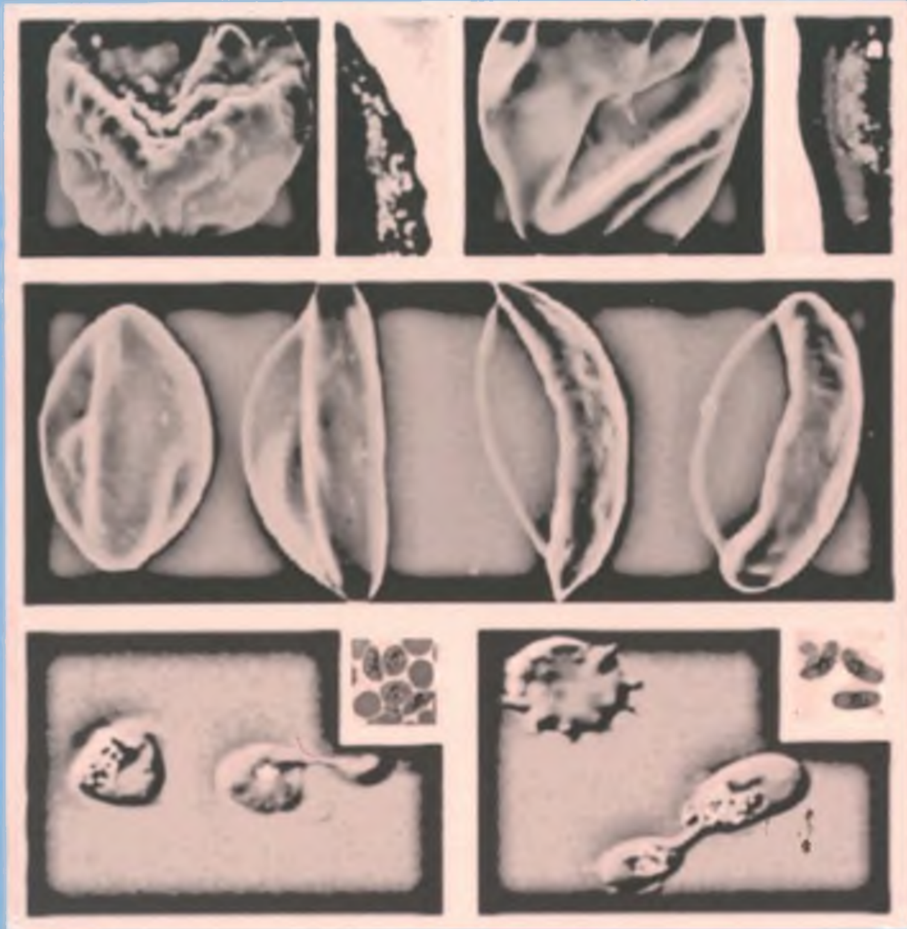

Targeting malaria transmission:

erythrocyte remodeling by *Plasmodium falciparum* in gametocyte-host interplay

Marta Lopes Tibúrcio

Targeting malaria transmission: erythrocyte remodeling by *Plasmodium falciparum* in gametocyte-host interplay

Doctoral Thesis

to obtain the degree of doctor
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on the authority of the Rector Magnificus prof. dr. S.C.J.J. Kortmann,
according to the decision of the Council of Deans
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at 10.30 hours

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volgens besluit van het college van decanen
in het openbaar te verdedigen op dinsdag 10 juni 2014
om 10.30 uur precies

door

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Chapter 1

Introduction

1.1 Malaria

1.1.1 Global burden of malaria

Malaria is a vector born parasitic infection disease caused by protozoan parasites belonging to the genus *Plasmodium*, one of the world's deadliest diseases. Formerly a global epidemic, with 77% of the world's population at risk of infection in the beginning of the 20th century, malaria is, a century after, limited to the tropics with, however, approximately half of the world's population still at risk of malaria [1]. Five *Plasmodium* species are currently recognized to be responsible for malaria in humans, *P. ovale, P. malariae, P. vivax, P. falciparum* and more recently *P. knowlesi.* Each of the different species present different parasite cell morphologies, geographical distribution and epidemiology, being *P. falciparum* the most virulent one, responsible for 90% of the deaths in Sub-Saharan Africa [2]. According to the 2011 WHO report, 216 million cases of malaria were estimated in 2010, 86% of which children under 5 years old, being 81% of the cases and 91% of 655.000 estimated deadly victims reported to have originated in the WHO African Region [3].

The global efforts to control malaria through the widespread use of bed nets, better diagnostics and a wider availability of effective medicines to treat malaria, allowed a 25% global decrease of malaria mortality in the last decade and a 33% reduction in the WHO African Region [3] . However, emerging threats such as the recurrent problem of parasite resistance to antimalarial medicines, more specifically a delayed sensitivity to artemisinin compounds, used in combination with other antimalarials recently reported in Cambodia-Thailand, Myanmar and Vietnam since 2009, can undermine and reverse the malaria control efforts, just like what happened with the spread of the resistance of *P. falciparum* to chloroquine and sulfadoxine-pyremetamine antimalarials in the 1970s and 1980s [3-5]. The absence of an efficient vaccine against malaria leaves chemotherapy as the only effective treatment, and the increasing drug resistance as a major health problem aggravated by cross resistance among drugs belonging to the same chemical family [3]. Another emerging problem is the

increasing resistance of mosquitoes to insecticides used for indoor residual spraying and long lasting insecticidal nets, where 45 countries from all the WHO regions, with exception of Europe, have reported insecticide resistance, 27 of which in the sub-Saharan Africa [3]. Eradication of malaria is a current major ambition. However, additional efforts will be required in order to tackle not only the pathology but also to interrupt transmission.

1.1.2 *Plasmodium falciparum* life cycle

Plasmodium falciparum presents a complex life cycle interacting with multiple tissues in two different hosts, the human and the *Anopheles* mosquitoe, the transmission vector. *P. falciparum* is transmitted to the vertebrate host after injection of sporozoites - pre-erythrocytic stages initially present in the mosquitoe salivary glands - into the human subcutaneous tissue, from where the parasite travels to the liver [6, 7] developing and multiplying inside the hepatocytes [8] into thousands of merozoites that are released into the blood circulation, ready to invade the host's erythrocytes and start the intra-erythrocytic development stage. During the intra-erythrocytic phase, *P. falciparum* can undergo a 48h cycle of asexual replication, associated with the clinical symptoms of malaria, or commit to sexual development, producing the gametocytes, the parasite stages responsible for the transmission to the *Anopheles* vector.

In the asexual cycle the newly invaded parasite matures from a ring form to a mature trophozoite that divides through multiple rounds of nuclear division until the formation of the mature schizont that will release into the peripheral circulation 16 to 32 new merozoites, ready to invade new host erythrocytes and restart the cycle. A fraction of the asexual parasites commits instead to sexual development, gametocytogenesis, undergoing a unique development pathway if compared to the other human malaria species. Female and male gametocytes require a period of maturation of 10 to 12 days in which they are sequestered away from the peripheral circulation in internal organs, from which they are released only at maturity, ready to

be uptaken by the *Anopheles* female during the mosquitoe blood meal (Figure 1). Environmental signals inside the mosquitoe midgut, such as a drop of temperature by approximately 5°C [9], and the presence of the mosquitoe-derived molecule xanthurenic acid (XA), a byproduct of eye pigment synthesis [10, 11], activate gametocyte egress resulting in the release of mature female and male gamete, fertilization and zygote formation. The zygote then becomes a motile and invasive ookinete that penetrates the midgut cell wall and develops into oocyst in the insect hemocel, where the sporozoites are produced. After oocyst rupture, sporozoites migrate to and invade the mosquitoe salivary glands and are now able to infect new vertebrate hosts, wide spreading malaria and restarting the new cycle of human host infection through the mosquitoe bite.

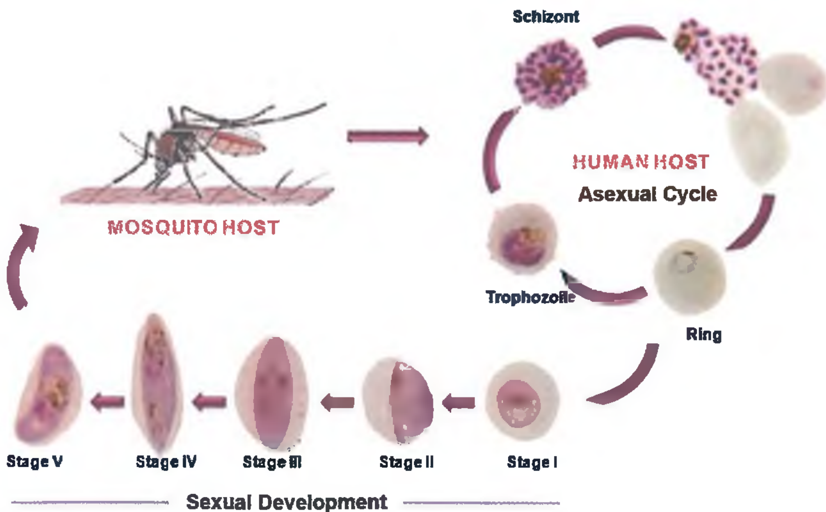

Figure 1. ***Plasmodium falciparum*** **intraerythrocytic life cycle.** Inside the human host, after the erythrocyte's invasion the parasite will undergo a 48h cycle of asexual reproduction, responsible for malaria pathology, where each merozoite will form 16 to 32 new merozoites. A fraction of the asexual parasites will commit to sexual development, which will happen through five different morphological stages, taking approximately 10 days. The mature sexual stages present in the peripheral circulation will then be uptaken by the mosquitoe in the blood meal.

1.1.3 *Plasmodium falciparum* transmission

The presence of the mature sexual stages in the peripheral blood circulation is a crucial aspect of the mosquitoe infectivity and the spread of malaria, as these are the stages uptaken in the blood meal. However, little information exists regarding the dynamics of gametocyte carriage in the areas characterized by different malaria transmission intensity. In such studies most of the evidence derives from analysis of the malaria symptomatic cases, although recently the contribution to transmission represented by asymptomatic carriers is increasingly recognized [12]. In high malaria transmission areas, evidences from microscopy and molecular quantification techniques show that a higher prevalence of gametocytes exist in younger individuals, while the ratio of gametocyte versus asexual parasites seem to increase with the age of the carrier [13]. Although it is generally accepted that high densities of gametocytes do not necessarily result in more efficient mosquitoes infections, a positive correlation between gametocyte density and proportion of infected mosquitoes has been described in both laboratory and natural *P. falciparum* infections [14]. On the other hand efficient malaria transmission can also occur at a lower density than the minimal theoretical gametocyte density threshold of 2 gametocytes per blood meal (2uL). In addition, transmission studies using membrane feeding assay (MFA) in areas of perennial and seasonal transmission showed that submicroscopic densities of gametocytes are able to infect mosquitoes [14]. Although in such cases the proportion of infected mosquitoes is lower when compared to microscopic detected gametocyte density, these evidences emphasize the importance and likely substantial contribution of submicroscopic gametocyte carriage in mosquitoe infection and the relevance of interfering with gametocyte maturation inside the human host.

1.2 *Plasmodium falciparum* gametocytogenesis

Plasmodium falciparum gametocytes originate from a small portion of asexual parasites that commit to sexual development. Unlike the other human malaria species, gametocytes develop through a unique morphological and structural reorganization lasting 10 to 12 days, a time 5 to 6 times longer when compared to the one required for the asexual cycle. During this maturation period gametocytes undergo five different morphological stages (I-V) [15] (Figure 2). Immature stage gametocytes (II-IV) are not visible in peripheral circulation as they mature sequestered in internal organs to be released in the peripheral circulation at maturity (stage V). In the peripheral circulation, *P. falciparum* gametocytes need another 2 to 3 days to become infectious to mosquitoes [16, 17] being the mean circulation time per gametocyte estimated, by microscopy and Pfs25 QT-NSBA, varies between 3.4 to 6.4 days [16, 18, 19].

1.2.1 Gametocyte development

After invasion of the host cell, sexually committed parasites induce profound modifications accompanied by several molecular and structural changes (Figure 2). The sexually committed ring progresses to a round shape gametocyte stage I, morphologically undistinguishable from the asexual trophozoites. Stage II gametocytes represent the first sexual stage morphologically recognizable, presenting a convex half-moon shape, 48 hours after erythrocyte invasion, due to the appearance of the sub-pellicular membrane complex underlying the parasite plasma membrane [20, 21]. This consists of a tri-laminar membrane structure, composed by flattened vesicles [22], interconnected with parallel longitudinally oriented and evenly spaced microtubules [21, 23, 24]. The microtubules begin to polymerize in a coat of spaced parallel microtubules longitudinally oriented and extend along with the subpellicular complex driving the elongation of the parasite [21, 25]. The elongated shape stage III

gametocytes appear around day 4, accompanied by male and female sexual dimorphism and the expression of sex specific markers. The sub-pellicular membrane complex continues to elongate to a straight spindle like form with pointed shape at the extremities at stage IV gametocytes, where sexual dimorphism is more evident. While male gametocytes have a large nucleus and a more diffused pigment, female gametocytes present a relative smaller nucleus and a concentrated pigment pattern, as well as a higher number of osmiofilic bodies, electron dense granules present in the periphery of the parasite's cytoplasm implicated in gamete emergence. During the final period of gametocyte maturation, depolimerization of the subpellicular microtubules occurs, giving rise to a relaxation of the parasite and consequently the erythrocytes that results in a crescent shape stage V gametocyte [24, 25].

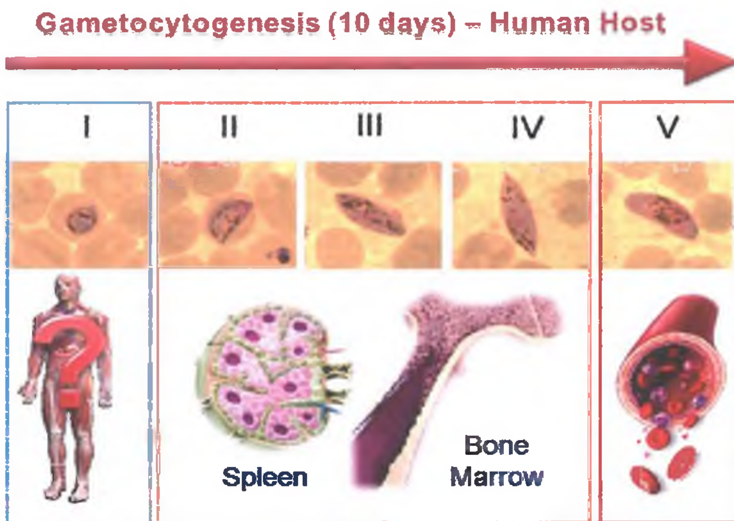

Figure 2. *Plasmodium falciparum* gametocytogenesis. Gametocytes develop through five different morphological stages during approximately 10 days. During this period, immature stages (II-IV) have been observed as preferentially sequestered in the spleen and bone marrow, before release to the peripheral circulation at stage V where they become available to be uptaken in the mosquitoe blood meal.

1.2.2 Sequestration and circulation of *Plasmodium falciparum*

A unique characteristic of *Plasmodium falciparum* among the human malaria species is the ability of asexual parasite-infected erythrocytes to sequester inside the microvasculature of the host internal organs, evading splenic clearance [26, 27]. The profound molecular and structural modifications induced by the parasite after erythrocyte invasion allow infected erythrocytes to efficiently bind to the endothelial cells of the microvasculature of several organs and sequester until completed the cycle and only ring stage parasites are seen in circulation. Among the molecules involved in this parasite adaptation, CD36 and ICAM-1 host cell receptors were reported to play a key role in the cytoadhesion process through the major family of antigenically variable surface proteins, PfEMP1, expressed and exposed on parasite induced modifications on the surface of infected erythrocytes - the knobs [28-31].

Alike asexual parasites, immature stage gametocytes (II-IV) have been reported to sequester in internal organs, being released to the peripheral circulation at maturity (stage V gametocytes). Nonetheless, unlike asexual stages, virtually nothing is known about the mechanism underlying gametocyte sequestration, except for few early observations on post-mortem specimens and a few recent field and clinical reports describing bone marrow and spleen as the organs where immature gametocytes (stage II to IV) predominantly sequester [32-35]. A small number of studies have addressed the gametocyte cytoadhesion. In such studies, however, conflicting data exists regarding the efficiency of cytoadhesion, as well as the involvement of the major parasite adhesion mediating with the host endothelial ligands, PfEMP1, or the host ligands possibly involved. Evidences show a consistent low binding efficiency of immature gametocytes (stage II to IV) to non-endothelial cells and host endothelial cells of different origins mediated through ligands such as ICAM-1 and CD36 [36-39]. Consistent with the low binding efficiency of sexual stages is the absence of knobs, structures thought to play a key role in strengthening the cytoadhesion under flow [40], in immature gametocyte stages [21]. Furthermore, the absence of positive immunoreaction by hyperimmune sera from Gambian children on the surface of

erythrocytes infected by immature stage gametocytes [41] also suggests the absence of antigenic molecules on the surface of immature gametocytes. Altogether these studies suggest that gametocytes remodel the host erythrocyte differentially from the asexual stages, which strongly suggests that the mechanism responsible for gametocyte sequestration is different from that described for asexual cytoadherence to endothelial cells mediated by PfEMP1. It is of crutial relevance to understand gametocyte-host interactions and the mechanism underlying gametocyte sequestration that allows the sexual stages to escape the spleen clearance.

1.2.3 Host cell specific molecular remodeling

Following merozoite entry in the new host cell, the parasite starts remodeling the host erythrocyte by the synthesis of novel structures and the export of hundreds of parasite encoded proteins to the erythrocyte cytoplasm. Such events translate into structural and functional modifications that will allow parasite growth, nutrient acquisition and survival. In the asexual stages the dynamic reorganization of the erythrocyte plasma membrane and submembrane cytoskeleton induced by the parasite is usually associated with changes in the host cell membrane permeability and viscoelastic properties [40, 42] as well as with immune evasion and pathology. The ability to cytoadhere to the microvasculature of several organs and ability to change the expression pattern of a vast repertoire of variant proteins exposed on the erythrocyte's surface allow the parasite to evade both spleen clearance and immune mediated immunity, contributing to the severe pathology associated.

The protein export and host cell remodeling induced by the sexual stages from the beginning of sexual commitment and their implications in host-parasite interaction are comparatively little explored. Alike asexual parasites, protein export is highly active from the onset of sexual differentiation, recruiting a significant set of gametocyte enriched proteins [43]. Some of the proteins previously reported to be expressed and exported beyond the parasite's vacuole membrane to the cell host cytoplasm, are

Pfpeg3, Pfpeg4, Pfg14_744, Pf14_748 [44], Rex3 [45] and PfGEXP10 [43]. Only few proteins have been reported to interact with the erythrocyte membrane during gametocyte maturation, such as MESA (mature-parasite-infected erythrocyte surface antigen), a high-molecular-mass protein that is expressed in both asexual and sexual stages that interacts with the cytoskeleton protein 4 [46, 47], REX2-4 [45] and the gametocyte-specific antigen Pf11-1, a megadalton protein that localizes to the host cell membrane during gametocytogenesis [48]. Furthermore there is evidence for the presence of members of the multigene families encoding RIFINs and STEVORs, associated with erythrocyte membrane at the mid-gametocyte stage (stages III-IV) [49, 50]. Understanding the gametocyte's host cell remodeling induced by the parasite will be fundamental in the understanding of gametocyte-host interplay and the players involved.

1.3 Thesis' aims and outline

Despite malaria being known for over 100 years, very little is currently known about the gametocyte induced host cell remodeling or gametocyte-host mediated interactions during gametocytogenesis. The main aim of this thesis is to explore different aspects of cellular, molecular and mechanical remodeling as well as parasite-host interaction induced by gametocytes during sexual development.

Aiming to study host-gametocyte specific interactions and to establish a reproducible *in vitro* cell binding assay, we address the binding efficiency of the different stage gametocytes to human endothelial cell lines, of different origins, comparing immature versus mature gametocytes and asexual stages (**Chapter 2**). Static cytoadhesion assays using different endothelial cell lines for asexual parasites as well as mid and late-stage gametocytes from the well characterized *P. falciparum* lines are described in **Chapter 2**. In particular,

attention was paid to PfEMP1 and KAHRP molecules because of their inconclusive presence and role of during gametocytogenesis. These molecules may be involved in immune mediated escaped mechanisms of the parasite and the spleen clearance. A novel protocol, previously described, was used for the isolation of the very early sexual stages, thus allowing the investigation of the expression and trafficking of KAHRP and PfEMP1 to the gametocyte infected erythrocyte membrane in early sexual development (**Chapter 3**).

P. falciparum gametocytes present a drastic morphological and structural development during a period of approximately 12 days, unique among *Plasmodium* species infecting humans. During this long period the parasites develop through five different morphological stages, but only the mature stage V parasites are seen in circulation. In **chapter 4** we address the mechanical properties of the gametocyte-infected erythrocytes at different developmental stages in ecktacytometry studies, using the laser-assisted optical rotational cell analyzer, and microsphiltration analysis that aim to study the role of this parameter during gametocyte sequestration and release.

Along with the morphological remodeling, protein export is very active at early stage gametocytes, as previously reported by a recent proteomic analysis, [43] reporting a set of 26 putatively exported proteins enriched in early stage gametocytes of *P. falciparum* (PfGEXP). Aiming to further address and understand the role of molecular remodeling at early sexual stages of development, we describe a novel exported protein and its detailed characterization in **Chapter 5**.

In **chapter 6** we review all aspects related to the gametocyte-infected host cell molecular and mechanical remodeling induced by the parasite during sexual development, including our recent achievements.

The final chapter of this thesis (**chapter 7**) contains the resume of the all the results obtained during the development of the doctoral thesis and the discussion of the implications of the novel data for future studies.

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Chapter 2

Differential adhesive properties of sequestered asexual and sexual stages of *Plasmodium falciparum* on human endothelial cells are tissue independent

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Abstract

The protozoan parasite *Plasmodium falciparum*, responsible of the most severe form of malaria, is able to sequester from peripheral circulation during infection. The asexual stage parasites sequester by binding to endothelial cell receptors in the microvasculature of various organs. *P. falciparum* gametocytes, the developmental stages responsible for parasite transmission from humans to Anopheles mosquitoes, also spend the almost ten days necessary for their maturation sequestered away from the peripheral circulation before they are released in blood mainstream. In contrast to those of asexual parasites, the mechanisms and cellular interactions responsible for immature gametocyte sequestration are largely unexplored, and controversial evidence has been produced so far on this matter. Here we present a systematic comparison of cell binding properties of asexual stages and immature and mature gametocytes from the reference *P. falciparum* clone 3D7 and from a patient parasite isolate on a panel of human endothelial cells from different tissues. This analysis includes assays on human bone marrow derived endothelial cell lines (HBMEC), as this tissue has been proposed as a major site of gametocyte maturation. Our results clearly demonstrate that cell adhesion of asexual stage parasites is consistently more efficient than that, virtually undetectable of immature gametocytes, irrespectively of the endothelial cell lines used and of parasite genotypes. Importantly, immature gametocytes of both lines tested here do not show a higher binding efficiency compared to asexual stages on bone marrow derived endothelial cells, unlike previously reported in the only study on this issue. This indicates that gametocyte-host interactions in this tissue are unlikely to be mediated by the same adhesion processes to specific endothelial receptors as seen with asexual forms.

Introduction

Plasmodium falciparum-infected erythrocytes are characterized by their ability to adhere to the endothelial cells lining the microvasculature of various organs. Adhesion of the asexual (trophozoite/schizont) stage-infected erythrocytes and their sequestration away from the peripheral circulation is implicated in malaria pathogenesis. *In vitro* binding assays with erythrocytes infected with asexual-stage parasites have revealed specific interactions between one or more receptors on the host endothelium and parasite-encoded ligands on the infected erythrocytes. Host cell receptors CD36 and ICAM-1 (CD54) are thought to be the major receptors in the adhesion of most *P. falciparum* isolates [1]. Members of the *P. falciparum* Erythrocyte Membrane Protein-1 (PfEMP-1) family of variable surface-expressed parasite antigens have been shown as parasite ligands mediating adhesion of asexual-stage-infected erythrocytes.

In *P. falciparum* not only asexual stages are able to sequester in internal organs. A portion of parasites in the bloodstream does not progress into the asexual cycle but differentiate into gametocytes, the parasite stages able to mature into gametes when engorged in the blood meal of a biting *Anopheles* mosquito, and therefore responsible of *Plasmodium* transmission from humans to the insect vector. *P. falciparum* gametocytes mature in about ten days, in an approximately five time longer period than asexual stages, in which they undergo morphological transformations classically divided in five distinct stages [2]. Only gametocytes at the last developmental stage (V) are normally detectable in peripheral blood of infected individuals. Immature gametocytes (stages I to IV), like asexual stages, have instead the ability to sequester in poorly defined body sites, from which they are released only when they reach maturity.

In contrast to the above described studies on asexual forms, the adhesion of erythrocytes infected with sexual-stage parasites has been poorly described. After early reports from the first years of malariology describing bone marrow and spleen as

the organs where all stages of gametocyte maturation are readily found, followed by few recent confirmations [3-5], systematic studies on sites of gametocyte sequestration are still not available. The only information currently available on gametocyte cytoadhesion is contained in a few reports using cell lines, on which binding of stages II to V gametocytes, the stages clearly recognizable by morphology, was measured. Gametocyte adhesion has been explored by Rogers [6] in a static-binding assay on C32 amelanotic cell line, which constitutively express both ICAM1 and CD36, reporting that mid-stage gametocytes (stage III-IV) are able to adhere to such cells, although at a lower level compared to asexual stages. In this study asexual and gametocyte adhesion was inhibited to a similar extent by anti-CD36 and anti-ICAM-1 antibodies, which led authors to suggest a common role of these receptors in mediating cytoadhesion. This conclusion was however not confirmed by the work of Day *et al.* [7], where adhesion phenotypes of different gametocyte stages of the parasite clone 3D7 (and of 6 additional isolates and clones) were assessed in binding assays on the above cell line and on purified ICAM-1 and CD36. No adhesion of mid-stage gametocytes was observed in that work neither on C32 cells nor on purified ligands. In another report specifically investigating the cellular interactions possibly governing gametocyte sequestration in bone marrow, Rogers *et al.* [8] used for the first time endothelial cell lines derived from human bone marrow (HBM) endothelium and stroma to compare binding of 3D7 asexual and mid- and mature sexual stages. Results were that mid-stage gametocytes showed a low binding affinity to these cell lines, which was nevertheless comparatively higher than that observed for asexual parasites. The main ligand involved in such binding, based on antibody inhibition experiments, was proposed to be ICAM1. Stage V gametocytes did not show any appreciable binding, consistent with their condition of being freely circulating cells.

In order to re-assess the controversial conclusions of the above reports, the present work provides a systematic comparison of asexual and sexual stage cell binding properties conducted on a panel of endothelial cell lines derived from different tissues and expressing different levels of the main ligands - ICAM1 and CD36 - proposed above to be implicated in gametocyte binding. Cell lines used were commercially

available primary lines HUVEC and HDMEC and two HBM-derived cell lines, HBMEC-60 and HBMEC-33 [9], used here for the first time in malaria research. The latter lines were developed and are currently used to study the cellular and molecular interactions between hematopoietic progenitor cells (HPC) and bone marrow endothelium responsible for homing, endothelial transmigration and interplay governing transplantation efficiency of hematopoietic precursors [10-12]. Analysis of baseline and induced expression of 44 surface molecules and receptors along several weeks of cultivation showed that such lines stably maintain the specific features of primary BM derived endothelial cells. In order to directly compare results with the above reports on gametocyte adhesion, most of the experiments were similarly carried out with the stable gametocyte producer parasite clone 3D7. Besides high gametocyte production, this clone stably retains the ability to adhere to C32 melanoma cells without selection by panning on host cells or ligands [6, 13]. Part of this remarkable phenotypic stability is probably due to the fact that long term propagation of this parasite clone is not accompanied by major chromosomal rearrangements [13], particularly subtelomeric deletions responsible for loss of knob production, cytoadhesion and gametocytogenesis [14]. Finally, in order to provide a dataset directly comparable to a variety of parasite cell binding studies in *P. falciparum*, adhesion experiments in this work were performed conforming to protocols currently used in studying *P. falciparum* asexual stage cytoadherence [15].

Materials and methods

Parasite culture

P. falciparum clones 3D7A [16], ItG [17] and isolate AQ104 were grown in 0+ red blood cells in RPMI 1640 plus hypoxanthine 50mg/ml, supplemented with 10% heat-inactivated 0+ human naturally-clotted serum, at 37° C, in a 2% O_2 and 5% CO_2 atmosphere. The cryo-preserved isolate AQ104 [18] was thawed by a stepwise replacement of glycerol with salt (NaCl) and systematically adapted to culture

conditions. Parasites were cultured in complete cell culture medium (RPMI supplemented with L-glutamine, 0,1 mM hypoxanthine, gentamicin, 2% heat-inactivated AB serum, and 5% albumax II) in the presence of O+ or A+ blood at 5% hematocrit and a gas mixture of 5% CO_2, 5% O_2 and 90% N_2. The parasite population was kept between 0.1 and 10% (parasites/erythrocytes) with regular change of medium and the addition of fresh blood.

For parasite synchronization, cultures at 8–10% parasitaemia at 10% haematocrit were centrifuged at 3000 rpm for 10 min through a 60% Percoll cushion and slow sedimenting schizonts used to reinvade fresh red blood cells. Prior to use, parasites were washed twice in binding buffer (RPMI 1640 medium, supplemented with 6mM glucose, pH 7.2) and re-suspended at 1% haematocrit and 3% parasitaemia (Giemsa staining). Induction of gametocytogenesis was performed as follows: 2% synchronous trophozoites were incubated overnight on a shaker to obtain around 10% ring stage parasites on day 1. On day 2 the culture was split to obtain a schizont parasitaemia of approximately 2%. About 40 h after reinvasion from merozoites produced by such schizonts stage I gametocytes were observable in culture amongst unhealthy asexual parasites. The latter were cleared by incubation with N-Acetylglucosamine for 48 h to obtain virtually pure gametocyte cultures, which were maintained with daily change of medium until they reached the required maturation stages.

Endothelial cells

Characterised human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HDMEC) were obtained from PromoCell, while human bone marrow endothelial lines, HBMEC-60 and 33 [9], were kindly provided by Dr. E van der Schoot (CLB, Sanquin Blood Supply Foundation). HUVEC and HDMEC cells were routinely grown in gelatin-coated culture flasks and in Endothelial Cell Grown Medium (PromoCell) supplemented with 10% (v/v) heat-inactivated FCS, while for HBMEC cells Lonza BulletKit® medium (Lonza AG) was used.

Flow Cytometry

The expression of endothelial cell markers was measured by fluorescence-activated cell sorting (FACS). Specific fluorescence-conjugated antibodies were used: APC Mouse anti-human CD54 (ICAM-1) and PE Mouse anti-human CD36 (Becton Dickinson, CA). Nonspecific fluorescence was assessed using corresponding isotype control antibodies. After 16 hours of cytokine activation, endothelial cells culture medium was gently removed and cell were washed once with fresh medium, followed by three washes with phosphate-buffered saline (PBS) to remove any adherent IEs. Endothelial cells were detached by gentle trypsin (Sigma) treatment, washed with PBS containing 1% BSA and stained with antibodies for 60 min on ice in 100 ml of the recommended dilution. Cells were washed twice in PBS with 1% BSA and a FACS analysis was carried out using a FACSCanto (Becton Dickinson, CA). The expression of surface molecules was indicated by geometric mean of the fluorescence intensity.

Static cell assay

Static cell binding assays were carried out using a modified version of a previously described method [14]. Endothelial cells (6th passage) were seeded onto 1% gelatin coated 13mm Thermanox coverslips (Nalgene, Nunc). Once confluent, they were incubated overnight at 37°C with or without 0.5 ng ml−1 rTNF-alpha or rIL-1beta (Invitrogen). Cells were washed with binding buffer supplemented with 1% (v/v) of naturally-clotted human serum and incubated with 0.5 ml of parasite suspension (3% parasitaemia, 1% haematocrit) for 1 h at 37°C, with gentle resuspension every 10 min. After washes in binding medium, coverslips were placed upside down in new clean microplate wells filled with fresh binding medium for 30 minutes. The remaining unbound cells detached from the endothelial layer sinking down by gravity. The procedure was repeated twice. After removal of unbound cells, adherent cells were fixed using 1% glutaraldehyde for 1 h and then stained with 5% Giemsa for 30 min. For cytokine stimulation, recombinant human SDF-1 alpha (CXCL12, PeproTech Inc.) was added at different concentrations to the parasites suspension during cells incubation for 2 hours at 37°C. Coverslips were dried and mounted on slides using

DPX mounting buffer (BDH Lab Supplies). For each condition/line bound parasites in triplicate wells were counted at 400X magnification and their numbers expressed as the number of infected RBC per mm^2. All experiments were repeated at least three times.

Results

In order to ensure comparability of the present experiments with state of the art cytoadhesion studies in *P. falciparum* [15], cell binding assays were performed using parasites directly taken from culture, rather than after Percoll or gelatin/plasmagel purification. Appropriate volumes of uninfected blood were used to adjust parasite numbers and culture hematocrit in comparing different parasite stages/samples. Two preliminary sets of experiments were performed with the HUVEC, HDMEC and HBMEC-60 endothelial cell lines. In the first, basal expression and TNF-alpha mediated upregulation of CD36 and ICAM-1 were measured with specific antibodies in the three endothelial cell lines. This experiment (Figure S1) confirmed that the host ligands were induced by the cytokine, and showed that HUVEC and HBMEC are essentially ICAM1-positive/CD36-negative, while HDMEC are ICAM1 /CD36-double positive [9, 19], thus providing cues to the functional role of such receptors in this comparative analysis. In the second asexual parasite binding efficiencies in presence/absence of TNF-alpha were measured. In these experiments the *P. falciparum* clone ItG, a reference clone in cytoadhesion studies whose stable cytoadherent phenotype is maintained by panning selection on HDMEC cells [17], and the gametocyte producer clone 3D7 were used. Endothelial cells were grown to confluence, and exposed to TNF-alpha (0.5 ng ml−1) for 12 h or left untreated. Same numbers of late trophozoites from synchronous asexual cultures of ItG and 3D7 were adjusted to 1% hematocrit, and incubated for 2h. After removal of unbound uninfected and infected erythrocytes, cell monolayers were fixed and stained by Giemsa, and the numbers of bound parasites per mm^2 of cell layer were counted.

Results of experiments (Figure 1) confirmed that TNF-alpha is a potent inducer of the lost ligands mediating asexual parasite adhesion, and suggested to undertake the subsequent gametocyte adhesion assays in TNF-alpha-stimulated cells. These experiments also showed that 3D7 asexual infected erythrocytes maintain a stable cytoadherent phenotype not only on C32 melanoma cells as mentioned above [13] but also on the panel of endothelial cells. Data on binding of 3D7 parasites to endothelial cells are scarce in the literature despite this being a reference clone in malaria research, and this experiment provides, to our knowledge, the first systematic comparison of adhesion of asexual stages of this clone and a clone ItG, widely used in adhesion studies. The comparison shows that 3D7 has a generally lower binding efficiency than ItG, which could be partly explained by the fact that 3D7 parasites used here were not routinely selected by panning. Another likely explanation is that 3D7 cytoadhesion is efficiently mediated by CD36, which is poorly expressed by HUVEC and HBMEC and is more abundantly produced on HDMEC cells, whilst it is reported that ItG binding relies on both CD36 and ICAM1, and the latter being efficiently stimulated by TNF-alpha on the surface of all the above endothelial cells [17].

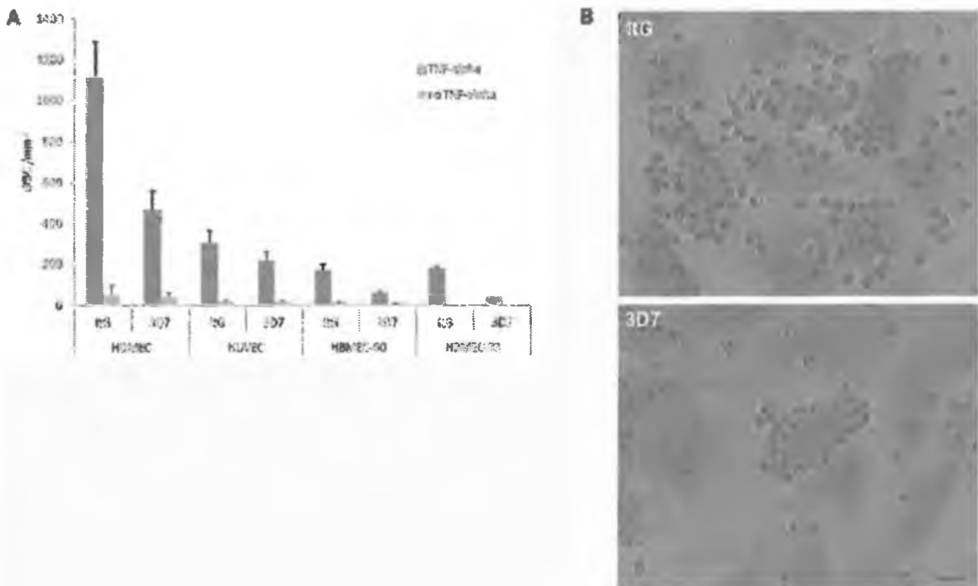

Figure 1 - Adhesion of asexual stages of ItG and 3D7 on stimulated and non stimulated HDMEC, HUVEC and HBMEC endothelial cell lines. A) Data shown are the mean number of iRBC per $mm^2 \pm$ S.E. of 3 to 5 biological replicates. Static assays were carried out as described in the Material and methods section. B) Giemsa-stained infected erythrocytes bound to TNF-alpha activated endothelial cells (HDMEC). Scale bar: 25mm.

Cytoadhesion of 3D7 asexual and sexual stages

Having confirmed the requirement of TNF to upregulate expression of the relevant host ligands, a comparative analysis of adhesive properties of asexual parasites and immature (stage III-IV) and mature (stage V) gametocytes was conducted on the above panel of endothelial cell lines. Experiments always included ItG asexual parasites as positive control, enabling expression of cell binding efficiencies of 3D7 asexual and sexual stages as percent of ItG cell binding (Figure 2). Unfortunately, the failure of the ItG clone to produce gametocytes prevented the possibility to directly compare asexual and sexual stage binding in this genetic background. One result of these experiments clearly showed that mature stage V gametocytes fail to bind to HUVEC, HDMEC and the newly used HBMEC lines to any measurable levels. This extends previous observations that mature gametocytes do not bind to C32 melanoma cells and the HBM endothelial and stromal cell lines used in [8] to the panel of endothelial cell lines used here. The most relevant result of this analysis is however that mid-stage gametocytes show a significantly lower adhesion to endothelial cells from all tissues/organs tested compared to asexual parasites, and that importantly this applies also to the bone marrow derived HBMEC cells. This observation is in contrast to that reported by the only available study addressing this issue [8], and does not support the hypothesis that specific host-gametocyte adhesive interactions are responsible for sequestration of immature sexual stages in bone marrow.

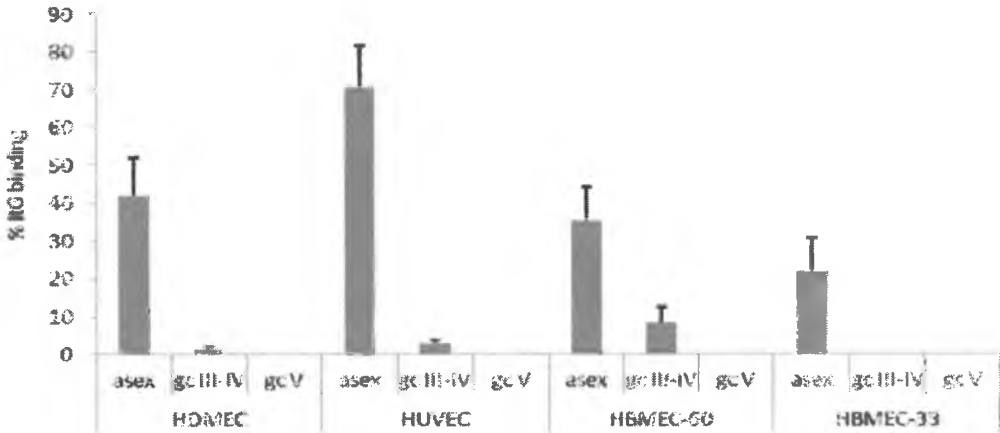

Figure 2 - Adhesion of asexual and sexual stages of 3D7 on TNF-alpha stimulated HDMEC, HUVEC and HBMEC cell lines. Static assays were carried out as described in the Material and methods section. The mean number of iRBC per mm^2 ± S.E. of 3 to 5 biological replicates were counted, and are expressed as % of bound parasites per mm^2 of the ItG control.

Parasite adhesion to endothelial cells in presence of Interleukin-1 beta and bone marrow Stromal-Derived Factor-1

In order to further investigate asexual stage and gametocyte binding to endothelial cells, the possible role of additional signaling molecules beside the inflammatory cytokine TNF-alpha was investigated. One experiment measured effect of Interleukin-1beta (IL-1beta), another inflammatory cytokine mediating host ligand upregulation [20, 21], on the binding of asexual parasites and mid-stage gametocytes. HDMEC and HBMEC cells were exposed to 10U per ml of IL-1beta for 6 h, and adhesion assays were performed as described above. Results of the experiment were that, although IL-1beta was able to stimulate asexual stage binding on HDMEC and HBMEC endothelial cells, as observed for TNF-alpha, no difference could be observed for gametocyte binding, which was virtually undetectable as observed in the same cells stimulated by TNF-alpha (Figure 3 A). In another experiment, binding of the same parasite stages was measured in response to increasing concentrations of Stromal-

Derived Factor-1 (SDF-1). This cytokine plays a role in bone marrow physiology as the major chemoattractant for homing, adhesion and extravasation of hematopoietic progenitor cells (HPC) [22]. For this reason only HBMEC cells were used in this experiment. Such cells were incubated with 0, 30 and 100 ng per ml of SDF-1 for 2 h, and asexual and sexual stage binding performed as above. Result of this experiment (Figure 3 B) showed that, in the conditions tested, SDF-1 had no effect in promoting adhesion of mid-gametocytes. In addition the experiment showed that, unlike observed with the above inflammatory cytokines, SDF-1 did not promote an increased binding of asexual stages.

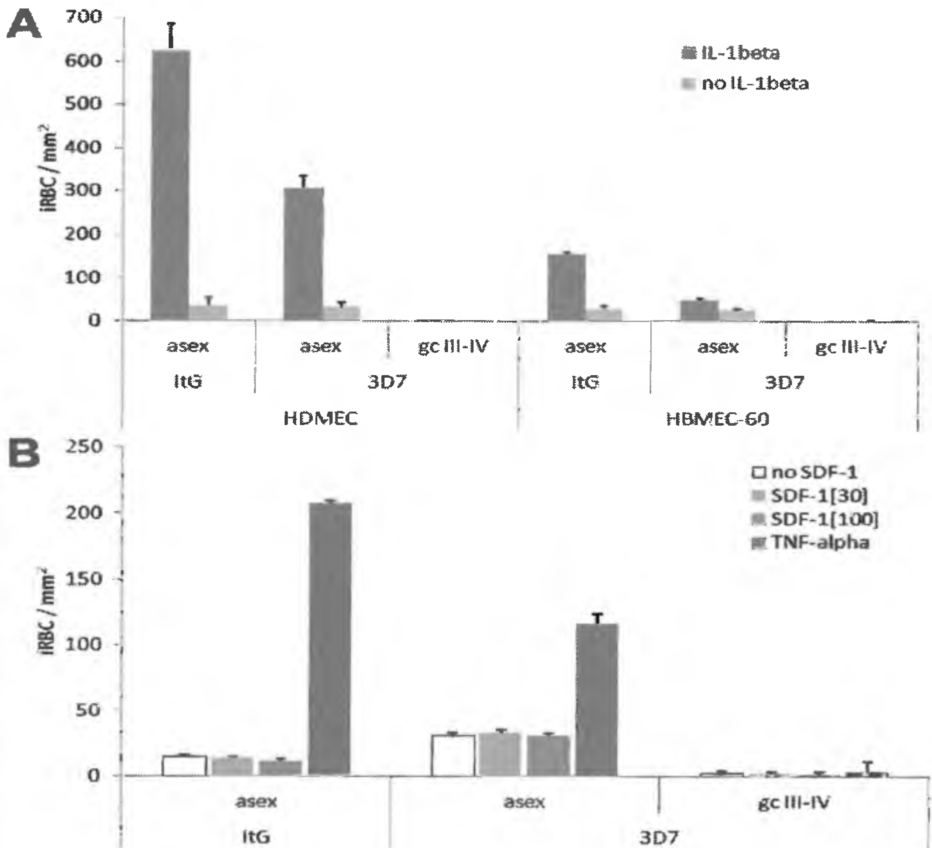

Figure 3 - Influence of IL-1beta and SDF-1 on adhesion of asexual and sexual stages of 3D7 on endothelial cells. A) Adhesion of asexual and sexual stages of 3D7 on HDMEC and HBMEC-60 stimulated with IL-1beta. B) Adhesion of asexual and sexual stages of 3D7 on HBMEC-60 with different concentrations of SDF-1. Static assays were carried out as described in the Material and methods section. Data shown are the mean number of iRBC per mm^2 ± S.E. of 2 to 5 biological replicates.

Adhesion of sexual and asexual stages of a gametocyte-producing parasite isolate.

The parasite clone 3D7 is able to successfully propagate through mosquitoes, and its use in human volunteer experimental infections indicates that this parasite is fully competent to infect humans [23, 24]. In order to nevertheless rule out that the observed failure of 3D7 gametocytes to appreciably bind to host cells was due to an unrecognized deficiency due to laboratory adaptation, a set of adhesion assays on the panel of endothelial cells was also performed with gametocytes from a recently, independently adapted parasite isolate with an unrelated genetic background. The African isolate AQ104 was obtained in the field study described in [18], and was observed to produce gametocytes for a few weeks of cultivation (Dr. S. Borrmann, University of Heidelberg, personal communication). It was thus possible to use freshly thawed parasite cultures of AQ104 to induce sexual differentiation and obtain mid-stage gametocytes for this experiment. Adhesion assays were performed on HDMEC, HUVEC and HBMEC with asexual and mid-stage gametocytes from AQ104, in parallel with asexual and sexual stages from clones 3D7 and ItG asexual parasites. Results of these experiments showed that asexual stages of AQ104 were able to adhere to endothelial cells of the different lines with efficiencies comparable to those shown by 3D7 (Figure 4).

Importantly, the binding of mid-gametocytes produced by AQ104 was invariably significantly lower than observed for asexual stages of the same line, and was hardly detectable as observed for the sexual stages produced by 3D7. This confirmed that, in the conditions of this experiment, mid-stage gametocytes from a genetically distinct isolate from 3D7 also exhibit minimal, if any, cytoadherence efficiency.

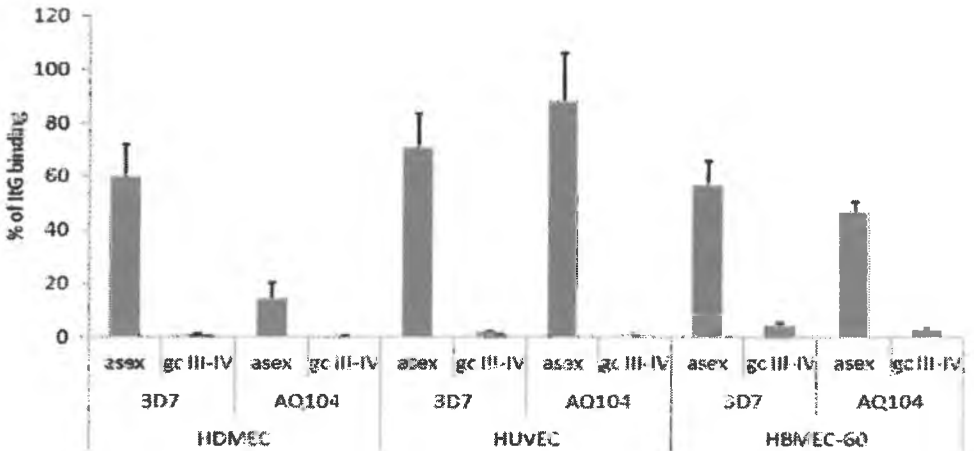

Figure 4 - Adhesion of asexual and sexual stages of AQ104 and 3D7 on TNF-alpha stimulated HDMEC, HUVEC and HBMEC cell lines. Static assays were carried out as described in the Material and methods section. Values are expressed as % of bound parasites per mm^2 of the ItG control. Data shown are the mean number of iRBC per mm^2 ± S.E. of at least 3 biological replicates was counted and expressed as % of bound parasites per mm^2 of the ItG control.

Discussion

Mechanisms of cytoadherence of asexual stages of *P. falciparum* have been extensively studied and different *in vitro* models to measure static and flow adhesion of these parasites to endothelial cells have been developed [25]. On the contrary, the hypothesis that sexual stages may interact through specific molecules with the human endothelium is still controversial, partly because several experiments measured

gametocyte adhesion on non-endothelial cell lines [6, 7]. The present work aimed to further address this issue by systematically comparing adhesion of asexual and sexual stages of *P. falciparum* on a panel of endothelial cell lines from various human organs/tissues. We dedicated special attention to specific aspects of the binding assay protocol. The first was to avoid artifacts in cell adhesion possibly introduced by parasite enrichment procedures using colloids such as Percoll [26]. We noticed that Percoll gradient purification of asexual or sexual stages was sometimes associated to detection of higher numbers of bound parasites of both stages compared to untreated parasite cultures. On the other hand use of magnetic columns (MACS) [27] for enrichment of pigmented asexual or sexual stage parasites often resulted in abundant presence of free haemozoin granules in the binding assays, particularly in the case of sexually induced cultures which need to reach high asexual parasitaemias. Free pigment was hardly removed by washing steps and was thought to possibly interfere with binding and with accurate parasite counts on the endothelial cell monolayers. For the above reasons and, importantly, to conform to routine protocols used to measure asexual stage adhesion, all cell binding assays were here performed by simply diluting cultures with parasites of the desired stages, and identifying the bound asexual and sexual cells by their typical morphologies. Other features of the experiments were to consistently expose the endothelial cell monolayers to equal numbers of parasites in all samples, and to routinely include in all binding assays the reference internal positive control of the ItG clone to confidently compare different experiments.

A major result of the present comparative analysis is that immature, mid-stage gametocytes, equivalent to those able to sequester in natural infections, consistently show a dramatically lower cell binding efficiency than isogenic asexual parasites on the entire panel of endothelial cell lines. An obvious difference between asexual stages and the immature gametocytes analyzed in this study (stage III-IV) is that the sexual stages do not modify the surface of the infected red blood cells with knobs [28], the cellular structures where the major parasite ligand PfEMP1 is exposed [14]. It should be however noticed that static binding assays such as those used in this study are able to detect the binding, albeit at lower efficiencies, of asexual parasites lacking

knobs but still expressing low levels of PfEMP1 [14]. Failure to detect gametocyte binding in the present assays therefore suggests that such a ligand, or alternative ligands with similar specificity, are likely to be absent from the surface of the red blood cells infected by stage III-IV gametocytes.

In this study asexual stage parasites showed different cell binding efficiencies, varying between parasite clones and endothelial cell lines used, whilst the poor binding values observed for sexual stages were independent from the endothelial cell lines used. Importantly, immature gametocytes from both the reference laboratory clone 3D7 and from a newly established parasite isolate do not exhibit a comparatively higher binding efficiency to HBM-derived endothelial cell lines compared to lines from other host tissues. This result does not support a previous hypothesis that sequestration of sexual stages in the bone marrow is possibly mediated by interactions between specific ligand on the host cells and the sexual stage parasites [8]. Although the question of the role of bone marrow as a major maturation site for *P. falciparum* gametocytes is awaiting a state of the art re-examination, several reports indeed stated that developing gametocytes are readily found in post mortem analyses and in aspirates from this tissue [3, 29]. In this respect it is interesting to mention that specific human cell types such as haematopoietic precursor cells (HPCs) and metastatic cells from some epithelial tumors, also find in bone marrow, a preferential homing/maturation site [30]. However, when HPCs were tested in cell adhesion assays on endothelial cell lines of different origin, they failed to show a higher binding efficiency on endothelial cells of HBM origin, suggesting that additional factors are needed to promote such specific interactions [9]. In the case of HPCs and of several of the above tumor cells, a major role as a chemoattractant is played by SDF-1 [31, 32]. A potential role of this factor in triggering the binding of gametocytes to HBM endothelial cells was tested in the present work. In this case, binding assays were performed in absence of TNF-alpha or IL-beta stimulation to mimic the non-inflammatory condition of bone marrow in a typical non symptomatic gametocyte carriers [33]. No effects of SDF-1 were however observed in promoting adhesion of gametocytes, and of asexual parasites, in the experimental conditions used here. An

additional consideration of the possible host-gametocyte interactions in the HBM environment is that blood circulation in this tissue occurs through sinusoids in which the discontinuous endothelium structure physiologically allows cells to pass in and out of circulation [34]. It is therefore conceivable that gametocytes have direct access to stromal cells with the absence of significant shear flow stress in this environment. In such a case gametocytes would not need to have asexual stage-like properties largely mediated by ICAM1 and CD36 for being retained in this environment, and/or could rely on different classes of ligands unrelated to those typically exposed on endothelial cells.

Cell binding assays cannot recapitulate the cellular and topological complexity displayed by human tissues, and that this caveat particularly applies to intricate microenvironments such as the bone marrow niche. The present work nevertheless confirms that asexual stages expose parasite surface molecules able to efficiently bind host ligands and to mediate adhesion like in their natural sequestration sites, and shows that in contrast gametocyte-infected red blood cells fail by the same criteria to appreciably show such a phenotype. The consequent hypothesis that minimal levels, if any, of parasite ligands are present on gametocyte-infected erythrocytes is in agreement with the recent observations that no specific antigens could be identified on the surface of immature gametocytes [35]. This on one hand may be consistent with the requirement of the developing gametocyte to keep a very low antigenic profile in the long period of its sequestered maturation. On the other hand this suggests that specific high affinity interactions between host and parasite encoded/induced ligands are not used by immature gametocytes as the major mechanism of parasite sequestration, in contrast to what is known for asexual stages. Speculation on additional, or alternative, mechanisms used by immature gametocytes to maintain sequestration may be suggested by recent observations that alteration of mechanical properties of erythrocytes infected by asexual parasites can affect the spleen retention process [36] and that red blood cells infected by immature gametocytes are significantly more rigid than those infected by mature gametocytes [37].

Elucidation of the mechanisms of gametocyte sequestration and identification of the involved body sites are obviously still awaiting definitive answers. The renewed interest in such fundamental issues of *P. falciparum* biology is however promising that understanding and interfering with such mechanisms will hopefully soon become an additional avenue in the effort to eliminate malaria.

Supporting Information

Figure S1 –Constitutive and TNF-alpha stimulated expression of endothelial cells markers ICAM-1 and CD36 in HUVEC, HDMEC and HBMEC-60 cell lines. Expression levels were determined by FACS and expressed as geometric means of fluorescence intensity and percentage of positive cells.

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Chapter 3

Early gametocytes of the malaria parasite *Plasmodium falciparum* specifically remodel the adhesive properties of infected erythrocyte surface.

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Abstract

In *Plasmodium falciparum* infections the parasite transmission stages, the gametocytes, mature in ten days sequestered in internal organs. Recent studies suggest that cell mechanical properties rather than adhesive interactions play a role in sequestration during gametocyte maturation. It remains instead obscure how sequestration is established, and how the earliest sexual stages, morphologically similar to asexual trophozoites, modify the infected erythrocytes and their cytoadhesive properties at the onset of gametocytogenesis. Here, purified *P. falciparum* early gametocytes were used to ultrastructurally and biochemically analyze parasite induced modifications on the red blood cell surface and to measure their functional consequences on adhesion to human endothelial cells. This work revealed that stage I gametocytes are able to deform the infected erythrocytes like asexual parasites, but do not modify its surface with adhesive "knob" structures and associated proteins. Reduced levels of the *P. falciparum* Erythrocyte Membrane Protein-1 (PfEMP1) adhesins are exposed on the red blood cell surface by these parasites, and the expression of the *var* gene family, which encodes 50-60 variants of PfEMP1, is dramatically downregulated in the transition from asexual development to gametocytogenesis. Cytoadhesion assays show that such gene expression changes and host cell surface modifications functionally result in the inability of stage I gametocytes to bind the host ligands used by the asexual parasite to bind endothelial cells. In conclusion these results identify specific differences in molecular and cellular mechanisms of host cell remodeling and in adhesive properties, leading to clearly distinct host parasite interplays in the establishment of sequestration of stage I gametocytes and of asexual trophozoites.

Introduction

The burden posed by malaria on human health results from the severity of the disease, annually causing almost a million deaths predominantly by *Plasmodium falciparum*, and by the efficient transmission of the parasite between humans and mosquitoes, responsible for 350-500 million new infections per year. The *P. falciparum* stages transmitted from infected humans to mosquitoes are the gametocytes, the specialized sexual cells formed in the bloodstream by a fraction of parasites which cease asexual propagation. *P. falciparum* gametocytes require around ten days to reach maturation, during which five morphological stages of development have been conventionally defined [1]. Such a long maturation period, compared to the 44-48h necessary to complete the asexual cycle, and the peculiar elongated shape assumed by gametocytes from stage II to V are unique features of this species amongst the human malaria parasites. In contrast to mid and late gametocytes, the early sexual cells at 24-30 h of maturation (stage I) are hardly distinguishable by morphology from the round shaped asexual trophozoites, although they already produce a set of abundant stage-specific proteins [2].

In *P. falciparum* infections, asexual and sexual stages sequester in the microvasculature of several organs during intraerythrocytic development, thus avoiding passage and clearance in the spleen. In the asexual cycle only the early asexual parasites ("ring" stages – until 24h after red blood cell invasion) are observed in the peripheral circulation while the late asexual stages (trophozoites and schizonts) cytoadhere to host receptors such as ICAM-1 and CD36 [3] on the vascular endothelium of several organs through the antigenically variable adhesins of the *Pf* Erythrocyte Membrane Protein 1 (PfEMP1) gene family. In the transition from ring stage to trophozoite a mechanism involving *var* allelic exclusion [4] ensures that only one *var* gene is transcribed at a time. The encoded PfEMP1 protein is exported in the erythrocyte cytoplasm and exposed on the erythrocyte surface in parasite induced host membrane modifications called knobs [5-7]. These structures appear 16 hours post-invasion, with the parasite Knob Associated Histidine Protein (KAHRP) as the main

component [8], and play a major role in the binding of the infected erythrocyte to endothelial cells and in the sequestration of asexual parasites in various organs.

In contrast with the above picture, virtually nothing is known about sites and mechanisms of gametocyte sequestration. Early observations on post-mortem specimens, confirmed by a few recent field and clinical reports, indicated that immature gametocytes (stage II to IV) predominantly sequester in bone marrow and spleen [9, 10, 11, 12] before being released in the peripheral circulations as mature stage V gametocytes. Knobs are absent from the surface of erythrocytes infected by stage II to IV gametocytes [13], indicating that the mechanism of sexual stage sequestration is distinct from that used by asexual stages. This is consistent with the observation that stage II-IV gametocytes show very low, if any, binding efficiency to purified host ligands such as ICAM-1 and CD36 and to host endothelial and non-endothelial cells [2, 14-17]. Failure of hyperimmune sera from gametocyte-infected individuals to stain the surface of stage II-IV casts doubt on the idea that immature gametocytes expose immunogenic adhesisns mediating cytoadhesion [18].

If the above evidence indicates that mid stage gametocytes maintain their sequestered state through different mechanisms than asexual stages, unanswered questions remain as to whether stage I gametocytes remodel the infected red blood cell surface and how do these parasites interact with host cells prior to sequestration. Very limited work has been done to investigate this issue, and conclusions from these studies have been conflicting. Two electron microscopy studies reported respectively absence [13] and presence [15] of knobs on the surface of erythrocytes infected by stage I gametocytes. The latter study concluded that stage I gametocytes and asexual parasites have indistinguishable adhesive properties on C32 melanoma cells and on purified CD36. Surface staining and agglutination studies with hyperimmune sera led to the proposal that PfEMP1 was present on the surface of stage I gametocyte-infected red blood cells, although a role of other antigenic molecules in such interactions was not excluded [19]. Expression of *var* gene mRNA in gametocytes was investigated in two separate studies which reached conflicting conclusions. One indicated that early and mid-stage

gametocytes express the same *var* genes of the asexual stages from which they derive [19], the other concluded that such an overlap is instead minimal, and that stage III gametocytes express a limited group of *var* genes irrespectively of the *var* transcript predominant in the preceding asexual stages [20].

A major reason for the limited body of data on stage I gametocytes is that physical purification of these cells is technically demanding, due to the difficulty of removing contaminating asexual trophozoites. A novel protocol achieving unprecedented purity in the isolation of the early *P. falciparum* gametocytes has been however recently described [2], and was used in this work to investigate red blood cell modifications and host-parasite interplay in the earliest detectable phase of *P. falciparum* sexual development.

Materials and methods

Parasites.

P. falciparum lines 3D7A [21], 3D7/pPfg27:GFP [2], ItG [8] were cultured in human 0^+ erythrocytes, kindly provided from Prof. G. Girelli, Dipartimento Biopatologia Umana, University of Rome "La Sapienza", at 5% hematocrit under 5% CO_2, 2% O_2, 93% N_2 [22]. Cultures were grown in medium containing: RPMI 1640 medium (Gibco) supplemented with 25 mM HEPES (Sigma-Aldrich), 50 micrograms per ml hypoxanthine, 0.25 mM $NaHCO_3$, 50 micrograms per ml gentamicin and 10% pooled heat-inactivated 0^+ human serum.

Purification of early gametocytes for scanning and transmission electron microscopy.

3D7/pPfg27:GFP Percoll purified schizonts were used to start a culture where gametocytogenesis was induced by parasite overgrowth and a 5% to 8% increase in

haematocrit. One day after stage I parasites were seen in Giemsa stained smears, a multilayer Percoll gradient was used to eliminate schizonts and mature gametocytes. The resulting erythrocytes, ring stages and early gametocytes were passed through a MACS (CS Miltenyi Biotech) column to retain the haemozoin-containing stage I-II gametocytes, which, eluted from the column, were FACS sorted to purify the fluorescent stage I-II gametocytes detecting GFP emission by a 530 nm band-pass filter, and gating on forward/side-light scatter. Parasites were purified, centrifuged in PBS-BSA 2% at 2000 rpm 20 minutes, and fixed for electron microscopy. Analysis of GFP fluorescence by the round shaped parasites obtained indicated that the preparation contained approximately 95% pure stage I-early stage II gametocytes.

Scanning and Transmission electron microscopy

Besides stage I gametocytes, purified as described above, asexual late trophozoites and schizonts for electron microscopy analysis were purified by Magnetic fractionation and gametocytes at stages IV and V of maturation were Percoll purified. All samples were processed for scanning electron microscopy (SEM) according to Bertuccini et al. [23]. Cells were fixed with 2.5% glutaraldehyde and $CaCl_2$ 2 mM in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C. Fixed parasites were let to adhere on poly-lysine coated glass coverslips for 4hs, washed in cacodylate buffer and postfixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer for an additional one hour at room temperature. Samples were then washed and dehydrated through a graded series of ethanol solutions (30–100% ethanol), critical point dried and gold sputtered (thickness 30nm), and examined by a Cambridge SE360 scanning electron microscope. At least two hundred cells were analyzed for each sample.

The same samples were processed for transmission electron microscopy according to de Koning-Ward et al. [24]. Cells were fixed overnight at 4°C with 2.5% glutaraldehyde, 2% paraformaldehyde and 2mM $CaCl_2$ in 0.1 M sodium cacodylate buffer (pH 7.4). Parasites were washed in cacodylate buffer and post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1h at room temperature, treated with 1% tannic acid in 0.05 M cacodylate buffer for 30 min and rinsed in 1% sodium sulphate

in 0.05 M cacodylate buffer for 10min. Fixed specimens were washed, dehydrated through a graded series of ethanol solutions (30–100% ethanol) and embedded in Agar 100 (Agar Scientific). Ultrathin sections prepared using a MT-2B Ultramicrotome (LKB – Pharmacia) were stained with uranyl acetate and lead citrate and examined using an EM 208 Philips electron microscope.

PfEMP1 trypsine cleavage assay

Early gametocytes were purified as described above, although FACS step was omitted, and asexual stages and stage IV gametocytes were similarly purified by Percoll and MACS. The trypsin cleavage assay was performed essentially as described in [25]: infected erythrocytes were treated using three different conditions for 1h at 37°C. Parasites were treated with TPCK-treated trypsin (1 mg/mL in PBS, Sigma) or with trypsin plus soybean trypsin inhibitor (STI) (5mg/mL in PBS), pre-incubated at room temperature five minutes before, or incubated with PBS only. The trypsin reaction was stopped by adding STI to a final concentration of 5mg/mL and incubation at room temperature for 15 minutes. Parasites were then fractionated using triton X-100 followed by SDS solubilization, as previously described [26] The Triton insoluble/SDS soluble fraction from approximately or $5x10^6$ parasites was used for Western blot analysis.

Production of anti-PFD0625c antibodies.

Production of recombinant PFD0625c protein fragment. The 3D7 PFD0625c DBLd domain was PCR amplified using primers 5'- CGGATCC C TGCAACACAGTGAAAACCGCACTCGAG and 3'- CTGCGGCCGCTACATGGATCACAATAATTCTCATGTC and subcloned into the baculovirus expression vector pAcGP67-A (BD Biosciences) modified to contain a His tag at the C-terminal end of the construct. Linearized BakPak6 Baculovirus DNA (BD Biosciences) was cotransfected with pAcGP67-A/DBLd into Sf9 insect cells for the generation of recombinant virus particles. High-Five insect cells grown in 600 ml of serum-free media (10486; GIBCO) were infected with 18 ml of the second

amplification of the recombinant virus particles. After 2 days of induction, the cells were centrifuged (8000g, 4 °C, 10 min), and the supernatant was filtered using two 10-kDa NMWC PES membranes (0.45 µm) (56-4112-04; GE Healthcare) with a total surface area of 200 cm^2. The supernatant was then concentrated to 30 ml and diafiltrated six times on an ÄKTA cross-flow (GEHealthcare) with buffer A (20 mM Tris and 500 mM NaCl). Retentate was recovered from the system and filtered (0.2 µm), yielding a final volume of 40 ml. Before loading onto a 1ml HisSelect column (Sigma-Aldrich), 150 µl of 1 M imidazole (pH 7.4; Sigma-Aldrich) was added to the sample, giving a final imidazole concentration of 15 µM. The bound protein was eluted with buffer A plus 200 mM imidazole. Verification of the recombinant protein was performed using SDS electrophoresis and Western blotting.

Rat immunizations and IgG preparations. Rat anti-sera were produced by injection of 30 µg of recombinant protein in Freund's complete adjuvant, followed by two booster injections of 15 µg of protein in Freund's incomplete adjuvant at 3-week intervals. Antisera were collected 8 days after the final boosting injection. All procedures regarding animal immunizations complied with European and national regulations. All immunizations induced antibodies against the recombinant proteins, as measured by ELISA of the final bleed. IgG was purified by manually passing 0.5 ml of rat immune serum through a column packed with Recombinant Gamma-Bind™G type 2 coupled to Sepharose™4B, in accordance with the manufacturer's recommendations (GE Healthcare), and bound IgG was eluted with Tris–glycine (pH 2.4) and dialyzed against phosphate-buffered saline.

Prior to Western blot analysis on parasite extracts, anti-PFD0625c antibodies were preabsorbed on uninfected erythrocytes and incubated overnight with a membrane containing Triton X100 insoluble/SDS soluble fraction of uninfected erythrocytes to eliminate cross reaction with erythrocytes proteins.

Flow cytometry

The surface staining of a FCR3 parasite line with rat anti-PFD0625c antibodies was assessed as previously described (Staalsoe T, et al. 1999). The data was acquired using a FC500 instrument (Beckman Coulter). Antibody surface reactivity to IEs was compared to reactivity with rat anti-VAR2CSA antibodies (a kind gift from Dr. Ali Salanti) and with the secondary FITC-conjugated goat anti-rat IgG (62-9511, Invitrogen) alone.

Western blot analysis

Proteins were separated by SDS-PAGE using *precast* polyacrylamide 4-12% Bis-Tris gels (Invitrogen) (Figure 2) or homemade 6% Tris-Glycine gels (Figure 4). The nitrocellulose membrane blotted from the precast gradient Bis-Tris gel was probed with the mouse monoclonal anti-ATS (Mab 1B/98-6H1-1) [27] 1:200 in an overnight incubation at 4°C. The membrane was subsequently probed with the mouse monoclonal anti-KAHRP mAb89 (1:1000) [28] and a rabbit anti-EF1alpha antiserum. The membrane blotted from the homemade 6% Tris-Glycine gel (Figure 4) was incubated with anti-PFD0625c, anti-KAHRP and anti-Hsp70 antibodies at 1:200, 1:1000 and 1:1000, respectively. Reaction was revealed using Supersignal West Pico Chemiluminescent (Thermo Scientific).

Quantitative reverse transcriptase-PCR (qPCR)

qPCR was performed as previously described [20, 29]. In short, total RNA was extracted from TRIzol (Invitrogen) preserved parasites according to the manufacturer's instructions. Following DNase I (Sigma-Aldrich) digestion of genomic DNA, cDNA was reverse transcribed from random hexamers, using Superscript II (Invitrogen) following the manufacturer's protocol. Quantitative PCR was performed on the Rotorgene 6000 (Corbett Research) in 20 ul reactions using Quantitec SYBR Green PCR master mix (Qiagen). *Var* sequences were amplified using previously validated *var* gene primers [30, 31] together with the novel *var* exonI primers listed in Table

S1. Additional control genes were amplified using published primers; *seryl-tRNA synthetase (*PF07_0073), *aldolase* (PF14_0425) [29], *kahrp* [32], and primers specific for *18S* (18SF ATTAATCTTGAACGAGGAATGC; 18SR TTCTTGTCCAAACAATTCATCA) *and 28S* (28SF TTTCGTGGAACATCTCCCTAGT; 28SR TAGGAGCGGCTAACTCTTGTTC). Adequately high (>95%) amplification efficiencies of new primer pairs were confirmed by qPCR measurements of serial 10 fold dilutions of 3D7 parasite genomic DNA. PCR cycling was 95°C for 15 mins, followed by 40 cycles of 95°C for 30 s, 54°C for 40 s and 68°C for 50 s, with final extension at 68°C for 40 s. Transcript abundance differences between ring stages and gametocytes were determined by comparing Ct values in each sample run with that of the endogenous controls *seryl-tRNA synthetase*, aldolase, 18S and 28S. Sense/antisense transcript abundance analyses were performed by first for each investigated gene, producing four cDNA preperations of equal volume using random hexamers, forward, reverse or no primers, respectively. Next qPCR measurements were performed on each sample with the same forward/reverse primer pair used for cDNA preparation.

Cytoadhesion assays

HDMEC endothelial cells (Promocell, 6th passage) were seeded onto 1% gelatin coated mSlides 8-wells (Ibidi). Once confluent (around $5x10^4$ cells per well), they were incubated 4 hours at 37 °C with 10 ng/ml rTNF-alpha (Invitrogen). After removal of culture medium, cells were washed once with binding buffer (RPMI 1640 supplemented with 6mM glucose and 10% human serum) and incubated with 0.3 ml of parasite suspension (3% parasitaemia, 1% haematocrit, $5x10^5$ parasites) for 2 h at 37°C, with gentle resuspension every 20 min. After washing by gentle pipetting to remove unbound cells, sample were fixed and used for Giemsa staining or IFA. Incubation in 1% glutaraldehyde for 1h was used for Giemsa staining (5% Giemsa for 30 min). For IFA analysis, a 4% paraformaldheyde incubation for 1h was followed by 0.1 % Triton X-100 permeabilization for 10’ and blocking with 3% BSA in PBS for 10min before antibody incubation. A serum against the gametocyte specific protein

Pfg27 (rat, 1:400) and an anti-KHARP serum (mouse, 1:200) were used to positively identify and count sexual and asexual stages, respectively. Levels of adhesion were quantified microscopically at 400x magnification as number of parasites per mm^2.

Results

Stage I gametocytes do not modify the erythrocyte surface with knobs

Purified preparations of stage I-early stage II gametocytes were obtained for ultrastructural analysis with a protocol combining multilayer Percoll gradient, MACS and FACS purification of gametocytes from a *P. falciparum* 3D7 transgenic line in which a Green Fluorescent Protein (GFP) is expressed from the onset of sexual differentiation, driven by promoter of the early gametocyte gene *pfg27* [2]. Preparations of early gametocytes, containing less than 5% contamination of asexual stages and uninfected red blood cells, were analyzed in scanning and transmission electron microscopy (SEM and TEM, respectively) to investigate structural modifications on the surface of the infected red blood cells.

In the SEM experiments control samples were represented by uninfected red blood cells and knobless stage IV gametocytes, which showed as expected a smooth erythrocyte surface, and by trophozoites of the cytoadherent parasite line ItG [8], which on the contrary clearly showed the presence of knobs on the surface of the infected erythrocytes (data not shown). SEM analysis then compared erythrocyte surface modifications induced by late trophozoites/schizonts and early stage gametocytes (stage I-early II) prepared from parallel cultures of the 3D7/pPfg27-GFP line. Results clearly showed a high density of knobs on the surface of the erythrocyte infected with asexual stages (Fig. 1A). In contrast, examination of the early gametocyte sample clearly showed that such cells do not produce knobs or any comparable structure on the surface of the host cell, despite the fact that they can

significantly deform the infected red blood cell like asexual trophozoites (Fig. 1B). In this analysis a smooth cell surface was observed both on the trophozoite-like round shaped stage I gametocytes (Fig. 1C) and on the early stage II, already characterized by an incipient crescent shape (Fig. 1D).

Similar preparations of asexual parasites and early stage gametocytes were analyzed by TEM. Knobs were clearly visible on the surface of erythrocytes infected by asexual parasites (Fig. 1E), whilst the surface of red blood cells infected by early gametocytes was devoid of any knob-like protrusions (Fig. 1F-I). An unambiguous identification of the early gametocytes in individual cell sections was provided by the detection of an incipient Inner Membrane Complex (IMC) (Fig. 1 H, I), a cellular structure which begins to be formed in this phase of sexual development [13, 33] .

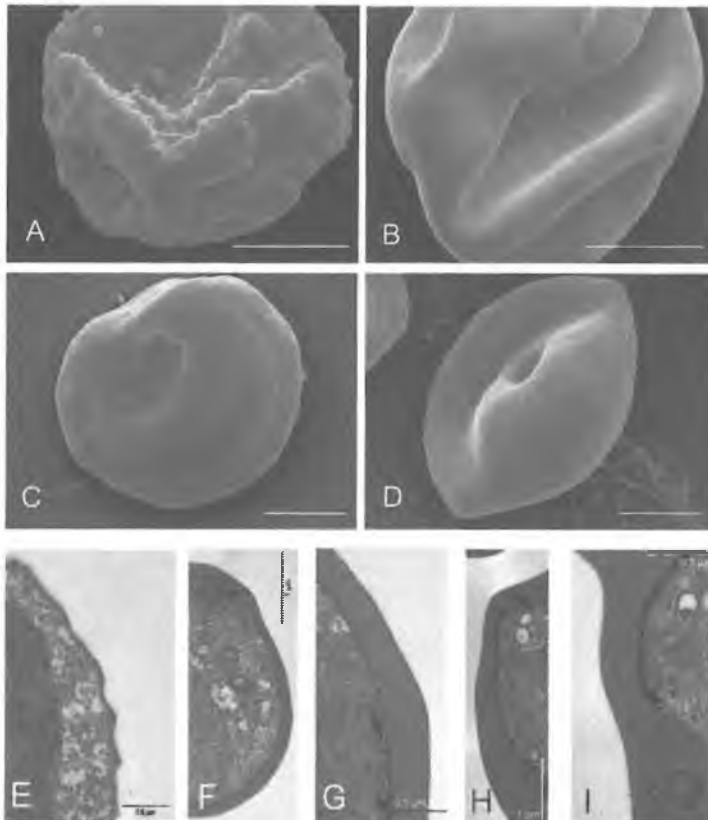

Figure 1. Scanning and transmission electron microscopy of the surface of erythrocytes infected by asexual parasites and by early gametocytes of line 3D7/pPfg27-GFP. A-D: Scanning electron microscopy images of A: asexual trophozoite; B: stage I-II gametocyte; C: stage I gametocyte; D: early stage II gametocyte. Bar size is 2 micron. E-I: Transmission electron microscopy images of the erythrocyte surface in E: an asexual parasite, showing knob structures; F-I: early stage gametocytes, where knobs are undetectable. In F and I the trilaminar structure of the incipient subpellicular membrane complex in stage I gametocytes is visible.

Analysis of surface exposure of PfEMP1 in early and mid-stage gametocytes

A biochemical analysis was conducted to investigate presence of PfEMP1 and of the essential knob component KAHRP [28] in early and mid-stage gametocytes and in asexual trophozoites. In order to specifically detect PfEMP1 molecules exposed on the red blood cell surface, purified preparations of the above stages were used in a biochemical assay in which trypsin digestion of surface exposed PfEMP1 produces a protected cytoplasmic fragment, which migrates as a specific band in Western blot analysis with a monoclonal antibody against the conserved PfEMP1 Acidic Terminal Section (ATS) [25, 34, 35] .

Analysis of trypsinized asexual trophozoites revealed the expected ATS-specific band, which was instead absent if trypsin activity was inhibited, confirming that surface exposed PfEMP1 molecules were readily detectable on these parasites (Fig. 2, marked by arrow). An intense band specifically reacting with the anti-KAHRP monoclonal antibody was also detected, confirming the presence of the knob-associated PfEMP1-KAHRP complex in asexual parasites. In the analysis of early gametocytes, specific care was taken to quantify contamination of asexual trophozoites in the three independent experiments performed on these stages. Counts of the Pfg27-GFP fluorescent parasites and immunofluorescence analysis with anti-Pfg27 antibodies indicated that asexual contamination did not exceed 5% of the cells analyzed. In these experiments the band corresponding to trypsine-cleaved PfEMP1 was reproducibly

detectable in the early gametocyte samples, albeit with a much reduced signal compared to that observed in asexual parasites (Fig. 2). Size of the ATS fragments in the early gametocyte and in the trophozoite samples was undistinguishable. The anti-KAHRP antibody failed to detect any band in Western blots of such samples (Fig. 2). As the assay routinely analyzes the TX100-insoluble/SDS-soluble fractions of parasite preparations, the TX100 soluble and the SDS insoluble fractions from early gametocytes were also analyzed, and no reactivity with the anti-KAHRP antibody was detected (data not shown). The formal possibility that the *kahrp* gene was deleted in gametocytes in the few days separating the sexual culture from the parallel trophozoite culture was also ruled out as *kahrp* genomic gene sequences could be readily PCR amplified by both asexual and purified early gametocyte samples (data not shown). These results suggest that very low levels of PfEMP1 are exposed on the surface of early gametocyte-infected red blood cells. Absence of KAHRP signal in the gametocyte samples and the observation that routinely >95% asexual parasites positively react with anti-KAHRP antibodies in IFA analysis (data not shown) support this conclusion, rather than attributing the ATS signal in the gametocyte sample to presence of contaminating asexual stages. Finally, the same experiment conducted on Percoll-purified stage III-IV gametocytes failed to detect any signals corresponding to both the PfEMP1 ATS fragment and the KAHRP protein (Fig. 2), indicating that absence of knobs in mid stage gametocytes is accompanied by absence of detectable levels of both KAHRP and surface exposed PfEMP1. In the above experiments the anti-ATS antibodies also detected, as expected, high molecular weight trypsin-resistant bands above the 191 kDa molecular marker produced by the pool of intracellular PfEMP1 molecules and by the previously described cross reactivity of the antibody with spectrin, the latter specifically showed in the sample of uninfected erythrocytes [8].

In conclusion, ultrastructural and biochemical analyses altogether clearly showed that knobs and the associated protein KAHRP are undetectable on the surface of early gametocyte-infected red blood cells, and that PfEMP1 adhesins are minimally present

on the surface of these cells. PfEMP1 was undetectable on the surface of the knobless and KAHRP-less stage III-IV gametocytes.

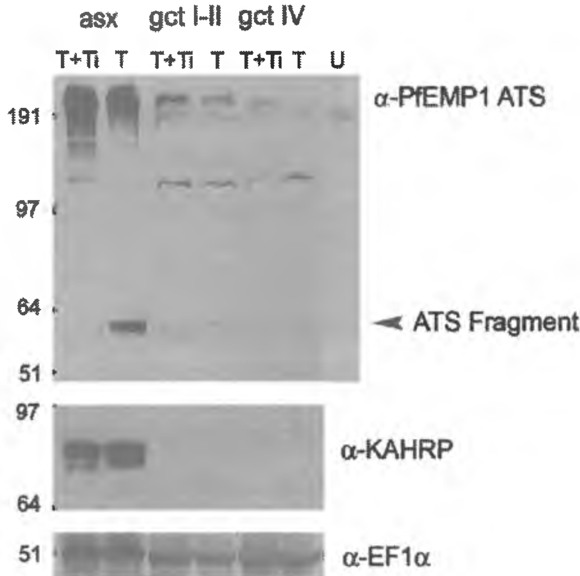

Figure 2. Analysis of surface expression of PfEMP1 in asexual parasites and early and mid-stage gametocytes. Western blot analysis of the Tx100 insoluble/SDS soluble fractions from $5x10^6$ parasites/lane subjected to the trypsine cleavage assay with trypsine alone (T) or trypsine plus its inhibitor (T+i). Lane U contain extract of the same number of uninfected erythrocytes. The same filter was sequentially reacted with the three antibodies indicated. Position of the ATS-specific band diagnostic of surface exposure of PfEMP1 is indicated by black arrow.

Var transcript production is generally downregulated at the onset of sexual differentiation

As the above analysis suggested that *var* genes are downregulated at the onset of sexual differentiation, expression of the 3D7 *var* gene repertoire in the transition from asexual to sexual development was investigated. Synchronous asexual ring stage

parasites of the 3D7/pPfg27-GFP line were sampled for *var* transcript analysis (sample R), whilst an aliquot was further grown to produce gametocytes. After clearing residual asexual parasites with 50 mM N-Acetylglucosamine, early stage II gametocytes were Percoll purified five days later yielding RNA sample G. The *var* transcript analysis of sample R and G was performed using quantitative RT-PCR (qPCR) with previously described primer pairs specific for each of the 58 active and two pseudo *var* genes of 3D7 [30, 31, 36] as well as with two pairs of primers, specific for the 5′ and 3′end of exonI, of 38 3D7 *var* genes (Table S1). The comparable amplification levels determined by the 5′and 3′ exonI primer pairs (Figure 3A, blue and red bars, respectively) showed that the ring stage parasites predominantly transcribed *var* gene PFD0625c and, at a lower level, PFL0030c-VAR2CSA. The qPCR analysis conducted on the G sample showed a markedly reduced overall *var* transcript level (~100 fold). In addition, for all *var* genes, except for PFD0625c, PF07_0051 and PFD0995c, the primer pairs targeting the 3′ region of exonI detected higher transcript levels than primer pairs targeting the exonI 5′region of the same gene (Figure 3B). It has previously been shown that *var* antisense mRNA molecules are transcribed from a bidirectional promoter located within the *var* intron between exonI and exonII [37]. In order to investigate if the unbalanced exonI 5′ vs. 3’ transcript levels in developing gametocytes indeed derived from antisense transcripts, qPCR was performed on cDNAs synthesized with strand-specific primers targeting the exonI 3′end. Analysis was conducted on three *var* genes showing the above unbalanced pattern, and on one *var* gene, PF07_0051, which showed instead a balanced pattern of mRNA production and was the second most abundant *var* transcript in the G sample after PFD0625c (Figure 3B, pie chart). This analysis showed that antisense transcripts were present in both the R and G sample (Figure 3C), but in ring stage parasites sense transcript levels were for all genes more abundant than antisense transcripts, whereas in gametocytes antisense transcripts were more abundant than sense transcripts, except for PF07_0051.

In conclusion these results showed, consistently with the above biochemical data, that the onset of gametocytogenesis is accompanied by the silencing of most *var* genes.

Importantly, this analysis revealed a generalized production of *var* antisense transcripts in sexual differentiation, with only a few upsC *var* genes still producing, at much reduced levels, mature sense transcripts.

Figure 3. *Var* transcript analyses of parasites in the transition from asexual to sexual development. A: *Var* transcript profile of ring stage parasites. Transcript levels are shown as transcript units relative to the averaged transcript level of 18S and 28S x10^7. Primer pairs targeting the 5′ and 3′ ends of individual *var* exon1 are shown in blue and red, respectively, while single primer pairs targeting a random position are shown in green (see table S1). Gene names and corresponding UPS group are given. The pie chart shows the relative *var* transcript distribution based on averaged transcript levels determined by the exon1 5′and 3′ targeting primer paris. B: *Var* transcript profile of early gametocyte stage parasites. Transcript levels are shown as transcript units relative to the averaged transcript level of 18S and 28S x10^7. Primer pairs targeting the 5′ and 3′ ends of individual *var* gene exonI sequences are shown in blue and red, respectively, while single primer pairs targeting a random position are shown in green. The pie chart reflects the *var* transcript distribution based on transcript levels averaged from 5′ exon1 primer pairs only. C: Fold difference of sense vs. antisense transcript levels of the 3′ region of four *var* exonI sequences measured on RNA from ring stages (Blue bars) and gametocytes (Red bars). Fold difference is positive if sense transcripts are more abundant than antisense transcripts, and negative in the opposite case.

The PfEMP1variant predominant in asexual stages is also produced in the derived early gametocytes

The above analysis indicated that the most represented *var* transcript in the early gametocyte sample, accounting for more than 25% of the *var* transcript pool, derived from gene PFD0625c (Fig. 3B), the *var* gene maximally expressed in the ring forms of the preceding asexual stages. Antibodies against PFD0625c were therefore produced and used on parallel preparations of 3D7 asexual stages and early gametocytes to investigate whether the PFD0625c protein was produced and possibly surface exposed in early sexual stages. In Western blot analysis the anti-PFD0625c antibodies clearly reacted with a band above the 191 kDa molecular marker in asexual trophozoite extracts, and also detected a fainter band at the same molecular weight in extracts from the same number of purified stage I-II gametocytes (Figure 4B). No band of similar size was instead detectable, at the same exposure used for the gametocyte

sample, in extracts from FCR3 trophozoites selected for expressing the VAR2CSA PfEMP1 variant (Supplementary Fig. S1A). Specificity of the anti-PFD0625c antibodies was also supported by the observation that they failed to stain the surface of VAR2CSA trophozoites in FACS analysis (Fig. S1B). This result Western Blot analysis indicated that early sexual stage produced the same PfEMP1 molecule predominant in the preceding asexual stages. Aliquots of the parasites used in this experiment were also used to analyze reactivity of the anti-PFD0625c antibodies on the surface of live infected red blood cells. This experiment showed that approximately 50% and 60% of the trophozoite and schizont infected erythrocytes, respectively, exhibited a dotted fluorescent pattern, diagnostic of the surface associated expression of PFD0625c. The experiment failed instead to detect any surface staining on erythrocyte infected by stage I-II gametocytes (Figure 4A). This result suggests that the PFD0625c molecules detected by Western blot in the early gametocytes are present in insufficient amount on the surface of these cells to produce a positive signal with the anti- PFD0625c antiserum.

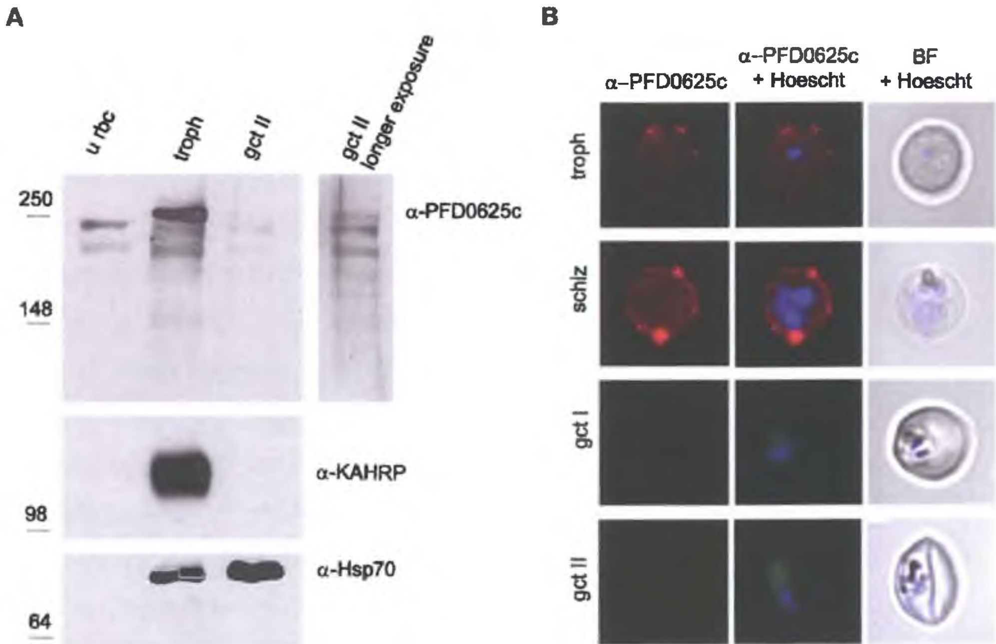

Figure. 4. Analysis of PFD0625c protein expression in sexual and asexual parasites.
A. Western blot analysis of trophozoites and stage II gametocytes with anti-PFD0625c antibodies. Tx100-insoluble/SDS-soluble fractions were run in SDS-PAGE and blotted from the following cells: 1.10^7 uninfected erythrocytes, 5.10^6 trophozoites (with $2.5.10^6$ erythrocytes contamination) and 5.10^6 stage II gametocytes (with 2.10^5 erythrocyte contamination). The filter was reacted with anti-PFD0625c, anti-KAHRP and anti-PfHsp70 antibodies, as indicated. Filter revealing the anti-PFD0625c-specific reaction was exposed for 10 s, and for 1 min in the longer exposure. **B.** Surface staining with anti-PFD0625c antibodies of live sexual and asexual parasites. Representative pictures of a trophozoite, a schizont, a stage I and a stage II gametocyte after surface staining with anti-PFD0625c (1:50).

Stage I gametocytes and asexual trophozoites have different adhesive properties on human endothelial cells

The above experiments showed a much reduced production of PfEMP1 in early gametocytes compared to asexual trophozoites, with a minimal exposure of such adhesins on the erythrocyte surface. Experiments were thus performed to functionally investigate whether such low levels of PfEMP1, exposed in the absence of knob structures, were nevertheless sufficient to bind host ligands. Cytoadhesion assays were performed on human dermal microvascular endothelial *cells* (*HDMEC*), expressing both major host ligands CD36 and ICAM1 [2, 17, 38], with the aim to compare cell binding efficiency of early stage gametocytes, of asexual trophozoites and of stage IV gametocytes, the latter reported to show minimal, if any, adhesion on human endothelial cells of various origins [17]. All parasite stages were used directly from culture to avoid artifacts due to enrichment procedures. After cell binding and wash of the unbound cells, identification of the parasites attached to TNF-activated endothelial cell monolayers was performed by IFA with anti-Pfg27 and anti-KAHRP antibodies, to positively identify round shaped stage I gametocytes and trophozoites, respectively (Figure 5). Results of two independent experiments clearly showed that only asexual stage parasites exhibited significant adhesion on HDMEC, while binding of early and mid-stage sexual stages was virtually absent. Importantly, the binding efficiency of

early stage gametocytes was indistinguishable from that of stage IV gametocytes, in which surface exposed PfEMP1was undetectable in the trypsinization experiment. This result indicates that the low level of PfEMP1 on the surface of early gametocyte-infected erythrocytes is not functionally sufficient to mediate detectable adhesion of these cells on HDMEC layers even in the permissive static cell binding assays.

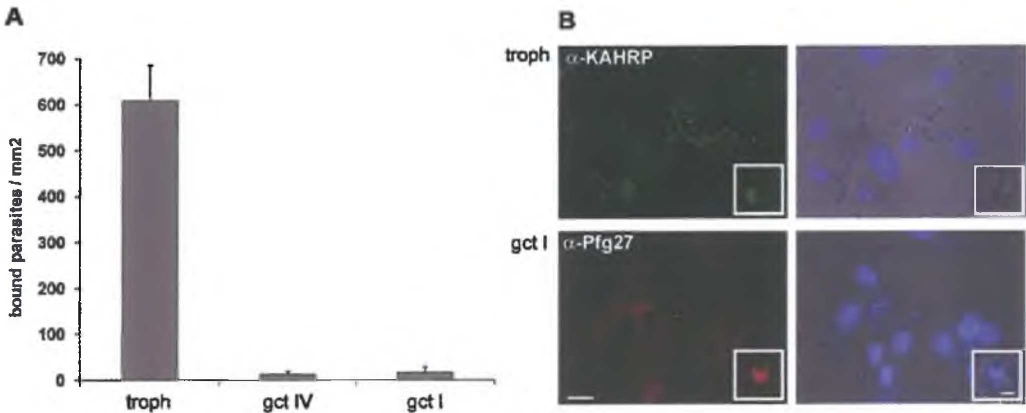

Figure 5. Static binding assay of asexual parasites, stage I and stage IV gametocytes on HDMEC. A: Cell binding of asexual trophozoites, early and mid-stage gametocytes on HDMEC cells. Histograms show mean number of bound parasites/mm^2 of HDMEC cell monolayer (two biological replicates, each with two technical replicates). B: Antibody-based identification of trophozoites (upper panel) and stage I gametocytes (lower panel) bound to the endothelial cell monolayer. Indent shows one asexual trophozoite and one stage I gametocyte. Bar in large panel: 20 micron; bar in indent picture: 2 micron.

Discussion

One of the most obscure aspects of the host-parasite interplay in *P. falciparum* infections is the ability of the immature gametocytes to develop for the exceedingly long period of about ten days sequestered from peripheral circulation. Despite the obvious difficulty to systematically analyze organs of infected individuals to define possible tissue preferences in gametocyte sequestration, evidence is accumulating to

confirm the early observations that bone marrow and spleen are major sites of gametocyte maturation [9, 11, 12, 39, 40]. The reduced blood flow characterizing both tissues has been proposed to be compatible with the presumably loose cellular interactions established by developing gametocytes from stage II to IV, lacking adhesive structures such as knobs on their surface [13] and showing minimal, if any, ability to cytoadhere to a variety of host cell and ligands [15-17]. Recently, analysis of rheological properties of infected red blood cells in the course of gametocyte maturation led to propose that rigidity of erythrocytes infected by immature gametocytes may contribute to maintain sequestration [33, 41, 42], and that an increase in infected cell deformability at gametocyte maturation may be responsible for release from sequestration sites and for stage V gametocyte ability to circulate through the spleen barrier [41].

The above picture however still provides no clues on how the gametocyte sequestration process starts, as conflicting results exist on fundamental issues such as whether the trophozoite-like stage I gametocyte modifies the surface of its host cell, and what are the adhesive properties of the earliest parasite sexual cell. This work presents results of ultrastructural, biochemical and molecular analyses on highly pure preparations of early gametocytes. Results show that between 30 to 48h from red blood cell invasion, the stage I – early stage II gametocytes significantly deform the host cell but do not modify its surface with knobs or any cellular structure able to morphologically alter its smooth surface. Consistently, such early sexual cells do not produce the KAHRP protein, the main structural component of knobs. The early gametocytes expose much reduced amount of PfEMP1 molecules on the surface of the host red blood cell, produced from the *var* gene predominantly expressed in the asexual parasites from which gametocytes are derived. In the parasites analyzed here this gene was PFD0625c, and the encoded protein was consistently detectable in stage I-II gametocytes in Western blot analysis, although it was probably exposed in insufficient amount on the infected erythrocyte surface to be revealed in live surface staining. It will be interesting to conduct this analysis on parasites with a different genetic background.

The low antigenic profile exhibited by early gametocytes is determined by regulatory mechanisms acting on the *var* gene repertoire in the transition from asexual to sexual development. The qPCR analysis of the entire 3D7 *var* gene family showed a dramatic reduction of *var* transcription in early gametocytocytes, and that the three genes with balanced 5′vs. 3′ exonI mRNA levels in such stages were all *upsC* type *var* genes, previously shown to dominate *var* transcripts in late stage gametocytes [20]. This analysis revealed that antisense transcription from the *var* introns, independent of *var* type or activation state, is readily observed in gametocytogenesis. Although the function of *var* antisense RNAs is still unknown, its association with the dramatically reduced *var* transcript levels observed in gametocytes may lead to speculate that these molecules play a regulatory role in *var* gene downregulation.

The present analysis indicates that PfEMP1 is minimally exposed on the surface of early gametocytes, in the latter case suggesting that the proposed mechanism to downregulate *var* gene expression may not be completely effective. Presence of low levels of PfEMP1 on parasitized erythrocytes in a knobless and KAHRPless context, as the one of early gametocytes, is reminiscent of what observed in asexual parasites where KAHRP production was ablated by spontaneous or targeted gene disruption. Interestingly, ability of such parasites to cytoadhere to human dermal endothelial cells (HDMEC) and on purified ICAM1 and CD36 purified ligands was unaffected [28] or reduced [43] in static binding assays, whilst in both studies it was virtually abolished if assays were conducted under flow conditions. Here, the functional consequences of the described host cell remodeling by the early gametocytes were directly evaluated, measuring ability of such cells to bind to human endothelial cells. Binding efficiency of stage I-II gametocytes on the ICAM1/CD36-double positive HDMEC cells was dramatically lower than that of asexual parasites in a static assay, and it was indistinguishable in these experiments from the virtually absent binding shown by the knobless, KAHRPless and PfEMP1-negative stage IV gametocytes. These experiments functionally indicate that the observed modifications induced on the erythrocyte surface by stage I gametocytes, including the possible residual exposure of PfEMP1, are unable to confer significant adhesive properties to such cells.

This work shows that mechanisms governing antigenic variation, host cell remodeling and adhesive properties in early gametocytes differ from those of asexual trophozoites, despite the morphological similarities between the two cell types. This result, obtained for the first time on highly purified early gametocytes, predicts that even the first steps in the sequestration process differ between asexual and sexual stage parasites. It is tempting to speculate that such differences may influence host tissue distribution in gametocyte sequestration, as the host cell modifications in stage I gametocytes may be nevertheless sufficient to establish preliminary loose interactions with host cells in tissues such as spleen and bone marrow, characterized by open circulation and low blood flow. In this hypothesis, further modifications of the gametocyte-infected erythrocyte, such as an increase in cell rigidity, and/or specific interactions with yet undefined host cell types in such tissues may consolidate sequestration of the elongated gametocyte until it reaches maturity.

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Chapter 4

A switch in infected erythrocyte deformability at the maturation and blood circulation of *Plasmodium falciparum* transmission stages

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Abstract

Achievement of malaria elimination requires development of novel strategies interfering with parasite transmission, including targeting the parasite sexual stages (gametocytes). The formation of *Plasmodium falciparum* gametocytes in the human host takes several days during which immature Gametocyte-Infected Erythrocytes (GIE) sequester in host tissues. Only mature stage GIEs circulate in the peripheral blood, available to uptake by the *Anopheles* vector. Mechanisms underlying GIE sequestration and release in circulation are virtually unknown. We show here that mature GIE are more deformable than immature stages using ektacytometry and microsphiltration methods, and that a switch in cellular deformability in the transition from immature to mature gametocytes is accompanied by the de-association of parasite-derived STEVOR proteins from the infected erythrocyte membrane. We hypothesize that mechanical retention contributes to sequestration of immature GIE and that regained deformability of mature gametocytes is associated with their release in the bloodstream and ability to circulate. These processes are proposed to play a key role in *P. falciparum* gametocyte development in the host and to represent novel and unconventional targets for interfering with parasite transmission.

Introduction

An essential step in the achievement of malaria elimination is to block the transmission of sexual stages parasites, the gametocytes, to the mosquito vector. In the case of *Plasmodium falciparum*, causing the most lethal form of malaria, gametocyte maturation requires the exceptionally long time of 8-10 days, compared to the 48h asexual cycle, and is conventionally divided in five different morphological stages (I-V) [1]. Only the mature stage V circulate in the peripheral blood, whilst immature Gametocyte-Infected Erythrocytes (GIE) from stage II to IV have been reported to sequester in internal organs such as the bone marrow and the spleen [2, 3]. Although profound morphological changes, accompanied by expression of 2-300 sexual stage specific transcripts and proteins, have been described in gametocytogenesis [4-6], the mechanisms of GIE sequestration, and the relative contribution of cytoadherence and changes in GIE deformability in this process are virtually unknown.

Proposed mechanisms of *P. falciparum* sequestration mainly derive from studies on the pathogenic asexual stages. These circulate in the bloodstream as "ring" stages in the first 24h post-erythrocyte invasion, and then sequester in various organs to complete maturation to schizont stages, which burst to produce the next generation of free circulating ring forms. Asexual parasite sequestration is mediated by parasite induced modifications of the erythrocyte surface called knobs, enabling the interaction of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) with host ligands on microvasculature endothelial cells. Absence of knobs in gametocytes from stage II to V [5] and their failure to adhere to endothelial cell lines [7] as well as failure to detect PfEMP1 on the surface of erythrocytes infected by stage III and IV gametocytes [8-11] suggest however that maintenance of sequestration of immature GIE is mediated by different mechanisms. Other families of genes involved in host cell modification such as RIFINs and STEVORs are expressed during gametocytogenesis [12, 13], but a functional role for such proteins in sexual differentiation has not yet been demonstrated. STEVOR proteins, produced by transcripts expressed early in gametocytogenesis, are trafficked to the infected erythrocyte membrane during

gametocyte maturation [12]. STEVORs have been recently shown to strongly impact deformability of erythrocytes hosting asexual *P. falciparum* parasites [14].

In this work we analyzed the rheological properties of GIE at various stages of development, complementing such observations with a molecular and cellular analysis of STEVOR expression and localization during gametocytogenesis. Ektacytometry and microsphiltration methods were combined here for the first time to measure GIE deformability and filterability, respectively, of *P. falciparum* gametocytes. Such technically diverse approaches indicated that immature GIE are poorly deformable and revealed that mature stage V GIEs are significantly more deformable than immature GIE. Moreover, we show that STEVOR proteins contribute to the overall stiffness of immature GIE, and that the observed switch in cellular deformability is linked to the de-association of STEVORs from the erythrocyte membrane in mature gametocytes.

Materials and Methods

Gametocyte culture and stage specific purification

The *P. falciparum* clonal lines 3D7, B10, H4 and A12 as well as the transgenic lines SFM (Stevor-Flag-c-Myc), 2TMFM (Pfmc-2TM-FLAG-myc) and 3D7GFP have been described elsewhere [4, 15, 16] All derive from the NF54 line. Parasites were cultivated *in vitro* under standard conditions using RPMI 1640 medium supplemented with 10% heat-inactivated human serum and human erythrocytes at a 5% haematocrit [17]. Synchronous production of gametocytes stages was achieved as described [18]. For the isolation of gametocytes, culture medium was supplemented with 50mM *N*-acetylglucosamine (GlcNAc) from day 0 onwards and medium replacement was continued for 2 to 5 days to eliminate the asexual stages. Gametocytes were enriched by Percoll gradient or by magnetic isolation using a MACS depletion column (Miltenyi Biotec) in conjunction with a magnetic separator.

Immature GIE from patient blood

A 42 year-old patient was treated with quinine for severe malaria attack with impaired consciousness, renal failure and 4% initial parasitaemia comprising a large proportion of mature trophozoites and schizonts and a smaller proportion of immature gametocytes. Howell-Jolly bodies, a marker of hyposplenism, were evident in 0.3% of red blood cells thereby explaining the very unusual aspect of the thin smear. Immunochromatography and PCR confirmed that the patient was infected exclusively with *P. falciparum*. At day 3 and day 4 of quinine therapy, blood was collected to assess parasite clearance and sent the same day to the National Reference Center for Malaria. Thin smears showed complete clearance of asexual stages and the persistence of immature GIE (stages II-IV). Blood samples were washed 3 times with RPMI medium to remove leucocytes and immediately submitted to microsphiltration as described. GIE were staged and enumerated on 200 high-power (x1000) fields and the retention rate calculated as described. Patient consent was obtained by the attending physician before blood collection as per National Reference Center standard operating procedure.

Ektacytometry measurement of GIE population elongation index

Deformability measurements of GIE populations were carried out using ektacytometry analysis via Laser-assisted optical rotational cell analyzer (LORCA) [19]. The extent of erythrocyte deformability, or elongation index (EI), was defined as the ratio between the difference of the 2 axes of the ellipsoid diffraction pattern and the sum of these 2 axes. Populations of GIE stage II/III and stage V, at 40% parasitaemia and 40% hematocrit in a final volume of 25 µL were diluted in isotonic solutions of polyvinylpyrrolidone (PVP) and were exposed to increasing shear stresses from 0 to 30 Pa, at 37°C, as described [19]. Gametocytes were enriched by multilayer percoll gradient from synchronized cultures. At least two independent experiments were performed for each GIE stage. Each independent experiment included a Percoll treated population of uninfected erythrocytes, from the same batch, and kept in culture for the same time of the GIE, at 40% hematocrit in a final volume of 25 µL. Direct

microscopic observation of GIE diluted in PVP was performed before and after LORCA measurements and excluded that cell aggregation was occurring in the experiments.

Microsphiltration

Calibrated metal microspheres (96.50% tin, 3.00% silver, and 0.50% copper; Industrie des Poudres Sphériques, Annemasse, France) with 2 different size distributions (5- to 15-µm-diameter and 15- to 25-µm-diameter) comprised a matrix used to assay infected erythrocyte deformability under flow, as recently described [14, 20]. Suspensions of synchronized cultures containing 2-5 % GIE were perfused through the microsphere matrix at a flow rate of 60 mL/h using an electric pump (Syramed µsp6000, Arcomed' Ag), followed by a wash with 6 mL complete medium. The upstream and downstream samples were collected and smeared onto glass slides for staining with Giemsa reagent and parasitaemia was assayed to determine parasite retention versus flow through. To visualize GIE shape during their flowing through the matrix, 1 mL of PBS/4% paraformaldehyde was added after perfusion of the GIE-containing culture on the microsphere matrix. After 5 min incubation, fixed GIE were separated from the microspheres by a 3-step decantation procedure, and GIE morphology was observed on a glass slide by light microscopy using a Leica DM 5000 B at 100X magnification.

Scanning Electron microscopy

Before gametocytogenesis induction, the *P. falciparum* B10 clone was selected by gel floatation during several cycles to select for knob-producing parasites. Gametocytes were purified by magnetic isolation and the cell pellets resuspended in 2.5% gluteraldehyde (EM grade) in sodium cacodylate 0.1M, pH 7.2, for 1 h at 4°C. Cells were washed 3 times in sodium cacodylate, transferred to polylysine-coated coverslips and incubated 1 h in 1% osmium tetroxide. After 3 washes in H_2O, samples were dehydrated (25%, 50%, 75%, 95%, 2×100%, 5 minutes each), incubated for 10 min. in acetone, subjected to critical point drying, and coated with platinum in a gun ionic

evaporator. Samples were examined and photographed with a JEOL 6700 F electron microscope operating at 2kV.

Immunostaining of fixed and live GIE

Parasites were washed in PBS, air-dried on glass blood smears and methanol- or acetone-fixed for 5-15 min. After 1h pre-incubation in 1% BSA, parasites were incubated with one of the following antisera: anti-STEVOR mouse antiserum (mouse anti-S2) diluted 1:400 [21], a pool of mouse antisera against four STEVOR proteins (anti-PFA0750w, -PFL2610w, -MAL13P1.7 or -PFC0025c) diluted 1:500 [22], an anti-Pfg27 rabbit antiserum diluted 1:100 [23], or an anti-c-myc rat monoclonal antibody diluted 1:500 (SantaCruz BiotechnoloGIE). After washes in 1X PBS, slides were incubated with Alexa Fluor conjugated secondary antibody against either rat, rabbit or mouse IgGs (Molecular Probes) containing DAPI (2µg/mL). Samples were observed at 100X magnification using a Leica DM 5000 B or an Olympus fluorescent microscope. Immunostaining of live GIE was performed on MACS purified GIE washed in RPMI, re-suspended in binding buffer (RPMI/10% FBS) and diluted to 5% parasitemia with uninfected erythrocytes. 50µL of cell suspension were incubated for 1h at 4°C in a rotating wheel with rabbit anti-STEVOR (rabbit anti-S2) [24] and mouse monoclonal anti-Glycophorin C (GPC, vCell Science, Singapore) diluted 1:400 and 1:500 respectively. For detection of Pfg27 and/or STEVOR internal proteins, infected erythrocytes were permeabilized by addition of 3-4 heamolytic units of Streptolysin O (SLO, Sigma Chemical Co, U.S.A.) as described [25]. After 3 washes in RPMI, parasites were incubated 1h with Alexa Fluor conjugated secondary antibody against either rabbit or mouse IgGs (Molecular Probes) containing DAPI (2µg/mL). Samples were mounted in vectashield onto a glass slide and visualized with Olympus fluorescence microscope at 100X magnification.

Immunoblotting analysis

GIE were purified by magnetic isolation and denatured in protein loading buffer 5 min at 100°C. Samples (5.10^6 parasites/lane) were separated by 4-12% SDS-PAGE, transferred to PVDF membrane and blocked for 1 h in 5% nonfat dry milk. Immunoblots were probed with a pool of mouse antiserum against STEVOR proteins (anti-PFA0750w, -PFL2610w, -MAL13P1.7 or -PFC0025c) at 1:3000 [22], a mouse mAb anti-HSP70 antibody at 1/5000, followed by 1 hour with horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies (Promega) at 1:25 000. Detection step was performed using the Pierce chemoluminescence system (Pierce) following the manufacturer's instructions.

Statistical analysis

Statistical significance for differences in elongation indexes and retention rates were established using Wilcoxon Mann-Whitney rank sum test. Statistical significance for differences in proportion of GIE showing different shape was established using a Chi-square test.

Results

Mature GIE are more deformable than immature GIE

Scanning electron microscopy of the conventionally described stages of gametocyte maturation[1, 5] showed the evolution from a convex half moon-like shape in stage II to an elongated shape in stage III, followed by a crescent shape with protruding extremities in stage IV, finally leading to an elongated and curved shape with smooth ends in the mature stage V (Figure 1A). Interestingly, the process is accompanied by an increasing transparency of the residual portion of erythrocyte cytoplasm (Laveran's bib), consistent with the reported decrease in hemoglobin concentration in developing

gametocytes [26]. To investigate whether these morphological changes are associated with changes in mechanical properties of infected cells, we used ektacytometry via Laser-assisted optical rotational cell analyzer (LORCA). In these experiments, the elongation index (EI) is measured in response to increasing shear stress (SS) from 0 to 30 Pa, with higher EI corresponding to increased GIE deformability. Cell samples containing 40% infected erythrocytes with either immature (II/III) or mature (V) 3D7 gametocyte stages were compared (Figure 1B). Result of these experiments was that mature GIE populations consistently showed higher EI values over all SS compared to the immature GIE samples in independent biological replicates (Figure 1C). In order to eliminate interference of factors such as blood source or culture time, the average ratio for EI of infected vs uninfected erythrocytes was calculated for each SS. In the SS range from 3 to 9,49 Pascal, more sensitive to detect differences in deformability between erythrocyte populations compared to higher SS [27], ratio was significantly lower (P =.0004) for stage II/III GIE compared to the mature stage V GIE (Figure 1D). Significantly different ratios persisted at higher SS (Supplemental Figure S1), although both cell types tended to reach closer EI values at maximum SS of 30 Pascal, a phenomenon previously observed in studies on blood diseases where erythrocytes present decreased deformability [27]. In summary, these results clearly indicate that mature GIE are significantly more deformable than erythrocytes containing the immature II/III sexual stages.

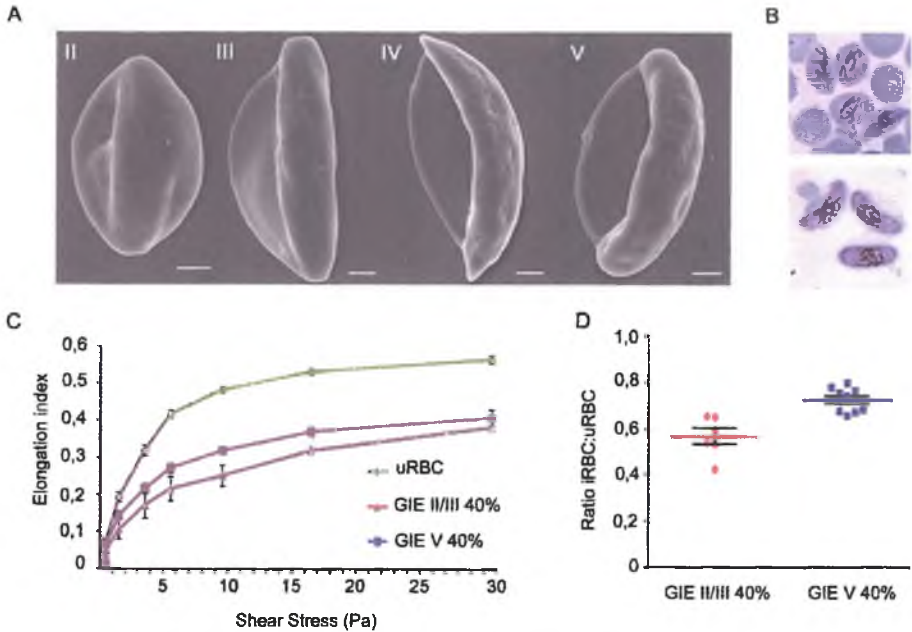

Figure 1. Scanning electron microscopy and ektacytometry analysis of immature and mature *P. falciparum* GIE. A: Scanning electron microscopy images of *P. falciparum* GIE (B10 clone) from stage II to V of maturation. The bars represent 1μm. B: Giemsa staining images of stage II/III (upper panel) and stage V (lower panel) GIE samples used in ektacytometry analysis. C: Response to increasing shear stress of erythrocytes infected by *P. falciparum* stage II/III (red line) and V (blue line) gametocytes (40% parasitaemia) and of uninfected erythrocytes (green line). The error bars indicate standard error. D: Ratios of Elongation Indexes of infected vs uninfected erythrocytes calculated from the 3-9.49 Pascal range of shear stresses in the ektacytometry analysis (C) showing a statistically significant difference between immature and mature GIE (Mann-Whitney rank sum test, $P = .0004$).

A switch in deformability and filterability of GIE occurs at the transition between immature and mature gametocytes

In order to evaluate GIE mechanical properties in an independent and technically diverse approach, the filterability of GIE using the microsphiltration method was analysed. This technique measures deformability of parasitized erythrocyte flowing

through a defined matrix of microspheres that contains narrow and short apertures, and was validated against *ex vivo* perfused human spleen [20, 28]. As this method mimics the physical constrains experienced by infected erythrocytes in the splenic microcirculation, it was used to investigate whether changes in GIE deformability during gametocytogenesis correlated with the ability of different gametocyte stages to circulate in peripheral blood. In this system, increased retention rates and impaired filterability correspond to decreased erythrocyte deformability [29]. Retention rates were monitored for synchronous cultures of stage II, III, IV, and V gametocytes from three different parasite clones (Figure 2A). In all parasite lines, immature GIE (stages II to IV) displayed high retention rates ranging from 72% to 96%. Experiments with immature GIE (stages II to IV) from the blood of a hyposplenic patient confirmed this high retention rate (75% to 100% retention), indicating that this property is not due to an *in vitro* artefact linked to gametocyte cultivation. In marked contrast, mature stages were substantially more deformable since less than 23% of stage V GIE were retained on the microspheres ($P < .00001$). Analysis of Giemsa stained smears of mature gametocytes upstream and downstream the microsphere matrix showed a similar male:female ratio for the two samples. To visualize the shape of GIE as they flow through the matrix we added a paraformaldehyde-fixation step in the microsphiltration experiment. This showed that 82% of observed immature stages maintained their convex shape, whereas 75% of mature gametocytes displayed a twisted, elongated dumbbell-like shape likely reflecting their ability to squeeze and slide between microspheres (Figures 2B and 2C). These results clearly confirm the observations obtained by ektacytometry that mature GIE are significantly more deformable than immature GIE. Importantly, microsphiltration experiments in addition reveal that a major switch in GIE deformability occurs at the transition between stage IV and stage V, which coincides with the release from the sequestration sites and the restored ability to circulate of the mature stages.

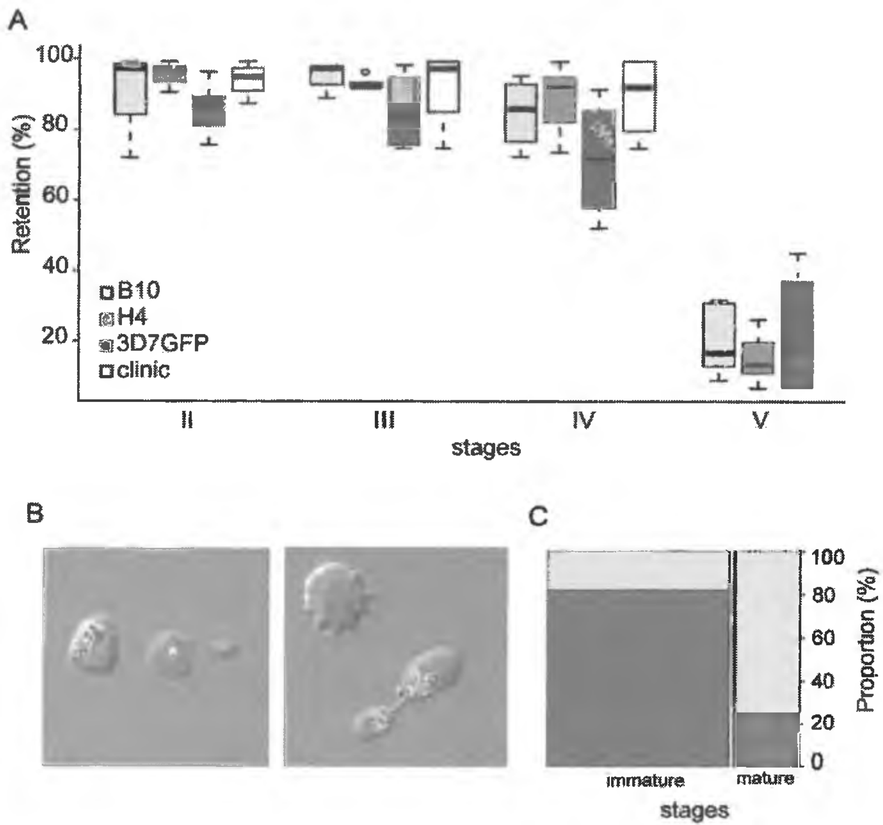

Figure 2. Retention in microsphilters of immature and mature GIE

A: Retention in microsphilters of stages II, III, IV and V GIE from different *P. falciparum* clonal lines (B10, H4, 3D7GFP) in culture or directly collected from the blood of a hyposplenic patient treated for a malaria attack (clinic). Immature GIE (stages II-IV) are retained by the microsphilters while mature GIE (stages V) flow through. B: Differential interference contrast images of paraformaldehyde-fixed GIE as they flow through the microsphilters. Immature stages (left panel) keep a convex oval or round shape, unlike uninfected erythrocytes (white star), whereas a majority of mature GIE are twisted and dumbbell-shaped (right panel). C. Graphical representation for the proportion of GIE showing a regular (dark grey) or twisted (light grey) shape in a population of immature (n= 100) and mature (n=36) GIE (Chi-square test, p=1e-9).

The switch in deformability correlates with a modified STEVOR localization in the erythrocyte

To investigate whether GIE deformability modifications are mediated by changes in expression or location of specific parasite proteins, we focused on parasite gene products known to be expressed during gametocytogenesis and associated with the erythrocyte membrane. Amongst them the members of the STEVOR multigenic family fulfilled these criteria [12, 13, 15, 24] and, importantly, STEVORs were recently shown in asexual stages to impact deformability of the infected erythrocytes [14]. Expression and cellular localisation of STEVORs during gametocyte development was investigated by western-blot and by immunofluorescence using different sets of antibodies. Western-blot analysis using mouse polyclonal sera against a semi-conserved region of STEVOR proteins [24] showed that STEVOR abundance was stable from stage II to V GIE (Supplemental Figure S2). Immunofluorescence analysis of acetone-fixed GIE using the same antibodies confirmed the presence of STEVOR from stage II to V GIE (Figure 3A). STEVOR-specific fluorescence was progressively exported to the erythrocyte cytoplasm of stage III and stage IV GIE, in which a dotted pattern co-localizing with the erythrocyte membrane was clearly visible. In stage V gametocytes the STEVOR signal was mainly associated to the gametocyte cytoplasm, co-localizing with the signal from the gametocyte internal protein Pfg27 (Figure 3A) [30]. Such a differential distribution of STEVORs in immature and mature GIE was confirmed on methanol-fixed GIE using a pool of mouse antisera against four STEVOR proteins (Figure 3 C and 5A) [22]. To gain further insights in STEVOR localisation, immunostaining of live GIE showed that STEVORs could be detected on the surface of stages III and IV but not of stage V GIE (Figure 3B). This observation was confirmed as anti-STEVOR antibodies gave a double positive signal with anti-Glycophorin C antibodies only on the surface of immature GIE and not on that of stage V GIE (Supplemental Figure S3). Furthermore, stage V gametocytes positively reacted with anti-STEVOR antibodies only after Streptolysin O permeabilization, confirming that the residual STEVOR-specific signal on mature GIE was due to internally located STEVOR proteins (Supplemental Figure

S2). In summary, our data show that the switch in cellular deformability at the transition between immature and mature GIE coincides with a moment in which STEVORs are no longer detectable in association with the erythrocyte membrane. This result suggests that STEVORs mediate a decreased erythrocyte membrane viscoelasticity and contribute to infected cell stiffness in immature GIE, similarly to what observed for erythrocytes infected with asexual stages [14].

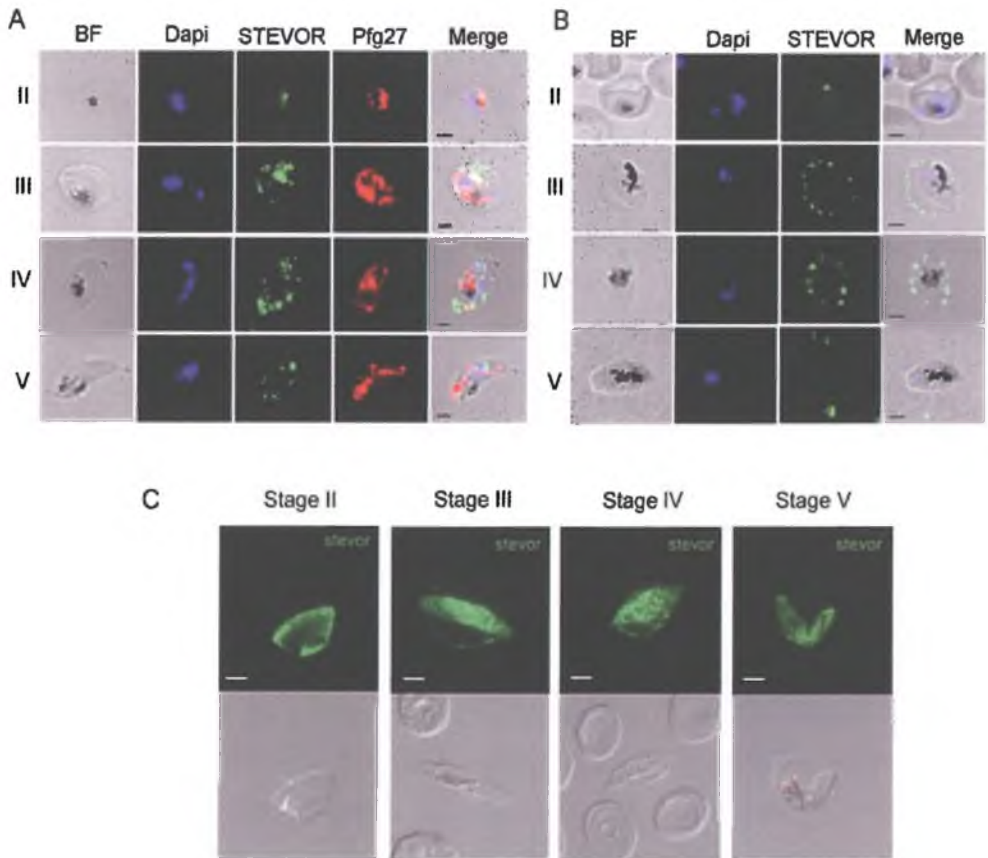

Figure 3. Immunofluorescence analysis of STEVOR proteins expression in GIE

A: Analysis of STEVOR proteins expression in fixed II-V-GIE preparations. GIE were acetone-fixed and co-stained with mouse anti-S2 (green) and rabbit anti-Pfg27 (red) followed by anti-mouse Alexa 488- and anti-rabbit Alexa 594-conjugated IgG. Parasite nuclei were stained with DAPI (blue). Bright field (BF) and merge images are shown. The bars represent 2 μm. B: Analysis of live GIE immunostained with rabbit anti-S2 (green) specifically recognizing native STEVOR on the surface of stages III and IV GIE. Bound antibody was detected with anti-rabbit Alexa 488-conjugated IgG. BF, nuclear staining (DAPI, blue) and merge images are shown. C: Immunofluorescence analysis of STEVOR expression in fixed II-V-GIE. GIE were methanol-fixed and stained with a pool of mouse polyclonal antibodies directed against STEVOR proteins followed by anti-mouse Alexa 488-conjugated IgG (green). The bars represent 2 μm.

STEVOR expression and localization contributes to GIE deformability

To test this hypothesis, stage-specific GIE deformability was analysed in gametocytes defective in the expression or, on the contrary, overproducing STEVOR proteins. In the first set of experiments the NF54 derived clone A12, in which mRNA production of all *stevor* genes is downregulated during asexual development[16] was used to produce gametocytes. Immunobloting and immunostaining with anti-STEVOR polyclonal sera on methanol-fixed GIE from clone A12 indicated that STEVOR expression was significantly decreased in A12 gametocytes compared to those produced by the sibling B10 and H4 clones (Figure 4A and 4B, supplemental Figure S4). Microsphiltration experiments on immature GIE from these clones showed similar retention rates in stages II but revealed a significant decrease in retention rates in A12 compared to B10 and H4 in stage III (68.2% vs 96% and 93.8%, P =.0051 and.0025) and stage IV GIE (65.8% vs 85.3% and 89%, P = .0086 and .0041). Mature GIE showed as expected similar low retention rates in all clones (Figure 4C). In the second set of experiments, STEVOR overproduction in gametocytes was achieved in the transgenic parasite line STEVOR-Flag-c-myc (SFM), which overexpresses a c-

myc-tagged copy of the PFF1550w *stevor* gene driven by the *hrp3* promoter [15]. Immunoblotting showed that STEVORs were more abundant in SFM than in wild-type gametocytes (Figure 5B). Importantly, immunostaining with anti-STEVOR polyclonal sera and an anti-c-myc mAb on methanol-fixed GIE in the SFM line revealed that the overexpressed STEVOR protein was partly localised at the infected erythrocyte membrane also in stage V GIE (Figure 5A). Microsphiltration analysis of synchronous wild-type and SFM gametocytes showed that retention rates were similar at the stages II and III, and slightly higher in SFM parasites at stage IV (P = .03). However, the retention rate of stage V GIE was substantially higher in SFM than in wild-type parasites (78% vs 23%, P = .00001), suggesting that STEVOR overexpression and its persistence at the erythrocyte membrane in stage V GIE significantly impaired the increase in deformability associated with wild-type GIE maturation (Figure 5C). In order to exclude that retention of SFM stage V was due to delayed sexual maturation, stage V gametocytes were tested for their exflagellation efficiency. Result was that SFM stage V exflagellated with the same kinetics and efficiency as wild-type gametocytes (not shown). To control for possible artifactual increase in the rigidity of SFM stage V GIE resulting from transgene overexpression, gametocytes overproducing a c-myc-tagged member of the *Pf*mc-2TM family, obtained from the transgenic line 2TMFM, were analysed. Retention rates for 2TMFM GIE were similar to those of the wild-type line at all stages, indicating that the increased retention rates of mature SFM GIE were specifically linked to STEVOR overexpression (Figure 5C).

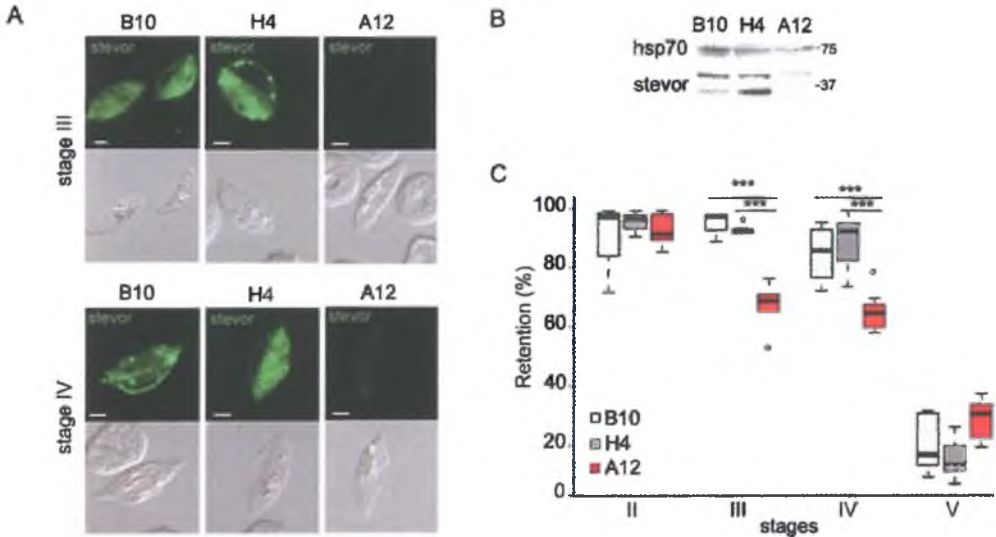

Figure 4. Decrease in STEVOR expression is associated with an increase in deformability of immature GIE

A: Immunofluorescence analysis of STEVOR expression in stage III and stage IV GIE from the B10, H4 and A12 clones. GIE were stained with a pool of mouse polyclonal antibodies directed against STEVOR proteins followed by anti-mouse Alexa 488-conjugated IgG. Signal intensity was analysed by ImageJ on at least 30 pictures taken under identical exposition conditions for each clone. The bars represent 2 µm. B: Western-blot analysis of STEVOR expression in stage III and stage V GIE from the wild-type and the SFM parasites. Immunoblots were probed with a pool of mouse polyclonal antibodies directed against STEVOR proteins and with a mAb directed against HSP70. C: Retention in microsphilters of stages II, III, IV and V GIE from the B10 (light grey), H4 (dark grey) and A12 (red) clones. Asterisks represent highly significant differences in retention rates ($P < .01$).

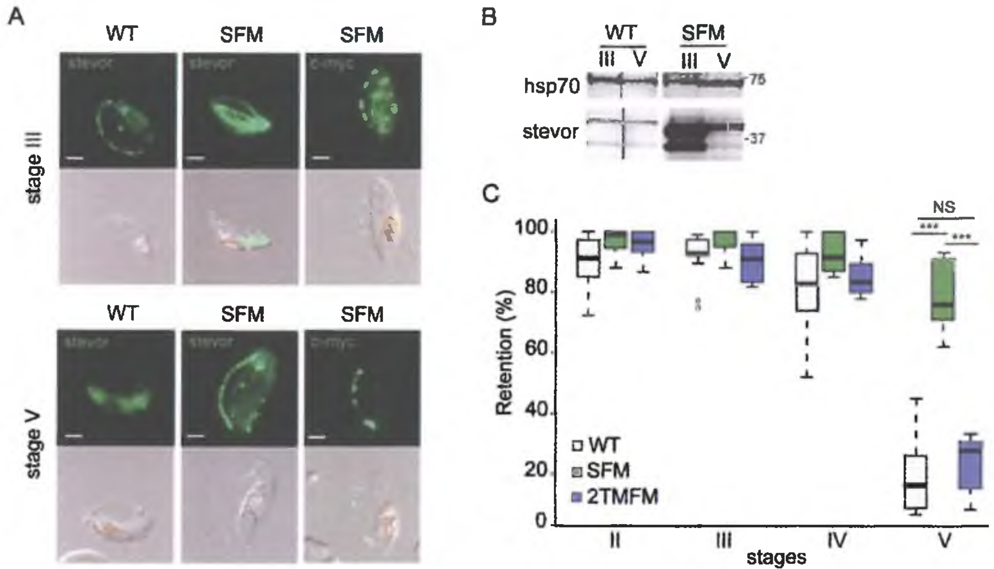

Figure 5. Stevor overexpression is associated with a decrease in deformability of mature GIE. A: Immunofluorescence analysis of STEVOR expression in stage III and stage V GIE from the wild-type (WT) and the SFM parasites. GIE were stained with a pool of mouse polyclonal antibodies directed against STEVOR proteins or with anti-c-myc mAb followed by anti-mouse Alexa 488-conjugated IgG. The bars represent 2 µm. B: Western-blot analysis of STEVOR expression in stage III and stage V GIE from the wild-type and the SFM parasites. Immunoblots were probed with a pool of mouse polyclonal antibodies directed against STEVOR proteins and with a mAb directed against HSP70. Vertical lines have been inserted to indicate a repositioned gel lane. C: Retention in microsphilters of stages II, III, IV and V GIE from different wild-type *P. falciparum* clonal lines (WT representing B10, H4, 3D7GFP clones, in grey), from the SFM parasite line overexpressing a c-myc-tagged copy of the PFF1550w *stevor* gene (green) and the 2TMFM parasite line overexpressing a c-myc-tagged copy of the PFA0680c *Pfmc-2TM* gene (blue). Asterisks represents highly significant differences in retention rates ($P < .01$). NS: Not Significant.

Discussion

We investigated changes in deformability and filterability of erythrocytes infected with immature and mature gametocytes of *P. falciparum*. Our results establish that immature GIE are poorly deformable, and that a switch in deformability occurs at the transition from immature to mature GIE. In human infections this developmental step coincides with the appearance of mature gametocyte in circulation after release from their yet unknown sequestration sites in the body, and with their restored ability to circulate in blood and to cross narrow capillaries and splenic slits. Microsphiltration results reported here establish that erythrocytes infected by immature gametocytes exhibit high retention rates comparable to those of mature asexual stages. In contrast, mature GIE displayed deformability properties similar to those of erythrocytes infected with the freely circulating ring stage parasites [14]. The independent technical approach of ektacytometry in our work and in a recent report [31] also showed that mature GIE are significantly more deformable than immature GIE, confirming the microsphiltration experiments. These results are altogether consistent with the need for mature gametocytes to cross the splenic slits in order to circulate, a prerequisite to be ingested by *Anopheles* and ensure transmission. We show here that the STEVOR family plays a role in this process, as immature gametocytes underexpressing such proteins display an increased deformability compared to wild type immature stages, and gametocytes overexpressing STEVORs remain poorly deformable also at the mature stage.

The integration in the present work of observations on whole GIE mechanical properties, gametocyte morphology, and dynamic association of parasite molecules with the erythrocyte membrane provides the first clues on the mechanisms governing the developmental changes in GIE deformability. Changes in mechanical properties of *P. falciparum* infected erythrocytes result from the combination of presence of the intracellular parasite and parasite-induced modifications of its erythrocyte host. Erythrocyte deformability is determined by three factors: the surface area and volume of the cell, the cytoplasmic viscosity and the membrane viscoelasticity [32]. The

altered morphology of GIE fixed inside the microsphere matrix shows that mature gametocytes themselves are, unlike the immature stages, highly stretched within the erythrocyte, suggesting an increase in intrinsic parasite deformability during sexual maturation. A process likely to be responsible for such a modification is the disassembly of the microtubular subpellicular network subtending the trilaminar membrane structure in the transition from stage IV to stage V gametocytes [5]. Recent cryo X-ray tomography analysis revealed that the surface area and the volume of GIE remain roughly constant during gametocyte development [26], suggesting limited impact of these factors on the observed switch in deformability at the transition from stage IV to stage V. The same study also showed that about 70% of the erythrocyte hemoglobin is digested during gametocytogenesis. As this decrease is progressive throughout sexual development, it is unlikely that changes in GIE cytoplasmic viscosity entirely account for the abrupt switch in deformability at the transition between stage IV and V GIE. It is therefore plausible that decreased membrane viscoelasticity contributes to stiffness of immature GIE, which, based on observations with asexual stages [33], is likely a consequence of mediated by parasite-encoded proteins associating with the erythrocyte membrane skeleton.

The findings that STEVORs associate with the erythrocyte membrane in immature GIE and that their disappearance from that site coincides with the increased deformability of mature GIE suggest that these trans-membrane proteins significantly contribute to the decrease in membrane viscoelasticity of immature GIE. The mechanisms underlying STEVOR-mediated stiffness remain elusive. A possible interaction with erythrocyte cytoskeletal proteins may induce spectrin cross-linking, as proposed for the plasmodial proteins KAHRP and PfEMP3 exported in asexual stages [34-36]. De-association from the erythrocyte membrane in mature stages may result from proteolytic cleavage or conformational changes upon post-translational modifications. Such events are likely responsible for the failure of our antibodies to detect STEVOR association with the erythrocyte membrane in mature GIE, although this was observed using a different set of antibodies raised to small peptides [12].

Which are the sites of *P. falciparum* gametocyte sequestration in the human body, how immature gametocytes hide in such sites for almost ten days, how do mature stages regain access to blood circulation and which are the mechanisms ensuring their persistence in peripheral circulation for several days are still major unanswered fundamental questions in the biology of malaria parasites. Although a mechanistic hypothesis on gametocyte sequestration is virtually impossible without the identification of the sites of sexual stage sequestration *in vivo*, our results allow us to speculate that immature GIE stiffening and the increase in GIE deformability at gametocyte maturation may be functionally linked with the dynamics of this phenomenon. Once sequestration is established by the round, trophozoite-like stage I gametocytes through yet elusive mechanism(s), we hypothesize that mechanical retention may significantly contribute to maintenance of sequestration throughout the ensuing maturation process of GIE. This is consistent with the fact that PfEMP1 and knob structures are absent from the surface of stage II to V GIE [5, 8], and that immature GIE show only weak, if any, binding interactions with endothelial host cells [7, 8, 11]. Observations from post mortem and *ex vivo* specimens and from rare clinical cases altogether indicating that bone marrow and spleen are the organs where immature gametocytes are more readily found [2, 3, 37], suggest that mechanical retention of immature gametocytes may be favoured in such tissues, characterized by an open and slow blood circulation. A role of adhesins in gametocyte sequestration cannot however be presently excluded. Recent findings that STEVORs have adhesive properties and are involved in rosetting in asexual stages (Niang et al, unpublished results), and our observation that STEVORs is accessible to antibodies on the GIE surface up to stage IV may suggest a more direct role of such proteins in GIE cytoadhesion, requiring further studies.

Our results suggest that the high deformability specifically characterising stage V GIE may facilitate release from sequestration sites. Importantly, we propose that such a property restores the ability of mature GIE to cross narrow apertures and allows them to escape mechanical retention in the spleen, thus contributing to their sustained circulation in the peripheral blood where they are available to the insect vector for

several days. We propose that interventions targeting these processes, thereby reducing the ability of mature GIE to circulate, open novel avenues in the present strategies to reduce parasite transmission.

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Chapter 5

The earliest landmark of *Plasmodium falciparum* sexual development is the export of protein PfGEXP5 in the gametocyte-infected erythrocyte cytoplasm.

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Abstract

Plasmodium falciparum sexual commitment and development plays a fundamental role in transmission and spread of malaria. In order to shed light on the early events in this still obscure process, in this study we characterize a novel parasite protein highly enriched in *P. falciparum* sexual stages, which is exported in the host erythrocyte cytoplasm. Analysis with specific antibodies and with gene fusions on synchronous gametocytes showed that PfGEXP5 starts to be actively exported to the host parasite cytoplasm at around 16 hours post-invasion and its presence in the red blood cell cytoplasm decreases over the gametocyte maturation period. The functional role of the early production and export of PfGEXP5 is however still not clear as *P. falciparum* lines where the gene was inactivated by gene disruption produce apparently normal gametocytes. The identification of a novel exported protein highly enriched in sexual stages and detectable as the earliest event in the formation of stage I gametocytes represent a new tool in the study of gametocyte commitment in its ability to identify subpopulations of newly invaded sexually committed parasites.

Introduction

Malaria is a vector born disease caused by the genus *Plasmodium* and responsible for an estimated 300 to 500 million clinical cases per year and approximately 1 million deaths affecting mostly pregnant woman and young children. Of the 5 currently recognized human malaria species, *P. falciparum* is the most virulent species, responsible for 90% of the disease victims from sub-Saharan Africa [1]. The huge burden posed by malaria is associated with the efficient transmission of the disease mediated by the invertebrate vector, the female *Anopheles* mosquito. Although only a fraction of asexual parasites commit to sexual differentiation, the inefficient gametocytocidal activity of the treatments currently used to fight malaria and the carriage of low numbers of infective sub-microscopic gametocytes in both treated and asymptomatic individuals still constitute a serious concern for malaria control strategies.

Within the human host, during the intra-erythrocytic life cycle, the asexual parasite can either undergo a 48h cycle of asexual replication, during which each asexual parasite can produce 16 to 32 new merozoites that will be released to invade new RBCs, or commit to sexual differentiation. Evidence indicates that induction and commitment to sexual development occur in the asexual generation preceding gametocytogenesis, where each individual schizont produces a progeny of merozoites that uniformly develop into either asexual or sexual parasites of the same sex [2-4]. The sexual parasite develops intraerytrocytically in 10 to 12 days, sequestered away from the peripheral circulation, where only mature gametocytes are detected.

Although several *in vitro* treatments have been reported to increase gametocyte production, the factors that trigger gametocytogenesis *in vivo* are still largely unknown. Part of the difficulty in efficiently measuring gametocyte conversion rates in such experiments is the lack of very early specific markers identifying the committed developing parasites. Recent studies on the early events in gametocyte formation are however contributing to generate such reagents and to understand

molecular events at the onset of sexual differentiation. Once one merozoite invades the new host cell, the parasite induces profound structural and molecular changes in the host red blood cell, required for the parasite's survival and development, by exporting proteins of parasite's origin beyond the parasitophorous vacuole. Such modifications have been extensively studied in asexual stage development, where such a molecular remodeling has been described to affect the mechanical properties of the infected RBC and results in the capacity to cytoadhere to the endothelial cells of the microvasculature to escape passage and clearance in the spleen. Alike asexual parasites, protein export is highly active in early stage gametocytes [5] and erythrocyte remodeling is observed, which is translated in modifications on the mechanical properties of the infected cell [6-8]. A comparative analysis of purified stage I gametocytes, mature gametocytes and asexual trophozoites revealed that 10% of the proteins preferentially or specifically upregulated at the onset of sexual differentiation are exported, and that cleavage and N-acetylation of the parasite export signal PEXEL/HT is readily detected in stage I gametocytes. Such proteins were defined as *P. falciparum* Gametocyte EXported Proteins (PfGEXP), some of which were further analysed with protein-specific reagents in different reports and shown to be exported in early gametocytes [9], 744 [10], PfGECO [11] , PfGEXP10 [5]. Nevertheless, very little is known about the role of these proteins in the gametocyte-host interaction, and importantly molecular markers are still missing of very early events in host cell remodeling in the newly formed gametocyte, with the earliest detectable event being the appearance of protein Pfs16 in the parasitophorous vacuole of the early gametocyte at about 24h post invasion [12].

The goal of this work was to identify and characterize a novel protein, specific of early stage gametocytes that we show here to be the earliest molecule so far described being exported beyond the parasitophorous vacuole of newly formed sexual stages.

Materials and Methods

Parasite culture

P. falciparum lines 3D7A [13] and were cultured in human 0+ erythrocytes, kindly provided from Prof. G. Girelli, Dipartimento Biopatologia Umana, University of Rome "La Sapienza" or from the Australian Red Cross Blood Service, at 5% hematocrit under 5% CO2, 1-2% O2, 93-94% N2 [14]. Cultures were grown in medium containing: RPMI 1640 medium (Gibco) supplemented with 25 mM HEPES (Sigma-Aldrich), 50 micrograms per ml hypoxanthine, 0.25 mM NaHCO3, 50 micrograms per ml gentamicin and 10% pooled heat-inactivated 0+ human serum. Transfected cultures were maintained on drug supplemented media at the following concentrations; WR99210 1X 5nM. Parasite growth was monitored by giemsa stained (10%) thin blood smears, viewed at X1000 magnification under oil immersion.

Production of GST Fusion Proteins and Mouse Immunization

A 483bp *pfGEXP05* (PFI1770w) fragment from the intronless genomic region of the gene–coding sequence was cloned in a BamHI/NotI-digested pGEX-6P-3 vector to produce the GST recombinant protein. PCR amplification–was carried out with Accuzyme DNA polymerase (Bioline) with-conditions described in Olivieri *et al. [15]* and PCR products purified with a PCR purification kit (QIAGEN) after which insertion was controlled by sequencing. Expression of GST from plasmid pGEX-6P-3 and of the GST-PfGEXP5 fusion protein was induced in *Escherichia coli* BL21 by 1 mM isopropyl-D-galactopyranoside for 2h at 37 °C. Protein extraction was performed by using BugBuster Protein Extraction Reagent (Novagen). SDS-Page analysis detected the protein of interest in the insoluble fraction, for which purification of the GST fusion protein was achieved by solubilization in 6M urea followed by SDS Page electrophoresis and electro elution (Mini Whole Gel Eluter) in tris-borate elution buffer, in denaturing conditions, for 45 minutes at 100mA. The eletroelution fraction containing the fusion protein was then dialysed (aiming to eliminate the denaturing conditions). Further SDS-PAGE analysis showed that the GST fusion protein could be

eluted as a highly purified protein. Immunizations were carried out as described in Silvestrini et al [5] .

Construction of the gene disruption vector of PfGEXP5

A vector containing a single targeting sequence corresponding to the 5' end of *PfGEXP5* was constructed to facilitate a targeted gene disruption (TGD) by single crossover recombination into the *PfGEXP5* locus. A 1000bp fragment from the 5'UTR and coding sequence (400bp) of *PfGEXP5* was PCR amplified from 3D7 genomic DNA (gDNA) using the GEXP5-5F (taattacatatgtctaggtgt) and the GEXP5-5R (**atcgat** ctacattacaattaccattat) primers. This fragment was directionally cloned into the AvrII and ClaI sites of the Gateway compatable pHH1-Destination vector (Invitrogen). This vector contains the positive selection cassette encoding resistance to the drug WR99210 and was utilised for this single cross over gene disruption experiment.

Drug selection cycling

Parasites transfected with knock out or targeted gene disruption vectors require cycling of the parasites on and off of the drug selection to force integration of the circular plasmid into the target locus [16, 17]. Parasites were removed from drug selection and cultured for a period of 3 weeks without the addition of drug to the cultures. This allows for the plasmid to be lost from parasites where integration has not occurred. The reintroduction of the selection pressure causes all parasites not carrying a plasmid to die leaving only those still containing a parasite which may include a population in which the plasmid has integrated. After the parasites have recovered from the reintroduction of the selection pressure the parasites are examined for integration events. If no integration events have occurred the process is then repeated until integration has occurred (~3 cycling rounds).

Cloning by limiting dilution

Parasites were cloned by the standard limiting dilution method [18]. The accurate parasitaemia of the culture was obtained and the parasites diluted to give 0.5 parasites per well at 2% haematocrit in 200μl of complete culture medium supplemented with the required drug. Parasites were aliquoted into a 96 well plate and maintained for a period of 21 days. From day 15 cultures were checked by examination of giemsa stained smears for the presence of parasites. Positive wells were expanded to larger volumes for analysis, and cryogenic preservation.

Isolation of RNA from P. falciparum

Parasite culture containing 5-10% parasitaemia of the desired stage parasite was harvested and centrifuged at 500 *g* for 3 minutes. The supernatant was removed and the IRBC pellet resuspended in 10 times the volume of pre-warmed (37oC) TRIzol (Invitrogen). The pellet was resuspended by shaking until all lumps had dissolved and incubated at room temperature for 5 minutes. At this stage the TRIzol mix could be frozen at –70oC for later use. Alternatively 2 pellet volumes of chloroform was added and the sample shaken for 15 seconds followed by incubation at room temperature for 3 minutes. Samples were then centrifuged at 16000 *g* for 30 minutes at 4oC. The upper aqueous layer was removed before being mixed with isopropanol to ½ the original volume of TRIzol. The solution was mixed well and incubated on ice overnight. The mixture was then centrifuged at 16000 *g* for 30 minutes. The supernatant was removed and the pellet allowed to air dry. The pellet was resuspended to 1/5 the original IRBC pellet volume in molecular grade formamide (100%) and placed at –20oC for storage.

Northern blot analysis of PfGEXP5

Total RNA was extracted from parasites as described previously. The gel apparatus including gel tray and combs were cleaned with RNase Zap (Ambion) washed in water and allowed to air dry prior to use. A 1% TAE agarose gel was prepared

containing a final concentration of 5mM guanidine thiocyanate. RNA samples were then heated to 65oC for 10 minutes before loading. A RNA molecular weight marker was also loaded along with a lane containing DNA loading Dye. The RNA was then run at 100 volts until the dye was 8-10cm from wells. The gel was removed from the electrophoresis tank and soaked in 1X TAE containing ethidium bromide (0.4ìg/ml) allowing visualization of RNA under UV light at 253nm and photographed for records. The gel was then soaked in 50mM NaOH for 30 minutes and transferred onto Hybond N+ nylon membrane (Amersham) by overnight capillary transfer, after which the membrane was allowed to air dry. DNA probes were prepared by PCR amplification, using the following primer pairs: *PfGEXP5F* (atgaaagatcagattgaatca), *PfGEXP5R* (taattacatatgtctaggtgt), Pfs16F (**agatct**atgaatattcgaaagttc), Pfs16R (**atgcag**agaatcatctccttcgtc), GFPF (**ctgcag**agtaaaggagaagaactt), GFPR (ggtaccttatttgtatagttcatc), 28sF (aatttaattaagcgcaggtaaacg) and 28sR (aaattagctcaatgtcacaagaga). DNA probes were prepared by labelling 10-100ng of purified PCR product with 32P-dCTP by using the DECAprime II random priming kit (Ambion) according to the manufacturer's protocols. After overnight hybridization the membrane was washed once for 15 minutes with NorthernMax low stringency wash buffer (Ambion), followed by two washes with NorthernMax high stringency wash buffer (Ambion). The washed membrane was then sealed in plastic and placed into a cassette containing an intensifying screen. The cassette was placed at -70oC and the film exposed for between 24-48 hours before developing with a Kodak Xomat imager.

Western blot analysis

Protein extracts from Percoll-purified gametocytes ($5x10^6$/lane) were obtained by lysis in 1% Triton X-100 or equanotoxin. Soluble and insoluble fractions were separated by gradient gel electrophoresis using *precast* polyacrylamide 4-12% Bis-Tris gels (Invitrogen). Transfer of separated proteins onto a nitrocellulose filter (Protran, 0.2 um) was performed by electroblotting for 1 h at 350mA. The nitrocellulose blotted membrane was probed with anti-PfGEXP5 (1:200 dilution) for 1 h, at room temperature. Horseradish peroxidase-conjugated secondary antibody against mouse

IgGs was diluted 1:10,000, and the chemiluminescent reaction was revealed with Supersignal West Pico Chemiluminescent (Thermo Scientific).

Immunofluorescence analysis

Parasites were fixed in suspension with 4% paraformaldehyde, 0.075% glutaraldehyde and permeabilized with 0.1% Triton X-100 as described in Tonkin *et al.[19].* For acetone fixation infected erythrocytes were washed in 1XPBS, the cells were resuspended to 2% haematocrit and 5-10 microlitersl placed on a glass slides and allowed to dry at room temperature. Air dried parasites were fixed in 100% acetone for 10 minutes prior to antibody incubations. After a 30-min preincubation in 3% BSA, parasites were incubated using the following primary antibodies: anti-PfGEXP5 (1:100), anti-GST mouse antibody (1:100), anti- Pfg27 (1:200), anti-Pfs16 (1:500), anti-RESA (1:200) or anti-GFP (1:200) antibodies for 1h. After incubation and washes in PBS, parasites were incubated with an affinity-purified rhodamine or fluoresceine conjugated secondary antibody IgGs diluted 1:200 for 1h and followed by 10 minutes incubation with Hoechst (1:200 dilution). Samples were observed at 100 times magnification using different microscopes and softwares. Immunofluorescence analysis performed with Leitz DMR fluorescence microscope, equipped with the filters BP 340–380 (Hoechst), BP 515–560 (rhodamine), and BP 470–490 (fluorescein), were and images collected with a Leica cooled charge-coupled device camera and deconvolved using the LAS V3.8.0 (Leica Application Suite 3.8.0) software.

Results and Discussion

Identification and characterization of a novel gametocyte exported protein

PfGEXP5 was identified among the set of predicted PfGEXPs putatively involved in cell remodeling and highly enriched in early stage gametocytes [5]. The *pfgexp5* gene (PFI1770w) is constituted by two exons and is located in the subtelomeric terminal region of chromosome 9, previously associated with deficient gametocyte production [20, 21]. The *pfgexp5* gene encodes a protein with a PEXEL/HT motif and a conserved PHIST domain approximately 150 amino acids in length. PfGEXP5 belongs to the PHIST subgroup B, whose members, along with members of subgroup C, have been characterized to peak during schizogony and after invasion in the ring stage parasites [22]. Transcriptomic data indicated an induction of *pfgexp5* transcript production in ring stages. *pfgexp5* upregulation in gametocytes compared to asexual rings suggested that the increased transcription might arise from parasites committed to gametocytogenesis [23-25].

To characterize the expression pattern and cellular localization of PfGEXP5 during the intraerythrocytic life cycle of *P. falciparum*, specific antibodies were generated, and a fusion protein PfGEXP5-GFP was produced and transfected in 3D7 parasites. Western blot and immunofluorescence analysis with the above antibodies on sexual stage parasites showed that the anti-PfGEXP5 anti-serum detected a specific band at approximately 25KDa, as expected after processing of the PEXEL motif (Figure 1A) and a double band in the transgenic line 3D7 GEXP5-GFP (data not shown) corresponding to the size of the endogenous protein and of the GFP fusion. Most of the protein specific signal was detected in the Triton X100- or Equanotoxin-soluble fractions. while a fainter signal was detected in the SDS-soluble or Equanotoxin-insoluble fractions, possibly suggesting an interaction of some of the PfGEXP5 protein with iRBC membranous structures (Figure S1). Immunofluorescence analysis was performed on gametocytes of the reference line 3D7 and the HB3 derivative

HB3sel4. Parasites were paraformaldehyde/gluteraldeide fixed and Triton X100 permeabilized, and antibodies against Pfg27, an early marker of sexual differentiation whose expression starts at approximately 30-40 hours post-invasion [26], were used to identify small round parasites as early gametocytes. Results show that in both 3D7 and HB3sel4, PfGEXP5 is clearly detected beyond the PVM and in the cytoplasm of the host cell of the Pfg27 positive parasites. Antibodies produced a strong positive signal in the RBC cytoplasm in early sexual stages (Figure 1B) and a comparatively much weaker signal during gametocyte maturation (Figure 1B). These observations were confirmed by confocal microscopy analysis (Figure S1).

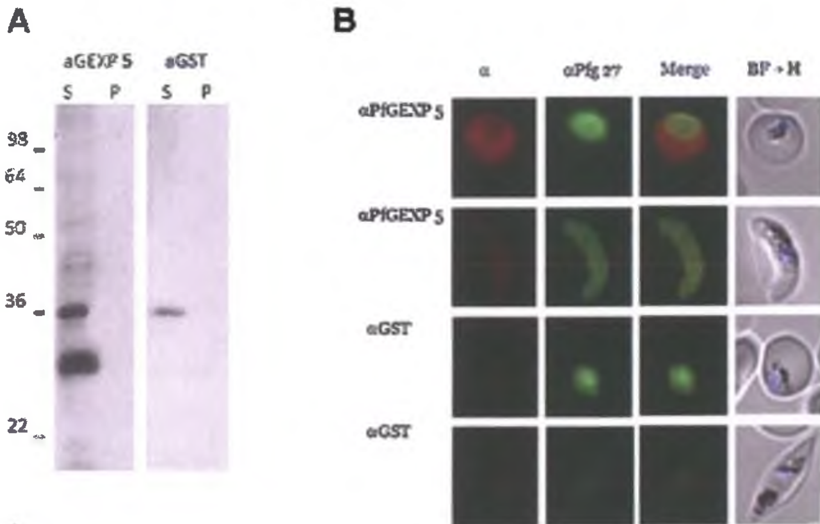

Figure 1. Characterization of PfGEXP5 expression pattern. (A) Western blot analysis on *P. falciparum* gametocyte extracts with anti-PfGEXP5 antibodies. Soluble (S) and insoluble (P) fractions were reacted with anti-PfGEXP5 and anti -GST serum, used as a negative control. (**B**) Immunofluorescence Analysis of PfGEXP5 in different stage gametocytes in the wild type 3D7 strain, in a double staining immunofluorescence analysis using a policlonal anti-PfGEXP5 (red) and anti-Pfg27 (green) and a double staining with anti-GST (red) and anti-Pfg27 (green) was used as a control. Anti Pfgexp5 serum detects the presence of PfGEXP5 in the cytoplasm of the iRBC, at a very early stage.

The results of the above analysis clearly showed that PfGEXP5 is exported beyond the parasitophorous vacuole at a very early stage of gametocyte development. Interestingly, in the above immunofluorescence experiments the anti-PfGEXP5 antibodies also stained a small percentage of RBCs infected with ring stage parasites, which however were not stained by the anti-Pfg27 antibodies. This suggested that such parasites could be early gametocytes which did not produce yet the early marker Pfg27.

PfGEXP5 is specifically expressed around 14 hours post-invasion of parasites committed to sexual development

In order to test this hypothesis an immunofluorescence analysis with anti-PfGEXP5 and anti-Pfg27 antisera was performed on synchronous parasites over 96 hours, across 2 parasite generations, sampling parasites every 12h during the first and every 24h during the second generation, .analyzing the presence and distribution of the Pfg27 and PfGEXP5 signals and of the nuclear stain DAPI. During the first generation, parasites were treated to induce sexual differentiation in order to analyze early gametocytes produced in the second generation. Parasitaemia in this experiment was 7% in the first generation and reached over 15% in the second. This analysis revealed a population of mononucleated parasites, negative for Pfg27, showing a distinct PfGEXP5 positive signal in the host cytoplasm (Figure 2A). Such parasites were first detectable at 12-16h post invasion, and their number increased to 10% of all the mononucleated parasites in the next 12h (Figure 2B). Later, at 36-40h post-invasion, a similar fraction of parasites was detectable, still positive for PfGEXP5, but at this time also positive for Pfg27, which identified them as stage I gametocytes. In the second parasite generation, at 12-16h post invasion the fraction of mononucleated cells which was Pfg27 negative and able to export PfGEXP5 in the host cell cytoplasm was at least 4 fold higher (>40%) than in the first generation. Similarly to what observed in the first generation, that fraction of cells became Pfg27 positive 24h later. In summary

this experiment indicated that from as early as 12-16h post invasion parasites that later became Pfg27 positive (i.e. early gametocytes) were already actively expressing and translocating in the host cell cytoplasm the PfGEXP5 protein. The time course experiment was repeated twice, and results were closely comparable in both biological replicates.

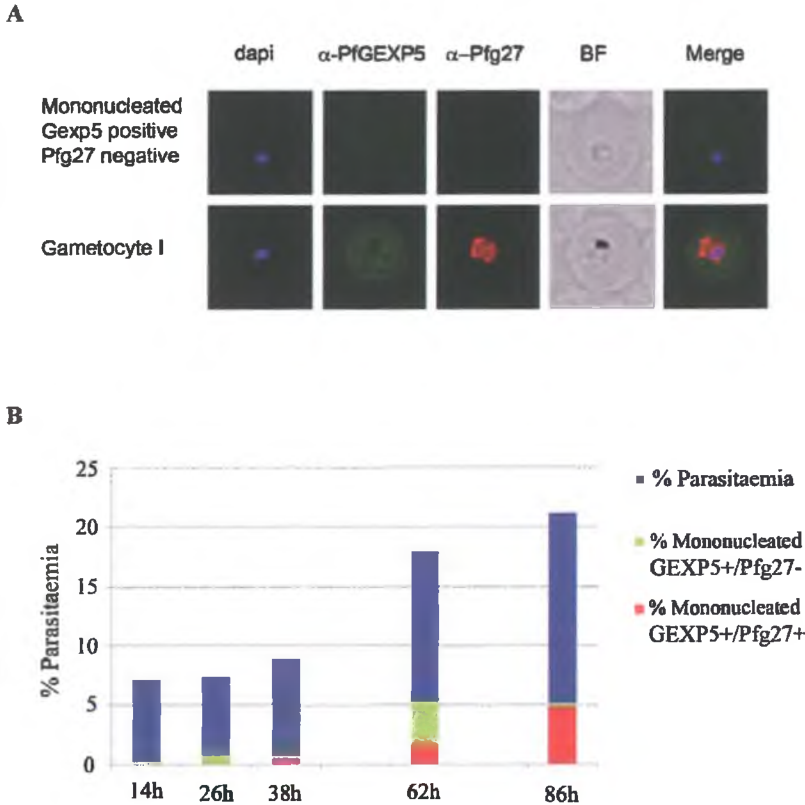

Figure 2. Time course analysis of PfGEXP5 expression. Representative images of PfGEXP5 positive parasites (green staining), negative (upper pictures) or positive (lower pictures) for Pfg27 (red staining), used to positively identify stage I gametocytes. The signal for PfGEXP5 is clearly localized in the cytoplasm of the parasite-infected erythrocyte. (B). Time course of the 96h parasite growth, showing the percent parasitaemia and the percent of the different classes of mononucleated parasites at each time point.

Considering that to date the earliest described event in sexual differentiation is the production of Pfs16 in the parasitophorous vacuole of the stage I gametocytes, at around 24h post-invasion [12] the result of the above experiments identify the export of PfGEXP5 as an even earlier event in sexual differentiation, and indicates that the PfGEXP5 positive ring stages are sexually committed parasites.

Early red blood cell remodeling events in gametocytogenesis are revealed by use of the PfGEXP5 marker

The possibility to mark sexually committed parasites from as early as 12-16h post-invasion provided the unprecedented possibility to investigate other events in host cell remodeling occurring at such an early time of sexual differentiation. One of the earliest protein released in the host cytosol by the invading merozoite is RESA, which associates with the RBC membrane through the spectrin network, where it remains detectable until 18-24 hours post invasion [27, 28]. Double IFA experiments were performed on synchronous parasites at 16-20h post-invasion using anti-PfGEXP5 antibodies either in combination with anti-RESA antibodies or with anti-Pfs16 antibodies. As the only available sera against PfGEXP5 and Pfs16 were raised in mice, it was impossible to unambiguously perform a double IFA with such antibodies.

Results of the anti-RESA/anti-PfGEXP5 IFA showed that in approximately 60% of the infected RBCs a RESA specific signal was associated with the host cell membrane, indicating that such parasites were younger than 24h. In such parasites a fluorescent signal was also commonly detectable on the parasite, possibly detecting a pool of parasite intracellular RESA or other cross reacting proteins. A fraction of 13% of the parasites where RESA was associated to the iRBC membrane also expressed and exported GEXP5, indicating that these represented sexually committed parasites (Figure 3, upper panel). Results of the anti-RESA/anti-Pfs16 IFA showed in contrast that none of the parasites showing a positive signal for Pfs16 was also showing a RESA-specific signal on the infected RBC membrane (Figure 3, lower panel). This

result suggests that at 24h, when Pfs16 starts to accumulate in the gametocyte PVM, RESA is no longer detectable in the RBC membrane of the developing gametocyte. Another result of the above double IFA experiments is consistent with this hypothesis. Amongst the RESA/PfGEXP5 double positive parasites 70% showed a weaker PfGEXP5 signal compared to the remaining 30% in which this was stronger, which further suggests that in the first 24h of gametocyte development the progressive disappearance of RESA is accompanied by the concomitant accumulation of PfGEXP5.

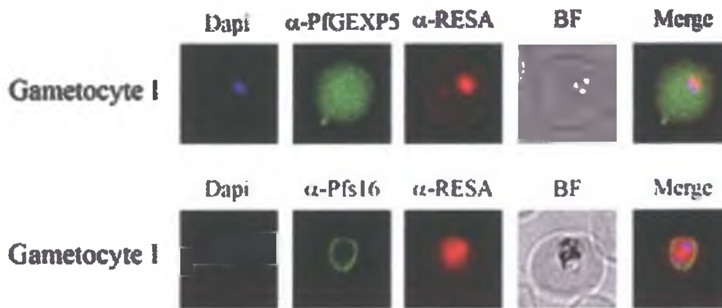

Figure 3. Immunofluorescence co-expression analysis of RESA and PfGEXP5 or Pfs16. Upper row. Representative images of RESA (red staining) and PfGEXP5 (green staining) co-expression at an early stage of sexual development. Lower row. Representative images of RESA (red staining) and Pfs16 (green staining) on the same parasite preparation as above. In the upper row PfGEXP5 is clearly localized in the cytoplasm of the parasite-infected erythrocyte with RESA associated to the erythrocyte membrane, as described for asexual parasites. The lower row is representative of the failure to detect RESA association to the erythrocyte membrane in Pfs16-positive stage I gametocytes.

KAHRP is another protein described to be expressed in asexual parasites from 16 hours post-invasion and absent in early and mid sexual stages [29] . Aiming to analyze the presence of KAHRP in very early gametocytes, double IFA experiments with anti-PfGEXP5 and anti-KAHRP antibodies were performed on sexually induced synchronous parasites at 12-16h and 24-28 hours post-invasion. The results clearly

showed that expression/export of PfGEXP5 and production of KAHRP were mutually exclusive in parasites at both time points (data not shown). These data confirm failure to detect KAHRP in stage I-II gametocytes and extend this observation to indicate that this protein is absent at earlier time of sexual differentiation.

In conclusion experiments using the very early gametocyte marker PfGEXP5 were able to positively identify the association of RESA with the RBC membrane in very early gametocytes until 24h post-invasion, and to reveal that RESA disappearance from the RBC membrane at 24h post-invasion temporally coincides with the localization of the Pfs16 protein in the newly formed parasitophorous vacuole membrane of the early gametocyte.

The developmental blockade of gametocyteless parasites is revealed using the PfGEXP5 marker

The unprecedented possibility to positively identify sexually committed parasites as early as 12-16h post-invasion by the export of PfGEXP5 was exploited to investigate the developmental blockade of a widely used gametocyteless clone of *P. falciparum*, F12. This clone was derived in a controlled long term propagation experiment from the reference line 3D7, and was shown to be unable to produce any stage of morphologically recognizable gametocytes nor the early stage I gametocytes identifiable only by the early Pfg27 marker [20] . The F12 parasites were however shown to be able to produce low levels of transcripts, but not of proteins, for the early gametocyte markers Pfs16 and Pfmdv-1/peg3 [30] , and to produce low level of PfGEXP5 peptides compared to purified stage I gametocytes [5] . These data suggested that F12 parasites either undergo some commitment to sexual differentiation and are blocked before stage I, or that the above sexual markers are abnormally expressed in F12 asexual stages.

In order to address these questions, F12 parasites were sexually induced and the expression of PfGEXP5 was analyzed by IFA and western blot. Immunofluorescence analysis on synchronous F12 parasites showed that approximately 18% of the parasites at 12-16 hours post invasion were positive for PfGEXP5, and that the protein was localized in the iRBC cytoplasm as in the case of the parental 3D7 parasites analyzed above (Figure 4, panel A). In F12 parasites, however, intensity of fluorescence for PfGEXP5 was generally lower than for 3D7 parasites. F12 parasites collected from the same culture at approximately 26-30 hours post-invasion were then analyzed by western blot, and compared to parental gametocyte-producing 3D7 parasites cultured in parallel and with purified stage II gametocyte. The same number of parasites were treated with Equinotoxin and soluble fractions of each sample were analyzed with anti-PfGEXP5 antibodies. Results showed that PfGEXP5 was detected at comparable levels in the sexually induced cultures of F12 and 3D7 parasites, in either case at lower levels than in purified stage II gametocyte (Figure 4, panel B).

Results of these experiments clearly show that F12 is able to produce sexually committed parasites expressing and exporting PfGEXP5 at 12-16h post-invasion in similar proportion than the parental line 3D7. This indicates that these parasites are still able to undergo commitment to sexual differentiation, although gametocytogenesis is then blocked before the expression of Pfg27, around 30-40h post-invasion. This results also pinpoint the time in which a developmental blockade occurs in this line, providing an essential functional information for further experiments aiming to elucidate the mechanism of sexual differentiation in *P. falciparum*.

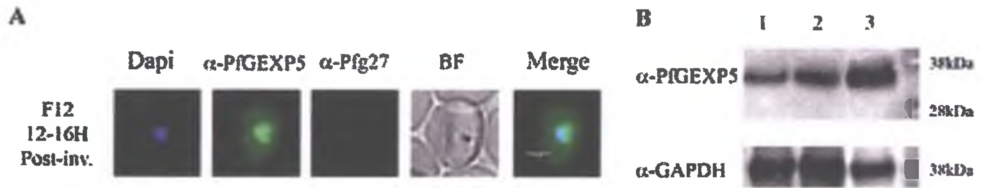

Figure 4. Immunofluorescence and western blot analysis of PfGEXP5 expression in F12. Representative images of PfGEXP5 positive parasites (green staining), negative for Pfg27 (red staining). The signal for PfGEXP5 is clearly localized throughout the cytoplasm of the parasite-infected erythrocyte. (B) Western blot analysis of sexually induced F12 (1) and 3D7 (2) parasites 26-30H post invasion and of 3D7 purified gametocytes stage II (3) treated with equinotoxin. Equinotoxin soluble fractions are represented in the upper panel probed with anti-PfGEXP5 and anti-GAPDH .

Functional characterization of the PfGEXP5 gene

Genetic disruption was performed to define the function of PfGEXP5. Disruption of the PfGEXP5 coding sequence was achieved by single homologous recombination and led to the production of two clones, A4 and C9, that failed to produce the *pfgexp5* transcript, as determined by Northern blot (Figure 5), and express PfGEXP5 protein, as determined in Western blot (not shown), unlike the 3D7 strain from which the KO clones were made. The disrupted parasites were assessed for their ability to enter gametocytogenesis using an immunofluorescence based assay based on reactivity for antibodies for the gametocyte specific Pfs16. Analysis of parasites from the above clones cultured for 96h failed to show any detectable PfGEXP5 or Pfs16 protein expression however, when compared to the original 3D7 strain which expressed PfGEXP5 after sexual induction. However, no Pfs16 expression was detected by western blot suggesting that gametocytogenesis is blocked before Pfs16 is expressed which was further confirmed by the absence of morphologically recognizable

gametocytes in the giemsa staining. Although the ability to commit and develop through gametocytogenesis was not possible to evaluate due to a deficiency in gametocyte development in the original 3D7 strain from which the KO were made, these results show that PfGEXP5 disruption is possible to achieve through single homologous recombination. Disruption of PfGEXP5 has been attempted again in high gametocyte producer 3D7 strain in order to characterize the role of PfGEXP5 during gametocytogenesis, being the new transfectants currently under selection.

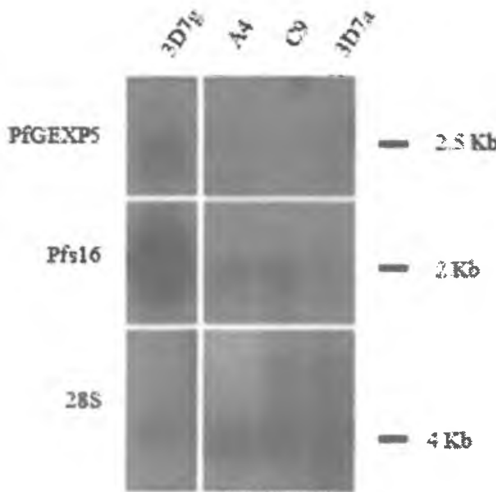

Figure 5. Characterization of the *pfgexp5*-disrupted parasite clones. (A) Northern blot analysis of the *pfgexp5* KO clones A4 and C9 with probes specific for the *pfgexp5* and the *pfs16* genes and for *28sRNA* as a loading control. No *pfgexp5* signal can be seen in the A4 and C9 KO clones nor in the asexual 3D7 RNA (3D7a), while a strong reaction can be seen to the *pfgexp5* transcript in the gametocyte 3D7 RNA (3D7g). Confirmation of the presence of gametocyte specific RNA in the TGD clones is seen in the next panel, probed with the gametocyte specific marker *Pfs16*. Stripping and re-probing with 28sRNA confirms the presence of equivalent amounts of RNA in each lane.

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Chapter 6

Specific remodeling of the infected erythrocyte during the development of the *Plasmodium falciparum* Gametocyte

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Abstract

Malaria is a vector born disease where the sexual stages of Plasmodium play a key role in the transmission of the parasite from human blood to mosquitoes and consequently in the spread of the disease. One of the most striking features of *Plasmodium falciparum* is the long development of its sexual blood stages - the gametocytes - which mature in 10-12 days in internal organs of the infected individuals. This review summarizes the most recent studies on the molecular and cellular remodeling of the host erythrocyte induced by the sexual parasite during gametocytogenesis and their differences from similar processes in the growth of asexual stage parasites. The review discusses the possible consequences of these host cell modifications on the establishment and maintenance of sequestration in internal organs of the immature sexual stages during development and on the release and ability of the mature gametocytes to circulate in the peripheral bloodstream.

Gametocytes in the Malaria Parasite Life Cycle

Malaria is a vector born disease caused by the genus *Plasmodium* and is responsible for an estimated 300-500 million clinical cases per year and approximately 1 million deaths mainly in pregnant woman and young children [1]. Six Plasmodium species have been so far identified to be responsible for malaria disease in humans, *Plasmodium ovale,* recently shown to be in fact constituted by the two sympatric species *P. ovale wallikeri* and *P. ovale curtisi [2], P. malariae, P. vivax, P. knowlesi and P. falciparum*, the latter being the most virulent, responsible for 90% of the deaths in Sub-Saharan Africa [3], where 81% of the cases of malaria are reported [4]. *P. falciparum* presents a complex life cycle involving parasite interactions with multiple tissues of two different hosts, the human and the mosquito. *Anopheles* mosquito transmits *P. falciparum* parasites feeding on humans through the injection of the sporozoites present in the salivary glands into the human subcutaneous tissue from where they travel to the liver. In this organ the parasite multiplies and differentiates inside the hepatocytes into thousands of merozoites that are released into the peripheral blood circulation, where they invade the host erythrocytes and start the intraerythrocytic development. During the intraerythrocytic phase, *P. falciparum* can either undergo a 48h cycle of asexual replication, during which each parasite can produce 16 to 32 merozoites that will invade other red blood cells, or commit to sexual differentiation and develop into male or female gametocytes, whose maturation requires 10 to 12 days. Sexual development has been traditionally divided into five stages distinguishable by morphological criteria [5], of which stage V represents the mature circulating gametocytes, able to further develop into gametes if engorged by a mosquito in a blood meal. Within minutes environmental signals in the mosquito midgut trigger gametocyte "rounding up", in which elongated gametocytes lose their distinguished shape and become gametes which egress from the human host erythrocyte. In this process male gametocytes undergo 3 rounds of DNA replication and coordinate the distribution of the resulting nuclei in 8 newly assembled flagella ("exflagellation"), while female gametes assume a spherical shape and disrupt the parasitophorous vacuole and the red blood cell surrounding membranes. Fertilization and zygote formation ensue, after which the zygote matures into a motile and invasive

ookinete that penetrates the midgut cell wall and develops into an oocyst, where the sporozoites are produced. After oocyst rupture, sporozoites migrate to and invade the mosquito salivary glands, able to infect new vertebrate hosts in the mosquito blood meal.

This review focuses on the cellular transformations accompanying the long gametocyte maturation in *P. falciparum*, and aims to highlight the differences in the intraerythrocytic development of *P. falciparum* sexual stages compared to asexual stages, playing special attention to the mechanisms developed by sexual stage parasites to remodel the host cell structural and mechanical properties to ensure their maturation, sequestration and the subsequent release from internal organs and ability to circulate.

Sequestration of *Plasmodium falciparum* gametocytes

Unlike other human malaria parasite species, both *P. falciparum* asexual and sexual stages do not freely circulate in the peripheral blood for the entire intraerythrocytic development, which instead requires sequestration of the parasite in internal organs of the infected individual. Indeed only stage V gametocytes and asexual ring stages are commonly seen in blood smears from peripheral blood of infected individuals. During the asexual cycle, only ring stage parasites are seen in circulation, while more mature stages efficiently bind to the endothelial cells of the microvasculature of several organs by cytoadhering to one or more host endothelial cells receptors through parasite encoded ligands exposed on the surface of the parasitized erythrocyte, evading in this way splenic clearance [6, 7]. CD36, ICAM-1 and, in the case of placental sequestration, CSA are host cell receptors reported to play a key role in cytoadhesion as the ligands of a major family of antigenically variable parasite proteins, PfEMP1, which are exposed on structures protruding from the surface of the infected erythrocyte, the knobs [8-10].

Immature gametocytes also sequester for 10 to 12 days in internal organs, from which they are released only at maturity. Virtually nothing is known about sites and

mechanisms of immature gametocyte sequestration. Only a few early analyses of post-mortem specimens and a few recent field and clinical reports provide morphological evidence indicating bone marrow and spleen as the organs where immature gametocytes (stage II to IV) predominantly sequester [11-15] (Joice et al, submitted; Aguilar et al, submitted). Once released in the peripheral circulation, *P. falciparum* mature gametocytes need another 2 to 3 days to become infective to mosquitoes [16, 17]. The mean circulation time per gametocyte is estimated to vary between 3.4 to 6.4 days by microscopy and the molecular method Pfs25 QT-NSBA [16, 18, 19].

Unlike the case of asexual parasites, the mechanism responsible for gametocyte sequestration is poorly understood. Few studies addressed the issue of gametocyte cytoadhesion, at times producing conflicting data on adhesion efficiency of sexual stage parasites and on the possible involvement in this process of PfEMP1 and host ligands. Most studies conducted on immature gametocytes of stage II to IV consistently showed low binding efficiency to host endothelial cells of different origins, to a few non-endothelial cells such as C32 melanoma or bone marrow stromal cell lines, and to purified host ligands such as ICAM-1 and CD36 [20-23]. Only two studies specifically investigated cytoadhesion of stage I gametocytes, partly for the difficulty in unambiguously distinguishing and efficiently purifying such stages from the morphologically similar asexual trophozoites. One study reported that stage I gametocytes produce knob structures on the surface of the infected red blood cell and bind to the CD36-positive C32 melanoma cells with the same avidity as asexual trophozoites [21]. The second study, which used a protocol to physically purify stage I gametocytes from asexual trophozoites [24], showed in contrast that early stage gametocytes show low if any binding to CD36-ICAM1-double positive endothelial cells [23], similarly to what observed in cytoadhesion studies with the elongated stages II to IV gametocytes [20-23]. Consistent with this observation, the above study confirmed absence of knobs on early gametocytes, in line with what previously reported by [25], and showed that the knob-associated protein KAHRP is absent in stage I and II gametocytes [26]. In addition the study showed that in the developmental transition from asexual proliferation to gametocytogenesis the parasite dramatically down-

regulates the expression of the entire *var* gene family encoding the PfEMP1 adhesins. The main conclusion of this study was that the mechanism responsible for the establishment of sequestration of the small, round shaped stage I gametocytes differs from the one enabling the PfEMP1 and knob mediated sequestration of the asexual stages from the very onset of sexual differentiation (Figure 1). Absence of knobs or similar structures from the surface of the gametocyte-infected erythrocytes through the subsequent stages of maturation and the low binding efficiency to endothelial cells of immature gametocytes (stages II-IV) indicate that also the maintenance of sequestration during gametocytogenesis does not rely on the mechanisms responsible for asexual stage cytoadhesion.

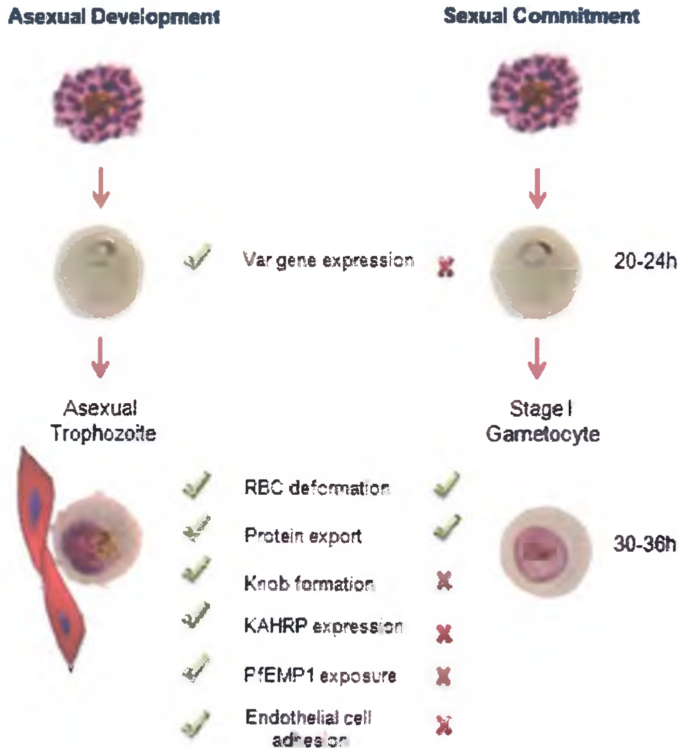


Figure 1. Host cell modifications at the onset of *P. falciparum* asexual and sexual development.

Cellular remodeling during gametocyte maturation

After invasion of the host cell, the sexually committed parasite undergoes profound molecular and cellular rearrangements, progressing from a round shaped stage I gametocyte, morphologically indistinguishable from an asexual trophozoite, to a half-moon form in stage II, to a symmetric elongated stage III, which further stretches and acquire pointed edges in stage IV, to finally become a gametocyte with a more blunted shape in the mature stage V. This dynamic morphological differentiation is accompanied by several molecular and structural changes such as the appearance from late stage I of a sub-pellicular membrane complex [27], the progressive production of about two hundred gametocyte-specific transcripts [28], the preferential or specific production of about 200 proteins as determined by comparing the proteomes of isolated stage I gametocytes, mature gametocytes and asexual trophozoites [24], and, later in development, the dissociation of the subpellicular microtubular network at the transition between stage IV and V [25, 29]. Of particular interest for the scope of this review is that the majority of the proteins identified as preferentially or specifically upregulated in early gametocyte contain the HT-PEXEL signature suggesting their putative export to the host cytoplasm, and that the above subpellicular membrane complex was recently shown to be molecularly similar to the inner membrane complex (IMC) so far described only in the parasite invasive and motile stages [29, 30]. Such observations altogether indicate that cellular and molecular processes along gametocytogenesis are likely to have a profound impact on the infected erythrocyte structure and conceivably on the mechanical properties of the gametocyte-infected red blood cell.

Mechanical properties of *P. falciparum* gametocyte-infected erythrocytes

In asexual stage development the parasite starts remodeling the host erythrocyte by synthesis of novel structures and the export of hundreds of parasite proteins to the

erythrocyte cytoplasm, which allow parasite growth, nutrient acquisition and survival. The dynamic reorganization of the erythrocyte plasma membrane and of the underlying cytoskeleton induced by the asexual parasite is associated with changes in the host cell membrane viscoelastic properties [31-33]. These changes, in addition to the presence of a large intracellular parasite impair infected erythrocyte ability to deform in response to an applied force and to circulate through the capillaries and the splenic sinusoids [34, 35]. The question of whether similar erythrocyte modifications are induced also by the developing gametocyte has been only recently addressed applying a wide variety of techniques (BOX1). Ektacytometry analysis was used to measure the rheological properties of purified gametocytes of stages III, IV and V, and described a progressive increase in deformability of the erythrocytes infected by gametocytes in the course of their maturation to stage V [29]. Another study combined the independent techniques of ektacytometry and microsphiltration to analyze changes in erythrocyte mechanical properties induced by stage II, III, IV and V gametocytes [36]. Experiments with both techniques showed that immature stages are less deformable than mature stages, and revealed that a distinct switch in deformability happens at the transition from stage IV to V gametocytes, leading mature gametocytes to be more deformable and significantly less retained when passing through a mesh of microspheres which mimic the splenic slits of the spleen sinus [36]. In another study micropipette aspiration was used to compare the viscoelastic properties of the membrane of erythrocytes infected by gametocytes at different maturation stages. Results showed a progressive decrease in membrane viscoelasticity from the early sexual stages to the elongated elliptical stage IV gametocytes, followed then by a significant increase in membrane viscoelasticity of red blood cells with mature stage V gametocytes [37]. All studies concluded that infection of *P. falciparum* immature sexual stages contributes to increase host cell rigidity, similarly to what observed for asexual parasites, and agreed that mature stages are more deformable than immature gametocytes. Amongst these observations, it is particularly intriguing that the switch in infected cell mechanical properties observed in the transition from stage IV to stage V gametocytes, resulting in more deformable mature sexual cells, coincides with the moment when gametocytes are released into the

peripheral circulation and are able to freely circulate for several days and to survive several passages through the spleen.

Several factors can determine the mechanical properties of erythrocytes, namely the membrane viscoelasticity, the cytoplasmic viscosity, the cell surface area to volume ratio [38, 39], besides, in the case of malaria, the intrinsic rigidity of the parasite cell. As the parasite develops within the erythrocyte it induces a decrease in surface area [40, 41] whereas the parasite volume increases and the infected cell volume remains almost constant [40, 42, 43]. This phenomenon triggers a decrease in the infected cell surface area to volume ratio and thereby a reduction of erythrocyte deformability. Despite the striking morphological changes observed during gametocyte maturation, the surface area to volume ratio remains roughly constant during gametocyte development, except for a transient decrease at stage IV, when the parasite is fully extended [37, 43]. Regarding the possible changes in cytoplasm viscosity due to hemoglobin digestion, it was described that 70% of the hemoglobin mass is digested during the development from stage II to stage IV gametocytes [43], suggesting that the steady progression of this phenomenon is unlikely to have an abrupt impact on cytoplasm viscosity at maturation. It should however be considered that the dissipation of the microtubules network at the onset of stage V may lead to a slight increase in the red blood cell volume with a consequent decrease of hemoglobin concentration [43]. Membrane viscoelasticity was addressed in the study using micropipette aspiration, whose results revealed a decrease in the erythrocyte membrane viscoelastic properties along maturation from stage II to stage IV [44]. This suggests that the gametocytes directly modify the host erythrocyte membrane affecting its mechanical properties. Identification of a large set of putatively exported proteins produced from the onset of gametocytogenesis [24], and the direct evidence that proteins of the STEVOR family show a dynamic association/dissociation with the erythrocyte membrane compartment during gametocytogenesis [36] are also consistent with the hypothesis that gametocyte proteins directly interact with host components to modify erythrocyte membrane viscoelasticity. It should however be noticed that microscopic analysis of gametocytes used in the above mentioned microsphiltration apparatus showed that the vast majority

of the stage V gametocytes able to pass through the microsphere filter exhibited a strikingly deformed shape compared to the retained immature stages. This clearly indicates that mature gametocytes are intrinsically much more deformable than their immature precursors, which contributes to increase the overall deformability of stage V infected erythrocytes [36].

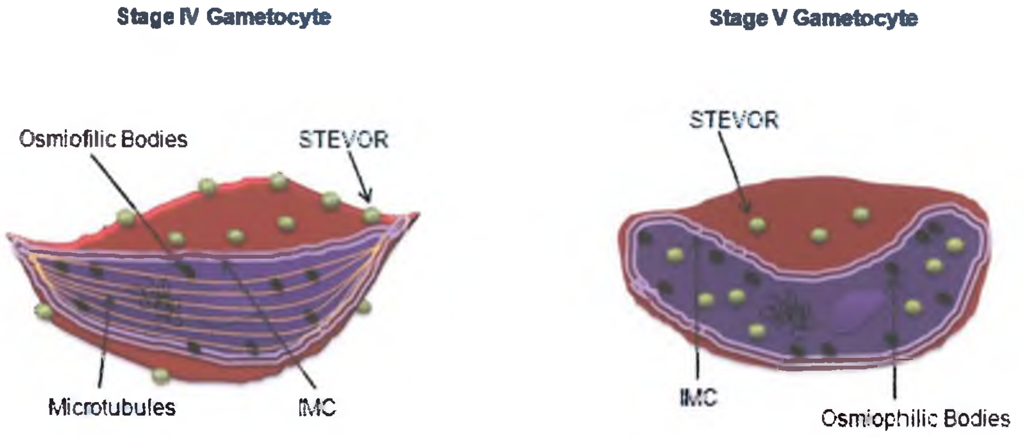
Molecular basis of parasite host cell remodeling

Several proteins of parasitic origin have been reported to participate in the host cell remodeling after *P. falciparum* invasion and to contribute to alter the mechanical properties of the newly invaded host cell. In asexual stages, RESA, a protein released by the invading merozoite and exported to the RBC membrane, was described to interact with the spectrin network, contributing to modifications in the membrane of erythrocytes infected with early ring stages leading to a reduced deformability of the host cell [33]. A moderately reduced deformability of ring stage infected erythrocytes has also been reported in another study using an *ex vivo* spleen perfusion system that showed that a small percentage of circulating ring stages can be retained in the spleen [45]. Other parasite exported proteins such as KAHRP and PfEMP3 were reported to decreased deformability in more mature asexual stages, in trophozoite and schizonts [31]. FIKK kinases have also been implicated in the mechanical remodeling of the host cell by phosphorylating different membrane skeleton proteins in a stage specific manner [46]. More recently, STEVOR multigene variant family has also been shown to contribute to the increased rigidity of the infected erythrocyte upon association with the erythrocyte membrane, which results in significantly decreasing the deformability and filterability of the infected erythrocytes [35].

As mentioned above, alike in asexual parasites, protein export is highly active from the onset of sexual differentiation, with a large set of gametocyte enriched proteins predicted or experimentally shown to be exported to the erythrocyte cytoplasm [24, 47, 48]. Only a few proteins have been however reported to directly interact with the

erythrocyte membrane during gametocyte maturation. Mature parasite infected erythrocyte surface antigen (MESA) is a high-molecular-mass protein exported to the red blood cell surface in both asexual and sexual stages. As MESA has been reported to interact with the host cell cytoskeleton in asexual parasites [49-52], it may possibly trigger alterations in erythrocyte membrane viscoelasticity also in sexual stages. The gametocyte-specific antigen Pf11-1, a megadalton protein that localizes to the host cell membrane during gametocytogenesis [53], is also an attractive candidate to participate in the altered membrane deformability of gametocyte-infected erythrocyte. Although much reduced levels, if any, of PfEMP1 are detected on the surface of erythrocytes infected with stage I-II gametocytes, and are virtually absent in further maturation stages [26], members of two other multigene families, STEVORs and RIFINs, are instead highly expressed during sexual differentiation and some were shown to be associated with the erythrocyte membrane in mid stage gametocytes (III-IV) [36, 54, 55]. Microsphiltration analysis was recently used to address the contribution of STEVOR to the infected erythrocyte mechanical properties during gametocytogenesis. Comparing isogenic parasite clones differing in STEVOR expression a significant decrease in retention rates, i.e. an increased deformability, was observed in stage III and IV gametocytes in which STEVOR expression was downregulated. Consistently, mature gametocytes overexpressing STEVOR, but not control gametocytes overexpressing another transmembrane protein, showed a much decreased deformability when compared to wild type stage V gametocytes [36]. In these experiments STEVOR was shown to be associated to the erythrocyte membrane in red blood cells containing stage III and IV gametocytes, while the STEVOR specific signal was no longer detectable on the membrane of red blood cells infected by stage V gametocytes. These experiments in conclusion indicate that STEVOR proteins impact upon the deformability of erythrocytes hosting stage III-IV gametocytes through a direct association with the host cell membrane, and their dissociation from the infected erythrocyte membrane at gametocyte maturation is likely to contribute to the observed switch in deformability at the transition between stage IV and stage V gametocytes [36]. The combined effects of STEVOR relocation and of the disassembly of the microtubule subpellicular network observed in the transition from stage IV to stage V [25 , 29] most

likely plays a major role in the increase in deformability marking the maturation of the *P. falciparum* gametocyte (Figure 2).



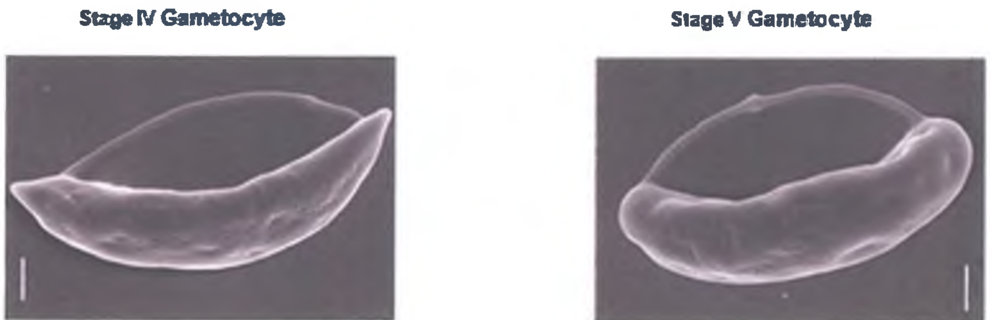


Figure 2. Cellular and molecular remodeling in the transition from stage IV to stage V (mature) gametocytes.

Mechanical properties of the gametocyte infected erythrocytes: which role in sequestration of the immature gametocytes and in ability to circulate of the mature stages?

The work summarized so far indicates that intracellular establishment and maturation of the *P. falciparum* gametocyte is accompanied by molecular and cellular changes that have distinct, stage specific effects on the mechanical properties of the infected erythrocytes in the long gametocytogenesis process of *P. falciparum*. Although no experimental data have been generated to directly address the following issues, this review will here discuss possible implications of such modifications in the key processes of sequestration of immature gametocytes, in their release at the end of maturation, and in the ability of stage V gametocytes to persist in peripheral circulation and survive repeated passages through the splenic slits.

Sequestration. The few studies on the sequestration sites of sexual stages in the infected body are essentially represented by early observations on post-mortem specimens and a few recent field and clinical reports, which altogether identify bone marrow and spleen as the organs where immature gametocytes (stage II to IV) are readily detectable [11-14, 15 , 56] (Joice et al, submitted; Aguilar et al, submitted). Despite the different physiological functions, some similarities exist in the blood circulation in the bone marrow and in the spleen, namely that in both organs part of the circulation occurs at a reduced flow rate in an open circulation microenvironment devoid of an endothelial barrier (Figure 3).

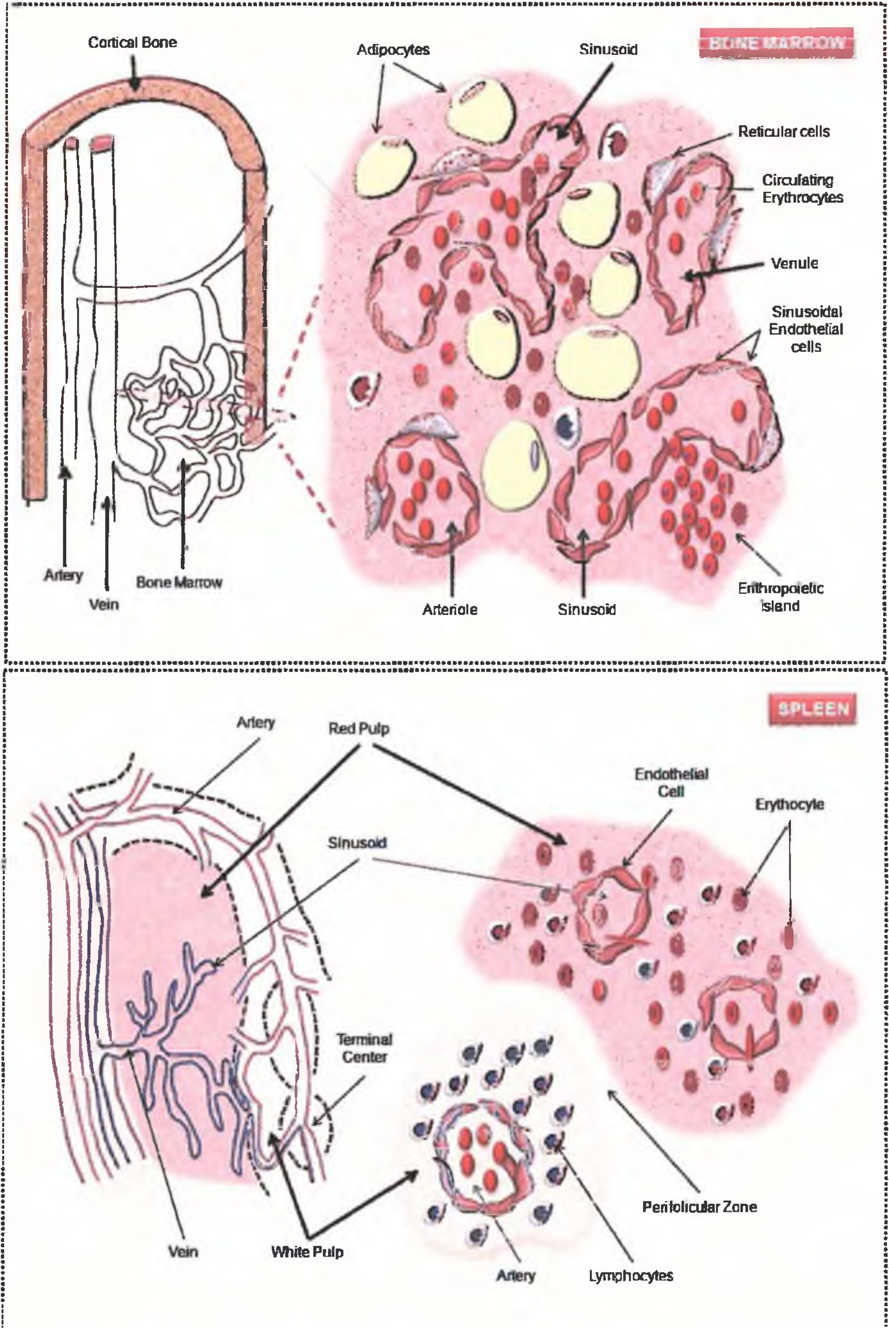

BONE MARROW
Cortical Bone
Artery
Vein
Bone Marrow
Adipocytes
Sinusoid
Reticular cells
Circulating Erythrocytes
Venule
Sinusoidal Endothelial cells
Arteriole
Sinusoid
Erithropoietic island
SPLEEN
Artery
Red Pulp
Sinusoid
Endothelial Cell
Erythocyte
Terminal Center
Vein
White Pulp
Artery
Lymphocytes
Perifolicular Zone

Figure 3. Figure 3. Schematic comparison of the histology of human bone marrow and spleen vascularisation and cellular microenvironment.

In the bone marrow, arterial vessels enter the tissue cavity dividing into several arterioles that span toward the endosteum, forming the thin walled and low pressure bone marrow sinusoids which are interconnected by the intersinusoidal capillaries radially distributed around the draining central vein [57, 58]. These vascular channels, constituted by a single layer of fenestrated endothelium devoid of a basement membrane, yield a structural configuration that allows a high surface area and a slower flow rate [57, 58]. In the spleen, the splenic artery branches into central arterioles from where 80-90% of the splenic flow engages in the fast "closed" microcirculation while the remaining fraction goes through the slow "open" microcirculation. In the fast microcirculation the erythrocytes pass directly from the perifollicular zone to the venous sinus, while in the slow circuit the red blood cells flow to the sinuses through the cords of the red pulp. The red pulp sinuses receive the blood from the cords through the sinus wall, formed by lining endothelial cells, positioned in parallel to the vase axis, which are connected by annular fibers running transverse to endothelial cells and stress fibers oriented along the endothelial cell long axis. The red pulp function is regulating the passage of the blood cells from the red pulp cords into the sinus and back to the venous system. Alike the bone marrow sinuses, the fenestrated splenic sinus do not present a basement membrane [59]. In sinuses blood flows at much lower velocity, 20 times slower, than in the fast microcirculation, in which blood flow is similar to that in capillaries [60].

Observation of immature and mature gametocytes in spleen specimens is usually not accompanied by information on the cellular microenvironment where these are detected, thus preventing to formulate hypotheses on the physiological relevance of such localization for development of the sexual parasites. As immature gametocytes of all stages can be observed in the spleen, it is nevertheless tempting to speculate that these are not only destined to be cleared by the macrophages crowding the red pulp cords, as is the case of asexual stages, but that these parasites may in fact develop in such an organ. Analysis of mechanical properties of gametocytes at different maturation stages indicates that mature gametocytes may pass through the splenic slits in order to circulate, but predicts that immature gametocytes will be retained. It is therefore likely

that, if gametocytes indeed develop in the spleen, the immature stages must avoid splenic passage and need appropriate microenvironment and host cell interactions preventing macrophage phagocytosis and ensuring progression of their maturation. Existence of a cryptic intrasplenic parasite growth has been hypothesized [60], which makes it tempting to further speculate that some fraction of such parasites enter sexual differentiation to produce the observed immature gametocytes. The field of parasite-spleen interactions is receiving a deserved increase in attention, and hopefully more experiments and observations will soon address this fascinating issue.

In the bone marrow, until recently, detection of immature gametocytes in this organ was similarly based on morphological analysis of bone marrow autopsy specimens or aspirates from infected individuals [11-13, 56]. A preferential localization of immature gametocytes in bone marrow aspirates compared to peripheral blood was recently confirmed in a survey of malaria infected children (Aguilar et al, submitted). In addition, two recent studies importantly conducted a morphological and immunohistochemical analysis of gametocytes in bone marrow biopsies in which tissue preservation enabled to examine in some detail the localization of gametocytes in these specimens. A clinical case report from a patient with sub-acute *P. falciparum* malaria revealed the presence of immature gametocytes in extravascular spaces of bone marrow [15], and a more systematic survey on 26 bone marrow specimens from fatal cases of *P. falciparum* malaria confirmed and extended this important observation showing that immature gametocytes, detected immunohistochemically by antibodies against the early gametocyte marker Pfs16, were present in significantly higher proportions in the extravascular compartment than intravascularly (Joice et al, submitted). Whether the above observations faithfully reflected the immature gametocyte localization in bone marrow, or were partly due to tissue modifications associated with the clinical symptoms of infections remains to be determined.

The features of the blood circulation in bone marrow and spleen may induce to hypothesize how immature gametocytes sequester, using the slightly more detailed information available on bone marrow to focus on this organ. Homing of circulating

early sexual stage parasites to bone marrow may occur if the residual PfEMP1 molecules detected on the surface of stage I gametocyte-infected red blood cell are still sufficient to mediate low affinity interactions with endothelial cells in the slow circulation in the marrow sinusoids. In this respect, a stage I gametocyte might be functionally similar to asexual trophozoites from natural or induced mutants of *P. falciparum* in which residual PfEMP1 presence in a knobless and KAHRPless context was shown to be sufficient to mediate cytoadherence of these parasites to CD36 and ICAM1 in static but not in "under flow" conditions [61]. In alternative, or in addition to this mechanism, yet unidentified gametocyte adhesins might mediate the binding of early sexual parasites to most likely non-endothelial cells in the bone marrow. In addition, rigidity of the immature gametocytes may also play a role in their retention in an area of low blood flow within bone marrow. It is noticeable that aggregates of cells with decreased deformability are prone to occur in low shear stress regions, and that similar events may represent a crucial hemorheological behavior in bone marrow sequestration [62]. The elongated red blood cells in Sickle Cell Disease were shown to aggregate and accumulate with an efficiency inversely related to the applied shear flow [63] and the onset of vasocclusion caused by sickle erythrocytes was reported to be associated with an enhanced ability of these erythrocytes to adhere to endothelial cells [64]. In summary, the increasing rigidity of gametocyte infected cell during development may either stabilize the initial low affinity binding interaction of the young stages, or even be sufficient to mediate an adhesion-independent sequestration solely due to mechanical properties. In this respect the cell elongation occurring during gametocyte maturation could produce a larger area of intracellular contact, strengthening such host-cell interactions and their retention during the 10 day long maturation.

The observations mentioned above from bone marrow biopsies that immature gametocytes are also, or preferentially, found in the extravascular compartment further complicate and enrich this picture. Albeit preliminary, those data are indeed compelling to propose that homing to bone marrow of immature gametocytes is followed by development in the extravascular compartment, where most likely they establish in this

environment modes of parasite-host cell interactions drastically different from those possible in the vascular compartment. If, also in this case, a role for yet unidentified immature gametocyte adhesins may not be formally ruled out, the role of cell mechanical properties in maintaining sequestration in the extravascular microenvironment is likely to be highly important. A complication in this model is that it predicts that the stage I gametocytes homing to bone marrow have to cross the microvasculature endothelium, presumably through the same "open" sites used in the opposite direction by the newly formed reticulocytes to enter circulation from the marrow stroma. The extravasation ability of stage I gametocytes is however difficult to reconcile with preliminary observations that such stages, identified by the specific expression of a fluorescent reporter, are rigid enough to be readily retained in microsphiltration experiments, and are so to a higher degree than asexual trophozoites (Alano, Deplain, Lavazec, unpublished). A possible alternative scenario is based on the hypothesis that asexual parasites already residing in bone marrow are able (or even induced) to produce a progeny of sexually committed parasites which are then retained in that microenvironment [65]. From the above biopsy observations Joyce and colleagues refined this hypothesis proposing that such asexual parasites in fact resided and produced their sexual progeny directly in the extravascular compartment (Joice et al, submitted). Although it might be still conceivable that sexually committed ring stages produced by schizonts in the vascular portion of the bone marrow would be still sufficiently deformable for extravascular migration shortly after invasion, this intriguing hypothesis would parsimoniously not rely on a step of early gametocyte extravasation. In either scenarios of the extravascular maturation of sexual stages, the appearance of mature gametocytes in circulation necessarily requires that such parasites eventually enter the vasculature by crossing the fenestrated endothelium. In this respect, the increase in gametocyte deformability associated to the transition from stage IV to the mature stage V is fully consistent with the hypothesis that such a change in cell mechanical properties is necessary, and maybe sufficient, for the entry in circulation from the extravascular gametocyte maturation site, possibly in a process similar to the entry in circulation of the highly deformable mature reticulocytes.

Circulability. After the entry in circulation, the ability of mature gametocytes to circulate for an average of 3-6 days [66] escaping the spleen mechanical retention represents one of the big challenges for gametocyte survival in the blood stream as well as it remains one of the most puzzling aspects of *Plasmodium falciparum* gametocyte biology.

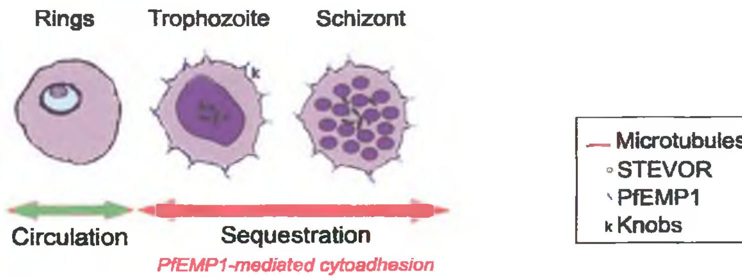
P. falciparum parasite stages with significantly decreased deformability such as the immature gametocytes [29, 36, 44], trophozoites [29, 35]and schizonts [35, 67]are likely or experimentally demonstrated to be readily retained in the narrow splenic slits of the sinusoids in that organ, is in contrast to the circulating mature gametocytes and ring stage parasites. Before being used on gametocytes of different stages as described above, ektacytometry, microsphiltration and micropipette aspiration analysis had shown that ring stage parasite infected red blood cells are significantly more deformable than those containing mature asexual stages [45 , 68]. Microsphiltration studies, where a mesh of microspheres mimic the narrow splenic slits of the sinusoids, have shown that a high fraction of rings can pass such narrow apertures. This result was confirmed with experiments conducted in an *ex vivo* spleen perfusion system which consistently showed that a large fraction of ring stage parasites were able to pass through the spleen tight circulation. It was recently showed that the main factors causing instead the retention of the remaining fraction of ring stages were an increased sphericity and a decreased surface area affecting a subpopulation of such parasites [41]. In the case of the circulating ring stage parasites the higher deformability of such cells is likely due to the still minimal remodeling induced to the host cell, the same explanation cannot hold for the morphologically much different mature sexual stages, in which the higher deformability must require different, sophisticated cellular and molecular modifications. The disassembly of the supporting parasite microtubule network mentioned above most likely results in the intrinsic high deformability of the stage V gametocytes. In addition, the dissociation of STEVOR proteins from the infected erythrocyte membrane may increase the membrane viscoelasticity and likely contributes to the switch in infected cell deformability observed at the transition between stage IV and stage V gametocytes. This results in a newly acquired filterability of mature gametocyte-infected

erythrocytes, allowing them to pass through the 1,89μm length and 0,65μm width interendothelial slits. These processes are predicted to be tightly regulated and some recent observations revealed that the switch in infected cell deformability is dependent on cyclic nucleotides signaling, and that inhibitors interfering with these pathways can drastically impair the filterability of mature gametocytes (C. Lavazec, unpublished results). Although the mechanisms allowing cyclic nucleotides-mediated changes in gametocyte-infected erythrocyte deformability remain to be deciphered, these results open avenues in the discovery of transmission-blocking drugs impairing the capability of transmission stages to circulate.

Conclusions

Despite the high relevance of sexual development in the transmission of malaria, a disease responsible for an average million deaths each year, gametocyte development in the human host has been long neglected. The few existing studies on gametocyte sequestration have until recently addressed this phenomenon under the influence of what is known of PfEMP1 mediated cytoadhesion described in the sequestration of asexual stages. It is however becoming more and more clear that although early stage gametocytes morphologically resemble trophozoites, the mechanisms used by asexual and sexual stages to remodel their host cells differs from the beginning of the respective developmental programs. While asexual parasites remodel the host erythrocytes in order to cytoadhere to the microvasculature to escape spleen retention, the gametocytes from the earliest stages of differentiation evolved different mechanism(s) to interact with the human host to ensure the same escape route. The described absence of antigenic molecules on the surface of erythrocytes infected by immature gametocytes [69, 70], likely to be advantageous for keeping a low immunogenic profile in the long gametocytogenesis process, most likely prevented the evolution of cytoadhesion mechanisms similar to those of asexual parasites. Mechanisms of host cell remodeling able to influence the mechanical properties of the gametocyte-infected red blood cells

and the fine-tuning of these properties along gametocytogenesis might have thus evolved to play a major role in governing specific gametocyte-host interplay. This ensures the sequestration of the immature sexual stages, the timely entry, or re-entry, in circulation of the mature gametocytes and their ability to freely circulate to maximize their chances of being engorged by a mosquito and to progress in the parasite life cycle (Figure 4).



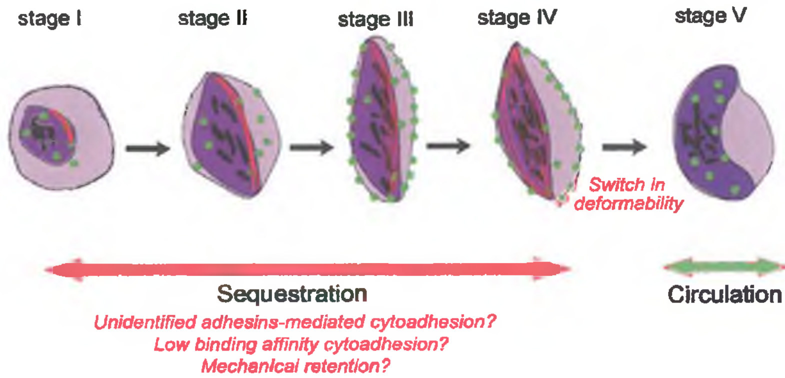


Figure 4. Differential host cell molecular development induced by sexual and asexual *P. falciparum* stage parasites

Although a significant progress has been recently made in our understanding of gametocyte mechanical and molecular remodeling of the host cell and of the impact of such modifications in gametocytogenesis, many questions remain to be answered to address the highly complex gametocyte-host interactions during sexual maturation.

Box1:

Experimental approaches to measure erythrocyte mechanical properties.

Several techniques have been developed to measure the rheological properties of red blood cells at the level of the individual cell or of the cell population

Micropipette aspiration.

This technique provides precise, quantitative information on the rheological properties of individual erythrocytes. By allowing measurements to be performed from different orientations, distinctive aspects of the individual cell mechanical properties can be addressed. Partial aspiration of a small portion of membrane into 1–2 μm pipettes allow the measurement of the membrane elastic shear modulus and area compressibility modulus, while aspiration of the entire cell into 3–5 μm pipettes gives measures the overall cell deformability [38]. Other parameters can be measured, such as the time constant for RBC shape recovery, and hence membrane surface viscosity, by extending then releasing a cell point-attached to a surface [38]. Furthermore, cell volume and membrane surface area can be determined by aspirating cells into small pipette to form a spherical outer portion and a tongue within the pipette [38].

Ektacytometry.

This technique was developed to quantitatively measure the average elongability of populations of cells at increasing shear stresses, which are applied to a diluted cell suspension in isotonic solutions (at about 30mPa viscosity) exposed to the movement of two concentric cylinders. A laser beam is directed through the sheared cell population that will generate a diffraction pattern, detected by a video camera, according to the elongation efficiency at increasing shear stresses. The extent of erythrocyte deformability, or elongation index (EI), is defined as the ratio between the difference of the 2 axes of the ellipsoid diffraction pattern and the sum of these 2 axes, $EI = (L - W)/(L + W)$, where L and W are the length and width of the diffraction pattern. The elongation index curve is obtained after the calculation of the EI at the different shear stresses applied [38, 71, 72].

Microsphiltration.

Microsphiltration is a recently developed technique based on filtration of erythrocytes through a mixture of Calibrated metal microspheres (96.50% tin, 3.00% silver, and 0.50% copper; Industrie des Poudres Sphériques, Annemasse, France) with 2 different size distributions (5- to 15-µm diameter and 15- to 25-µm-diameter) that mimic the geometry of inter-endothelial splenic slits, generating 1,85 µm wide aperture [67, 73]. The filterability of a population of cells through this matrix is measured under flow and its readout is the "retention rate" versus the flow through, which positively correlates with the rigidity of a cell population causing inefficient passage through the microsphere matrix.

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Chapter 7

General Discussion

For several years study of the host cell remodeling and parasite-host interaction in sexual stages of *Plasmodium falciparum* were neglected, especially concerning the early stage gametocytes. This was not only because of the general difficulty of reproducibly achieving experimental sexual induction but also due to the resemblance of stage I gametocytes to trophozoite asexual stages and the absence of molecular tools that would allow an accurate recognition of the sexual parasites from the very beginning of gametocytogenesis. The main goal of this thesis was to characterize the host cell and molecular remodeling imposed by the sexually committed parasite after invasion, aiming to explore the mechanisms involved in gametocyte sequestration and host interaction during the maturation and release of the infective mature stages.

Plasmodium falciparum sexual stages are virtually unable to bind to human endothelial cells

It has already been widely described that asexual parasites sequestration is mediated through efficient and specific cytoadhesion between endothelial cell ligands and members of the well characterized PfEMP1 multigenic variation family however, almost nothing is known about how gametocyte sequestration is mediated. The few existent studies addressing the morphologically recognizable mid stage gametocytes binding efficiency to endothelial cell ligands mediating PfEMP1 cytoadhesion in asexual stages suggest a very poor binding efficiency of those stages when compared to the asexual parasites. Only one study had used human endothelial cell lines, specifically from bone marrow origin, one of the organs previously reported as a preferential location for gametocytes sequestration, reporting the existence of specific binding efficiency to HBMEC [1]. In order to gain a deeper insight into the gametocyte's ability to interact with human endothelial cell (EC) lines, in **Chapter 2** we explored the binding efficiency of mid stage gametocytes to a panel of human EC lines with origin in different organs, differently expressing the some of the main ligands involved in *P. falciparum* asexual parasite's cytoadhesion, including two

different cell lines of human bone marrow origin, used here for the first time. Our data clearly show that mid stage gametocytes from the reference parasite clone 3D7 and from a wild isolate present a very inefficient binding profile to any of the EC lines. Our main conclusion is that maintenance of immature gametocyte sequestration is most likely not mediated by specific interactions with human bone marrow endothelial cells.

Another aspect of gametocyte sequestration that was virtually unanswered was how sequestration is established by the very early stage gametocytes. For several years it was postulated that early stage gametocytes might behave like trophozoites and sequester by efficient PfEMP1-mediated cytoadhesion. Using new molecular tools that allow the identification and purification of the "trophozoite like" stage I gametocytes, static cytoadhesion assays on endothelial cell lines expressing both CD36 and ICAM1, two of the main endothelial cell receptors for PfEMP1, were performed and analysed by immunofluorescence analysis with anti-Pfg27 antibodies, one of the earliest sexual marker so far described [2]. In **chapter 3** we show that stage I gametocytes are virtually unable to cytoadhere to endothelial cells expressing CD36 and ICAM1. Consistent with the poor binding efficiency during sexual development in the early stage gametocytes is the absence of knobs or similar structures and of the knob-associated protein (KAHRP) described in **chapter 3** for first time, and the evidence of the dramatic down-regulation of the expression of the entire *var* gene family encoding the PfEMP1 adhesins. Our data strongly suggests that sexual stages likely behave differently from the beginning of gametocytogenesis onwards with so far unidentified mechanisms in gametocyte-host interaction and sequestration. Cytoadhesion is unlikely to play the key role in gametocytes sequestration, as the sexual stages do not interact with the host vasculature via the well-characterized avidity and specificity of asexual stages. However, we cannot exclude the presence of low affinity interactions between the gametocytes and the endothelia and their importance in a more complex environment such as bone marrow. It could be possible that different aspects of gametocyte molecular and structural remodeling are working simultaneously during gametocyte sequestration.

Unique and specific molecular and structural erythrocyte remodeling by *P. falciparum* gametocytes impacts infected-host cell mechanical properties

After invasion of the host cell, the sexually committed parasite undergoes profound cellular rearrangements accompanied by several molecular and structural changes such as the appearance from late stage I of a sub-pellicular membrane complex [3], the progressive production of about two hundred gametocyte-specific transcripts [4], the preferential or specific production of about 200 proteins [5], and, later in development, the dissociation of the subpellicular microtubular network at the transition between stage IV and V [6, 7]. Aiming to address how the structural and molecular reorganization induced by the gametocyte would impact the host red blood cell (rbc), ektacytometry and microsphiltration analyses were used to investigate changes in erythrocyte mechanical properties during gametocyte maturation [8]. Experiments with both techniques showed that immature stages are less deformable than mature stages, and revealed that a distinct switch in deformability happens at the transition from stage IV to V gametocytes, leading mature gametocytes to be more deformable. The overall conclusion of this work is that infection of *P. falciparum* immature sexual stages contributes to increase host cell rigidity, similarly to what was previously observed for asexual parasites. Increase in infected cell deformability coincides with the moment of sexual stage maturation in which gametocytes are released into the peripheral circulation proposes that switch in mechanical properties may play a major role in the process of “de-sequestration” and in the subsequent ability to freely circulate for several days and to survive several passages through the spleen.

In **chapter 4** a role for the STEVOR multigene variant family was shown in determining the increased rigidity of the infected erythrocyte upon association with the erythrocyte membrane, and in the switch in deformability, coinciding with STEVOR de-association from the rbc membrane in mature gametocytes. The

combined effects of STEVOR relocation and of the disassembly of the microtubule subpellicular network observed in the transition from stage IV to stage V [6, 7, 9] most likely play a major role in the increase in deformability marking the maturation of the *P. falciparum* gametocyte. In **chapter 5** we also show that RESA, a protein released by the invading merozoite and exported to the rbc membrane, previously described to interact with the spectrin network in asexual parasites and contribute to a reduced deformability of the host cell [10].

All together these results seem to suggest that molecular remodelling is likely to play a role in the alteration of the mechanical properties from the beginning of the gametocyte induced host remodelling. In addition, rigidity of the immature gametocytes may also play a role in their initial retention in an area of low blood flow within bone marrow. The increasing rigidity of gametocyte infected cell during development may either stabilize the initial low affinity binding interaction of the young stages, or even be sufficient to mediate an adhesion-independent sequestration solely due to mechanical properties, as aggregates of cells with decreased deformability are prone to occur in low shear stress regions and may represent a crucial hemorheological behavior in bone marrow sequestration.

In this respect the cell elongation occurring during gametocyte maturation could produce a larger area of intracellular contact, strengthening such host-cell interactions and their retention during the ten days long maturation.

Export of a newly described exported protein is the earliest molecular event detectable in *P.falciparum* gametocytogenesis

Ten percent of the proteins preferentially or specifically upregulated at the onset of sexual differentiation are exported, and that cleavage and N-acetylation of the parasite export signal PEXEL/HT is readily detected in stage I gametocytes. Such proteins were defined as *P. falciparum* Gametocyte EXported Proteins (PfGEXP), In **chapter**

5 we report the identification and characterization of a novel protein, specifically exported beyond the parasitophorous vacuole of newly formed sexual stages, the PfGEXP5 protein. Analysis with specific antibodies and with gene fusions on synchronous gametocytes showed that PfGEXP5 starts to be actively exported to the host parasite cytoplasm around 16 hours post-invasion and its presence in the rbc cytoplasm decreases over the gametocyte maturation period. Although the functional role of the early production and export of PfGEXP5 is still not clear and functional studies are currently underway, this represents the earliest event so far described in the formation of stage I gametocytes and reagents specific for this gene product represent new tools in the study of sexual parasite stages such as the to address the sexual induction after the administration of malaria drug treatment.

Although PfGEXP5 is so far the earliest marker and exported protein in gametocytes, protein export seems to be very active in early stage gametocytes and the identification of other exported candidates and their roles during the young gametocyte induced remodeling would likely shed some more lights into the rbc remodeling and gametocyte-host interactions.

Final considerations and future directions

The work presented in this thesis consitutes a significant progress in the understanding of gametocyte mechanical and molecular remodeling of the host cell and of the impact of such modifications in gametocytogenesis, shedding light in long neglected aspects of the biology of the parasite's transmission stages. It is becoming more and more clear that although early stage gametocytes morphologically resemble trophozoites, the mechanisms used by asexual and sexual stages to remodel their host cells differs from the beginning of the respective developmental programs. While asexual parasites remodel the host erythrocytes in order to cytoadhere to the microvasculature to escape spleen retention, the gametocytes from the earliest stages of differentiation evolved different mechanism(s) to interact with the human host to ensure the same escape

route. The described absence of antigenic molecules on the surface of erythrocytes infected by immature gametocytes [11, 12], likely to be advantageous for keeping a low immunogenic profile in the long gametocytogenesis process, most likely prevented the evolution of cytoadhesion mechanisms similar to those of asexual parasites. On the other hand, mechanisms of host cell remodeling able to influence the mechanical properties of the gametocyte-infected red blood cells and the fine-tuning of these properties along gametocytogenesis might have thus evolved to play a major role in governing specific gametocyte-host interplay. Remodeling of the mechanical properties might play a crucial role in the sequestration of the immature sexual stages, the timely entry, or re-entry, in circulation of the mature gametocytes and their ability to freely circulate to maximize their chances of being engorged by a mosquito and to progress in the parasite life cycle.

A number of open questions remain to be answered: where do gametocytes sequester in the bone marrow; what is the exact mechanism responsible for gametocytes sequestration; where does sexual commitment happens, in the peripheral circulation or in specific organs/environment; how can we interfere with gametocyte development, aiming to prevent gametocyte maturation. Sexual parasites seem to develop through a specific and unique process, way different from that known and reported during asexual development. It is clear that such complex questions need to be addressed independently from what we know about asexual stage parasites. As such, it is fundamental that the next models used to address gametocyte sequestration/desequestration can reflect the environment preferred by developing sexual stages. Furthermore, it will be of extreme importance to identify new molecular markers for early gametocyte commitment to further explore the very initial steps of gametocytogenesis and its relevance during sexual development and in the establishment of sequestration.

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Summary

Malaria is one of the world's deadliest diseases, having been estimated approximately 207 million cases of malaria in 2012 and 627.000 deaths. Resistant parasites have been reported for all current drugs and there is no sufficiently effective vaccine available. One of the major problems in eradicating human malaria is the efficient transmission of sexual stages, the gametocytes, and identification of novel targets for transmission blocking strategies against malaria will be essential. *Plasmodium falciparum* gametocytes present a unique development through five different morphological stages (stages I-V), during approximately 12 days, and only at maturity those stages are observed in circulation. The ability to sequester during the long maturation process and its ability to circulate in the peripheral circulation at maturity, undetected by the mechanical spleen retention, represents a key aspect to the parasite's sexual development. This thesis addresses several basic and largely neglected questions related to host-cell remodeling, parasite-host interactions and gametocyte sequestration during the development of the sexual stages.

In **chapter 2** we address the ability of immature gametocytes (stage II to IV) to cytoadhere to human endothelial cells from different tissues, in comparison to highly cytoadhesive asexual parasite stages. We show that the cytoadhesive capacity of immature gametocytes is relatively poor and independent of host cell origin. Unlike previously reported, sexual immature stages do not show a preferential binding to human bone marrow endothelial cells. We conclude that gametocyte sequestration in the human host is only marginally, at best, mediated through ligand-specific cytoadhesion to the endothelial cells.

For several years it has been postulated that early stage I gametocytes behave like trophozoites and sequester by efficient PfEMP1-mediated cytoadhesion. In **chapter 3** we address this virtually unanswered aspect by studying expression of PfEMP1 and knobs/KAHRP during the early stage I gametocytes and its potential role during the establishment of early sexual stages sequestration. Static cytoadhesion assays of early

stage I gametocytes on endothelial cell lines expressing both CD36 and ICAM1 (two of the main PfEMP1 endothelial cell receptors) show that those stages are virtually unable to cytoadhere. Furthermore we show the absence of knobs, or similar structures, and of the dominant constituent of knobs, *knob-associated histidine-rich protein* (*KAHRP*), in "trophozoite like" stage I gametocyte. Moreover, our work provides evidence of a dramatic down-regulation of the entire *var* gene family encoding the PfEMP1 adhesins. Our data strongly suggest that sexual stages likely behave differently from asexual parasites directly from the beginning of sexual development.

In the absence of an identified mechanism responsible for gametocyte-host interaction and sequestration, we question the possible functional role for the striking and unique morphological development during gametocyte maturation. In **chapter 4** we report how the structural and molecular reorganization in gametocytes may play a role during sequestration, release, and circulation of the mature stage V parasites. Studying deformability of gametocyte-infected by ektacytometry and microsphiltration analysis we show that immature forms are less deformable than mature stage V gametocytes. There appears to be a switch in erythrocyte host cell deformability from stage IV to stage V gametocyte. Increases in infected cell deformability coincide with release of mature gametocytes into the peripheral circulation. These observations strongly suggest that a switch in the mechanical properties plays a major role in the process of "de-sequestration" and subsequent ability to freely circulate for several days, surviving several passages through the spleen. We also find that the mechanical switch correlates with the loss of parasite-derived STEVOR proteins localized in the infected erythrocyte membrane, suggesting that the gametocyte actively remodel the host cell erythrocyte.

In **chapter 5** we show that the host cell remodeling induced by the gametocyte starts in the first hours after invasion and report the earliest event so far described in the formation of stage I gametocytes. Approximately ten percent of the proteins preferentially upregulated in the early gametocyte proteome are represented by

putatively exported proteins defined as *P. falciparum* Gametocyte EXported Proteins (PfGEXP). Characterization of expression and localization of PfGEXP candidate proteins, using specific antibodies, allowed the identification a novel exported protein – PfGEXP05. This protein is expressed and exported to the host erythrocyte cytosol at approximately 16 hours post-invasion, representing the earliest molecular event identified in the early "ring" stage gametocyte.

Several aspects of the sexual development of *P. falciparum* in the human host have been largely neglected, especially concerning the early stage gametocytes. Our data brings novel and relevant information to the understanding of the host cell erythrocyte mechanical and molecular remodeling induced by the parasite during sexual maturation and the importance of the parasite induced modifications during maturation of malaria transmission stages.

Samenvatting

Malaria is een van de dodelijkste ziektes ter wereld en wordt geschat ongeveer 207 miljoen gevallen te hebben veroorzaakt in 2012, resulterend in 627.000 doden in dat jaar. Resistente malaria parasieten komen voor tegen alle tegenwoordig gebruikte drugs en er is nog geen goed werkend vaccin tegen. Een van de grootste problemen bij het uitbannen van humane malaria is de efficiënte transmissie van de sexuele stadia, ofwel de gametocyten. Identificatie van nieuwe targets bruikbaar voor transmissie blokkade strategieën is daarom noodzakelijk. *Plasmodium falciparum* gametocyten vertonen gedurende ongeveer 12 dagen een unieke ontwikkeling door vijf verschillende morfologische stadia (stadium I-V), waarvan alleen de rijpe stadia waarneembaar zijn in de circulatie. Het vermogen om te sekwestreren gedurende het lange rijpingsproces en zijn perifere circulatie vermogen van de volwassen stadia, waarbij deze onopgemerkt blijven voor de mechanische milt retentie, zijn kernaspecten van de sexuele ontwikkeling van de parasiet. In deze thesis wordt ingegaan op verschillende basis en grotendeels genegeerde vragen gerelateerd aan gastcel remodellering, parasiet-gast interacties en gametocyt sekwestratie gedurende de ontwikkeling van de sexuele stadia.

In hoofdstuk 2 wordt ingegaan op het cyto-adherentie vermogen van onvolwassen gametocyten (stadium II tot IV) aan humane endotheelcellen van verschillende weefsels, in vergelijking tot de hoog cyto-adherente asexuele parasiet stadia. We laten zien dat het cyto-adherentie vermogen van onvolwassen stadia vrij laag is en onafhankelijk van de oorsprong van de gastcel. In tegenstelling tot wat eerder gerapporteerd is, vertonen sexueel onvolwassen stadia geen preferentiële binding aan humane beenmerg endotheelcellen. We concluderen dat gametocyt sekwestratie in mensen slechts

marginaal plaatsvindt via ligand specifieke cyto-adherentie aan de endotheelcellen.

Sinds verscheidene jaren wordt gesuggereerd dat de vroege stadium I gametocyten zich gedragen als trofozoieten en sekwestreren via efficiënte PfEMP1 gemedieërde cyto-adherentie. In hoofdstuk 3 gaan we in op dit virtueel onbeantwoorde aspect door de expressie van PfEMP1 en knobs/KAHRP gedurende de vroege stadium I gametocyten, en hun potentiële rol gedurende het optreden van vroege sexuele stadium sekwestratie, te bestuderen. Statische cyto-adherentie assays met vroeg stadium I gametocyten op endotheelcellijnen die zowel CD36 en ICAM1 (twee van de belangrijkste PfEMP1 endotheelcelreceptoren) tot expressie brengen, laten zien dat stadium I gametocyten vrijwel niet in staat zijn om over te gaan tot cyto-adherentie. Verder laten we zien dat de zogenaamde "knobs", of soortgelijke structuren, en het daarbij dominante bestanddeel van deze "knobs", -knob geassocieerd histidine rijke eiwitten (KAHRP), afwezig zijn in "trofozoiet-achtige" stadium I gametocyten. Bovendien levert ons werk het bewijs voor een dramatische down-regulatie van de gehele var gen familie, welke coderen voor de PfEMP1 adhesines. Onze data wijzen er sterk op dat sexuele stadia zich waarschijnlijk anders gedragen dan de asexuele parasieten die net aan hun sexuele ontwikkeling zijn begonnen.

Vanwege het ontbreken van een geïdentificeerd mechanisme dat verantwoordelijk is voor gametocyt-gastheer interacties en sekwestratie, zijn we nieuwsgierig naar de mogelijke functionele rol van de opvallende en unieke morfologische ontwikkeling gedurende gametocyt rijping. In hoofdstuk 4 beschrijven we hoe de structurele en moleculaire reorganisatie in gametocyten een rol kunnen spelen tijdens, sekwestratie, het vrijgeven van, en circulatie van volwassen stadium V gametocyten. Door het bestuderen van gametocyt

geïnfecteerde erytrocyten met behulp van ektacytometrie en microsphiltratie analyse laten we zien dat onrijpe vormen minder vervormbaar zijn dan volwassen stadium V gametocyten. Er lijkt een schakelaar aanwezig te zijn in erytrocyt-gastcel deformatie van stadium IV naar stadium V gametocyten. Verhoogde celvervormbaarheid van geïnfecteerde cellen, valt samen met de release van volwassen gametocyten in de perifere circulatie. Deze waarnemingen wijzen er sterk op dat een schakelaar in de mechanische eigenschappen een belangrijke rol speelt in het proces van "de-sekwestratie" en vervolgens het vrij kunnen circuleren in de perifere circulatie, gedurende enkele dagen, waarbij de parasiet meerdere passages door de milt kan overleven. We hebben ook gevonden dat de mechanische schakelaar correleert met het verlies van een lid van de STEVOR familie, gelokaliseerd in de erytrocyt-gastheercel membraan, wat erop wijst dat de gametocyt actief de erytrocyt-gastheercel remodelleert.

In hoofdstuk 5 laten we zien dat gastheercel remodelling door de gametocyt geïnduceerd wordt tijdens het eerste uur na invasie en raporteren we over het tot dusver vroegst beschreven moment van de stadium I gametocyt formatie. Meer dan tien procent van de eiwitten die verrijkt zijn in het vroege gametocyt proteoom worden vertegenwoordigd door eiwitten waarvan men denkt dat ze geëxporteerd worden, ookwel gedefinieerd als *P. falciparum* Gametocyt geëxporteerde eiwitten (PfGEXP). Karakterisering van expressie en lokalisatie van PfGEXP kandidaat eiwitten met behulp van specifieke antilichamen, heeft geleidt tot de identificatie van een nieuw geëxporteerd eiwit – PfGEXP05. Dit eiwit wordt tot expressie gebracht en geëxporteerd naar het cytosol van de gast erytrocyt ongeveer 16 uur na invasie en vormt hiermee de vroegst ontdekte moleculaire gebeurtenis gedurende het vroege "ring" gametocyt stadium.

Verschillende aspecten van de seksuele ontwikkeling van *P. falciparum* in de menselijke gastheer hebben tot dusverre weinig aandacht gehad, vooral waar het, het vroeg gametocyt stadium betreft. Onze gegevens brengen nieuwe en relevante informatie aan het licht betreffende het begrijpen van de door de parasiet, tijdens sexuele rijping, geïnduceerde mechanische en moleculaire remodelling van de erytrocyt-gastheercel, en het belang van de door de parasiet geïnduceerde veranderingen tijdens de rijping van de malaria transmissie stadia.

Sumário

A malária é uma das doenças parasitárias mais mortíferas do planeta, tendo o relatorio anual da Organizacao Mundial da Saude apontado para uma estimativa de aproximadamente 207 milhões de vítimas,em 2012, das quais 627.000 mortais. Casos de resistência contra os tratamentos actualmente existentes têm vindo a ser reportados e ainda nao existe disponível uma vacina suficientemente eficaz. Um dos maiores problemas para a erradicação da malária nos seres humanos reside na eficácia da transmissão dos estádios sexuais, os gametócitos, pelo que é fundamental a identificação de novos alvos terapêuticos para o estabelecimento de estratégias inovadoras contra esta doença . Os gametócitos da espécie *Plasmodium falciparum* apresentam um desenvolvimento único através de cinco estádios morfológicos distintos (estadio I-V), que dura aproximadamente 12 dias, e em que apenas os estádios V maduros sao observados em circulacao. O sequestro dos gametócitos durante o longo período de maturação e a capacidade para circular na circulação periférica depois de atingir a maturidade, passando despercebidos pelo baço, representam um aspeto chave no desenvolvimento sexual do parasita. Esta tese aborda diversas questões básicas e largamente negligenciadas no âmbito de estudos sobre malária, relacionadas com a remodelação da célula hospedeira, a interacção parasita-hospedeiro e o sequestro dos gametócitos durante o desenvolvimento dos estádios sexuais.

No **capítulo 2,** aborda-se a capacidade de citoadesão dos gametócitos imaturos (estadios II a IV) às células endoteliais humanas de diferentes tecidos, em comparação com os estádios assexuados altamente citoaderentes. Este trabalho mostra que a capacidade de citoadesão dos gametócitos imaturos é relativamente baixa e independente da origem das células endoteliais. Ao contrário de estudos anteriores, os estádios sexuais imaturos não mostram uma

citoadesão preferencial às células endoteliais da medula óssea humana. Concluimos que o sequestro dos gametócitos no hospedeiro humano é, na melhor das hipóteses, apenas marginalmente mediada pela citoadesão a ligandos específicos das células endoteliais.

Durante vários anos foi postulado que gametócitos do estádio I poderiam comportar-se como trofozoitos e estabelecer o sequestro através de uma citoadesao eficiente mediada pelo PfEMP1. No **capítulo 3**, aborda-se este aspecto através do estudo da expressão do PfEMP1 e dos knobs/KAHRP durante o estádio I e o seu potencial papel no estabelecimento do sequestro dos estádios sexuais. Os ensaios de cito-adesão estática dos gametócitos I em linhas de células endoteliais expressando CD36 e ICAM1 (dois dos principais receptores de células endoteliais para PfEMP1) mostram que gametócitos nesta fase são virtualmente incapazes de cito-aderir. Além disso, mostram que a ausência de knobs, ou estruturas similares, e dos componentes dominantes dos knobs, *knob-associated histidine-rich protein* (KAHRP), nos gametócitos I. Mais ainda, este trabalho fornece evidências para a existência de uma dramática regulação negativa de toda a família dos *var* genes, que codifica o PfEMP1. Estes dados sugerem fortemente que as fases sexuais se comportam de maneira diferente dos parasitas assexuais desde o início do desenvolvimento sexual.

Na ausência da identificação de um mecanismo responsável pelo sequestro e interacção gametócito-hospedeiro, questionou-se ainda sobre o potencial papel do desenvolvimento morfológico único durante o desenvolvimento dos gametócitos. No **capítulo 4**, mostramos o impacto que a reorganização estrutural e molecular do eritrócito infectado, induzida pelos gametocitos, desempenha durante o sequestro, libertação e circulação dos estadios maduros V. O estudo da deformabilidade de gametócitos infectados através de análises

ectacitométricas e de microfiltração, permitiu demonstrar que formas imaturas são menos deformáveis do que gametócitos maduros V. Parece pois, haver uma alteração na deformabilidade do eritrocito infectado na passagem do estádio IV para o estádio V. O aumento da deformabilidade nas células infetadas coincide com a libertação de gametócitos maduros para a circulação periférica. Estas observações sugerem fortemente que a mudança nas propriedades mecânicas desempenham um papel importante no processo de libertação dos estádios maduros e a consequente capacidade para circular livremente durante dias, sobrevivendo a várias passagens pelo baço. Constatou-se também que esta alteracão mecânica está correlacionado com proteínas da família STEVOR, produzindas pelo gametocito, localizadas na membrana do eritrócito, sugerindo que o parasite remodela activamente a célula hospedeira.

No **capítulo 5**, mostramos que a remodelação da célula hospedeira induzida pelo gametócito começa logo nas primeiras horas após a invasão do parasita e relatamos o mais recente evento molecular durante a formação do gametócitos no estádio I. Aproximadamente dez por cento das proteínas enriquecidas no proteoma inicial do gametócito I são representadas por proteínas exportadas definidas como *P. falciparum* Gametocyte EXported Proteins (PfGEXP). A caracterização da expressão e localização de algumas PfGEXPs, utilizando anticorpos específicos, permitiu a identificação de uma nova proteína exportada – a PfGEXP05. Esta proteína é expressa e exportada para o citosol do eritrócito hospedeiro aproximadamente 16 horas após a invasão, representando o acontecimento molecular mais precoce identificado até ao momento na fase inicial de "anel" do estádio sexuado.

Diversos aspetos do desenvolvimento sexual do *P. falciparum* em hospedeiros humanos têm sido amplamente negligenciados, especialmente em relação aos gametócitos na sua fase inicial de desenvolvimento. Os dados aqui

apresentados trazem novas e relevantes informações para a compreensão da remodelação molecular e mecânica da célula hospedeira, induzida pelo parasita durante a fase de maturação sexual, e da importância das modificações induzidas pelo parasita durante a maturação dos estádios de transmissão da malária.

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me strength through this journey. Thank you for your support, friendship and love!

Curriculum Vitae

I was born on November 18th 1982 in Leiria, Portugal. Since I can remember I have always wanted to be a biologist and in July 2004 I graduated in Biology - Field of Applied Scientifc Technological Animal Biology – at Oporto University, Portugal. As I became more interested in genetics and molecular biology applied to biomedical research, after graduation I started a six months internship in the field of Cytogenectics and Prenatal screening at Clinical Genetic Center which which was followed by a second internship in the Oncology Department of Instituto de Oncologia Francisco Gentil, in Portugal. In October 2005 I was awarded a one-year scholarship to work on a project that aimed to identify specific inhibitors of transthyretin dissociation that is responsible for amyloid fibril formation.

Aiming to pursue a career in biomedical research, I joined a two years Master program in Molecular Genetics, in October 2006. My master thesis focused on the correlation of the expression patterns of two important protein receptor kinases in invasive breast carcinomas with prognostic parameters.

Soon after obtaining my Master degree in Molecular Genetics in December 2008, I was awarded a Marie Curie Scholarship to start a PhD project that focused on the translation of fundamental knowledge on malaria biology into antimalarial intervention strategies, under the supervision of Dr. Pietro Alano and Professor Robert Sauerwein. During the past five years we, in collaboration with other groups, have addressed fundamental and largely neglected aspects of *Plasmodium falciparum* parasite stages responsible for transmission.

Since February 2014 I started working in the group of Dr. Moritz Treeck at the Department of Parasitology in the National Institute for Medical Research in London, UK, on the characterization of kinase mediated cell signaling in *Plasmodium falciparum*.