface and intracellular markers of PBMCs segregated by children that got malaria (MAL) or not (no MAL) in the ensuing transmission season.

Parameter	n	n no MAL	no MAL Mean (SD)	n MAL	MAL Mean (SD)	p value	test
CD3 (% of Lymphocytes)	252	138	71.67 (9.79)	114	72.69 (6.28)	0.8786	Mann-Whitney
CD4 (% of CD3 T cells)	235	128	62.13 (6.16)	107	62.38 (6.69)	>0.9999	Kruskal-Wallis
CD8 (% of CD3 T cells)	252	137	29.05 (5.16)	115	29.7 (6.02)	>0.9999	Kruskal-Wallis
NKT cells (% of CD3 T cells)	252	137	2.49 (1.72)	115	2.63 (1.71)	>0.9999	Kruskal-Wallis
Granzyme B (% of CD8 T cells)	97	52	22.95 (12.56)	45	22.2 (12.83)	>0.9999	Kruskal-Wallis
Granzyme B (% of NKT cells)	97	52	45.61 (21.88)	45	40.75 (21.64)	0.6723	Kruskal-Wallis
Granzyme B (Geo Mean of CD8 T cells)	97	52	3661 (3354)	45	3057 (2545)	>0.9999	Kruskal-Wallis
Granzyme B (Geo Mean of NKT cells)	97	52	15769 (16607)	45	20170 (20118)	>0.9999	Kruskal-Wallis
T-bet (% of CD4 T cells)	76	47	0.13 (0.22)	29	0.15 (0.54)	0.5259	Kruskal-Wallis
T-bet (% of CD8 T cells)	80	48	3.531 (4.63)	32	2.06 (4.09)	0.1546	Kruskal-Wallis
T-bet (% of NKT cells)	47	24	0.3 (0.39)	23	0.3 (0.42)	>0.9999	Kruskal-Wallis
T-bet (Geo Mean of CD4 T cells)	74	45	1483 (522.3)	29	3913 (9493)	0.1722	Kruskal-Wallis
T-bet (Geo Mean of CD8 T cells)	77	47	1386 (459.8)	30	1381 (228.6)	>0.9999	Kruskal-Wallis
T-bet (Geo Mean of NKT cells)	77	47	1407 (260.3)	30	1538 (461.8)	0.7347	Kruskal-Wallis
IL2 (% of CD4 T cells)	71	44	0.086 (0.039)	27	0.097 (0.105)	>0.9999	Kruskal-Wallis
IL2 (% of CD8 T cells)	92	47	0.08 (0.039)	45	0.082 (0.085)	>0.9999	Kruskal-Wallis
IL2 (% of NKT cells)	92	47	3.337 (4.456)	45	2.677 (3.5)	>0.9999	Kruskal-Wallis
CD25 (% of CD4 T cells)	133	71	3.161 (2.58)	62	2.92 (2.14)	>0.9999	Kruskal-Wallis
CD25 (% of CD8 T cells)	144	76	0.029 (0.035)	68	0.024 (0.03)	>0.9999	Kruskal-Wallis
CD25 (% of NKT cells)	144	76	0.55(0.57)	68	0.47 (0.396)	>0.9999	Kruskal-Wallis
CD25 (Geo Mean of CD4 T cells)	133	71	2804 (414.9)	62	2861 (440.7)	>0.9999	Kruskal-Wallis
CD25 (Geo Mean of CD8 T cells)	119	64	3885 (7734)	55	2997 (1401)	>0.9999	Kruskal-Wallis
CD25 (Geo Mean of NKT cells)	137	72	4252 (3024)	65	5447 (5220)	0.3069	Kruskal-Wallis
IL2 (Geo Mean of CD4 T cells)	82	43	5406 (1649)	39	5615 (2054)	>0.9999	Kruskal-Wallis
IL2 (Geo Mean of CD8 T cells)	91	47	5239 (1578)	44	5222 (1699)	>0.9999	Kruskal-Wallis
IL2 (Geo Mean of NKT cells)	91	47	5595 (3902)	44	6362 (5795)	>0.9999	Kruskal-Wallis
CD19 (% of Lymphocytes)	249	135	15.86 (4.39)	114	16.28 (3.72)	0.3966	Mann-Whitney
Activated MBCs (% of CD19 B cells)	225	118	4.071 (2.6)	107	3.52 (1.81)	>0.9999	Kruskal-Wallis
Classical MBCs (% of CD19 B cells)	225	118	13.96 (5.77)	107	14.35 (6.11)	>0.9999	Kruskal-Wallis
Naive (% of CD19 B cells)	225	118	69.24 (9.85)	107	71.09 (8.81)	>0.9999	Kruskal-Wallis
Atypical MBCs (% of CD19 B cells)	225	118	12.73 (6.41)	107	11.05 (4.94)	>0.9999	Kruskal-Wallis
FCRL5 (% of Activated MBCs)	50	21	20.15 (9.97)	29	19.77 (8.00)	>0.9999	Kruskal-Wallis
FCRL5 (% of Classical MBCs)	50	21	2.13 (1.77)	29	2.21 (1.57)	>0.9999	Kruskal-Wallis
FCRL5 (% of Naive MBCs)	50	21	0.51 (0.43)	29	0.48 (0.71)	>0.9999	Kruskal-Wallis
FCRL5 (% of Atypical MBCs)	50	21	24.4 (10)	29	22.3 (10.65)	>0.9999	Kruskal-Wallis
Granzyme B (% of Activated MBCs)	97	52	24.64 (19.09)	45	22.89 (20.87)	>0.9999	Kruskal-Wallis
Granzyme B (% of Classical MBCs)	97	52	8.63 (13.47)	45	4.48 (8.17)	0.2632	Kruskal-Wallis
Granzyme B (% of Naive MBCs)	97	52	2.95 (6.20)	45	2.33 (5.89)	>0.9999	Kruskal-Wallis
Granzyme B (% of Atypical MBCs)	97	52	6.84 (9.41)	45	7.14 (13.37)	>0.9999	Kruskal-Wallis
IL2 (Geo Mean ofActivated MBCs)	91	47	869.7 (273.6)	44	859.8 (296.4)	>0.9999	Kruskal-Wallis
IL2 (Geo Mean ofClassical MBCs)	91	47	778.9 (243.5)	44	776 (261.7)	>0.9999	Kruskal-Wallis
IL2 (Geo Mean ofNaive MBCs)	91	47	573.1 (171.4)	44	573.7 (184.7)	>0.9999	Kruskal-Wallis
IL2 (Geo Mean ofAtypical MBCs)	91	47	674.8 (213.7)	44	675.4 (216.4)	>0.9999	Kruskal-Wallis
Granzyme B (Geo Mean of Activated MBCs)	96	52	4009 (3263)	44	4127 (4147)	>0.9999	Kruskal-Wallis
Granzyme B (Geo Mean of Classical MBCs)	97	52	2650 (2231)	45	2100 (1689)	>0.9999	Kruskal-Wallis
Granzyme B (Geo Mean of Naive MBCs)	97	52	2051 (1659)	45	1638 (1284)	>0.9999	Kruskal-Wallis
Granzyme B (Geo Mean of Atypical MBCs)	97	52	2245 (1755)	45	2206 (2322)	>0.9999	Kruskal-Wallis
CD25 (Geo Mean of Activated MBCs)	91	47	91.96 (33.99)	44	90.55 (32.47)	>0.9999	Kruskal-Wallis
CD25 (Geo Mean of Classical MBCs)	91	47	164.9 (29.07)	44	164 (27.46)	>0.9999	Kruskal-Wallis
CD25 (Geo Mean of Naive MBCs)	91	47	56.25 (15.02)	44	56.28 (17.03)	>0.9999	Kruskal-Wallis
CD25 (Geo Mean of Atypical MBCs)	91	47	40.88 (11.28)	44	37.58 (9.47)	>0.9999	Kruskal-Wallis
CD56-CD16+ (% of CD3-CD19-	249	135	20.51 (14.23)	114	19.5 (12.97)	>0.9999	Kruskal-Wallis
CD56dimCD16- (% of CD3-CD19-)	249	135	2.16 (2.68)	114	1.729 (1.21)	>0.9999	Kruskal-Wallis
CD56dimCD16+ (% of CD3-CD19-	249	135	42.21 (20.65)	114	46.38 (18.41)	>0.9999	Kruskal-Wallis
CD56brightCD16- (% of CD3-CD19-)	249	135	3.3 (2.56)	114	3.155 (2.04)	>0.9999	Kruskal-Wallis
CD56brightCD16+ (% of CD3-CD19-	249	135	1.43 (1.35)	114	1.41 (1.35)	>0.9999	Kruskal-Wallis
Granzyme A (% of CD56-CD16+)	50	21	19.27 (19.15)	29	34.61 (30.18)	0.2815	Kruskal-Wallis
Granzyme A (% of CD56dimCD16-)	50	21	10.04 (8.16)	29	19.28 (16.75)	0.1758	Kruskal-Wallis
Granzyme A (% of CD56dimCD16+)	50	21	23.65 (21.51)	29	37.63 (25.21)	>0.9999	Kruskal-Wallis
Granzyme A (% of CD56brightCD16-)	50	21	4.61 (5.71)	29	8.539 (10.82)	0.2781	Kruskal-Wallis
Granzyme A (% of CD56brightCD16+)	50	21	11.98 (14.05)	29	21.26 (20.06)	0.3723	Kruskal-Wallis
Granzyme B (% of CD56-CD16+)	97	52	56.81 (22.59)	45	52.64 (20.57)	>0.9999	Kruskal-Wallis
Granzyme B (% of CD56dimCD16-)	97	52	17.07 (10.95)	45	15.46 (12.89)	>0.9999	Kruskal-Wallis
Granzyme B (% of CD56dimCD16+)	97	52	74.37 (21.23)	45	72.71 (20.03)	0.8453	Kruskal-Wallis
Granzyme B (% of CD56brightCD16-)	97	52	8.17 (8.13)	45	4.25 (5.1)	>0.9999	Kruskal-Wallis
Granzyme B (% of CD56brightCD16+)	97	52	25.95 (25.11)	45	21.86 (21.97)	>0.9999	Kruskal-Wallis
MΦ(CD14 +) (% of PBMCs)	244	135	6.022 (4.311)	109	6.0 (4.76)	0.6688	Mann-Whitney
Classical MΦ (% CD14 +)	244	135	72.33 (15.96)	109	72.58 (13.93)	>0.9999	Kruskal-Wallis
Inflammatory MΦ (% of CD14 +)	244	135	13.22 (8.55)	109	12.45 (7.06)	>0.9999	Kruskal-Wallis
Patrolling MΦ (% of (CD14 +)	244	135	13.57 (10.97)	109	13.81 (10.85)	>0.9999	Kruskal-Wallis
Activated (% of B cells)	52	33	6.99 (5.59)	19	4.62 (4.29)	>0.9999	Kruskal-Wallis
Classical (% of B cells)	52	33	18.77 (4.54)	19	18.55 (7.14)	>0.9999	Kruskal-Wallis
Atypical (% of B cells)	52	33	13.4 (6.34)	19	9.46 (3.67)	0.7894	Kruskal-Wallis
Naive (% of B cells)	52	33	60.83 (12.57)	19	67.37 (8.68)	>0.9999	Kruskal-Wallis
Pf specific (IgM+ of non-naive mature B cells)	52	33	0.17 (0.16)	19	0.18 (0.13)	0.4143	Mann-Whitney
HA specific (IgM+ of non-naive mature B cells)	52	33	0.046 (0.045)	19	0.05 (0.04)	0.2505	Mann-Whitney
Pf specific (IgG+ of non-naive mature B cells)	52	33	0.162 (0.22)	19	0.15 (0.16)	0.9887	Mann-Whitney
HA specific (IgG+ of non-naive mature B cells)	52	33	0.13 (0.15)	19	0.14 (0.12)	0.8542	Mann-Whitney
Pf specific (% IgG+ of Activated)	52	33	0.22 (0.29)	19	0.26 (0.34)	>0.9999	Kruskal-Wallis
Pf specific (% IgG+ of Classical)	52	33	0.21 (0.33)	19	0.15 (0.17)	>0.9999	Kruskal-Wallis
Pf specific (% IgG+ of Atypical)	52	33	0.06 (0.07)	19	0.083 (0.082)	>0.9999	Kruskal-Wallis
HA specific (% IgG+ of Activated)	52	33	0.18 (0.54)	19	0.25 (0.663)	>0.9999	Kruskal-Wallis
HA specific (% IgG+ of Classical)	52	33	0.098 (0.078)	19	0.12 (0.102)	>0.9999	Kruskal-Wallis
HA specific (% IgG+ of Atypical)	52	33	0.14 (0.17)	19	0.17 (0.16)	>0.9999	Kruskal-Wallis

3.4 Genetic diversity of *P. falciparum* parasites is maintained during the year

We wondered if *P. falciparum* parasites that survive during the dry season could be genetically distinct from the ones causing clinical malaria during the transmission season. To answer this, we measured the size of the merozoite surface protein 2 gene (*msp*2), which is a very polymorphic gene that is often used to discriminate different *P. falciparum* genotypes, through nested PCR followed by capillary electrophoresis (Liljander et al., 2009; Snounou et al., 1999). We compared paired samples from 93 individuals carrying *P. falciparum* asymptomatic infections at the beginning (January) and end (May) of the dry season, with 136 samples from clinical malaria episodes in the following transmission season. We observed that the number of clones detected per individual, and the percentage of individuals with different number of clones was similar between samples isolated during the dry season or during clinical malaria in the ensuing transmission season (Figure 9 a and b). Moreover, the size and distribution of msp2 clones was identical between samples isolated during the dry season or during clinical malaria in the transmission season, with the most prevalent clone sizes being the same at any of the time-points analysed (Figure 9 c and d). We observed that *P. falciparum* genetic diversity circulating during the dry season is similar to the one causing clinical disease in the following transmission season.

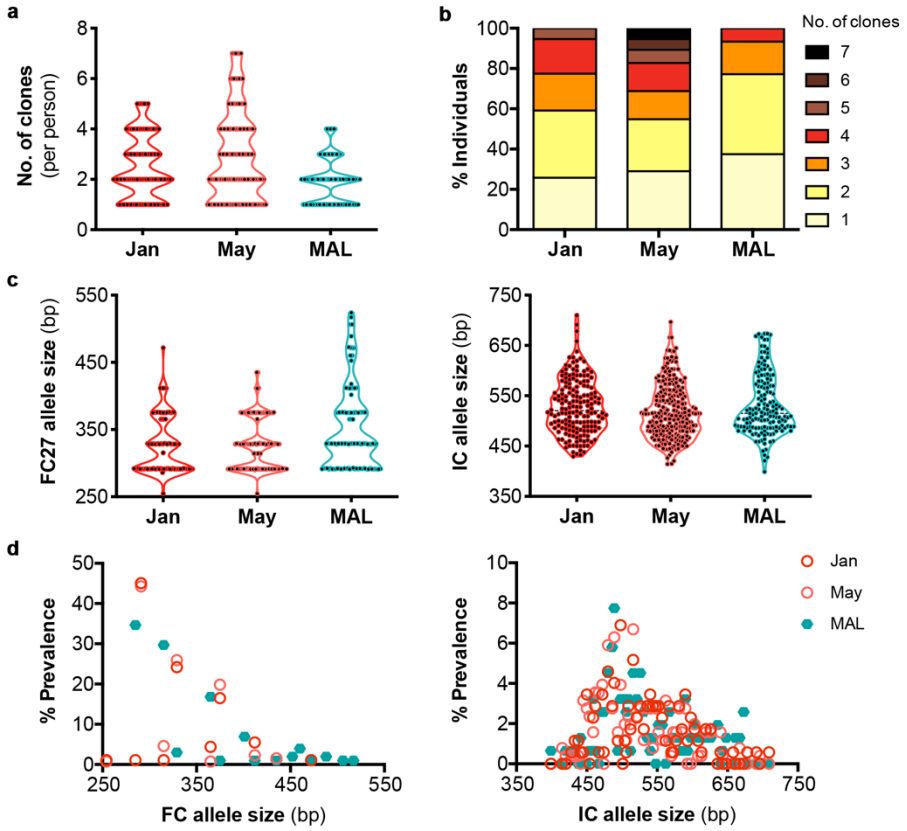

Figure 9 | *P. falciparum* genetic diversity in dry season and clinical malaria determined by *msp*2 capillary electrophoresis

a, Number of *P. falciparum* clones in asymptomatic children in the beginning (Jan) and end (May) of the dry season, and at first clinical malaria case (MAL) in the ensuing transmission season **b**, Proportion of individuals with different numbers of *P. falciparum* clones in the beginning (Jan) and end (May) of the dry season, and during first clinical malaria (MAL). **c**, Size of the FC27 (left) and IC/3D7 (right) alleles in the beginning (January) and end (May) of the dry season and clinical malaria cases (MAL). **d**, Prevalence of *msp2* clone sizes of FC27 (left) and IC/3D7 (right) allelic families in January (n = 93), May (n = 93) and clinical malaria (MAL, n = 136). All violin plots show all data points; Mood's median test. Figure from Andrade *et al.,* 2020.

3.5 Transcriptome of circulating *P. falciparum* at the end of the dry season differs from that of malaria-causing *P. falciparum* during transmission season

To understand if the transcriptome of circulating *P. falciparum* in the dry season was different from that of parasites causing clinical malaria during transmission season, we performed RNA sequencing of leucocyte-depleted blood from 12 children with persistent asymptomatic

P falciparum at the end of the dry season (May), and from 12 age- and gender- matched children presenting with their first clinical malaria case in the ensuing transmission season (MAL). Principal component (Figure 10 a) and unsupervised clustering (Figure 10 b) analyses showed segregation of transcription profiles based on seasonality. We found 1607 differential expressed genes (DEGs), 1131 transcripts up-regulated, and 476 transcripts down-regulated in the dry season compared to clinical malaria samples, at a false discovery rate threshold of 5% (Figure 10 c). We validated these results by RT-qPCR of eight high expressing DEGs with variable functions and observed that correlation between the two methods was highly significant (r^2= 0.929, Figure 10 d). Functional and gene ontology analyses revealed a significant enrichment of transcripts involved in cellular processes related with several metabolic pathways and also with phagosome, DNA replication or homologous recombination of the dry season DEGs (Figure 10 e). 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 compared to parasites from clinical malaria (MAL) in the transmission season (Figure 10 f). To understand if the host environment could promote the different metabolic differences observed at the end of the dry season and malaria-causing *P. falciparum*, we used liquid chromatography–mass spectrometry to profile both hydrophilic and hydrophobic metabolites from the plasma of 12 asymptomatic 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 (Figure 10 g). However, it was difficult to conclude what could be seasonal or parasite induced metabolic alterations, due to the very high parasite burden in clinical-malaria samples (Figure 6 d) and difficult normalization of metabolite levels to parasite burden.

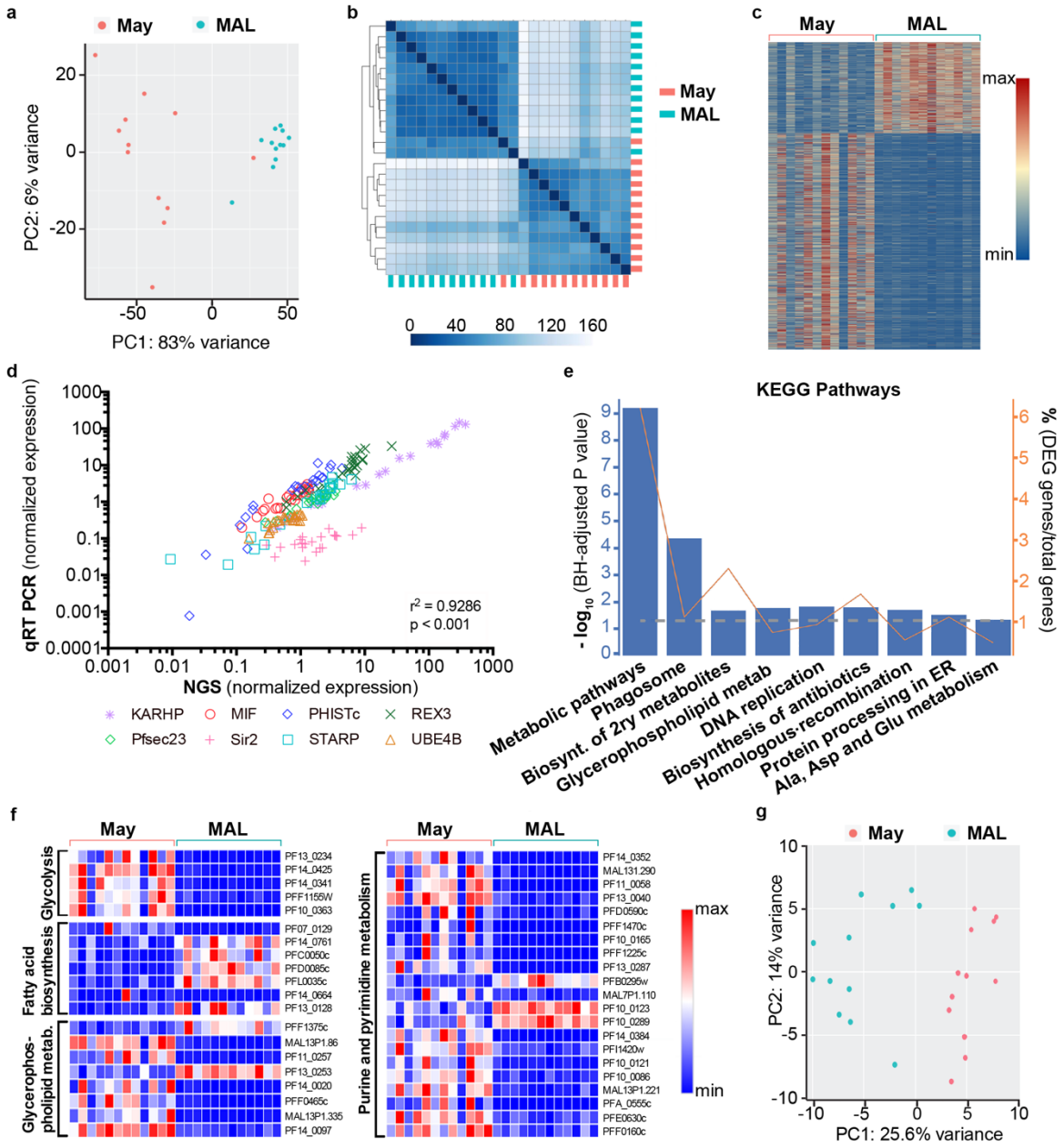

Figure 10 | Transcriptome of circulating *P. falciparum* at the end of the dry season and from malaria-causing P. falciparum during transmission season.

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) and at the first clinical malaria case (MAL) in the ensuing transmission season. **d**, RT-qPCR validation of RNA-Seq data for eight labelled DEGs (n= 24). *P* and r^2 determined by Pearson correlation. **e**, Summary of KEGG pathways significantly enriched with DEGs. Blue bars indicate *P*adj for enrichment of each pathway. Grey dashed line indicates the threshold *P* = 0.05. Orange line indicates the percentage of genes in each pathway present in the DEG list. **f**, Heatmap showing normalized reads of DEGs involved

in different metabolic pathways (rows) for each subject (columns) from *P. falciparum* at the end of the dry season (May, n=12) and at the first clinical malaria (MAL, n=12). **g**, Principal components analysis of target metabolites' data of plasmas from asymptomatic *P. falciparum* carriers at the end of the dry season (May, n=12) and from clinical malaria cases (MAL, n=12). Adapted from Andrade et al., 2020.

3.6 *P. falciparum*'s replication is not impaired during the dry season and parasites are more developed than *P. falciparum* during clinical malaria

Since *P. falciparum* parasitaemias do not increase to cause clinical disease during the dry season, we inquired if parasite replication ability could be decreased during this period. To this end, we cultured *P. falciparum* parasites from individuals found rapid diagnostic test (RDT) + in the beginning (January), middle (March) and end (May) of the dry season and during the first clinical malaria episode in the ensuing transmission season. We measured both parasitaemia and parasite development by flow cytometry at 0, 16, 24, 30, 36 and 48 h of in vitro culture of samples collected at the end of the dry season in ~40 individuals and from clinical malaria in ~30 individuals. We observed that at any given time-point during the year parasitaemia increased between 2 and 5 – fold: 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, we observed that the number of hours in cultured needed for *P. falciparum* to increase its parasitaemia was shorter in *P. falciparum* collected during the dry season than in clinical malaria cases' samples (Figure 11 a). In agreement with this observation, we were able to observe mature schizonts after 16 or 24 h of culture and ring stages after 30 or 36h in the dry season, whereas in *P. falciparum* parasites from malaria patients in the transmission season, we could only identify mature schizonts after 36 h in culture and ring stages after 48 h in culture (Figure 11 b). Moreover, when we calculated the number of hours in culture at which the highest increase in parasitaemia could be detected, we observed that this value decreased during the dry season and was highest during malaria cases in the transmission season – January 26.4 h, 95% CI (24.5, 28.3); March, 24.9h, 95% CI (23.9, 25.9); May, 22.7h, 95% CI (20.8, 24.6); MAL 44.0 h, 95% CI (41.5, 46.6) (Figure 11 c). We then determined the number of merozoites per multinucleated schizont by flow cytometry and observed that this number was similar in dry season and malaria-causing *P. falciparum* (Figure 11 d). Additionally, we applied a maximum likelihood estimation (MLE) statistical method to determine the average age of

parasites in each sample in hours post invasion (hpi) (previously described in Lemieux et al., 2009) to the RNAseq data described in Figure 10. This method estimated that the parasites circulating during the dry season had a transcriptional profile of ~17 hpi, 95%CI (14.05, 20.8), whereas the ones causing clinical malaria in the transmission season had a transcriptional profile of ~7 hpi 95% CI (6.5, 7.7) (Figure 11 e). In agreement with an older hpi transcriptional profile in the dry season, we observed by light microscopy of Giemsa-stained thick blood smears that samples collected during the dry season had more developed trophozoite stages, whereas only small ring stages were found circulating during clinical malaria in the transmission season (Figure 11 f and g). Accordingly, we also determined by flow cytometry that the percentage of non-ring *P. falciparum* parasites circulating during the dry season was higher than during clinical malaria in the ensuing transmission season (Figure 11 h). We further performed an in vitro experiment to mimic the narrow and short inter-endothelial slits of the human spleen and observed that malaria-causing *P. falciparum* fails to be retained in the spleen-like filter at 0, 6 and 18 h in culture, whereas *P. falciparum* collected at the end of the dry season in May is retained immediately after blood draw at 0 h (data not shown, Andrade et al., 2020). Further mathematical modelling has shown that differences in parasite age in circulation can be explained by a difference in cyto-adhesion (data not shown, Andrade et al., 2020) The circulation of older parasites within their ~48 h intraerythrocytic developmental cycle without adhesion to the endothelium during the dry season allows for a higher splenic clearance maintaining parasitaemia low and below the immunological radar. In summary, we observed that replication of *P. falciparum* is not impaired during the dry season. We observed that dry season *P. falciparum* increased their parasitaemia in culture faster than malaria-causing ones, due to being more developed within their intra-erythrocytic cycle in circulation.

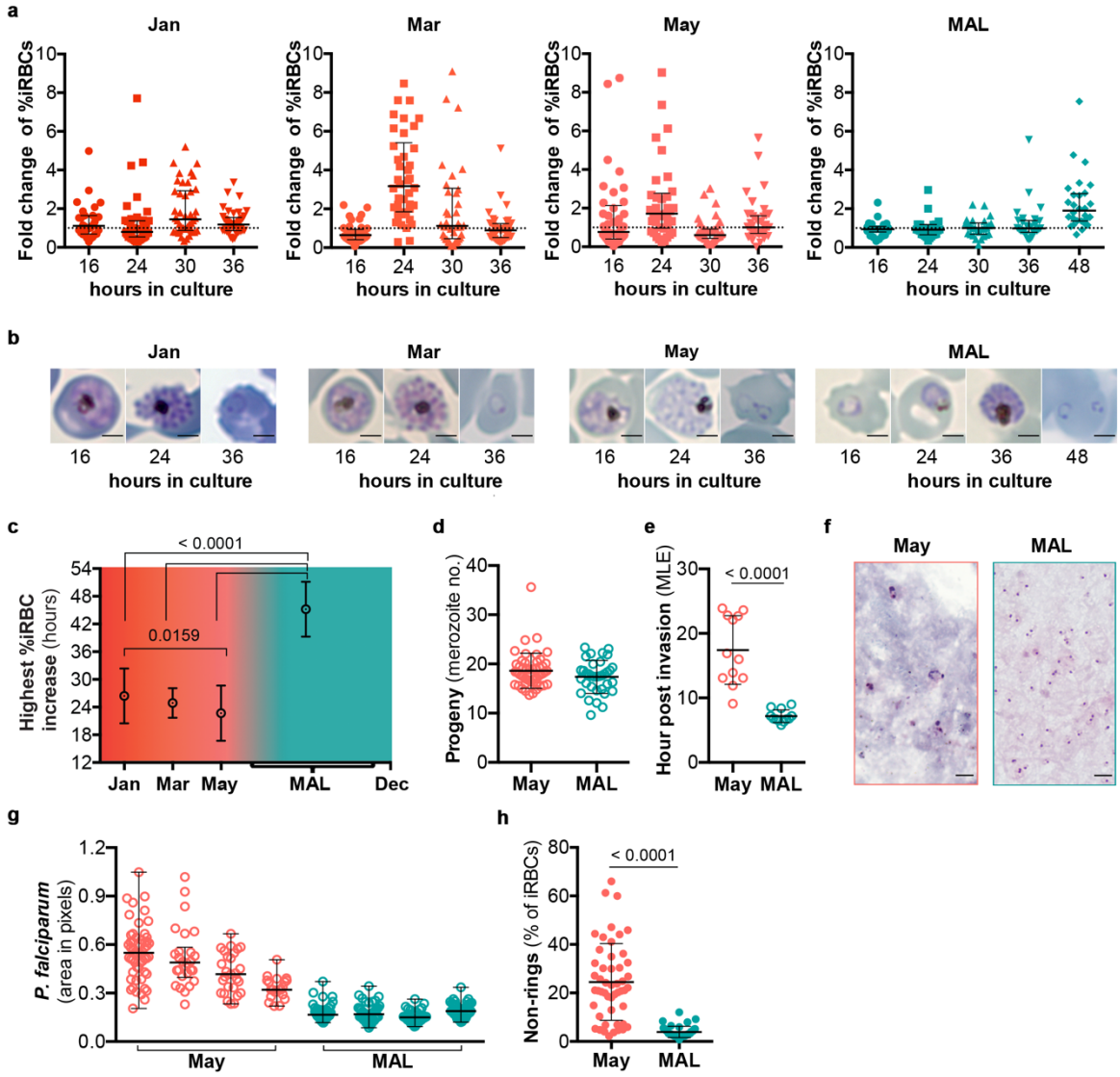

Figure 11 | Replication and developmental stages of *P. falciparum* during the dry season and clinical malaria

a, Parasitaemia (infected red blood cells, iRBCs) fold change determined by flow cytometry at 16, 24, 30, 36 and 48 h after culture of *P. falciparum* collected at the beginning (January), middle (March) or end (May) of the dry season and during 1st clinical malaria case (MAL) in the transmission season. Fold change is defined as %iRBC t(n)/%iRBC t(n−1). Data indicate median ± IQR. Dashed line at fold change of 1. **b**, Light microscopy of giemsa-stained thin blood smears of *P. falciparum* parasites cultured for 16, 24, 36 or 48 h during the dry season (January, March and May) or clinical malaria cases (MAL). Scale bar, 2 µm **c**, Time of highest increase of *P. falciparum* parasites' parasitaemia during in vitro culture from children in January (n = 39), March (n = 42) and May (n = 40) during the dry season, and clinical malaria cases (MAL, n = 27). Data indicate mean ± SD; one-sided Dunn's Kruskal–Wallis multiple

comparisons test. **d**, Number of merozoites determined by flow cytometry at the end of the dry season (May, *n* = 50) and clinical malaria (MAL, *n* = 35) during transmission season. Data indicate mean ± SD; two-tailed Mann– Whitney test. **e**, Maximum likelihood estimation (MLE) of the hpi of dry season (May, *n* = 12) and clinical malaria (MAL, *n* = 12) parasites. Data indicate mean ± SD.; two-tailed Mann–Whitney test. **f**, Light microscopy of giemsa-stained thick blood films of *P. falciparum* parasites collected from children at the end of the dry season (May) and at their first clinical malaria (MAL). Scale bar, 5 µm. **g**, Area of *P. falciparum* measured from Giemsa-stained thick smears at the end of the dry season (May) and in malaria cases (MAL) in the transmission season. Data indicate median ± IQR; two-tailed Mann–Whitney test, $P < 0.0001$. IQR, interquartile range. **h**, Percentage of circulating non-ring stage iRBCs at the end of the dry season (May, n= 50) and during 1st malaria case during transmission season (MAL, n=39) determined by flow cytometry. Adapted from Andrade et al., 2020

3.7 *P. falciparum* growth and host cell remodelling is similar in different season plasmas

Our data indicates that *P. falciparum* parasites collected during the dry season and parasites that cause malaria during transmission season have very different transcriptional profiles (Figure 10 and Andrade et al., 2020), and processes such as DNA replication appear affected in dry vs. transmission season in *P. falciparum*. Although these differences might be a result of circulation of more mature parasites during the dry season, we wondered what impact the host environment would have in driving less virulent parasites in this time. To have a better insight as to how the host environment affects parasite growth, we cultured *P. falciparum* 3D7 in low-glucose RPMI medium, in which the glucose concentration is close to the reference value in human plasma, and we replaced the lipid source (Albumax II) usually added to *P. falciparum* in continuous culture by human serum or plasma. To allow *P. falciparum* to retrieve maximal nutrient availability from the host plasma, we then determined the highest concentration of plasma we could use to culture *P. falciparum* in vitro. We tested several different concentrations of human plasma in culture. We first cultured *P. falciparum* 3D7 parasites in regular RPMI or in low glucose RPMI, supplemented with either Albumax II, 10% German adults' plasma or 10% Malian children plasma. We measured parasitaemia after one cycle and observed that *P. falciparum* parasites increased their parasitaemias in all conditions, albeit a

lower increase was seen when parasites were grown in plasma collected from Malian children than in plasma from German donors or complete RPMI (Figure 12 a). Then, we cultured 3D7 *P. falciparum* in 20, 50, 70 or 98% of either German or Malian plasma. We observed that the increase in parasitaemia of *P. falciparum* is inversely proportional to the percentage of plasma supplementation (Figure 12 b). Having this in mind and knowing that plasma concentration in the human host is ~50%, we then aimed to understand the optimal concentration of plasma between 20 and 50%. To this end, we cultured 3D7 and Pf2004 *P. falciparum* strains in 20, 30, 40 or 50% supplementation with German and Malian plasmas. For both strains, we observed that the increase in parasitaemia was higher when grown with German plasma. Albeit not statistically significant, we observed that both strains had showed a lower increase in parasitaemia as plasma concentration increased in culture (Figure 12 c and d). Due to low availability of plasma volume and the observed optimal concentration between 20 and 50% of supplementation, we decided to use 25% plasma supplementation for all further experiments.

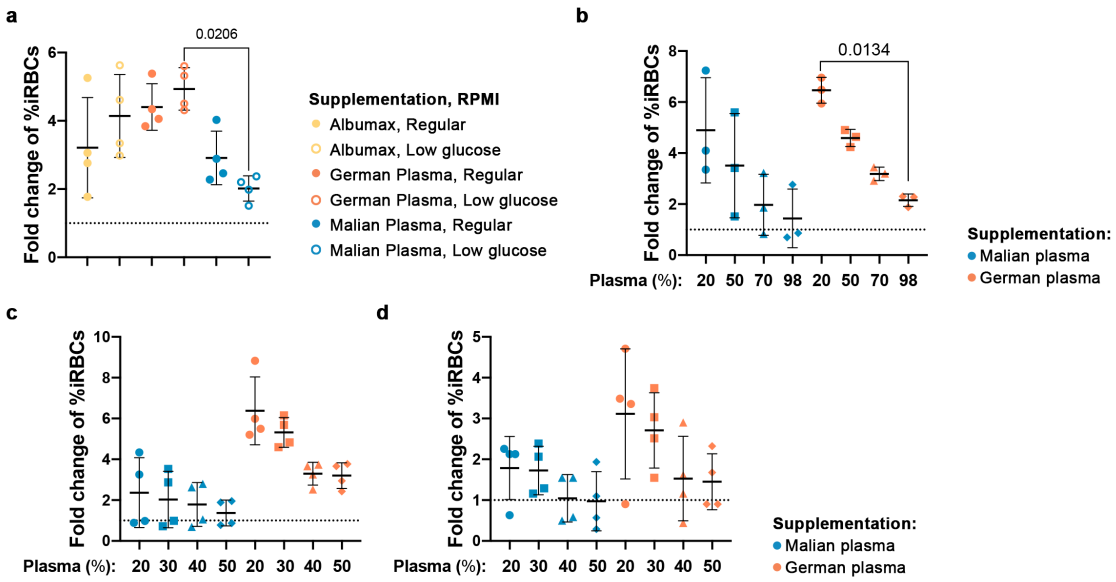

Figure 12 | *P. falciparum* growth in different RPMI media and plasma concentrations

a,3D7 *P. falciparum* strain grown in regular or low glucose RPMI, supplemented with Albumax II, or 10% Malian or German plasma. **b,** 3D7 *P. falciparum* strain grown in low glucose RPMI supplemented with 20,50,70 or 98% Malian or German plasma. **c,** 3D7 *P. falciparum* and **d,** Pf2004 *P. falciparum* grown in low glucose RPMI supplemented with 20,30,40 or 50% Malian or German plasma; Fold change of %iRBCs is **a,** %iRBCs at 46h divided by %iRBCs at 0h; **b, c,** and **d,** %iRBCs at 55h divided by %iRBCs at 0h; Error bars indicate mean ± s.d.; one-sided Dunn's Kruskal–Wallis multiple comparisons test.

We cultured several *P. falciparum* laboratory-adapted strains in low-glucose RPMI supplemented with 25% plasma collected from children at the beginning (January) and end of the dry season (May) in 2012 for one replicative cycle, measuring parasitaemia and development by flow cytometry following 52-62 h in culture. We observed no differences when 3D7 parasites were grown in December or May plasma (Figure 13 a). 3D7 is a strain that was adapted to the laboratory conditions in 1980's (Walliker et al., 1987)and since then has suffered mutations and deletions in its genome (Gardner et al., 2002). To overcome this potential problem, we used a more recently adapted *P. falciparum* strain, Pf2004, isolated from a patient in Ghana (Elliott et al., 2007) and reported to sense changes in human serum that induce gametocytaemia (Brancucci et al., 2017). We grew Pf2004 parasites in low-glucose RPMI supplemented with 25% plasma, and we did not observe any differences in growth between any of the conditions tested (Figure 13 b). We proceeded with growth of Dd2 and HB3 *P. falciparum* parasite strains in plasmas collected at the beginning or end of the dry season. Dd2 and HB3 are *P. falciparum* strains isolated from different geographic regions that differ in their drug resistance phenotypes. Dd2 is resistant to chloroquine, whereas Hb3 is fully sensitive (Wellems et al., 1990). We did not observe any differences in growth when Dd2 or HB3 parasites were grown with 25% of December or May plasma supplementation (Figure 13 c and d). In 2018, we laboratory-adapted and cloned one *P. falciparum* strain collected from a malaria case from Kalifabougou, Mali- PfMali2k1. Since this was the most recently adapted strain we could use, and it originated from the same cohort study the plasmas used, it was ideal to perform these experiments. We observed that although parasites could grow in most of the Malian plasmas, we observed no differences when parasites were grown in December, May or German plasma (Figure 13 e). As culture for only one cycle could possibly not be enough to induce a parasite encoded mechanism allowing *P. falciparum* to sense the presence of the different plasmas in culture, we decided to culture Pf2004 *P. falciparum* for 3 replicative cycles (166 hours). We observed that Pf2004 parasitaemia increased more when cultured in German plasma than December or May Malian plasmas. However, after 3 cycles in culture, we did not detect any differences in parasitaemia when parasites were grown in December or May plasmas (Figure 13 f). We then decided to compare *P. falciparum* in vitro growth in May (end of the dry season) or October (peak of transmission season) plasmas to test whether *P. falciparum* can detect seasonality and control its growth accordingly. We cultured ex vivo *P. falciparum* freshly collected from asymptomatic children in the beginning (January) and end

(May) of the dry season, and during the ensuing transmission season when children reported their 1st malaria case. We cultured these parasites for 48 h in low glucose RPMI supplemented with 25% antibody-depleted plasma from a pool of matched 27 donors from the end of the dry season (May) or peak of transmission season (October), or in complete RPMI supplemented with Albumax II. We observed that parasites collected during the dry season increased their parasitaemia before 48 h in culture (Figure 13 g), in accordance with our previous results showing that dry season parasites circulate longer than the ones causing clinical malaria in the transmission season (Figure 11). We did not observe differences in parasitaemia growth or parasite development when *P. falciparum* parasites collected either during the dry season or malaria cases in the transmission season were grown in plasmas from the end of the dry season or peak of transmission season plasma (Figure 13 g). Then, we inquired if we could detect alterations in host cell remodelling when parasites were cultured in plasmas from different seasons. We cultured the knobby-rich *P. falciparum* FCR3 parasites in a pool of antibody-depleted plasmas from the end of the dry season (May) or peak of transmission season (October). We performed both scanning and transmission electron microscopy. We found no differences in knob density or diameter, number of Maurer's clefts, length or distance to the plasma membrane in *P. falciparum* parasites grown in May or October antibody-depleted plasma (Figure 13 h, i, j, k, and l). In summary, in the conditions tested, we could not detect any difference in replication, development, or host cell remodelling when *P. falciparum* parasites were grown in plasmas from the beginning or end of the dry season, or peak of transmission season plasmas. In the conditions tested, we found no differences in growth and host cell remodelling when different *P. falciparum* strains or field isolates were grown in dry or transmission season plasmas.

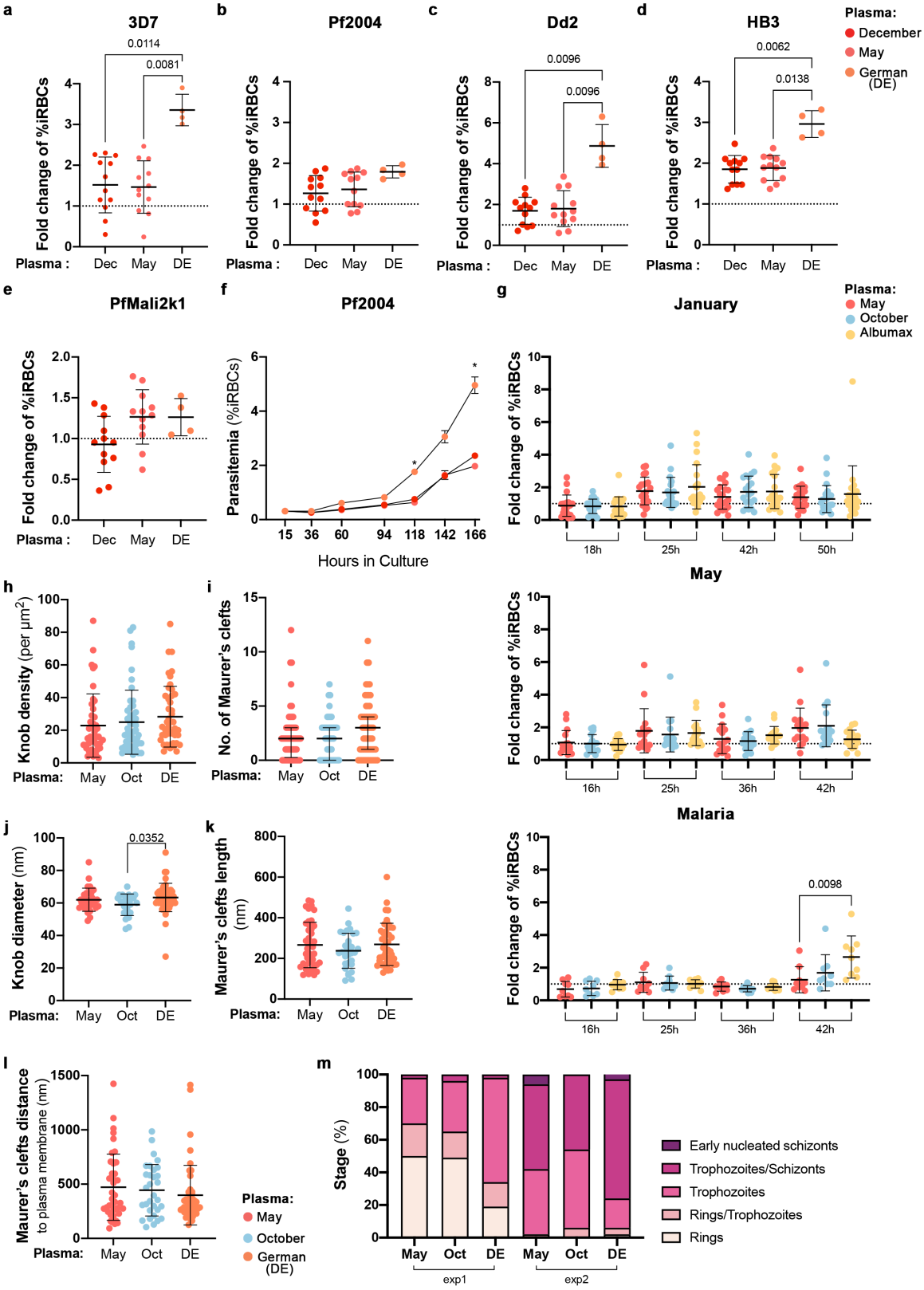

Figure 13 | *P. falciparum* cultured in plasmas from different time-points

P. falciparum growth over 1 cycle in culture with plasma from beginning (December, n=12) or end (May n=12) of the dry season or German (n=4) plasma in **a,** 3D7; **b,** Pf2004; **c,** Dd2; **d,** HB3; and **e**, PfMali2k1;

Each dot corresponds to growth in one individual plasma **f**, Pf2004 *P. falciparum* growth for over 3 cycles in culture with pooled plasma from 12 children in beginning (December) or end (May) of the dry season or from 4 German adults' plasma; Fold change of %iRBCs is **a,** and **b,** %iRBCs at 60h divided by %iRBCs at 15 h. **c,** and **d,** %iRBCs at 52h divided by %iRBCs at 2 h. **e,** %iRBCs at 62h divided by %iRBCs at 0h; Dashed line at fold change of 1. Error bars indicate mean ± s.d.. **f,** * shows a p value = 0,0219 between German and May plasma at 118 and 166h in culture; one-sided Dunn's Kruskal–Wallis multiple comparisons test. **g**, Parasitaemia fold change of *P. falciparum* collected in a, January (n=21), May (n=16) or during 1st malaria case during transmission season (n=9) when grown in May or October 25% antibody-depleted plasma. Fold change is defined as %iRBC t(n)/%iRBC t(n−1). Error bars indicate mean ± s.d.; one-sided Dunn's Kruskal–Wallis multiple comparisons test within each group of hours in culture. Dashed line at fold change of 1. **h**, Knob density measured by scanning electron microscopy. **i**, Number of Maurer's clefts; **j**, knob diameter. **k**, Maurer's cleft length; and **l**, Maurer's cleft distance to the plasma membrane measured by transmission electron microscopy in *P. falciparum* parasites grown in May, October or German (DE) 25% antibody-depleted plasma. **m,** Developmental stages determined by Giemsa-stained light microscopy of the two independent experiments used for electron microscopy analysis. **h**, **j**, **k**, **l**, Mean ± SD. **i**, Median ± IQR. One-sided Kruskal-Wallis multiple comparisons test **(h- m)** n= 2 independent experiments, with 1 replicate analysed each.

3.8 *P. falciparum* clones maintained asymptomatically in dry season do not cause clinical malaria in the ensuing transmission season

Although the risk of clinical malaria is reduced in individuals carrying asymptomatic infections at the end of the dry season (Portugal et al., 2017; Sonden et al., 2015), these children can still became ill with malaria in the ensuing transmission season. We wondered if the parasites that were carried asymptomatically during the 6-month dry season would be present in the same host at their first clinical malaria episode in the ensuing transmission season. To answer this question, we first compared *P. falciparum mps*2 allele sizes by capillary electrophoresis and *ama*1 sequences by amplicon sequencing in children that were infected at the end of the dry season and that had a clinical malaria case in the ensuing transmission season. We used 7 samples from children that were PCR+ positive at the end of the dry season in 2012 and became ill with malaria in the ensuing transmission season to compare the *P. falciparum mps*2 allele sizes, and despite the low sample size we observed that in 6 of the 7 children (85,7%) malaria

was caused by clones that were not previously present in May, at the end of the dry season (Supplementary Figure 3). However, in one child we found a common clone between May and malaria samples, and also two clones were found during clinical malaria that had not been there in the end of the dry season, so it is not clear which clone might have caused disease. Moreover, this clone that was present in the same child at both time-points was the most frequent clone at any given time-point analysed (FC 291 bp), suggesting that re-transmission was likely to occur.

With the *ama*1 amplicon sequencing approach we compared the *P. falciparum* clones present in children at the end of the dry season in May 2013 and in clinical cases of malaria during the ensuing transmission season in 64 children with paired samples and observed that in 52 of these children (81,3%) malaria was necessarily caused by a new clone (Supplementary Figure 4), as none of the clones present at the end of the dry season were detected when the clinical malaria occurred. Moreover, of the 12 children sharing one or more clones at both time points, 7 of them had malaria within 3 months after the dry season (58,3%), suggesting that it could be a clone that persisted during this time, and not re-transmitted. However, of the total of 17 clones that were present in both time-points in these children, 16 were haplotypes that were found frequently (3,5-9% of all infections), whereas only 1 child had one clone that was considered "rare", only found in 0,4% of all infections. A more frequent haplotype at the population level, might be indicative of re-transmission. Nonetheless, none of the clones that were present at both time-points, were single during a malaria case, making it difficult to define if they were causing disease or if disease was caused by another clone. Our data indicates that it is unlikely that *P. falciparum* parasites that are kept asymptomatically during the dry season cause clinical malaria in the transmission season.

Apart from the 64 paired samples, we also compared the number of clones present in in an extra 42 children at the end of the dry season (total 106) in May 2013 with the 64 samples from clinical cases of malaria during the ensuing transmission season to compare the number of clones present at either time-point. In agreement the *msp*2 capillary electrophoresis presented in section 3.4 (Figure 9 and Andrade et al., 2020), the number of clones detected by *ama*1 amplicon sequencing per child did not significantly differ between samples collected at the end of the dry season or during clinical malaria nor did the percentage of individuals with different number of clones (Figure 14).

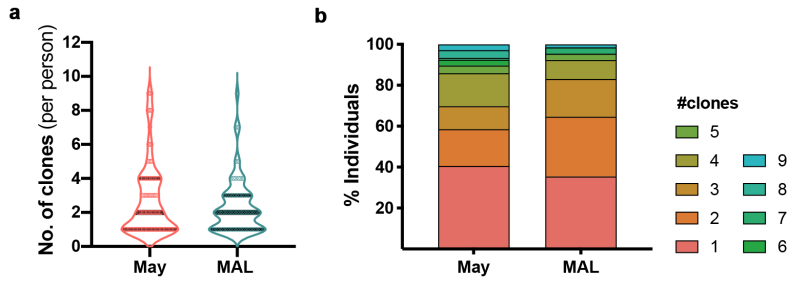

Figure 14 | *P. falciparum* complexity of infection in dry season and clinical malaria determined by *ama*1 amplicon sequencing

a, Number o f *P. falciparum* clones and **b**, Proportion of individuals with different numbers of *P. falciparum* clones at the end (May) of the dry season and clinical malaria cases (MAL). Two-tailed Mann-Whitney test.

3.9 *P. falciparum*'s asymptomatic infections during transmission season are similar to asymptomatic dry season infections

We observed that *P. falciparum* asymptomatic infections collected during the dry season were not impaired in replication in vitro (Figure 11), and we observed older parasites within the intra-erythrocytic development circulating. We questioned if this phenotype was exclusive to the dry season or if it could be found in parasites from asymptomatic carriers during the transmission season. To this end, we cultured *P. falciparum* collected during a cross-sectional time-point in October (transmission season) from RDT+ asymptomatic individuals and measured parasitaemia increase and determined parasite development by flow cytometry after 0, 16, 24, 30, 36 and 48 h of in vitro culture. During the 48 h in vitro culture we observed that *P. falciparum* collected in October, increased 4.8-fold, 95% C I (3.4, 6.2), similar to the 2 and 5 – fold increase observed in other time-points during 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); 2.8-fold in malaria cases, 95% CI (2.1, 3.5)]. We observed that parasitaemia of asymptomatic individuals during transmission season, albeit sometimes higher, resembled the values found during the dry season and not the ones seen clinical malaria in the transmission season (Figure 15 a). Moreover, we observed that *P. falciparum* collected in October from asymptomatic individuals

could increase parasitaemia as early as after 24 hours in culture, similar to dry season samples (Figure 15 b), and accordingly we could frequently identify mature schizonts after 16 h on Giemsa smears from these samples (Figure 15 c). When we calculated the number of hours in culture at which the highest increase of parasitaemia could be detected – 27.6 h , 95% CI (23.8, 31.34) - it resembled more the values found in the dry season [January, 26.4 h, 95% CI (24.5, 28.3); March, 24.9h, 95% CI (23.9, 25.9); May, 22.7h, 95% CI (20.8, 24.6)] than the ones observed during malaria cases in the transmission season [MAL 44.0 h, 95% CI (41.5, 46.6)] (Figure 15 d). Nevertheless, when we measured the developmental stage of the parasites circulating in asymptomatic infections in the transmission season, we observed that, although a wide range of parasite stages were present, there were mostly young stages circulating, similar to was seen during clinical malaria cases (Figure 15 e). We also found that the developmental stage observed was correlated with the parasitaemia found in all time-points – January ($r= 0.57$, $p= <0.0001$), May ($r= 0.38$, $p= 0.0025$), MAL ($r=-0.52$, $p=0.0006$) and October ($r=0.61$, $p=<0.0001$). (Figure 15 f). Asymptomatic infections during transmission season had characteristics similar to those of dry season and malaria-causing parasites regarding replication and development.

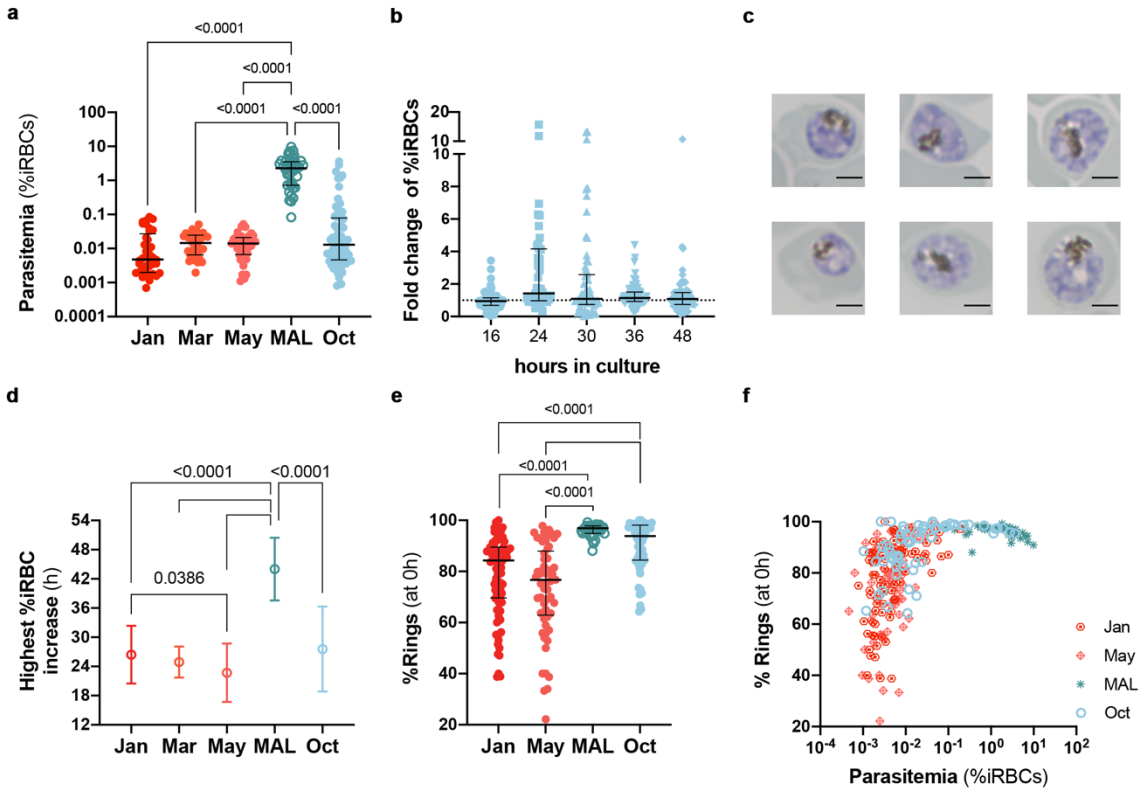

Figure 15 | Asymptomatic infections during transmission season

a, Parasitaemia detected by flow cytometry in the beginning (Jan, n=37), middle (Mar, n=36) or end (May, n=41) of the dry season and during first clinical malaria (MAL, n=39) and asymptomatic infections in October (Oct, n=69) in the ensuing transmission season. **b**, Parasitaemia fold change at 16, 24, 30, 36 and 48 h after culture of parasites collected from children at different times during the dry season (January, March and May) and clinical malaria cases (MAL). Fold change is defined as %iRBC t(n)/%iRBC t(n−1). Data indicate median ± IQR. Dashed line at fold change of 1. **c**, Giemsa-stained thin blood smears 16 h after culture of *P. falciparum* parasites collected from asymptomatic children during transmission season. Scale bar, 2 µm. **d**, Time of highest increase in parasitaemia detected during in vitro culture of *P. falciparum* parasites from children in January (n = 39), March (n = 42) and May (n = 40) during the dry season, clinical malaria cases (MAL, *n* = 27) and asymptomatic infections (Oct, n=23) in the ensuing transmission season. Data indicate mean ± s.d.; one-sided Dunn's Kruskal–Wallis multiple comparisons test. **e,** Percentage of circulating ring stage iRBCs determined by flow cytometry at the beginning (Jan, n=90) and end (May, n=61) of the dry season and during malaria cases (MAL, n=39) or asymptomatic infections (Oct, n=67) in the ensuing transmission season. Mean ± s.d.; one-sided Dunn's Kruskal–Wallis multiple comparisons test. **f**, Percentage of circulating ring stage iRBCs (y axis) and Parasitaemia (x axis) determined by flow cytometry at the beginning (Jan, n= 90) and end (May, n= 61) of the dry season and during malaria cases (MAL, n= 39) or asymptomatic infections (Oct, n= 67) in the ensuing transmission season. Two-tailed Spearman correlation.

3.10 Duration of *P. falciparum* asymptomatic infections

Our previous data obtained through microsatellite and *msp*2 capillary electrophoresis analyses indicate that parasites persisting at the end of the dry season in May had been carried since the beginning of the dry season in January (Portugal et al., 2017). However, it was unclear when had these parasites been transmitted in the preceding transmission season, and how long they had remained in the host without being cleared or causing disease. To determine *P. falciparum* infection length or longevity we now did retrospective PCRs from filter papers collected bi-weekly, from 92 children aged 6-12 years old that were PCR positive in May 2013 (Table 8). We performed quantitative PCR (qPCR) of *var* gene acidic terminal sequence (*var*ATS) on all samples from each donor preceding the beginning of that dry season until they were found PCR negative for 4 consecutive time-points, received malaria treatment, or until the end of the previous dry season (May 2012) (Figure 16). We then performed *ama*1 deep amplicon sequencing on all *var*ATS PCR positive samples from these children.

Table 8 | Characteristics of study participants.

n	Age, y, Median (95%CI)	female (%)
92	9 (8,0;9,0)	50

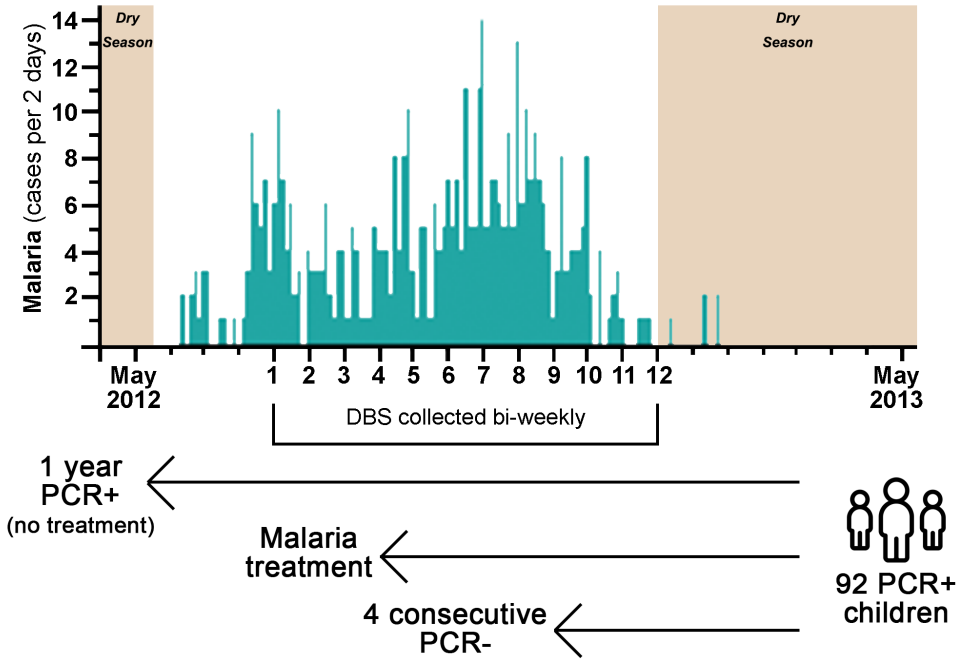

Figure 16 | Study design to determine duration of dry season asymptomatic infections.

Histogram represents clinical malaria frequency measured every 2 days from May 2012 until May 2013. Dried blood spots (DBS) were collected in May 2012. During transmission season, DBS were collected every 2 weeks 8 weeks after May 2012, and then in May 2013.

We first determined which samples were PCR+ from these 92 children across 13 time-points by *var*ATS qPCR (Figure 17 a).We observed that PCR+ children at the end of the dry season had been infected for a median of 254 days, with a minimum of 144 days consecutively positive and a maximum of 364 days (Figure 17 b), and albeit the narrow age groups analysed, there were no differences observed (Figure 17 c and Table 9) nor duration of infections based on gender (Figure 17 d and Table 10).

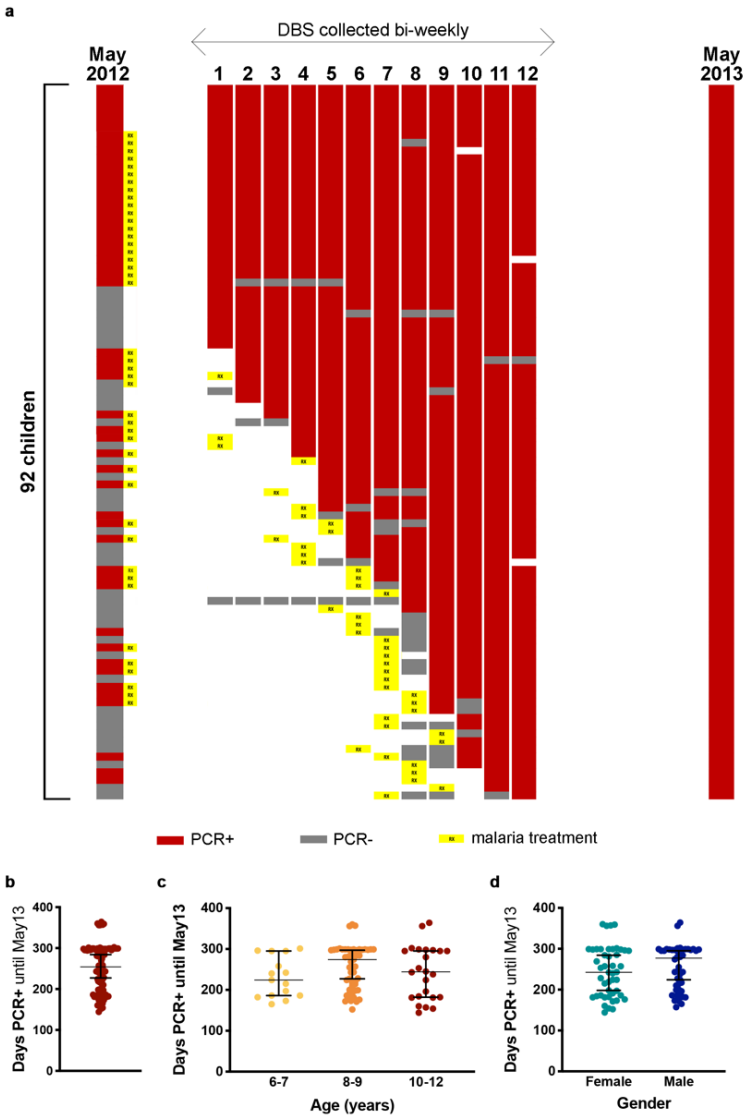

Figure 17 | Parasite detection by varATS qPCR of 92 children throughout one year period from dried blood spots

a, Each column is sorted so that each child is represented in the same row at all time-points. Blank spaces represent data not measured or not available. **b**, Days PCR+ of all 92 children. **c**, Days PCR+ stratified by age group. one-sided Dunn's Kruskal–Wallis multiple comparisons test. **d**, Days PCR+ stratified by gender. Two-tailed Mann–Whitney test; Data indicates median ± 95% CI.

Table 9 Days PCR+ stratified by age group

Gender	n	Days PCR+, Median (95% CI)	Female (%)
6-7 years	15	224 (186;295)	66,7
8-9 years	53	274 (227;297)	52,8
10-12 years	24	244 (182;295)	33,3

Table 10 |Days PCR+ stratified by sex

Gender	n	Days PCR+, Median (95% CI)	Age, y, Median (95%CI)
Female	46	242,5 (198;284)	8,5 (8;9)
Male	46	277 (224;295)	9 (8;10)

*ama*1 amplicon sequencing requires 3 PCR runs prior to the actual sequencing (see 2.2.20 *ama1* heminested PCR for details) and a different number of cycles is recommended based on parasite density, thus we determined parasite density in all samples by *var*ATS qPCR. In the 92 children we observed that parasite density fluctuated during the year, and then we stratified children by when they last became PCR+ or when they last received malaria treatment in the preceding transmission season. We observed that children that became positive or received treatment later in the year, had lower parasitaemias during this period than children that remained positive for most of the year (Table 11 and Figure 18).

Table 11 | Parasite density determined by *var*ATS qPCR in 92 children, stratified by time-point

	all samples		positive until T6		positive after T6		positive until T6 vs positive after T6	
Time-Point	n	Median (95% CI)	n	Median (95% CI)	n	Median (95% CI)	p value	test
May12	5	62,11 (0,09;808,7)	5	62,11 (0,09;808,7)	0	n/a	n/a	n/a
1	34	743,8 (261,2;2310)	34	743,8 (261,2;2310)	0	n/a	n/a	n/a
2	41	450 (82,55;1422)	41	450 (82,55;1422)	0	n/a	n/a	n/a
3	43	326,8 (96,14;1075)	43	326,8 (96,14;1075)	0	n/a	n/a	n/a
4	48	419,2 (263,5;884,9)	48	419,2 (263,5;884,9)	0	n/a	n/a	n/a
5	55	530,4 (93,71;1715)	55	530,4 (93,71;1715)	0	n/a	n/a	n/a
6	61	447,1 (105,9;757,6)	61	447,1 (105,9;757,6)	0	n/a	n/a	n/a
7	64	346,7 (103,8;746,1)	61	347,6 (168,5;746,1)	3	3,47 (1,45;8463)	ns	Mann-Whitney
8	68	646,5 (258,8;1358)	61	813 (262,4;1366)	7	225,7 (0,07;20280)	ns	Mann-Whitney
9	80	556,6 (228,8;1050)	61	560,1 (324,3;1466)	19	128,2 (2,55;803,1)	0,0177	Mann-Whitney
10	86	713,7 (368,9;2263)	60	771,3 (357,9;1887)	26	602,4 8 (113;1163)	ns	Mann-Whitney
11	91	379,8 (225;854,6)	61	815,5 (311,2;1484)	30	195,1 (123,4;351,5)	0,0185	Mann-Whitney
12	90	611,9 (356; 899,9)	60	814,8 (395;1737)	30	276,4 (104,2;888,7)	ns	Mann-Whitney
May13	92	227,5 (123,3;384,6)	61	310,6 (135,4;795,7)	31	123,3 (76,37;229,6)	ns	Mann-Whitney

CI: confidence interval; n/a: not applicable; ns: not significant

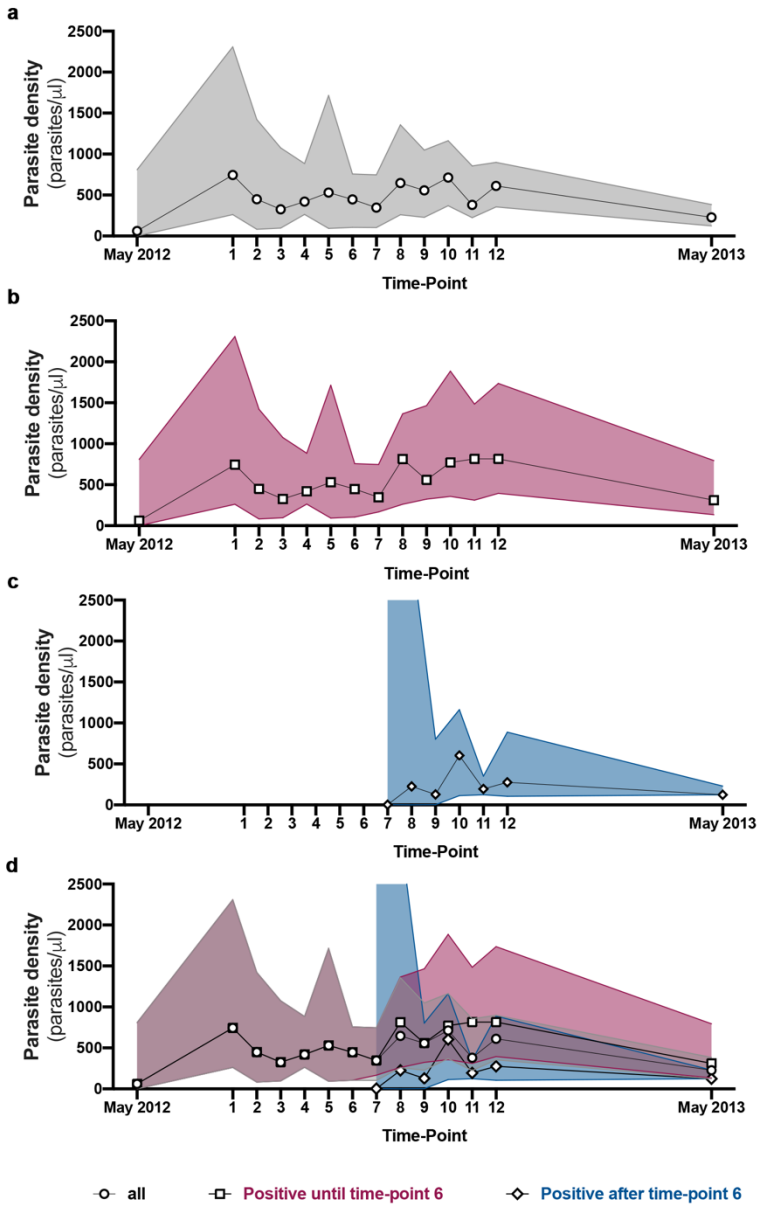

Figure 18 | Parasite density determined by varATS qPCR throughout 1 year period.

a, Parasite density of all 92 children during 1 year period; **b**, Parasite density of children that were positive the entire year, became positive before time-point 6 or last malaria treatment was before time-point 6. **c**, Parasite density of children that were positive or received treatment after time-point 6. **d**, Parasite density of all 3 conditions analysed in a, b and c. Data represents median at each time-point, shadow area represents 95% CI.

Next, we determined the number of clones present at the end of both dry seasons (May 2012 and 2013) and during the 2012 transmission season. The number of *ama*1 haplotypes was maintained throughout the year in these children (Figure 19 a and b and Table 12).

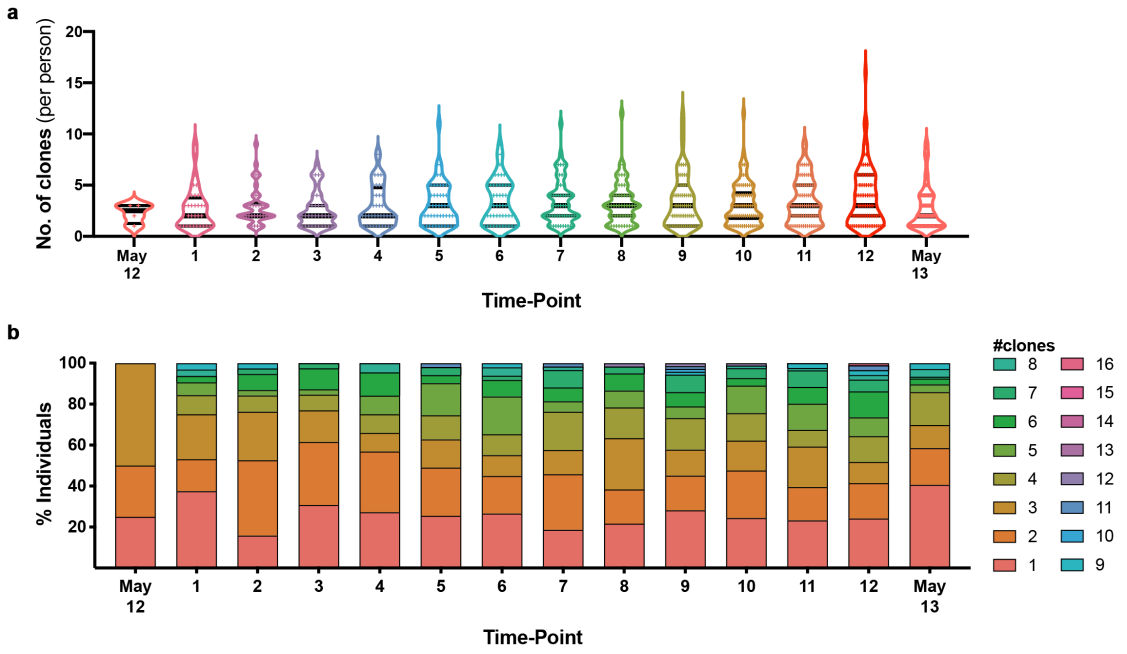

Figure 19 | Complexity of infection throughout the year determined by *ama*1 amplicon sequencing

a, Number of *P. falciparum* clones and **b**, Proportions of individuals with different numbers of *P. falciparum* clones at the end of the dry season in 2012 (May), throughout transmission season (1 to 12) and at the end of the dry season in 2013 (May 2013); One-sided Dunn's Kruskal-Wallis multiple comparisons test; Violin plots indicate mean ± IQR.

Table 12 | Complexity of infection determined by *ama*1 amplicon sequencing, stratified by time-point

Time-Point	n	No. Clones, Median (Interquartile range)
May 12	4	2,5 (1,25;3)
1	32	2 (1;3,75)
2	38	2 (2;3,25)
3	39	2 (1;3)
4	44	2 (1;4,75)
5	51	3 (1;5)
6	49	3 (1;5)
7	59	3 (2;4)
8	60	3 (2;4)
9	71	3 (1;5)
10	82	3 (1,75;4,25)
11	86	3 (2;5)
12	87	3 (2;6)
May 13	106	2 (1;4)
MAL 13	65	2 (1;3)

We next determined the length of infection of each individual clone infection using the same DBS collected every two weeks apart. Out of the 92 children analysed, 4 children had infections that on the earliest timepoint analysed in May 2012, of which we detected 9 haplotypes carried to the ensuing transmission season. Since we did not have data prior to May 2012, these haplotypes were excluded of further analysis. Altogether we identified 74 unique haplotypes throughout the year. It is possible that some clones present in the children were not detected either due to sequestration to the vascular endothelium, or due to levels below the limit of detection. For this reason, we assumed that some missing clones at some timepoints were likely present, to which we called “skips”. The number of clonal infections counted decreased as the number of skips increased, we identified 982 infections allowing for only 1 skip, versus 799 infections when we allowed 4 skips (Table 13). This reduction is because missing clones in between positive timepoints may break a long infection in 2 shorter ones when a stricter number of skips was used. The minimum number of days of an infection was 6.5 days and did not differ between analyses with different skips. The maximum number of days varied between 313 days (1 and 2 skips) and 316 days (3 and 4 skips). The average number of days of infections varied from 66.5 days (1 skip) to 80.1 days (4 skips) (Table 13). We also observed that in all analysis, the majority of infections (20-30%) were short infections, present only for 1-2 weeks (Figure 20).

Having in mind that the village from the cohort-study is under intense *P. falciparum* transmission during the rainy season, we assumed that a clone was cleared if it was missed in two consecutive timepoints (1 month – 2 skips), and that it was transmitted again upon its re-detection. all further analyses were performed allowing for a maximum of 2 skips.

We separated infections in those that did or did not reach the end of the dry season in May 2013, and then calculated the number of days that each haplotype was present in the host. We measured the number of days each haplotype was present in the host, and we grouped together haplotypes that were present in less than 3 infections for simplicity of display. We observed that the number of days each haplotype was present did not differ in infections that were only found during the transmission season and that did not reach the dry season (Figure 21 a and Table 14). However, in infections that reached the end of the dry season, less frequent haplotypes (present in less than 3 infections) were associated with shorter infections when compared with 2 of the more frequent haplotypes (Figure 21 b and Table 14).

We then averaged the number of days of all infections present in each child, separating our analysis by infections that reached or not the end of the dry season in May 2013. For infections that did not reach the end of the dry season, we determined that during transmission season clones were present for an average of 35.3 days per child, with an averaged minimum of 6.5 days and persisted for an averaged maximum of 127 days (~ 4 months) per child. Infections that survived until the end of the dry season persisted in the human host for an average of 180.9 days, the shortest infections being the ones assumed to have been transmitted at the beginning of the dry season (see 2.2.21 Haplotype length analysis for details) and lasting an averaged minimum of 126 days. The longest infections observed that persisted until the end of the dry season lasted an average maximum of 293 days (~9 ½ months) (Figure 21 c and d and Table 15). Furthermore, the duration of infections was similar in all included age groups, gender and haemoglobin type both for infections reaching or not the end of the dry season (Figure 21 e and j).

Lasting an average length of 180.9 days (or 180.3 days average in all infections in the dry season) highlights that clones surviving until the end of the dry season were mainly transmitted in the middle of November during the preceding transmission season.

We then questioned if the likelihood of a clone reaching the end of the dry season was affected by its transmission date. We included in the analysis only children that were PCR+ for the majority of the time (at least since August 2012), excluding children that either got treatment

or were PCR- after August 2012, and we calculated the odds of clones transmitted earlier (before October 2012) or later (after October 2012) in the transmission season to reach the end of the following dry season, through an odds ratio (OR). We observed that the odds to reach the end of the dry season in May 2013, of a clone transmitted earlier in the preceding transmission season was much lower than that of clones transmitted later in the transmission season, OR = 0.2155 (95% CI 0.13;0.36), suggesting that the presence of a clone before October 2012 reduces the odds of its survival until May 2013 (Table 16).

Table 13 | Infection length when allowing for different number of skips

	1 skip	2 skips	3 skips	4 skips
n (infections)	982	892	832	799
n (haplotypes)	74	74	74	74
Minimum # days	6.5	6.5	6.5	6.5
Maximum # days	313	313	316 *	316 *
Average # days	66.5	71.8	76.3	80.1
Median # days	35.0	36.0	48.0	48.5.0
SD	70.0	73.1	75.6	77.6

* including May 2012: 345 days

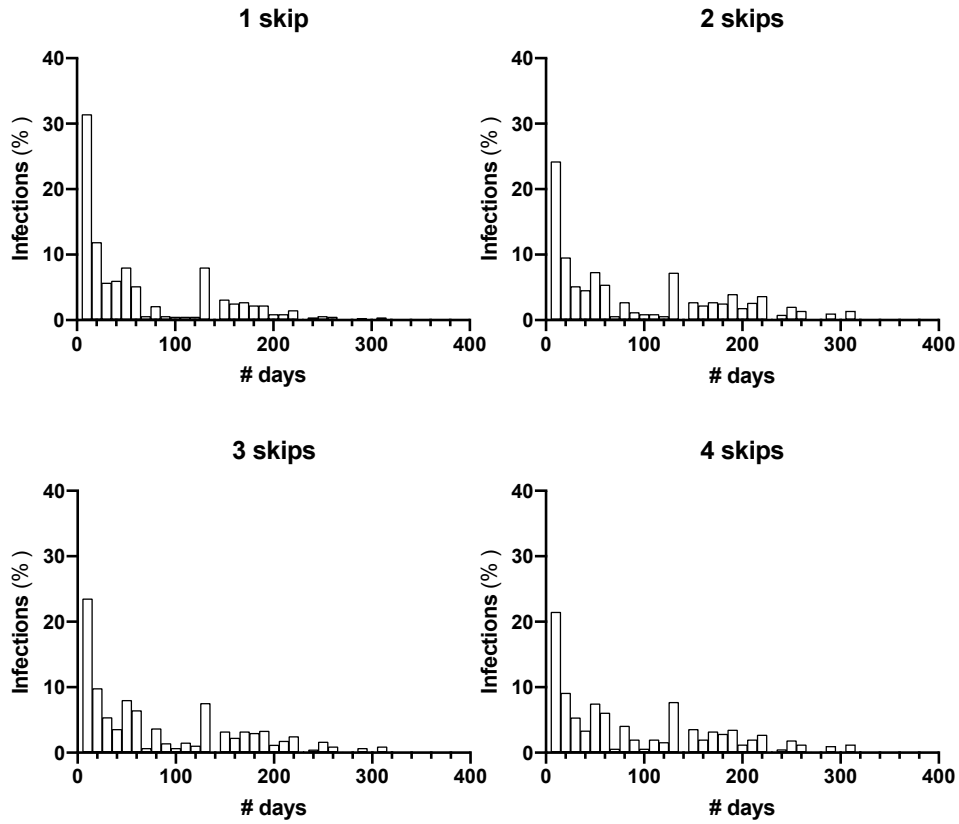

Figure 20 | Infection length when allowing for a different number of skips

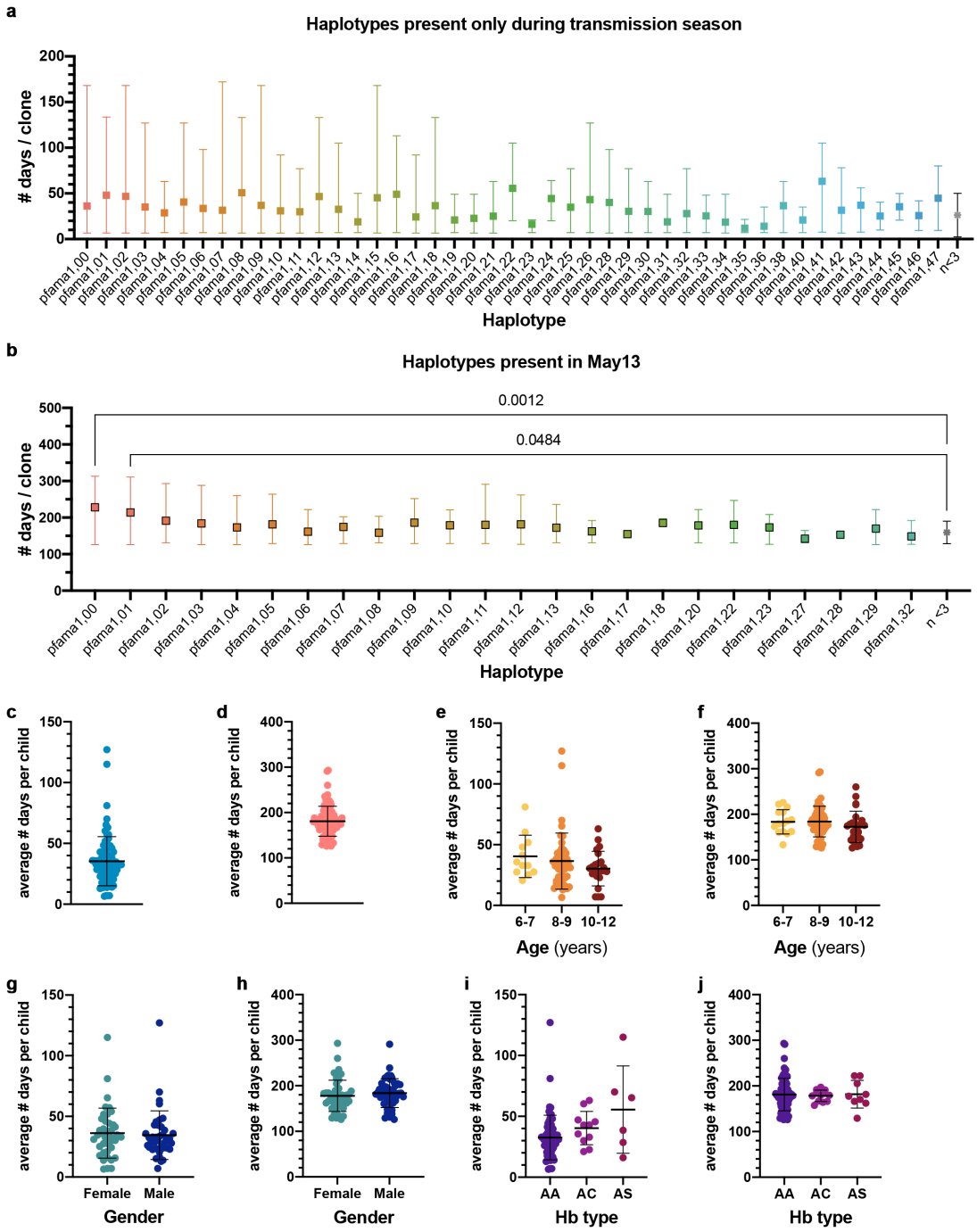

Figure 21 | Duration of infections by *ama*1 sequencing

Number of days each haplotype is present when found **a)** only in the transmission season or **b)** during the dry season. Data represented as mean with range; Haplotypes that were found in less than 3 infections (n <3) were grouped; Average number of days per child with haplotypes found **c)** only in the transmission season or **d)** during the dry season; Average number of days per child with haplotypes stratified by age found **e)** only in the transmission season or **f)** during the dry season; Average number of days per child with haplotypes stratified by gender found **g)** only in the transmission season or **h)** during the dry season; Average number of days per child with haplotypes stratified by hemoglobulin

(Hb) type found **i)** only in the transmission season or **j)** during the dry season; **(c-j)** Data represented as mean ± SD. (Individual data per child can be found on Table 15). **(a-b, e-f, i-j)** One-sided Dunn's Kruskal-Wallis multiple comparisons test; (**g-h**) two-tailed Mann– Whitney test.

Table 14 | Duration of infections by *ama*1 haplotype

	Infections during tranmission season				Infections during dry season			
Haplotype	n (infections)	Average # days (SD)	Minimum # days	Maximum # days	n (infections)	Average # days (SD)	Minimum # days	Maximum # days
pfama1.00	39	36,33 (38,72)	6,5	168	22	228,2 (64,7)	126	313
pfama1.01	40	48,06 (36,28)	6,5	133,5	15	214,1 (53,45)	126	311
pfama1.02	39	46,73 (40,23)	6,5	168	16	191,5 (53,74)	131	293
pfama1.03	39	35,02 (32,47)	6,5	127	21	184 (47,19)	126	288
pfama1.04	30	28,75 (20,6)	7	63	16	173,2 (41,73)	126	260
pfama1.05	25	40,54 (36,5)	6,5	127	19	181,4 (37,64)	129	264
pfama1.06	39	33,62 (23,8)	7	98	8	161,4 (34,52)	126	222
pfama1.07	35	31,64 (39,37)	6,5	172	11	174,3 (21,98)	129	202,5
pfama1.08	28	50,71 (34,92)	6,5	133	7	158,7 (28,17)	131	203,5
pfama1.09	23	36,9 (44,54)	6,5	168	9	186,1 (34,84)	129	252
pfama1.10	20	30,86 (29,39)	6,5	92,04	14	179 (28,29)	129	221
pfama1.11	26	29,86 (24,57)	6,5	77,04	10	180,2 (54,93)	129	291
pfama1.12	20	46,66 (34,63)	7	133	8	181,5 (46,42)	127	262
pfama1.13	19	32,64 (29,9)	7	105	4	172,5 (51,19)	131	236
pfama1.14	17	18,91 (16,4)	7	50,04	2	192 (1,414)	191	193
pfama1.15	13	45,24 (45,69)	6,5	168	1	221 (0)	221	221
pfama1.16	7	49,08 (43,19)	7	113	6	162,2 (21,74)	131	192
pfama1.17	14	24,19 (25,5)	6,5	92,04	3	155 (7)	150	163
pfama1.18	8	36,64 (42,69)	6,5	133	3	185,3 (10,31)	177,5	197
pfama1.19	5	20,9 (19,71)	7	49	2	193 (38,21)	166	220
pfama1.20	7	22,79 (18,62)	6,5	49	3	178,5 (45,65)	131	222
pfama1.21	7	25,01 (22,62)	6,5	63,04	2	189 (0)	189	189
pfama1.22	4	55,64 (37,47)	20	105	4	180,3 (48,64)	131	247
pfama1.23	3	16,17 (7,943)	7	21	4	173 (40,05)	127	208,5
pfama1.24	3	44,35 (22,39)	20	64,04	1	179,5 (0)	179,5	179,5
pfama1.25	5	34,9 (32,73)	7	77	1	179,5 (0)	179,5	179,5
pfama1.26	6	43,18 (43,36)	7	127	2	167,5 (28,99)	147	188
pfama1.27	2	63,02 (0,02946)	63	63,04	3	142,3 (19,63)	131	165
pfama1.28	8	40 (32,34)	6,5	98	3	153,3 (6,807)	148	161
pfama1.29	3	30,35 (40,44)	7	77,04	3	170 (48,52)	126	222
pfama1.30	6	30,26 (25,96)	7	63,04	1	131 (0)	131	131
pfama1.31	7	18,93 (16,86)	7	49	2	131 (0)	131	131
pfama1.32	4	27,89 (33,38)	7	77,04	3	148,6 (37,53)	127	192
pfama1.33	3	25,33 (20,84)	7	48	2	140,5 (16,26)	129	152
pfama1.34	6	18,59 (16,45)	6,5	49,04	1	127 (0)	127	127
pfama1.35	3	11,84 (8,384)	7	21,52	2	146,5 (21,92)	131	162
pfama1.36	5	14 (12,12)	7	35	2	179 (19,8)	165	193
pfama1.37	2	31,5 (24,75)	14	49	1	129 (0)	129	129
pfama1.38	4	36,5 (27,23)	7	63	1	151 (0)	151	151
pfama1.39	2	73,52 (4,979)	70	77,04	2	163 (2,828)	161	165
pfama1.40	3	20,83 (14)	7	35	1	127 (0)	127	127
pfama1.41	3	63,2 (50,21)	7,521	105	1	127 (0)	127	127
pfama1.42	4	31,51 (33,67)	6,5	78,04	1	126 (0)	126	126
pfama1.43	4	37,02 (21,32)	7,521	56	1	162 (0)	162	162
pfama1.44	5	25,2 (15,22)	7,521	49	1	129 (0)	129	129
pfama1.45	3	35,35 (14,52)	21	50,04	0	-	-	-
pfama1.46	3	25,68 (16,21)	7	36,04	0	-	-	-
pfama1.47	3	44,69 (35,33)	7	77,04	0	-	-	-
pfama1.48	1	64,04 (0)	64,04	64,04	0	-	-	-
pfama1.49	0	-	-	-	1	235 (0)	235	235
pfama1.50	1	35 (0)	35	35	1	148 (0)	148	148
pfama1.51	1	16 (0)	16	16	1	190 (0)	190	190
pfama1.52	1	7 (0)	7	7	1	126 (0)	126	126
pfama1.53	3	11,17 (7,654)	6,5	20	0	-	-	-
pfama1.54	3	20,84 (23,97)	7	48,52	0	-	-	-
pfama1.55	4	15,63 (13,01)	7	34,5	0	-	-	-
pfama1.56	2	14 (9,899)	7	21	0	-	-	-
pfama1.57	2	14 (9,899)	7	21	0	-	-	-
pfama1.58	1	34,5 (0)	34,5	34,5	0	-	-	-
pfama1.59	2	20,75 (19,45)	7	34,5	0	-	-	-
pfama1.60	1	91,04 (0)	91,04	91,04	0	-	-	-
pfama1.61	1	48,52 (0)	48,52	48,52	0	-	-	-
pfama1.62	1	22,04 (0)	22,04	22,04	2	128,5 (3,536)	126	131
pfama1.63	0	-	-	-	1	179,5 (0)	179,5	179,5
pfama1.64	1	49 (0)	49	49	0	-	-	-
pfama1.65	1	7 (0)	7	7	0	-	-	-
pfama1.66	2	6,75 (0,3536)	6,5	7	0	-	-	-
pfama1.67	1	7 (0)	7	7	1	130 (0)	130	130
pfama1.68	0	-	-	-	1	161 (0)	161	161
pfama1.69	1	21 (0)	21	21	0	-	-	-
pfama1.70	1	21,52 (0)	21,52	21,52	0	-	-	-
pfama1.71	2	7 (0)	7	7	0	-	-	-
pfama1.73	1	7 (0)	7	7	0	-	-	-
pfama1.74	1	7 (0)	7	7	0	-	-	-

Table 15 | Duration of infections per child

				Infections during tranmission season			Infections during dry season	
Child	Age	Gender	Hb type	Average # days (SD)	n (infections)	n (haplotypes)	Average # days (SD)	n (haplotypes)
kali0061	10	Male	AA	13,8 (7,7)	6	6	148 (0)	2
kali0067	10	Male	AA	30,6 (17,3)	7	7	162 (0)	1
kali0070	10	Female	AA	54 (47,6)	7	6	194 (39,6)	2
kali0071	12	Female	AA	34,5 (0)	1	1	181,2 (9,5)	2
kali0072	10	Male	AA	22,9 (30,7)	11	11	186,5 (67,3)	3
kali0081	10	Male	AA	48,4 (37,1)	8	8	175,7 (39,3)	2
kali0084	10	Male	AS	28,5 (10,6)	2	2	181,5 (0)	1
kali0086	10	Male	AA	28,3 (26,1)	6	6	143,5 (24,75)	2
kali0095	10	Male	AA	35,8 (33,8)	19	14	175,7 (9,546)	2
kali0096	10	Female	AA	7 (0)	1	1	161 (0)	1
kali0106	10	Female	AA	7 (0)	5	5	146 (12,12)	3
kali0107	10	Male	AA	25,2 (27,8)	7	7	126 (0)	2
kali0109	10	Female	AA	-	-	-	136,5 (14,85)	2
kali0110	10	Female	AA	31 (0)	1	1	174 (0)	1
kali0112	10	Male	AA	27,9 (24,3)	4	4	174,6 (25,2)	4
kali0113	10	Female	AA	46,1 (43,7)	5	4	260 (0)	1
kali0115	10	Male	AC	42,8 (40,6)	19	18	178,1 (45,1)	7
kali0119	10	Male	AA	24 (15,1)	9	8	223,2 (88,43)	4
kali0122	10	Male	AA	28,4 (29,2)	16	13	239 (0)	1
kali0126	10	Female	AA	31,9 (28,1)	16	13	151 (0)	1
kali0137	10	Male	AA	26,1 (27,9)	17	12	129 (0)	3
kali0143	10	Male	AC	63 (49,1)	16	12	164 (0)	1
kali0144	10	Female	AA	-	-	-	150 (0)	1
kali0147	10	Male	AA	33,5 (37)	20	15	221 (0)	4
kali0148	10	Male	AA	7 (0)	1	1	131 (0)	1
kali0162	9	Female	AA	24,3 (14,5)	2	2	165 (0)	1
kali0170	9	Male	AA	30 (29,3)	3	3	291 (0)	1
kali0175	9	Female	AC	30 (27,6)	17	15	166,9 (35,32)	7
kali0183	9	Male	AA	23,4 (22,5)	5	5	217,3 (56,6)	3
kali0184	9	Female	AC	42,9 (28,8)	15	14	179,7 (47,28)	4
kali0186	9	Female	AA	34,7 (38)	14	10	156,5 (44,18)	3
kali0192	9	Male	AA	32,4 (29,8)	6	6	161 (0)	1
kali0193	9	Female	AC	35,6 (26,3)	12	10	166 (0)	1
kali0198	9	Male	AS	70 (29,7)	2	2	177,5 (0)	1
kali0203	9	Female	AA	41,3 (9,6)	2	2	188 (0)	1
kali0206	9	Female	AS	65,3 (59,6)	3	3	166 (0)	2
kali0209	9	Male	AA	-	-	-	168 (0)	1
kali0211	9	Female	AA	14 (9,9)	2	2	178,5 (0)	1
kali0214	9	Female	AA	37,9 (33,4)	7	4	126 (0)	4
kali0224	9	Male	AA	-	-	-	198,8 (58,28)	9
kali0236	9	Female	AA	20 (0)	1	1	165,3 (24,25)	4
kali0249	9	Male	AA	31 (31,6)	17	16	138 (12,12)	3
kali0250	9	Female	AA	13,8 (9,6)	2	2	155 (44,82)	9
kali0255	9	Female	AA	38,4 (42,1)	19	15	170,2 (35,21)	8
kali0256	9	Female	AA	51,7 (28,6)	8	8	179,8 (27,89)	5
kali0258	9	Male	AC	45,6 (30,5)	20	16	191,7 (48,62)	4
kali0259	9	Male	AC	22,7 (20,3)	7	7	183,8 (16,18)	3
kali0260	9	Female	AA	57,2 (37,8)	7	7	293 (0)	1
kali0277	8	Male	AA	16,6 (11,2)	10	9	193 (0)	1
kali0280	8	Female	AS	115 (0)	1	1	222 (0)	1
kali0282	8	Male	AA	-	-	-	192 (85,82)	4
kali0284	8	Female	AA	39,7 (35,3)	3	2	228 (89,12)	2
kali0285	8	Male	AA	34,9 (28)	24	18	206,8 (49,38)	6
kali0286	8	Male	AS	38,6 (37,3)	4	3	205,3 (81,23)	3
kali0288	8	Female	AA	38,3 (30,5)	8	8	184,3 (21,35)	5
kali0290	8	Male	AC	32,9 (22,9)	8	8	197 (0)	1
kali0291	8	Female	AA	57,7 (8,4)	3	3	189 (0)	1
kali0303	8	Female	AS	-	-	-	170,5 (26,42)	6
kali0304	8	Male	AA	-	-	-	183,3 (36,73)	4
kali0308	8	Male	AA	42,9 (30,2)	6	5	214,7 (75,72)	4
kali0315	8	Female	AA	30,2 (22,7)	13	12	213,1 (29,1)	4
kali0316	8	Male	AA	127 (0)	1	1	167,2 (10,25)	2
kali0317	8	Male	AC	47 (45,6)	7	7	191 (0)	1
kali0325	8	Male	AA	35,9 (23,4)	15	10	146,5 (24,75)	2
kali0332	8	Female	AA	15,7 (9,47)	11	11	184,3 (37,99)	8
kali0333	8	Female	AA	44,2 (38,3)	6	6	134 (0)	1
kali0336	8	Male	AA	23,9 (19)	21	16	217,5 (40,33)	2
kali0337	8	Female	AA	6,5 (0)	1	1	129 (0)	2
kali0338	8	Female	AA	32,4 (23,2)	5	4	183,1 (17,46)	4
kali0343	8	Male	AA	12,9 (13,1)	7	6	139,7 (17,5)	4
kali0344	8	Male	AA	24,5 (23,4)	8	7	200,2 (10,27)	2
kali0345	8	Female	AC	21 (0)	2	2	179,5 (0)	1
kali0346	8	Male	AA	-	-	-	202,5 (0)	1
kali0347	8	Male	AA	15,2 (18,3)	5	5	176,5 (0)	1
kali0351	8	Female	AS	16 (0)	1	1	129 (0)	1
kali0354	8	Female	AA	17,7 (12,7)	8	5	235,6 (55,71)	4
kali0355	8	Male	AA	31,5 (28)	14	12	176,5 (19,09)	2
kali0376	7	Female	AA	48 (0)	1	1	215,8 (29,64)	3
kali0394	7	Female	AA	35,8 (40)	2	2	204,5 (76,18)	4
kali0395	7	Male	AA	32,8 (20,1)	13	11	160,4 (59,06)	9
kali0406	7	Male	AC	60,3 (56,4)	23	19	185 (65,61)	6
kali0414	7	Female	AA	51,8 (50,1)	5	5	173,6 (38,71)	3
kali0417	7	Female	AA	32,9 (18,5)	7	7	179,5 (0)	1
kali0430	7	Male	AS	-	-	-	222 (0)	1
kali0438	7	Female	AC	-	-	-	157,4 (24,72)	5
kali0440	7	Female	AA	25,2 (16,2)	3	3	225,7 (24,55)	4
kali0448	7	Female	AA	81 (45,3)	2	2	133 (0)	1
kali0457	7	Male	AA	41,3 (38)	10	9	188 (0)	1
kali0473	6	Female	AA	27,5 (29)	2	2	179,5 (0)	1
kali0480	6	Male	AA	20,5 (0,7)	2	2	204,5 (0)	1
kali0481	6	Female	AS	-	-	-	162 (0)	1
kali0490	6	Female	AA	27,5 (9,9)	2	2	162 (0)	1

Table 16 | Contingency table to calculate the odds ratio of a clone that is present in May 2013 to have been transmitted before October 2012

	reaches May 13	does not reach May 13	
haplotype transmitted before October 2012	20	202	222
haplotype transmitted after October 2012	116	252	368
	136	454	590

Odds ratio: 0.2155 (95% CI: 0.13;0.36)

CHAPTER

DISCUSSION 4

4.1 Immune system is minimally affected by asymptomatic infections during the dry season

Previous studies have shown that carrying asymptomatic infections during the dry season associate with a lower risk of developing clinical malaria in the ensuing transmission season (Crompton et al., 2008; Portugal et al., 2017; Sonden et al., 2015). However, treatment of these infections at the end of the dry season did not increase the risk of falling ill with malaria in the following transmission season, suggesting that it may not be the presence of these parasites that is protecting against clinical disease, but rather that the presence of these parasites is a marker of previous higher exposure to *P. falciparum* (Portugal et al., 2017). Moreover, studies from our laboratory have showed that maintaining an infection during the dry season, does not prevent loss of antibodies, and that this loss occurs similar to what is seen in uninfected individuals as well (Portugal et al., 2017). Since immunity to *P. falciparum* is intrinsically related to exposure, children that have been more exposed to *P. falciparum* have a higher immunity to disease (Crompton et al., 2010). Likewise, previous studies from our laboratory have shown that infected children at the end of the dry season have a higher antibody breadth and magnitude against *P. falciparum* specific antigens (Portugal et al., 2017) and we now showed that this higher breadth of antibodies was associated with a lower risk of developing clinical malaria in the ensuing transmission season (Figure 8). Higher breadth of *P. falciparum* antibodies against merozoite antigens has been associated with protection against clinical malaria (Stanisic et al., 2009) and also when in association with the presence of multiclonal infections (Rono et al., 2013). We now show that this is also true for the *P. falciparum* antibody breadth present at the end of the dry season. Moreover, when we further look at the dynamics of *P. falciparum* specific antibody breath from the beginning till the end of the dry season, we observe that children that have clinical malaria in the ensuing transmission season have a

higher loss of antibody breadth during the dry season. Notably, it is not clear what the contribution of maintaining an asymptomatic infection during the dry season is, since infected children have a higher antibody breadth during the dry season compared to non-infected children.

Nevertheless, we observed that infected children consistently had higher *P. falciparum* specific antibodies (Figure 8) (Portugal et al., 2017, Andrade et al., 2020) and higher *P. falciparum* memory B cells (MBCs) (Figure 7). Moreover, ours and previous studies have reported that asymptomatic infections are more frequent in older children (Figure 6) (Andrade et al., 2020; Goncalves et al., 2017; Portugal et al., 2017), consistent with age-dependent acquisition of immunity against *P. falciparum* (Crompton et al., 2010; Tran et al., 2013).

Asymptomatic infections during the dry season do not seem to elicit any detectable inflammation. Only one chemokine, CXCL1, a pro-inflammatory chemokine known to recruit neutrophils, which 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 (Figure 7 and Table 6). Furthermore, peripheral blood mononuclear cells (PBMCs) subpopulations and intracellular activation markers were similar between infected and non-infected children at the end of the dry season (Figure 7 and Table 4). This differs from the potent cellular immune responses elicited in naïve volunteers in controlled human malaria infections (CHMI) with a low infected erythrocytes inoculum (Pombo et al., 2002). Albeit challenge was performed with a homologous strain, this study suggests that even at a low parasitaemia, it is still possible to elicit an immune response in non-immune individuals capable of protection from disease. The low levels of parasitaemia found in the dry season, however, were not capable of eliciting a different immune response in infected individuals when compared to non-infected individuals.

A certain level of immunity might be required to sustain asymptomatic infections during the dry season. Studies in endemic areas have reported that with constant exposure, the host shifts from pro-inflammatory to anti-inflammatory immune responses. A study in Kenya compared the number and proliferation of $CD4^+$ T cell in PBMCs from children from an area with continuous *P. falciparum* exposure or an area where transmission was significantly reduced. Authors show that although the absence of continuous exposure for 8 years does not change the proportion of circulating $CD4^+$ T cells, the proliferation of these cells in vitro and their

cytokine production upon *P. falciparum* stimulation is significantly different. With interruption of transmission to *P. falciparum,* $CD4^+$ T cells from these children proliferate more and produce higher quantities of pro-inflammatory cytokines in vitro (Bediako et al., 2016). With continuous exposure to *P. falciparum,* the host shifts for a more immunoregulatory response that avoids over reaction of the immune response, leading to the cytokine storm associated with clinical malaria (Crompton et al., 2014). Likewise, a study from Mali has isolated monocytes from Malian children and adults and US adults and stimulated them in vitro with *P. falciparum* infected erythrocytes. They observe that production of pro-inflammatory cytokines such as TNF, IL-1b and IL-6 occur is observed mainly in Malian children and US adults, and decreases with age. Anti-inflammatory cytokine IL-10 production is mainly observed in Malian adults, and increased with age, whereas young Malian children and US adults had comparable low levels of IL-10. These results suggest that indeed, with cumulative exposure to *P. falciparum*, the immune response shifts from pro-inflammatory to immunoregulatory. Moreover, authors observe that *P. falciparum* successive infections induce epigenetic changes in the monocytes of malaria exposed individuals, making these monocytes more tolerant to infection (Guha et al., 2020).

Naturally, one cannot exclude that there might be subtle differences in cellular populations or their activity that this study was not capable of detecting in both children. Nonetheless, our current data on the immune system dynamics during the dry season leads us to propose that parasites that are kept asymptomatically during the dry season are less able to drive an immune response in a semi-immune host. This agrees with what is observed in response to other chronic stimuli, when the immune system is continuously presented with the same low-level stimulus, the immune effector response declines. Authors of the discontinuity theory of immunity suggest that the immune system recognizes sudden changes in the organism and react to those (Pradeu & Vivier, 2016). A chronic infection such as the one observed during the 6-month dry season is less effective at driving antigenic stimulation that would drive an immune response.

Antibodies against *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) appear to be acquired sequentially, where domains responsible for severe disease are acquired early in life. Antibodies against PfEMP1s can prevent adhesion of infected erythrocytes and mediates immune responses against infected erythrocytes (Cham et al., 2010; Obeng-Adjei et al., 2020). Even though the antibody responses against the 35 PfEMP1 domains we could measure

decreased during the dry season, regardless of the children's infection status (Figure 8). It is possible that in a chronic infection, with continuous asexual replication, there is a sequential presentation of variant surface antigens (VSA) that leads to the gradual acquisition of antibodies, that would favour a less virulent parasite in the dry season, that would be unable to reach levels capable of triggering an immune response. In fact, a recent study of CHMI including malaria-naïve and semi-immune individuals has shown that clinical disease was seen in malaria-naïve individuals while chronic infections appeared in individuals with intermediate antibody levels (Bachmann et al., 2019). Longitudinal sequencing of the PfEMP1 variants of *P. falciparum* parasites circulating in these children might elucidate on the hypothesis that sequential presentation of different VSAs might be at play when entering the dry season, leading to a less virulent VSA being favoured during this period.

4.2 Transcriptome of *P. falciparum* during dry season and longer circulation of infected erythrocytes in peripheral blood

Our results show that the transcriptome of *P. falciparum* collected in the dry season segregates from that of malaria-causing *P. falciparum* during transmission season (Figure 10). We identified several differential expressed genes up-regulated in asymptomatic infections in the dry season vs malaria-causing *P. falciparum* in the transmissions season. Using a bioinformatic method that estimates the age in hours post invasion of the circulating parasites (Lemieux et al., 2009), based on the peak of gene expression (Bozdech, Zhu, et al., 2003), we concluded that *P. falciparum* parasites during the dry season corresponded to older and more developed parasites when compared to the ones causing clinical malaria during transmission season (Figure 11). We further confirmed these results by microscopy and flow cytometry. Older parasites circulating in the dry season is likely the main driver of the separation of the transcriptional profiles based on seasonality (Figure 11). However, albeit most genes following the expected peak expression for the estimated hours post-invasion, this is not true for all genes, and single-cell RNA sequencing will be necessary to properly compare same-stage parasites and characterize which genes are differentially expressed in *P. falciparum* asymptomatic infections in the dry season.

Several studies have addressed the transcriptome of *P. falciparum* in different malaria severity outcomes. A study from Senegal with samples from clinical malaria samples was able to

segregate patient samples into 3 different clusters based on the similarity of their transcriptional profile. Interestingly, authors report that one of the clusters has parasites that resembled more the ring-stage in vitro than the other two clusters, based on their transcriptional profile (Daily et al., 2007). A study from Cameroon compared the transcriptome of *P. falciparum* infected asymptomatic patients and patients with uncomplicated or cerebral malaria. Authors found that transcriptional profiles segregated based on disease (asymptomatic vs symptomatic) but not based on disease severity (uncomplicated vs cerebral malaria), and found upregulation of genes involved in adhesion and pathogenesis in patients with cerebral malaria when compared with asymptomatic patients. Notably, authors report to have discarded samples that contained trophozoites, and only samples with 100% ring-stage were included for further analysis (Almelli et al., 2014). Would have been interesting to know how different the transcriptional profiles would have segregated should these samples have been included in the analysis, and how they would compare with the observations we made with dry season asymptomatic infections. Another study from Papua New Guinea compared *P. falciparum* infection in uncomplicated and severe malaria. The authors report different metabolic pathways to be differentially expressed between the two disease outcomes. They also report a bias towards early trophozoite-stages in samples isolated from uncomplicated malaria patients, whereas severe malaria patient samples appeared mainly composed of ring-stage parasites (Tonkin-Hill et al., 2018). A study with Gambian children with severe and uncomplicated malaria has shown also several differential expressed genes between the two disease severities. In severe malaria, upregulated genes were mainly associated with cyto-adhesion (Lee et al., 2018). In our laboratory, we revisited publicly available transcriptional profiles of parasites from different studies based on different clinical outcomes of malaria and parasite densities. Interestingly, we observed that transcripts upregulated in high parasite densities were of genes with an early expression peak in the 48-hour blood-stage development, whereas in low parasite densities, upregulated transcripts belonged to genes peaking later in the 48-hour blood-stage developmental cycle. Moreover, when we applied a whole-transcriptome algorithm approach to determine the age of parasites in hours post infection Lemieux et al), we consistently estimated older parasites in infections of lower parasite densities. We further validated these results by microscopy and found more developed parasites circulating in lower parasitaemias' infections and in mild malaria when compared to severe malaria (Thomson-Luque *et al.,* submitted).

Parasites circulating longer than 24 hours in the peripheral blood have been described in *P. falciparum* genotyping studies as well. In a study from the high endemicity area of Tanzania, authors genotyped parasites every 6 hours during 5 days in 1 asymptomatic child. They found that some parasite genotypes were not restricted to the 24 hour time frame circulation in the peripheral blood, and also report to have found mature trophozoites in circulation (Farnert et al., 2008). A study from Senegal that genotyping parasites between 4-12 hours apart in 21 asymptomatic children has found that although some parasites display the synchronized disappearance from peripheral blood with sequestration, some clones remained stable during the entire study (Jafari-Guemouri, Boudin, Fievet, Ndiaye, & Deloron, 2006). Naturally, in all these studies, *P. falciparum* clones might only be asynchronous and not necessarily be in circulation for longer periods due to a lower sequestration. Nonetheless, revisiting blood smears from these studies might be interesting, to see if a longer persistence of a determined clone (within the 48-hour replicative cycle) in the blood would associate with more developed circulating parasite. *P. falciparum* infections can be asynchronous or synchronous depending on the percentage of rings and other stages found in circulation. A study with symptomatic children which identified parasite stages by microscopy and different parasite clones by gel electrophoresis of 3 different *P. falciparum* polymorphic genes has found that synchronicity of *P. falciparum* in vivo is strongly associated with a monoclonal infection (Toure-Ndouo, Zang-Edou, Bisvigou, & Mezui-Me-Ndong, 2009). Having in mind that with this genotyping technique, the amplification of a high-density clone masks the amplification of low-density ones (Contamin et al., 1995), this high synchronicity in monoclonal infections is likely to be due to a fast-growing clone that is present at a higher parasite-density. Fast-growing clones are likely to have a more efficient binding to the endothelium, and therefore avoid splenic clearance. Circulating antibodies in the semi-immune host may control growth of the parasites, biasing towards the low adhesion phenotype we observe. The low adhesion of these parasites will therefore increase splenic clearance and hinder growth of parasitaemias. Further studies evaluating the expression of variant surface antigens in *P. falciparum* during the dry season, as well as the gradually acquisition to humoral responses against these parasites' variable antigens will help elucidate how low adhesive parasites are selected in asymptomatic infections during the dry season.

Seasonal malaria chemoprevention (SMC) is usually administered during transmission season to prevent clinical malaria (B. Greenwood, 2006). During the dry season, transmission of *P.*

falciparum is interrupted and the parasites that remain in the human host are likely to be the reservoir that restarts transmission upon mosquitos' return. Elimination of these parasites at the end of the dry season has not increased the risk of clinical malaria in the ensuing transmission season (Portugal et al., 2017) indicating that this period might be an interesting timeframe to achieve local elimination of *P. falciparum* reservoir. Notably, artemisinin and its derivatives – the first line of treatment to uncomplicated malaria in the African region (World Health Organization, 2020) – have been shown to be more active on young ring-stage parasites (Klonis et al., 2013). Therefore, our results on a longer circulation of *P. falciparum* parasites during the dry season might be important to delineate elimination campaigns in areas of seasonal transmission.

4.3 *P. falciparum* ability to sense the season

In the work presented here, we have tried to elucidate if *P. falciparum* was able to sense the season and adapt its behaviour according to presence or absence of mosquitoes. We have cultured different *P. falciparum* parasite strains to elucidate if it would have the ability to sense environmental cues in its hosts and adapt accordingly. We have not found differences in growth or host cell remodelling when *P. falciparum* laboratory-adapted strains or field isolates were grown in medium supplemented with plasmas collected at different times of the year in any of the conditions tested (Figure 13).

P. falciparum in vitro culture conditions are very distinct from the ones found in vivo. In the human host, *P. falciparum* has to adjust to a multitude of factors such has host inflammation markers, immune cell populations, antibodies, hormones, different metabolites, and daily nutrient fluctuations (reviewed in LeRoux, Lakshmanan, & Daily, 2009). We attempted here to recreate the environment in the different seasons in the conditions we deemed most appropriate. *P. falciparum* was continuously adapted to culture in vitro in 1976 by Trager and Jensen (Trager & Jensen, 1976) Since then, RPMI 1640, has been widely used to continuously grow *P. falciparum* in vitro and ex vivo. RPMI 1640 is an extremely rich medium that contains nutrients necessary for parasite growth in supraphysiologic concentrations when compared to the concentrations present in human plasma (reviewed in Desai, 2013). To allow *P. falciparum* to the derive the maximum from the culture media, we have performed our experiments in

low-glucose media, in which the glucose concentration is equivalent to the average glucose present in healthy adults. Hypoxanthine is a purine derivative commonly added to culture in serum-free conditions, since it is essential for parasite nucleic acid synthesis (Asahi, Kanazawa, Kajihara, Takahashi, & Takahashi, 1996). However, since it is present in human serum/plasma, we did not add it to our cultures. Still, even with these less rich conditions supplemented with human plasma, we did not observe any differences when parasites were cultured with different season plasmas. RPMI 1640 has also supraphysiological conditions of several amino acids. Early studies in which authors removed individual amino acids from culture identified 7 essential amino acids for *P. falciparum* culture (Divo, Geary, Davis, & Jensen, 1985), and bioinformatics analysis of *P. falciparum* genome identified 6 amino acids that *P. falciparum* can synthesize *de novo* (Payne & Loomis, 2006). However, a different study has identified isoleucine as the single amino acid to be detrimental to *P. falciparum* culture. In the absence of this amino acid *P. falciparum* parasites enter a hibernating state, that can be reversed with the re-supplementation of isoleucine within 72 hours (Babbitt et al., 2012). The ideal culture conditions may be detrimental for *P. falciparum* in studies trying to understand the interplay between the parasite and the environment. For instance, the plasmodial surface anion channel (PSAC) increases RBC permeability to many solutes in *Plasmodium* and it has been shown that PSAC inhibition differs when normal RPMI or a lower nutrient media is used (Pillai et al., 2012). Thus, assuming the possibility there is a sensing mechanism, the composition of the culture media we used for our experiments might still be too rich to detect potential environmental cues in the human host.

In vitro culture has surely a severe impact on *P. falciparum* parasites. Comparison of laboratory-adapted strains and field isolates has shown that transcript abundance found in chromosomal clusters to be attributable to copy number variations, where genomic duplications or deletions occur (Mackinnon et al., 2009). Another study has found that culture adaptation of *P. falciparum* parasites led to nonsense mutations, such as a mutation in the transcription factor ApiAP2, that plays an important role in gametocytogenesis (Claessens, Affara, Assefa, Kwiatkowski, & Conway, 2017). Remodelling of the host cell is also affected in long-term culture of *P. falciparum* strains, where knob density in the infected erythrocyte decreases with culture time in vitro (Quadt et al., 2012). Drug sensitivity also is found to decline in culture-adapted laboratory strains (Chaorattanakawee et al., 2015). Furthermore, most of the variant surface antigen families were found overexpressed in field isolates compared to laboratory adapted *P.*

falciparum strain 3D7 (Bachmann et al., 2012). To overcome limitations of long-term culture, we have cultured field isolates for only one cycle after blood collection in medium supplemented plasmas from different times of the year, and although many samples show parasitaemia increase in the first 48h, not all increase their parasitaemia during culture, possibly due to a failure in culture adaptation (Figure 13). Accordingly, failure to adapt to culture has been reported from several studies trying to lab-adapt field isolates (Nsobya, Kiggundu, Joloba, Dorsey, & Rosenthal, 2008).

In our experiments, we set 25% as the optimal plasma supplementation concentration, since we observed that at a higher concentration, *P. falciparum* growth was deterred, even in plasmas where *P. falciparum*-specific antibodies were absent (Figure 12). This concentration might not have been sufficient to allow the parasite to detect possible environmental cues present in the plasma, since the concentration present in the human body is close to 50%. However, in a study linking plasma nutrients and sensing in *P. berghei* and *P. falciparum*, authors have shown that with 10-25% plasma supplementation to their in vitro cultures, they could induce a lower number of merozoite per schizont when parasites were grown in supplementation with plasma from calorie restricted mice (Mancio-Silva et al., 2017).

We did observe differences in the metabolites in the plasma between plasma samples collected during the dry season or at clinical malaria case in the transmission season (Figure 10). However, due to the very high parasite burden in clinical malaria samples, it is complicated to disentangle what could be seasonal or parasite induced. Studies have shown that the nutritional status changes seasonally, being more rich during the dry season (Brown, Black, & Becker, 1982 Roba, O'Connor, Belachew, & O'Brien, 2015). It would be of interest to perform metabolomics and proteomics analysis on plasmas collected seasonally in infected and non-infected individuals, and from clinical malaria patients to investigate if there are seasonal host environment changes that can influence *P. falciparum* growth and development. We have recently sent samples collected during the dry season (March and May), at peak of transmission season (October) or during clinical malaria, in both asymptomatic, symptomatic (malaria cases only) and uninfected children to analyse through liquid chromatography mass-spectrometry. These results will aid us understand if the environment changes seasonally or in response to clinical disease and/or parasite density.

Transcriptomic studies are also a powerful tool to investigate possible sensing encoded mechanisms in *P. falciparum*'s genome. Would be interesting to laboratory-adapt *P. falciparum* isolates collected at different times of the year and supplement these with plasmas collected in different seasons. Performing RNA sequencing of these parasites might elucidate into pathways that might be differentially regulated when parasites are cultured in dry or transmission season plasmas that might translate into an adaptation of sensing the season.

In addition, sensing of seasonality might be of interest to define when it is time to commit and transmit to the mosquito vector. Data from our laboratory not shown in this work indicates that there are fewer total gametocytes in the dry season, but when in proportion with asexual stages, the proportion of gametocytes is higher in the dry season than during clinical malaria cases (van Djik and Lima et al, *in preparation*). Lysophosphatidylcholine (LysoPC) concentration has also been associated with sexual differentiation. A high concentration of LysoPC represses sexual differentiation (Brancucci et al., 2017). Our data indicates that although LysoPC concentration is lower in clinical malaria cases than during the dry season, it is still in higher levels that the ones required to induce gametocytogenesis in vitro. An interesting approach to the sensing experiments would be to measure gametocytogenesis in *P. falciparum* when grown in different seasonal plasmas.

Alternatively, *P. falciparum* sensing mechanism could be not of sensing the host environment *per se*, but rather the return of the mosquito vector. In fact, in an avian malaria study, chronic *Plasmodium relictum* parasites respond to the bites of uninfected mosquitoes, increasing their replication and number of gametocytes, increasing transmission (Cornet, Nicot, Rivero, & Gandon, 2014). Due to the variety of factors that could influence these results, such as inflammation in the host due to mosquito bites, it is a very complex experiment to attempt to replicate in vitro. However, it could be of interest to perform such experiments in the human host and measure asexual and sexual *P. falciparum* stages in the peripheral blood longitudinally after uninfected *Anopheles* mosquito bites.

4.4 Clinical malaria is caused by a recently transmitted clone

If a sensing mechanism would regulate asymptomatic infections in the dry season, it could also regulate *P. falciparum* increase during transmission season upon mosquitoes return. To

understand the latter, we genotyped and compared *P. falciparum* genotypes at the end of the dry season with genotypes found during clinical malaria.

Our results show that it is unlikely to *P. falciparum* to cause clinical malaria in the ensuing transmission season, since in the majority of the children analysed, clinical malaria was accompanied with circulation of new clones. Furthermore, in the children that had common clones at both time-points, these were frequently seen genotypes that are likely to be more prevalent at the population level.

Studies have shown that clinical malaria is likely caused by a recently transmitted clone (Babiker et al., 1998) and that successive malaria episodes are accompanied by different clones in the same child (Contamin et al., 1996) . A study from Kenya performed agglutination assays to detect recognition by children's plasma of homologous *P. falciparum* strain during the malaria episode and 3 weeks thereafter has shown that induction of antibodies against this homologous strain occur in those 3 weeks (Bull et al., 1999). Ours and others results suggest that the acquisition of antibodies against a specific clone, will prevent it from causing malaria in the same child.

Another interesting question would be to determine if *P. falciparum* parasites that persist asymptomatically during the dry season are able to cause clinical malaria, in a different child or if these parasites have lost the capacity of causing disease, regardless of previous acquired immunity. In fact, a recent opinion argues that in the low transmission area of Zanzibar, where the majority of people have a low acquired immunity to *P. falciparum*, asymptomatic infections are common due to being less virulent than the ones found in high transmission areas. The authors propose that an antigen shift to a less virulent one, would allow for parasite persistence in an area of low transmission (Bjorkman & Morris, 2020).

4.5 Asymptomatic infections during transmission season

We observed that asymptomatic infections in the transmission season are comprised of a wide range of parasitaemias, ranging from levels found in both clinical malaria and in asymptomatic infections during the dry season. However, the parasitaemias were more closely related to the

ones found in the dry season, in agreement with parasitaemias below the clinical radar, hence asymptomatic infections. We found however, that asymptomatic infections in the transmission season are characterized by a higher percentage of rings circulating in the peripheral blood than asymptomatic infections in the dry season (Figure 15). This might be the result of more recently transmitted *P. falciparum* clone/s that have variant surface antigens being expressed that allow for a higher adherence to the endothelium. Nonetheless, we also observe that the highest increase of *P. falciparum* asymptomatic infections in the transmission season occurs similarly as dry season ones, suggesting that older parasites in circulation are also observed in asymptomatic infections during transmission season. However, older parasites are still observed in circulation in asymptomatic infections during transmission season indicating that this does not occur exceptionally during the dry season (Figure 15). Moreover, asymptomatic infections during transmission season may be a mixture of old and new infections. This would mean that in culture, a mixture of different parasite strains with different ages. Older parasites in culture might translate into a faster increase in parasitaemia, as we observed, while the more recently transmitted would account for the large proportion of young forms seen in circulation. Furthermore, we also observed that the proportion of rings in culture is associated with a higher parasitaemia (Figure 15), in agreement with our results on the revisiting of old transcriptomic studies (Thomson-Luque et al., submitted).

Due to the high entomological inoculation rate experienced during transmission season, it is difficult to discern when exactly were these clones transmitted. To understand if older *P. falciparum* in circulation might be associated with longer infections, we further aimed to determine the duration of *P. falciparum* asymptomatic infections.

4.6 Duration of *P. falciparum* infections

Although we could not show that *P. falciparum* parasites detect the different seasons, the presence of dry season-like parasites in asymptomatic carriers during transmission season suggests there is not a dry season-unique phenotype, therefore there might not exist the necessity of a season sensing mechanism.

We aimed to understand if *P. falciparum* virulence might be gradually reduced with the length of an infection, as they enter the dry season. Although we could not answer this question specifically, a gradual decrease in virulence would be associated with a determined length of an infection. In fact, we observed that it is highly unlikely that a clone transmitted early in the transmission season will reach the end of the dry season (Table 16). These data, plus the fact that we observed older parasites within their erythrocytic cycle in circulation at the end of the dry season suggests that *P. falciparum* parasites might transition gradually to a less adhesive phenotype as the infection progresses. *P. falciparum* parasites transmitted late in the transmission season, are more likely to reach the end of the dry season.

We have found that the clones that reach the end of the dry season, have been on average transmitted around the mid of November. Moreover, we have determined that if a clone is transmitted before October, it is unlikely to survive until the end of the dry season in the next month of May (Table 16). Interestingly, a mathematical model of the duration of infections from malaria therapy data estimates that, in multiclonal infections, parasites that infect very early or late in an infection are at a disadvantage compare to the ones in intermediate time points. Whereas parasites that infect early activate the innate immune system, parasites that infect later are under pressure of an already established adaptive immune response. In the in-between time after the initial innate immune response and before the adaptive response, newly transmitted parasites are more likely to produce lengthy infections (Childs & Buckee, 2015). This model suggests that there might be an ideal time for a clonal infection to be established, and that outside this optimal window, short infections may be more common.

We also observed that short infections (<1 month) are common (Figure 20 and Figure 21). We wonder how much the within-host competition of different clones might reduce the duration of infections. With different clones being constantly transmitted, competition within the host occurs. As the end of transmission season approaches, and transmission of new *P. falciparum* parasites becomes rarer, it could favour longer infections in the host. Interestingly, the impact of multiclonal infections has been studied in relation with their transmission capability, where multiclonal infections were associated with longer infections and gametocyte production (Nassir et al., 2005).

A study has observed that shorter infections are common and that are not a result of naturally acquired immunity, since all age groups had similar duration of infections (Bretscher, Maire, Felger, Owusu-Agyei, & Smith, 2015). This agrees with previous results showing that children

and adults that start the transmission season PCR- take approximately the same time to become PCR + (Tran et al., 2013). Nevertheless, would still be interesting to relate our data on the duration of infections with the results from the *P. falciparum*-specific antibody profile from these children in the previous dry season (Portugal et al., 2017) to elucidate if the loss or level of antibody breadth in the previous dry season might influence clonal length and parasite densities during transmission season.

Our results are from an endemic area with high transmission and a sharply demarcated rainy and dry season, of ~6-month each. Seasonal transmission might range from several dry seasons per year, or having dry season last for a different number of months, or have year-round transmission, where no dry season is observed (Gemperli et al., 2006; Ryan et al., 2020).

Assuming *P. falciparum* has a hardwired gradual transition into a lower adhesion phenotype, where increase in splenic clearance will occur, then it could be expected that the duration of infections would be similar, regardless of the duration of the dry season. However, *P. falciparum* survival during the dry season is crucial to ensure transmission upon mosquitoes return. Having this in mind, perhaps the duration of *P. falciparum* infections during the dry season might be intrinsically related with the duration of the dry season itself, where a short dry season would result in shorter infections and a longer dry season would result in survival of *P. falciparum* individual clonal infections longer than the ones observed in our study. The contribution of transmission of new parasite strains cannot be ignored, and possibly the competition of the persistent clones and recently transmitted ones might be at play in the survival of individuals genotypes. It will be of great interest to compare results from this study with areas of different seasonal transmission to understand how the dry season duration affects the length of *P. falciparum* infections.

In rare cases, *P. falciparum* parasites can stay in the human host for years after transmission is interrupted (Ashley & White, 2014), and in areas with continuous transmission there is a rapid turnover of *P. falciparum* clones (Daubersies et al., 1996). A comparison between study sites with continuous transmission or absent transmission has shown that in continuous transmission, parasite clones fluctuate in the host, lasting a few days to 50 days (over a study period of 3 months), whereas in the absence of transmission parasite clones remained stable for the entire duration of the study (Daubersies et al., 1996). Moreover, the same study has shown that the rapid turnover of parasite clones occurred similarly in all age groups analysed, although children carried higher parasite densities (Daubersies et al., 1996). This agrees with

what we observed here, that during transmission season there is fast acquisition and loss of clones, whereas in its absence, during the dry season, clones are stable for several months (Figure 20 and Figure 21). Notably, in our study design we only analysed children that were positive at the end of the dry season. Data from children that cleared their infections prior or at the beginning of the dry season is missing to be able to conclude about clone stability.

A study from Sudan, where transmission season lasts 2-3 months has found that individuals can be infected with the same clone for over one year (Babiker et al., 1998) although sampling was done only every month. Another study from Uganda with the same technique applied in this work, has described one child to be infected for over 400 days with the same clone. Since transmission is lower in the analysed area, the authors allowed for a maximum of 3 "skips", meaning a parasite clone could be undetected for ~1 ½ months to still be considered present as long as it would be present again later (Briggs et al., 2020). In our study design we had only 4 children continuously infected through the entire year, and none of these had clones present for more than 1 year. Moreover, in our study, the odds of a clone reaching the dry season if it was transmitted early in transmission season are very low (Table 16).

A study from Senegal has shown that malaria cases are accompanied by a rapid increase in parasitaemia, suggesting an uncontrolled parasite growth (Contamin et al., 1996). Interestingly, a recent study from Mali has shown that children fall ill with malaria either when becoming *P. falciparum* PCR+ or in the following 14 days (Tran et al., 2019). This could suggest that upon acquisition of a new clone, increasing parasite densities cause clinical malaria, or at least poke the immune system to mount an immune response that might control these parasites forcing them to remain at low level in the host. We could revisit our data of parasite densities and analyse if peaks of parasitaemia are always accompanied by acquisition of a new clone, and if these would influence clearance of previously present clones.

Our data suggests that *P. falciparum* parasites survive a maximum period that allows for maintenance of the reservoir during the dry season. However, the importance of all these parasites to restart transmission has not been clearly defined, and they might not all contribute similarly to resuming transmission. Recent studies have described the capacity to determine the relatedness between distinct *P. falciparum* clones, from co-transmission or geographical hotspots (Nkhoma et al., 2020,Briggs et al., 2021). It will be of interest to apply this technique to understand which of the parasites that persist in the human host during the dry season are restarting transmission upon the return of mosquitoes.

Finally, analysis of blood smears collected at the different time-points used for amplicon sequencing is of extreme interest to integrate the results of the duration of *P. falciparum* infections with the circulation of more developed parasites. Assuming *P. falciparum* gradually transitions to a less adhesive phenotype, circulation of more developed parasites should be reflected in longer clonal infections when compared with recently transmitted ones.

4.7 Conclusion

In this study we aimed to understand how *P. falciparum* infections persist during the dry season, without increasing to levels that cause disease and without being cleared by the host.

We have shown that *P. falciparum* parasites induce a minimal immune response. Furthermore, we have shown that although the *msp*2 genetic diversity is maintained during the year, the transcriptome of *P. falciparum* parasites collected at the end of the dry season vs parasites causing clinical malaria in the transmission season are very different. These differences are likely to be the result of the circulation of more developed *P. falciparum* parasites in the dry season. These more developed parasites are subjected to increased splenic clearance, contributing to the low parasitaemias observed in the dry season.

We were not able to show that *P. falciparum* is capable of sensing seasonality and respond accordingly. However, we have observed circulation of older *P. falciparum* parasites in asymptomatic infections, suggesting that a lower adhesive phenotype is a central feature in asymptomatic infections, regardless of the season.

We further determined the duration of *P. falciparum* infections that reach the end of the dry season and observed that the clones that reach the end of the dry season are more likely to have been transmitted later in the transmission season, while clones transmitted early in the transmission season were unlikely to survive until the end of the dry season.

The results from this study have begun to elucidate on the mechanisms of how *P. falciparum* asymptomatic infection are kept bellow the immunological radar, allowing for persistence in the dry season and bridging transmission seasons several months apart.

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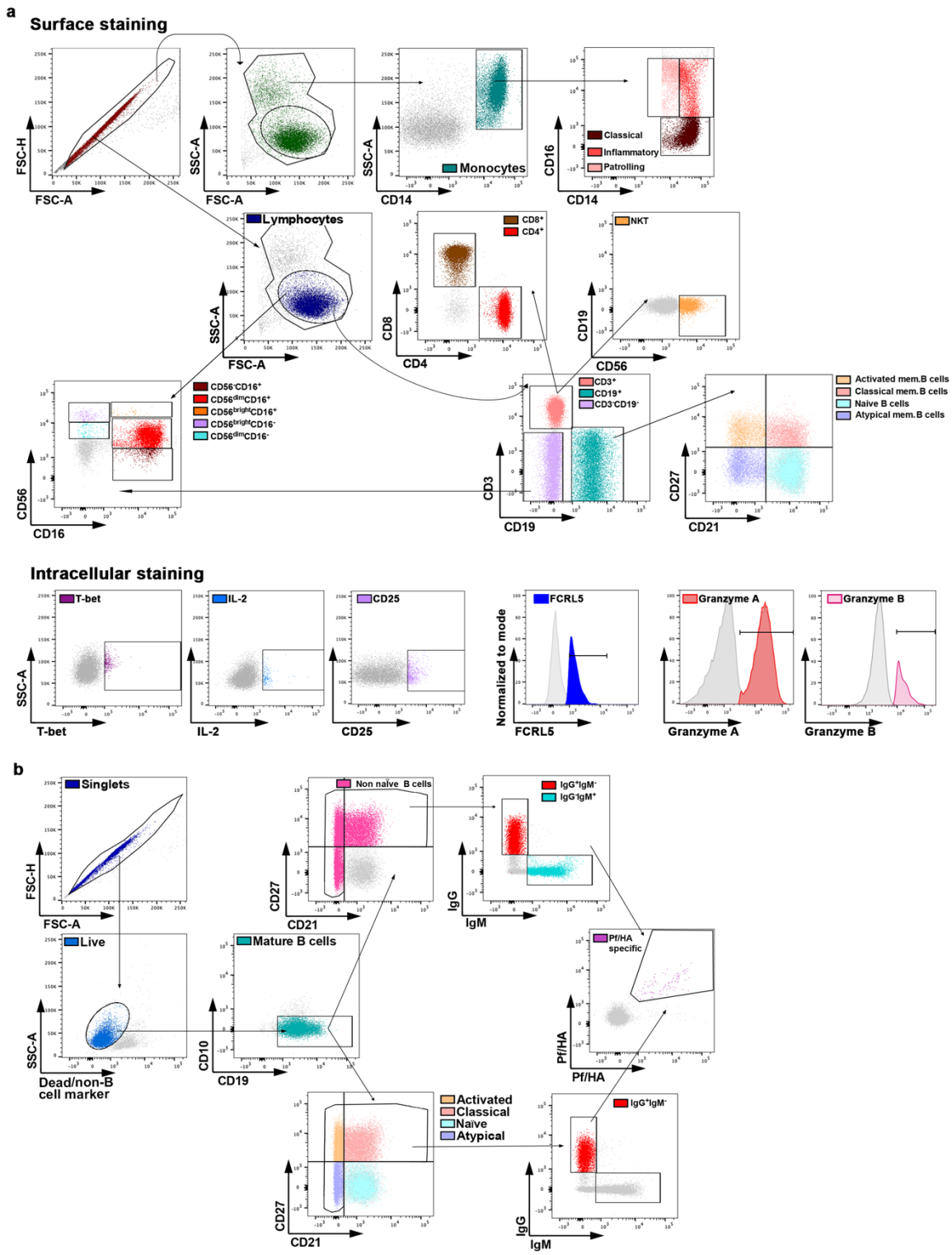
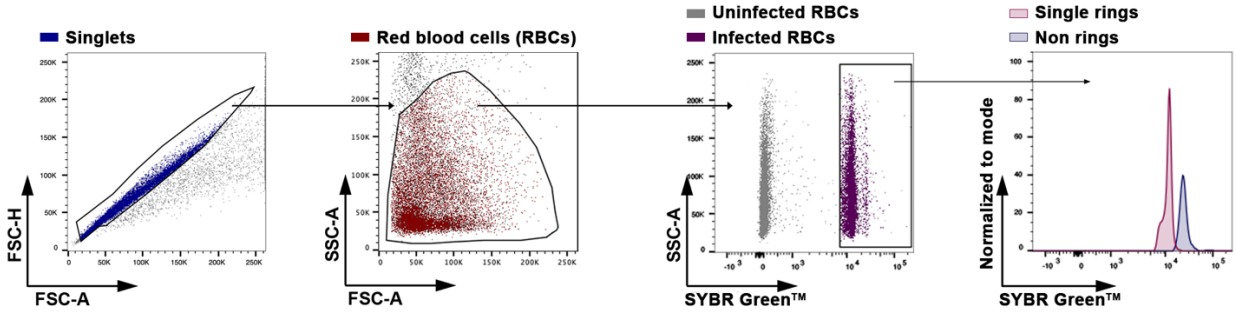
APPENDIX 6

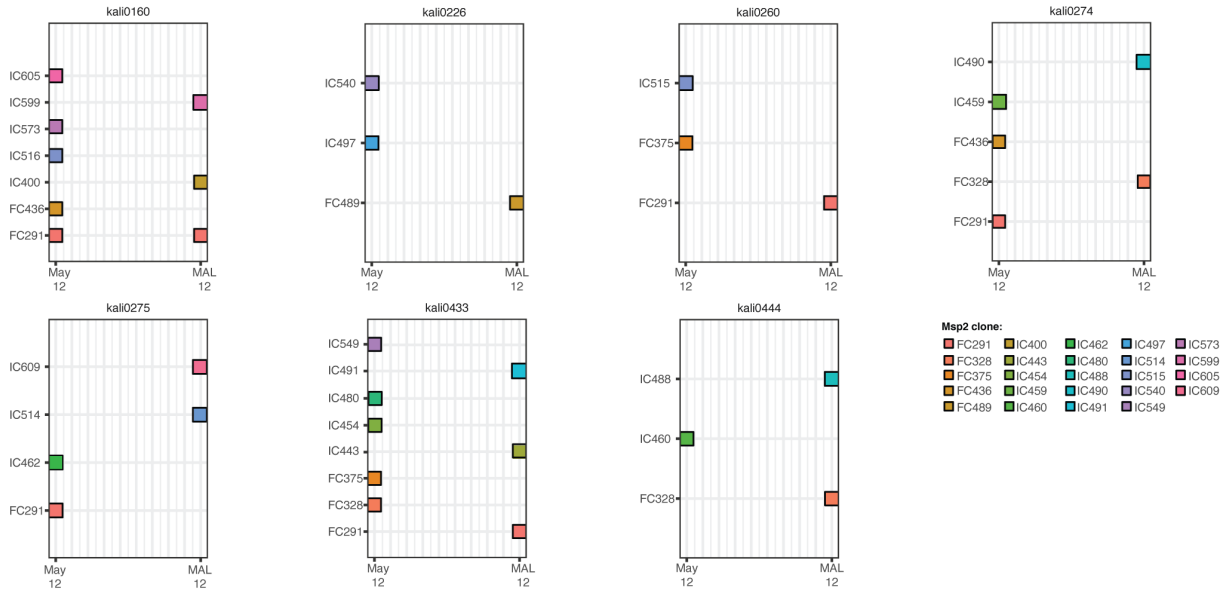


Supplementary Figure 1 Flow cytometry PBMCs gating strategies.

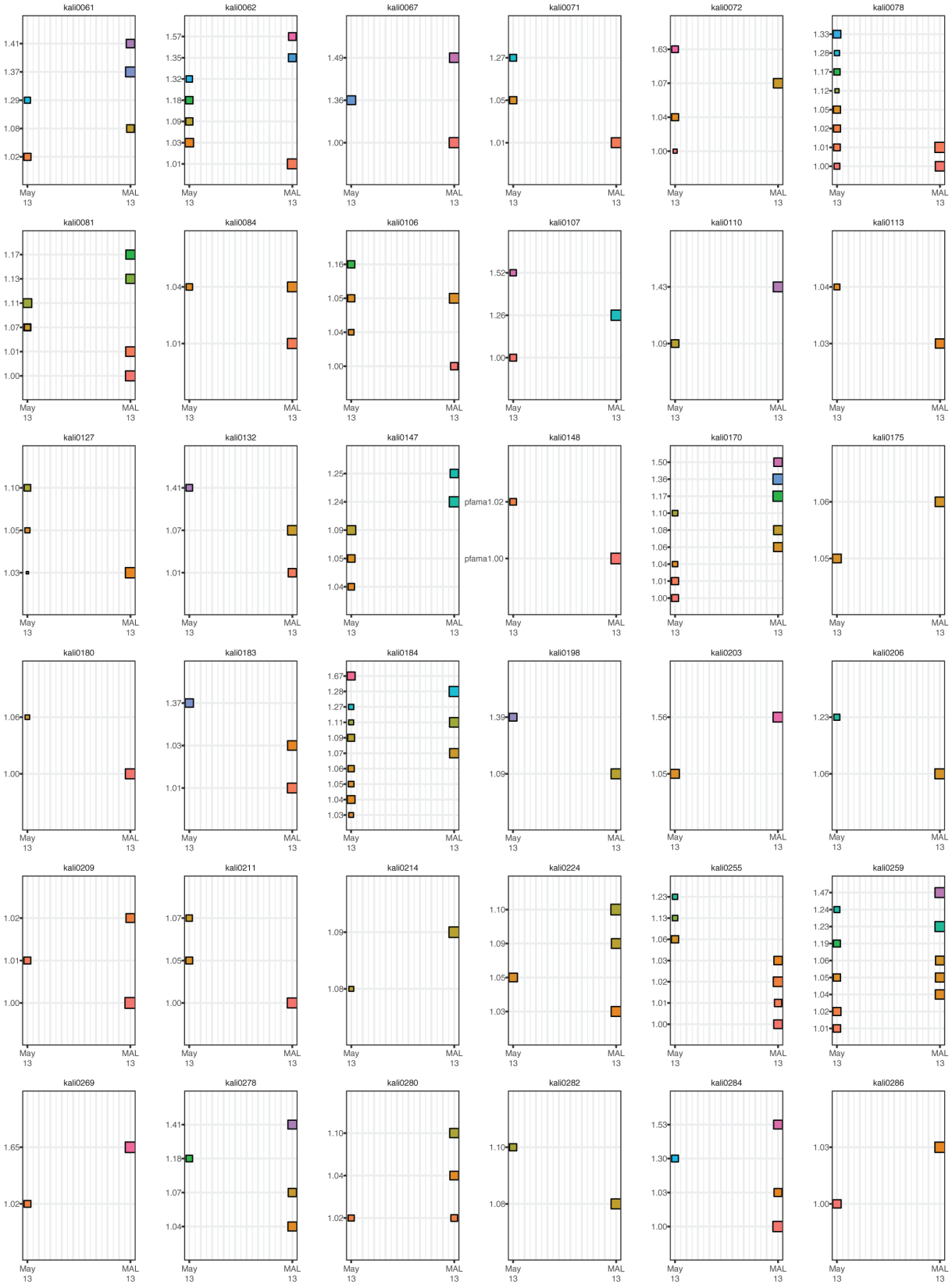
a, Major leucocyte populations from frozen and fresh PBMCs (Surface staining) and fresh PBMCs intracellular content (Intracellular staining). **b**, *P. falciparum*-specific memory B cells in frozen PBMCs at the end of the dry season of children with or without subclinical *P. falciparum* infection. Figure from Andrade et al., 2020.



Supplementary Figure 2 Gating strategy for *P. falciparum* analysis by flow cytometry



Supplementary Figure 3 *P. falciparum* genetic diversity determined by differences in size of a polymorphic region of msp2 at the end of the dry season (May 12) and at first malaria episode in the ensuing transmission season (MAL 12) in 7 children.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May
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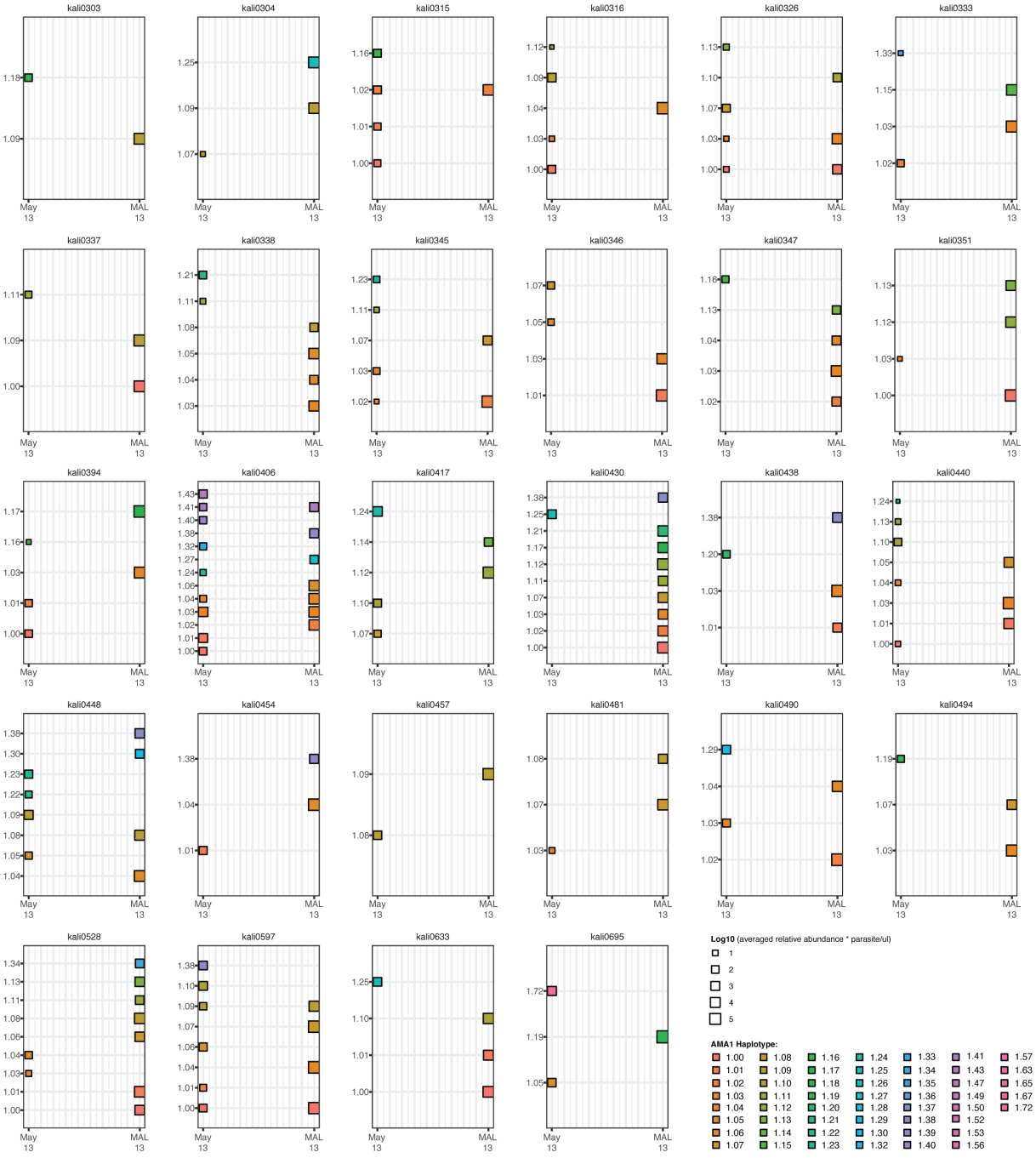
MAL
13



Supplementary Figure 4 *P. falciparum* genetic diversity determined by amplicon sequencing of the polymorphic gene of *ama*1 at the end of the dry season (May 13) and at first malaria episode in the ensuing transmission season (MAL 13) in 64 children.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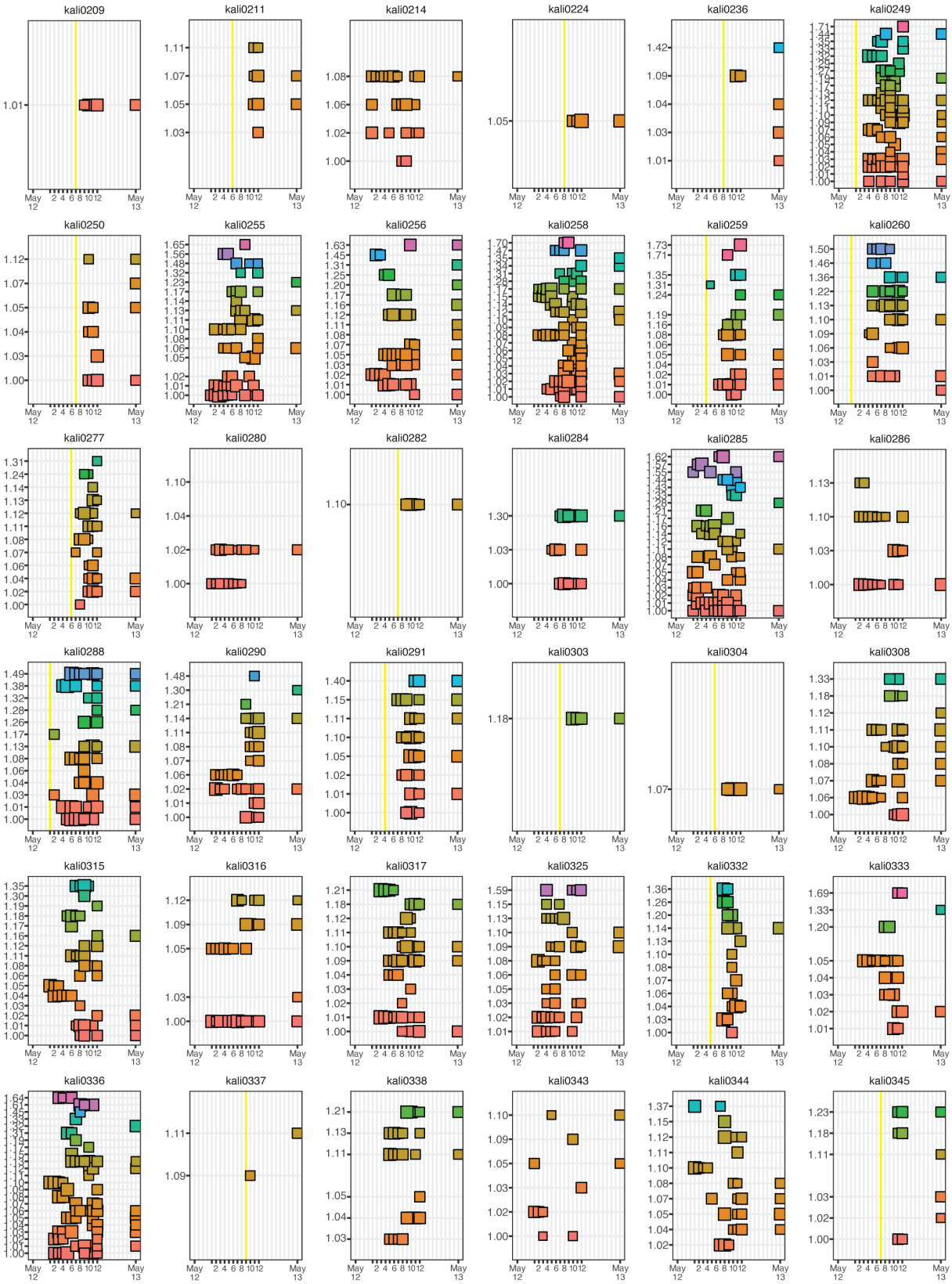
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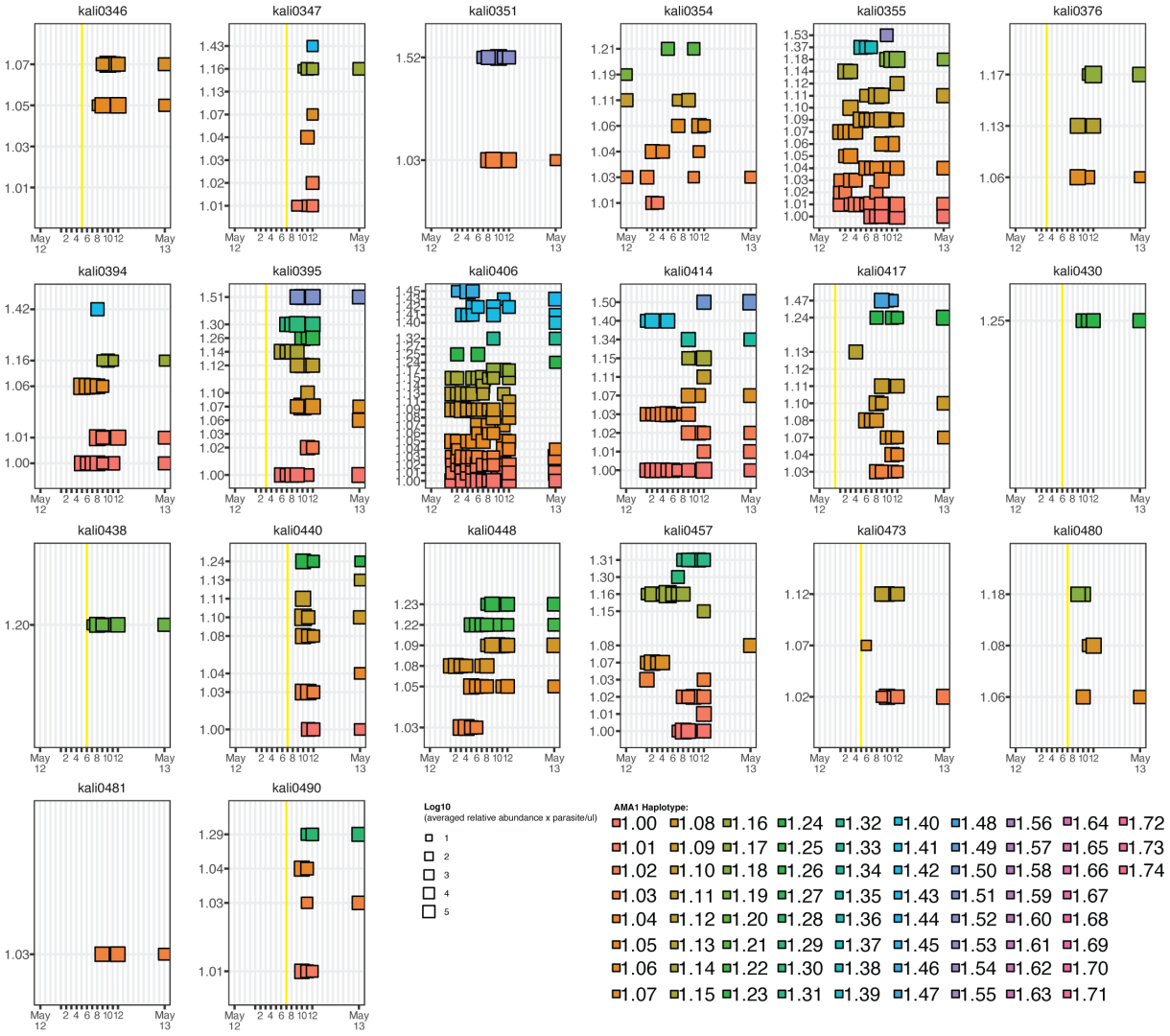
May 12
May 13



Supplementary Figure 5 *P. falciparum* genetic diversity determined by amplicon sequencing of the polymorphic gene of *ama*1 for one year

Starting at the end of the dry season (May 12), during transmission season (1-12 time-points) and at the end of the following dry season (May13) in 92 children that were positive at the end of the dry season in 2013. Yellow lines represent last malaria treatment before the 2013 dry season.