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Congress Abstracts

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01

Deciphering the stimuli inducing gametogenesis in the malaria parasite Plasmodium falciparum following its transmission to the mosquito

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The transmission of the malaria parasite *Plasmodium falciparum* from the human host to the mosquito vector during a blood meal is mediated by sexual precursor cells, the dormant gametocytes. When entering the mosquito midgut, the gametocytes become activated by environmental stimuli, which include a drop of temperature and the contact of the parasite with the mosquito-derived molecule xanthurenic acid (XA). Gametocyte activation initiates gametogenesis, and within 15 min, the crescent gametocytes round up and egress from the enveloping red blood cell (RBC), while forming male and female gametes. It was the aim of this study to investigate in detail the initial contact of the intraerythrocytic gametocytes with XA and to identify additional factors involved in gametocyte activation. A combination of exflagellation (= microgametogenesis) inhibition assays and mass spectrometric analyses were used to identify the modes of action of different external and intrinsic factors of gametocyte activation. We show that RBCs accumulate XA in their cytosol independent from infection. Inhibitor studies suggest that XA uptake by the RBCs is mediated by organic anion transporter peptide (OATP)-type transporters. When life gametocytes were liberated from the enveloping RBC, they remained sensitive to XA-induced activation, indicating that the XA receptor is located on the gametocyte plasma membrane. We also showed that the ionophores nigericin and valinomycin are able to induce exflagellation in the absence of XA, indicating that a change in RBC potassium levels plays a role in gametogenesis. Interestingly, the herbicide fluridone, which was shown to inhibit the synthesis of the egress molecule absisic acid (ABA) in *Toxoplasma gondii*, is able to block exflagellation in vitro, and fluridone-mediated inhibition of gametogenesis can be reversed by external addition of ABA. Our data demonstrate that XA is being taken up by RBC transporters of the OATP-type and indicate that the XA-receptor is located on the gametocyte plasma membrane. Additional factors playing a role for the induction of gametogenesis include the host cell potassium levels and the intrinsic molecule ABA. This molecule is known to induce the production of the second-messenger cADPR, which in turn controls release of calcium from internal stores during egress. Due to the importance of calcium in egress and the previously shown involvement of phospholipase C in gametocyte activation, we are currently currently investigating the potential involvement of plasmodial G protein-coupled receptors in perceiving the XA signal.

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02

Antimalarial resistance in Kilifi: an update

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Drug resistance is a disastrous but unavoidable result of evolution of pathogens in the face of drug pressure. We have embarked on a comprehensive long-term study to identify and track early signs of reduced susceptibility of *Plasmodium falciparum* to artemisinins. Here we will present an update on both clinical studies and whole-genome scans designed to provide an extensive set of baseline data.

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03

Deoxyhypusine hydrolase from Plasmodium vivax, the neglected human malaria parasite: molecular cloning, expression and functional activity

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Primaquine, a 4-aminoquinoline, is the only drug which cures the dormant hypnozoites of persistant liver stages from *P. vivax*. Increasing resistance needs the discovery of alternative pathways as drug targets to develop novel drug entities. Deoxyhypusine hydroxylase (DOHH) completes hypusine biosynthesis in eukaryotic initiation factor (eIF-5A) which is the only cellular protein known to contain the unusual amino acid hypusine. Modified EIF-5A is important for proliferation of the malaria parasite. Here, we present the first successful cloning and expression of DOHH from *P. vivax* causing tertiary malaria. The nucleic acid sequence of 1041bp encodes an open reading frame of 346 amino acids. Histidine tagged expression of *P. vivax* DOHH detected a protein of 39.01 kDa in *E. coli*. The DOHH protein from *P. vivax* shares significant amino acid identity to the simian orthologues from *P. knowlesi* and *P. yoelii* strain H. In contrast to *P. falciparum* only four E-Z-type HEAT-like repeats are present in *P. vivax* DOHH with different homology to phycocyanin lyase subunits from cyanobacteria and in proteins participating in energy metabolism of *Archaea* and *Halobacteria*. However, phycocyanin lyase activity is absent. The *dohh* gene is present as a single copy gene and transcribed throughout the whole erythrocytic cycle. Specific inhibition of recombinant *P. vivax* DOHH is possible by complexing the ferrous iron with zileuton, an inhibitor of mammalian 5-lipoxygenase (5-LOX). Ferrous iron in the active site of 5-LOX is coordinated by three conserved histidines and the carboxylate of isoleucine[673]. Zileuton inhibited the *P. vivax* DOHH protein 5.3 fold at a concentration of 100 nmol. By contrast, the human orthologue is only less inhibited suggesting a selective iron-complexing strategy for the parasitic enzyme.

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04

CDK-like kinases of the Plasmodium falciparum blood stages are involved in phosphorylation of splicing factor

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The kinome of the human malaria parasite *Plasmodium falciparum* comprises representatives of most eukaryotic protein kinase groups, including kinases which regulate proliferation and differentiation processes. Despite extensive research on most plasmodial enzymes, little information is available regarding the four identified members of the cyclin¬dependent kinase-like kinases (CLK) family. In other eukaryotes, CLKs regulate mRNA splicing through phosphorylation of Serine/Arginine-rich proteins. Here we investigate the PfCLKs, the Lammer kinase homologue PfCLK-1 (PF3D7_1445400), PfCLK-2 (PF3D7_1443000), PfCLK-3 (PF3D7_1114700) and PfCLK-4/SRPK-1 (PF3D7_0302100). All four PfCLKs show homology with the yeast Serine/Arginine protein kinase Sky1p and are transcribed throughout the asexual blood stages as well as in gametocytes. PfCLK-1/Lammer possesses two nuclear localization signal sites and PfCLK-2 possesses one of these signal sites upstream of the C-terminal catalytic domains. Indirect immunofluorescence and Western Blot Data confirm that the kinases are primarily localized in the parasite nucleus and in the cytoplasm. *In vitro* kinase assays show substrate phosphorylation by the PfCLKs, including the Sky1p substrate, yeast splicing factor Npl3p, and the plasmodial splicing factors PfASF-1 (PF3D7_1119800), PfSR-1 (PF3D7_0503300) and PF3D7_1022400. Inhibitors of human/microbe CLKs inhibit growth of the *P. falciparum* blood stages in the low micromolar range. In addition, preliminary findings show inhibitory effects of these inhibitors on parasite gametogenesis. Our data indicate a crucial role of PfCLKs predominantly for malaria blood stage parasites, presumably by participating in gene regulation through the post-transcriptional modification of mRNA.

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05

Unravelling the role of erythrocyte deformability in Plasmodium falciparum transmission

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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.

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06

Single, white, female – a tale of P. falciparum sex

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Gametocytes, the transmissible forms of the malaria parasites, represent a significant bottleneck in the parasite's life-cycle. The formation of these forms is tightly regulated, however, the exact molecular mechanism is poorly understood. Here we present the characterization of a gametocyte specific transport molecule that plays a role in the genesis of these forms.

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07

Genetic epidemiology of Malagasy Plasmodium falciparum in pregnant women in two different transmission strata

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Malaria epidemiology in Madagascar is classified into four different strata, ranging from unstable seasonal transmission to hyperendemic perennial transmission areas. Most malaria studies in Madagascar are focused on children. However because of the low transmission in some areas with correspondingly low challenge of the immune system, adults and specifically pregnant women are also at risk.

The objective of our study was to gain information on the genetic epidemiology of malaria infections in pregnant women in order to provide information for malaria control and elimination programs in Madagascar.

We collected 1,244 blood samples from healthy pregnant women from April to July 2010 at six locations in two of the four different malaria epidemiology strata: three sites in the equatorial east coast area with perennial transmission of malaria (Mananjary, Manakara and Ifanadiana) and the three others, in the highland with an unstable seasonal transmission of malaria (Tsiroanomandy, Moramanga and Ambositra). Species of Plasmodium were detected using a Real-Time PCR. In *P. falciparum* positive samples (n=233), the prevalence of common antimalarial drug resistance markers as pfcrt K76T, pfmdr1 N86Y, pfdhfr (at codons N51I, C59R, S108N, and I164L) and pfdhps (at codons A437G and

K540E) was determined by PCR-RFLP. Finally, the multiplicity of infection was evaluated by genotyping 6 different neutral microsatellites (TA60, TA81, TA87, ARAII, PolyAlpha, PfPK2).

As expected, the prevalence of *P. falciparum* infections was higher in the coast areas (0.10 to 0.26) than in the highlands (0.08 to 0.14). Eleven samples were infected by non-falciparum species: 9 samples with *P. malariae* (8/9 in the coast), one with *P. ovale* in the coast and one with *P. vivax* in the highlands. We are currently analysing the data from the drug resistance markers and microsatellites genotyping. We will discuss the prevalence of the drug resistance markers regarding the policy of antimalarial drug use established in Madagascar; the multiplicity of infection and the structure of Malagasy *P. falciparum* populations among the 6 different locations of the study.

Our findings will enhance the comprehension of malaria genetic epidemiology in two different transmission strata in Madagascar with a fine description of *P. falciparum* infections among a neglected population at risk.

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08

Human genetic epidemiology of falciparum malaria

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The impact of host genetics on resistance and susceptibility to *Plasmodium falciparum* malaria has been intensively investigated over the past two decades. There is increasing evidence that malaria parasites have shaped the human genome in endemic regions with strong selective pressure. Various genes have been identified which are associated with malaria phenotypes. Factors that promote manifestations of malaria comprise parasitemia level, parasite induced inflammation, sequestration and anemia of infected erythrocytes in organ microvasculature, in particular in the brain. Advances in genomic research technologies such as genome-wide association studies (GWAS) and fine genotyping techniques have enabled the uncovering of a number of genetic polymorphisms that justify further studies in host-parasite interactions.

Human gene variants identified so far will be described and discussed that have been demonstrated to be associated with protection or susceptibility to *falciparum* malaria. Although some polymorphisms have a significant impact for the course of *Plasmodium* infection and malaria, other findings are indecisive or even contradictory and should be considered with caution. The discovery of genetic polymorphisms associated with various diseases phenotypes may help to understand the pathophysiology of malaria and facilitate the development of prevention measures or treatments. Heterogeneity of human populations as well as environmental effects can affect the diversity of clinical malaria, thus warranting further research with the aim of developing and evaluating new interventions, therapies and better management against f*alciparum* malaria.

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09

MMV's drug development pipeline and strategies to move novel compounds into the clinic

J. J. Möhrle

Medicines for Malaria Venture, Geneva, Switzerland

The global antimalarial pipeline has been strengthened in recent years with the delivery of new artemisinin combination therapies, promising new clinical candidates and early stage discovery projects. This talk will focus on the challenges that remain and the strategy adopted by Medicines for Malaria Venture (MMV) in targeting eradication. MMV's focus has shifted from the treatment of blood stage malaria infections and now also includes relapse prevention, blocking of transmission and the protection of vulnerable populations in areas with little or no transmission.

New target product profiles and target candidate profiles defining the specific characteristics of individual molecules for asexual blood stage cures, transmission blocking, *vivax* and chemoprotection will be described.

Finally strategies will be presented how obtain earlier information and confirmation on antimalarial activity in humans.

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10

Post-treatment anaemia in African children with severe malaria after treatment with parenteral artesunate – a prospective observational study

T. Rolling[1,2], D. Spahlinger[1,2], T. Agbenyega[3], S. Issifou[4], S. Bolte[5], P. G. Kremsner[5], G. D. Burchard[1,2], B. Mordmüller[5], J. P. Cramer[1,2]

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Introduction: Parenteral artesunate is now considered to be the first-line treatment for severe malaria because of its superiority when compared to parenteral quinine. However data on medium to long-term side effects of parenteral artesunate are scarce so far. As parenteral artesunate has been implemented as the treatment of choice for imported severe malaria in a growing number of centres in Europe, first reports have revealed cases of post-treatment haemolysis in a significant proportion of patients. Whether this potential side-effect is also clinically relevant in African children is unknown. We therefore designed a prospective observational study to detect any haematological alterations after parenteral artesunate in the endemic setting of Sub-Saharan Africa.

Methods: Children in Lambaréné, Gabon and Kumasi, Ghana between the age of six months and ten years were eligible for inclusion if they presented with Plasmodium falciparum malaria warranting hospitalization. Patients were treated with a total of 12 mg/kg body weight of parenteral artesunate. Clinical parameters and blood samples were obtained at presentation (day 0) and at follow-up visits on days 7 (±2), 14 (±3) and 28 (±4).

Results: A total of 102 patients were recruited. 31% of children experienced a decrease in haemoglobin (Hb) larger than 0.5 g/dl between days 0 and 14, while the mean rise in Hb for all children was 1.2 g/dl. 28 children received a blood transfusion at some point during hospitalization, thereby influencing the haemoglobin levels. 42% of children who did not receive any blood transfusion had a decrease of at least 0.5 g/dl between days 0 and 14, while the mean decrease in this group was 0.1 g/dl. When controlling for baseline Hb, the difference in Hb between day 0 and day 14 negatively correlates with geometric mean parasite density.

Conclusion: A significant proportion of African children treated with artesunate have not recovered from their malaria-induced anaemia two weeks after treatment has been initiated. Hyperparasitaemita seems to be a relevant risk factor for not recovering from anaemia. In how far this effect is due to haemolysis or impaired erythropoiesis has to be determined in our subsequent analyses.

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11

Methylene Blue as a Prodrug of Azure B

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MB-based drug combinations against schizonts and gametocytes of *Plasmodium falciparum* have been tested in clinical trials conducted by Müller, Meissner and Coulibaly in Nouna, Burkina Faso [1], [2], [3]. *In vitro* and even more so *in vivo*, methylene blue is readily demethylated to give trimethylthionine (azure B) [4]. This finding could be due to first-pass metabolization in the liver by N-demethylases and/or to enhanced intercellular exchange of demethylated, neutral quinoneimine-forming products of MB [1]. Recently, interest in MB and its metabolites was reinforced by C. Wischik's group because of their possible protective role in Alzheimer's disease (see [5] for a review).

We had already shown that MB is a ligand of different redox flavoenzymes such as glutathione reductases and *Plasmodium falciparum* lipoamide dehydrogenase [1]. Consequently, we have studied now azure B with the same enzymes. The fact that azure B was a substrate and a better ligand for all tested flavoenzymes was supported by x-ray diffraction analysis of a glutathione reductase/azure B complex, where the phenothiazine was localised to an aromate-binding subsite that is possibly too small for the bulkier MB molecule. If MB is a prodrug and azure B the major active agent, it will be necessary to focus not only on methylene blue but even more so on azure B.

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12

Novel FAS II inhibitors as multistage antimalarials

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In order to cure not only malaria but prevent transmission as well, a drug must target both, the blood-stage and the pre-erythrocytic stages of the parasite. PfENR is a key enzyme of plasmodial type II fatty acid biosynthesis (FAS II). It has been shown to be essential for liver-stage development of *P. berghei* and is therefore qualified as a target for true causal chemoprophylaxis. By virtual screening based on two crystal structures of PfENR, we identified a structurally novel class of FAS inhibitors. Subsequent chemical optimization yielded two compounds, which are effective against both pre-erythrocytic and blood-stage malaria parasites. Two of the most promising derivatives were found to inhibit multiple stages of *Plasmodium*. These compounds inhibit blood-stage parasite growth with IC50s of 1.7 and 3.0 µM and lead to a more prominent developmental attenuation of liver-stages than the standard primaquine. Both compounds display very low cytotoxicity (CC50 HeLa >80 µM).

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13

Hsp70, an important cog in the malaria parasite's protein folding machinery

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Heat shock proteins (Hsps) play a central role as part of the cell's molecular chaperone machinery. Hsp70s are proteins that are characterized by a highly conserved ATPase domain and a peptide binding domain (PBD). Hsp70 is capable of binding hydrophobic patches that are displayed by misfolded proteins. This allows it to stabilize unfolded proteins, facilitating their refolding. *Plasmodium falciparum* encodes 6 Hsp70s which are located in various cellular organelles. This report analyses the specialized roles of these proteins and discusses their central role in protein folding in the malaria parasite. *Plasmodium falciparum* Hsp70-1 (PfHsp70-1), is a stress-inducible cytosol/nuclear localized protein. Evidence for the chaperone role of this protein is presented. Furthermore, insights into its interaction with other molecular chaperones and co-chaperones such as PfHsp90, and Hsp40 co-chaperones will be discussed.

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Detection of P. falciparum and P. vivax gametocytes in field surveys

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Background: Measurements of the prevalence of Plasmodium sp. gametocytes may serve as a tool to monitor the success of antimalarial interventions, in particular in the context of renewed efforts to eliminate malaria. We developed tools for detection and quantification of *P. vivax* and *P. falciparum* gametocytes in field surveys, and evaluated several markers for differentiating individual gametocyte-producing clones of *P. falciparum*.

Methods: In cross-sectional samples from 315 Papua New Guinean children aged 5–10 years, three RNA sampling methods were tested in parallel: whole blood into RNAprotect (Qiagen), whole blood on Whatman 3MM paper stored in TRIzol reagent, and whole blood on Whatman FTA filter cards. Sequential qRT-PCR analyses were performed to gain prevalence data for asexual parasites and for sexual stages. We also assessed the genetic diversity of pfs230 and pfg377 in a set of 80 DNA samples by nested-PCR and subsequent sizing by capillary electrophoresis. We also investigated novel size-polymorphic gametocyte markers, such as PF11.1 (PF10_0374), PF11_0214, PFI1210w, PFL0545w. A gametocyte trendline was used to evaluate the detection limit of the nested-RT-PCR of theses markers.

Results: Sampling and storing finger prick blood in RNAprotect was found to be most sensitive and suitable for high sample throughput. Of 76 *P. falciparum* positive study participants, 31 (40.8%) carried gametocytes. Of 121 *P. vivax* positive samples, 44 (36.4%) were gametocyte positive. For our gametocyte-genotyping markers we observed high genetic diversity with pfs230 (He=96.3) and pfg377 (He=89.4). 17 and 13 different alleles were found for pfs230 and pfg377, respectively. The multiplicity of infection (MOI) of these stage-specific markers was lower than MOI by marker msp2, but showed the highest discriminatory power for gametocytes in literature.

Conclusion: Although prevalence of gametocytes was lower in *P. vivax* than in *P. falciparum* infections, the reservoir of asymptomatic schoolchildren carrying gametocytes was 11.2% (33/295) and 7.5% (22/295) for *P. vivax* and *P. falciparum*, respectively. The contribution of these asymptomatic children to the overall transmission needs to be considered in antimalarial interventions. Gametocyte typing of field samples requires RNA sampling and high gametocyte-specific expression of the genotyping marker. We discuss the application of high-resolution gametocyte genotyping for studies on malaria transmission.

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The role of proteases during the egress of malaria gametocytes from the enveloping erythrocyte

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The transmission of the malaria parasite *Plasmodium falciparum* from the human to the mosquito is mediated by sexual precursor cells, the intraerythrocytic gametocytes which become activated in the mosquito midgut by environmental stimuli and then undergo gametogenesis. Because gametocytes are the only life-cycle stages of the parasite that are able to establish an infection in the mosquito, they play an important role in spreading the tropical disease. Gametocyte egress from the enveloping erythrocyte is a crucial step for the parasite to prepare for fertilization, but the molecular mechanisms are not well understood. We show that *P. falciparum* gametocytes exit the erythrocyte by an inside-out type of egress. The parasitophorous vacuole membrane (PVM) ruptures at multiple sites within less than a minute after activation. Following PVM rupture the inner membrane complex begins to disintegrate, while the gametocyte is in the process of round up. At approximately 15 min post-activation, the erythrocyte membrane ruptures with the formation of a pore. We show that erythrocyte rupture can be inhibited by the cysteine/serine protease inhibitors TLCK and TPCK. Inhibitors directed against the cysteine protease dipeptidyl aminopeptidase PfDPAP3, on the other hand, prevent the rupture of the PVM. PfDAP3 was previously shown to activate the subtilisin protease PfSUB1 during egress. It is now our aim to investigate the function of PfSUB1 in gametocyte egress by the use of loss-of-function mutants, which will be generated via a dominant negative expression approach. Identifying the role of proteases will lead to a better understanding of the molecular mechanisms behind gametocyte egress and may point to new targets for transmission blocking strategies.

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