

## Introduction

The work of the Molecular Parasitology – Epidemiology falls into two main categories: (i) research on the basic molecular biology of parasites and (ii) molecular epidemiological studies using findings from basic research. Within our unit we try to link bench results to observations made in the field. For this reason, many of our studies are conducted at our partner institutions, such as the Ifakara Health Institute (IHI) in Tanzania and the Papua New Guinea Institute of Medical Research (PNG IMR). Our research group is closely interwoven with the Molecular Diagnostics unit at the STI, with shared staff, laboratories, research projects and publications.

The main focus of our research is the malaria parasite *Plasmodium falciparum* with particular emphasis on early events occurring after invasion of the parasite into the host erythrocytes. We are especially interested in the variable antigens (PfEMP1s) and their corresponding genes (*var* genes), which enable the parasite to stick to host cells (known as cytoadherence) and are considered to be the major virulence factors. We are investigating the expression of these genes in naturally infected individuals. Our previous identification of two membrane-associated, histidine-rich proteins (MAHRP1 and MAHRP2) has prompted us to investigate the transport and trafficking of proteins, including PfEMP1, located at the Maurer's clefts. We hope to understand the function of the variable antigens in their capacity to confer cytoadherence. Moreover, using transfection technology and transgenic parasites, we hope to discover how the variable antigens interact with other proteins outside the infected cell as well as within the infected erythrocytes. These efforts should enable us to identify means of blocking transport of these antigens from the parasite to the surface.

Using similar cell biological and molecular techniques, we are also studying new vaccine candidates which have been identified using an innovative bioinformatics approach. This includes localisation studies, episomal tagged protein expression and gene knockout technology.

Having developed an innovative microarray-based tool to analyse drug resistance-associated single-nucleotide polymorphisms (SNPs) to monitor drug resistance dynamics at the molecular level, we applied this tool extensively to samples from epidemiological studies from endemic countries. We have also now further developed this microarray to include SNPs identified in cytochrome (Cyp) and N-acetyltransferase (NAT) genes involved in drug metabolism. We would like to use our findings to test how much the host metabolism contributes to the modulation of drug efficacy.

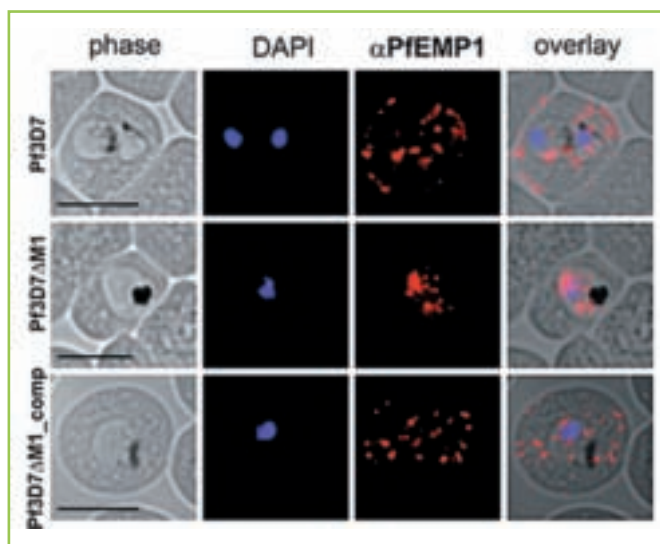
By analysing these aspects of *Plasmodium* molecular biology, and by improving our understanding of its epidemiology at the molecular level, we hope to gain knowledge about the parasite-host interaction that will contribute to fighting this devastating disease. Another aim of

our work is to identify innovative approaches for controlling malaria in endemic areas.

### 1.1 *var* genes and PfEMP1

Most studies of *var* gene expression and switching have been performed on cultured material, and few groups have attempted to study *var* gene expression and cytoadherence *ex vivo* (i.e. directly in blood samples from naturally infected individuals). Because there are significant technical difficulties in undertaking such studies on site in endemic areas, little is known about the hierarchy and programme of *var* gene expression within a parasite isolate. Following the intriguing observation of genetic structuring of the *var* gene repertoire defined by their upstream region, and the development of robust reverse transcriptase-polymerase chain reaction (RT-PCR) techniques, we conducted studies in PNG and Tanzania on *var* gene expression *ex vivo*.

We carried out cross-sectional, longitudinal and case-control studies in PNG, and a case-control study in Tanzania to analyse the expression pattern of *var* genes. From isolated *var* gene messenger RNAs (mRNAs), we performed both real-time quantitative PCR for the various *var* gene subgroups and cloned the complementary DNAs (cDNAs) for sequencing. We found that *var* gene transcription is highly complex when analysed longitudinally, with many *var* gene transcripts detected in one sample at any one time. The occurrence of a malaria episode is accompanied by a major shift in the expression pattern of the *var* gene. In PNG, a major downshift of *var* group C genes and increased expression of *var* group B genes was observed. In Tanzania this pattern was even more pronounced, with additional upregulation of *var* group A genes.



Immunofluorescence images of *P. falciparum*-infected erythrocytes using antibodies against PfEMP1. (Upper panel) 3D7 wild-type parasite. (Middle panel) 3D7 parasite with MAHRP1 knocked down. (Lower panel) Knockout parasites complemented with episomally expressed MAHRP1. Blue dye indicates nuclei.

We generated a large number of *var* gene sequences which are being further analysed in silico to test whether certain sequences or sequence tags cluster within a particular clinical presentation. Through this approach we are now in the position to test the degree of diversity in different epidemiological settings (e.g. Tanzania and PNG) and observe an as yet unlimited repertoire of expressed genes in naturally infected individuals even in PNG, which has a lower endemicity than Tanzania. In addition to sequencing *var* genes expressed in naturally infected individuals, we also studied *var* gene expression in culture to determine switching rates in the absence of selection pressure. For this, and to provide a rapid screening system for the large and diverse group of *var* genes, we developed a GeneMapper-based gene identification system. In the 3D7 clone/strain, we were able to achieve a high discrimination power and to quickly identify a switch from one *var* gene to another.

Because we realised that generating expression data alone might not provide the complete picture of sequestration and cytoadherence, and to create better tools for protein analysis, we have recombinantly expressed several *var* gene domains in *Escherichia coli*. Here we are using two approaches, cloning (i) DBL (Duffy binding-like) fragments of expressed and sequenced *var* genes and (ii) fragments from full-length published *var* sequences that span the entire protein. All protein fragments are then used for sero-epidemiological studies, employing sera from various endemic areas, different age groups and different clinical presentations. Here we can show that domains derived from severe malaria cases are more strongly and more frequently recognised by adult sera than domains deriving from asymptotically infected children.

In a further attempt to understand PfEMP1 interactions with host cells, we are collaborating with the Institute of Biochemistry, ETH Zurich (Dr Hans Lutz), and the Karolinska Institutet, Stockholm (Prof. Mats Wahlgren), to study serum factors which mediate rosetting. We were able to show that this process involves human serum albumin, naturally occurring antibodies against band 3 and complement factor D. We have good evidence that factor D cleaves a large molecule, perhaps PfEMP1, on the surface of infected erythrocytes.

We are also finalising our initial studies on *var* gene promoter activity and transfected 3D7 parasites with constructs carrying various truncated domains of the upsB and upsC upstream region in order to identify minimal promoter elements and potential interaction sequences for *var*-intron-mediated silencing of expression. We now have a suite of transfected deletion clones which also carry the *dhfr* cassette for stable episomal transfection. All clones are full-length or carry fragments of the 2.5-kb upstream region that controls the expression of luciferase. We have pinpointed domains which contain promoter repressor elements and domains which are important for promoter activity. In addition, we have zoomed in on the area which presumably mediates the interaction with the intron.

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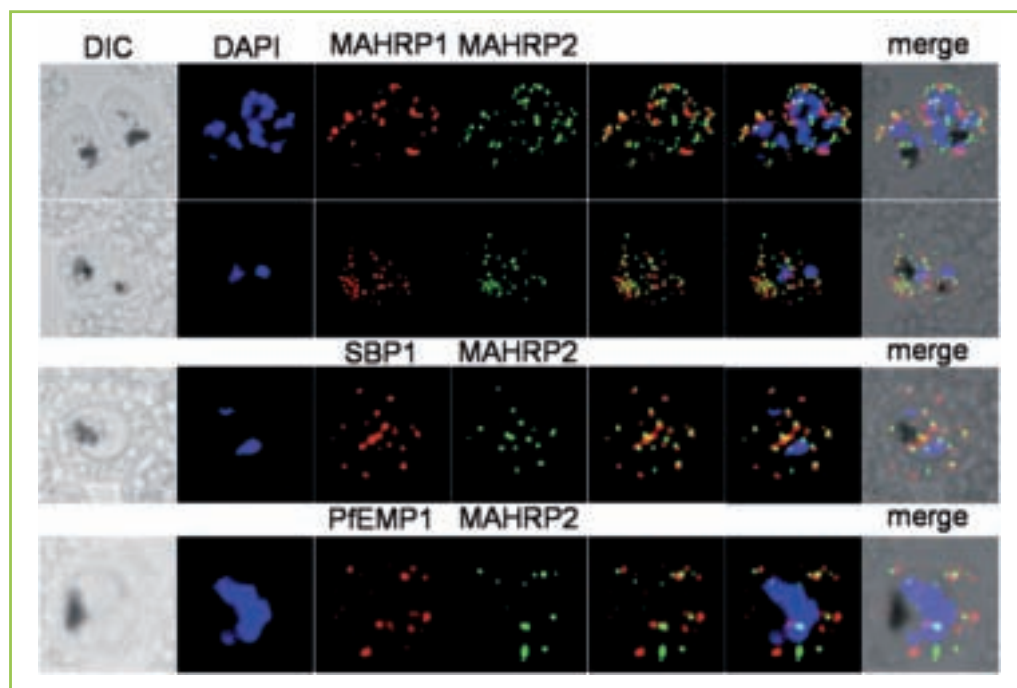
### 1.2 Stage-specifically regulated genes in *P. falciparum*

We have continued to study our previously identified histidine-rich transmembrane proteins, MAHRP1 and MAHRP2. Both genes are transcribed exclusively in early ring-stage parasites. Analysis of the predicted amino acid sequences of both cDNA fragments revealed a similar structure comprising a transmembrane domain and a histidine-rich region. One protein contains histidine-rich repeats at its 3' end, while the other contains a histidine-rich domain at its 5' end. The only polymorphism found in several *P. falciparum* strains was for the number of histidine-rich repeats in MAHRP1.

MAHRP1 and MAHRP2 were shown to locate to Maurer's clefts. We have now generated recombinant transfection constructs comprising either the transmembrane domain or the complete or partial N- and C-terminal domains of MAHRP1 and MAHRP2 fused to green fluorescent protein (GFP) to study the minimal requirements for MAHRP1 and MAHRP2 trafficking by live fluorescence imaging. With these GFP-tagged proteins, we were able to show an accumulation of MAHRP1 at the parasitophorous vacuole, suggesting that Maurer's clefts are formed at the vacuole membrane and might bud off as vesicles. In collaboration with Prof. A. Cowman [Walter and Eliza Hall Institute (WEHI), Melbourne] we have produced an MAHRP1 knockout clone, in which PfEMP1 transport is blocked and the protein is retained within the parasite's confines. This knockout clone does not show any cytoadherence nor is PfEMP1 present on the surface of the infected cell. For MAHRP2 we are also trying to generate knockout clones, and for both MAHRP1 and MAHRP2 we now have triple hemagglutinin-tagged constructs to use in pull-down and affinity chromatography experiments.

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 Funding: SNSF, COST action 587 through the SER (State Secretary for Education and Research)

Immunofluorescence colocalisation of *P. falciparum*-infected erythrocytes using antibodies against MAHRP2 and MAHRP1 (a Maurer's cleft resident protein, upper two rows), against SBP1 (another Maurer's cleft resident protein, middle rows) and against PfEMP1 (a transient Maurer's cleft protein) (bottom row).



### 1.3 *P. falciparum* genome-based antigen discovery and vaccine development

This project focuses on a search for new malaria vaccine candidates targeting the *P. falciparum* erythrocytic stage. Selection of new antigens was performed using a genome-wide approach complemented with high-throughput peptide synthesis. We first identified potential protein antigens present in the asexual malaria blood stages through bioinformatics as well as published transcriptome and proteome analyses. Among the proteins identified, we selected those that contain predicted coiled-coil regions, which are generally short and structurally stable as isolated fragments. To test the feasibility of this approach, we selected 39 segments, 30–40 amino acids long with the highest coiled-coil score, present either in the same protein or in different ones. All selected antigens were recognised to various degrees (5–60%) by a panel of sera from donors living in endemic areas.

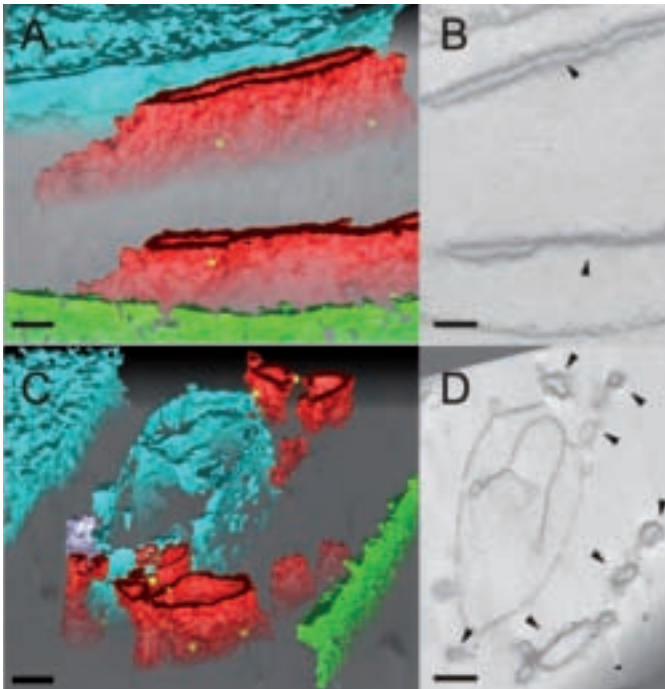
In collaboration with the University of Lausanne and the Pasteur Institute, Paris, we are currently identifying the most promising candidates for further vaccine development by conducting a series of preclinical vaccine evaluation steps. At the STI we are focusing on cytological localisation of the parasite proteins which are represented by these synthetic peptides, and on functional analyses of the respective proteins. The genetic diversity of the selected candidate genes is assessed by sequencing in a small-scale molecular epidemiological study.

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### 1.4 Drug resistance monitoring using a microarray-based SNP analysis system

Previously, we reported the development of a microarray-based analysis system for single-nucleotide polymorphisms (SNPs). This system allows the simultaneous determination of SNPs described in parasite genes associated with drug resistance against antimalarial drugs. We have included in the analysis the *dhfr*, *dhps*, *crt*, *mdr* and *atpase 6* genes. We have now used this microarray system intensively in PNG and in Tanzania. All data have been analysed and compiled for publication. The main objectives of this project were (i) to estimate frequencies of relevant molecular markers in the parasite population circulating in several endemic communities, and to compare them with the incidence risk of treatment failure in the nearby health centres, and (ii) to investigate whether molecular marker (and haplotype) frequencies reproducibly predict the risk of treatment failure in different areas and at different time points. We have analysed data from in vivo and community cross-sectional surveys from PNG and Tanzania. These studies confirm on a molecular level that the efficacy of standard treatment for uncomplicated malaria, i.e. chloroquine + sulphadoxine-pyrimethamine (SP) or amodiaquine + SP in PNG, and SP alone in Tanzania, is compromised. The results support current changes in treatment policy towards artemisinin-containing combination therapies.

To test how fast drug resistance develops, we used historical samples from Tanzania which were collected before, during and after a clinical trial in which SP was also given in an area where it had not been used as an anti-malarial drug. Surprisingly, we found an already high baseline frequency of *dhfr*108 mutations. Even more surprising, however, was the significant increase in drug



Tomographic images of Maurer's clefts in 3D7 wild-type parasites (upper panel) and MAHRP1 knock-down parasites (lower panel). The erythrocyte membrane is artificially coloured in green, the parasitophorous vacuole membrane in blue and the Maurer's clefts in red. Arrows indicate gold labelling of SPB1, a resident Maurer's cleft protein.

resistance-associated *dhfr* and *dhps* mutations after only 6 doses of SP given to 600 children. This very convincing finding suggests that resistance against SP develops rapidly once the drug is employed.

We also conducted a historical study in PNG where we determined drug resistance-associated SNP frequencies over 10 years and compared the result with drug consumption through the health centres as a surrogate marker for antimalarial treatment. We were able to show that molecular markers indeed provide evidence for failing drugs and as such constitute a tool for drug resistance monitoring in sentinel sites.

In the light of a move towards artemisinin-containing combination therapy as a first-line treatment in PNG, we also tested to what degree gene duplication of *pfmdr* has already occurred. Because such an analysis cannot yet be performed by the microarray system, in collaboration with the University of Heidelberg we used real-time PCR. However, no gene duplication was observed in more than 400 samples, confirming that mefloquine has not yet been widely used in the country.

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### 1.5 Fitness costs of antimalarial drug resistance

The emergence of drug resistance is a major problem in malaria control. To be able to mathematically model the transmission and spread of drug resistance, the determining parameters need to be identified and measured. The underlying hypothesis is that mutations associated with drug resistance incur fitness costs to the parasite in the absence of drug pressure. Accordingly, we investigated the distribution of haplotypes based on their drug resistance associated SNPs in different subsets of the host population. In particular, newly acquired haplotypes after radical cure were characterised and compared with haplotypes from persistent infections.

We have genotyped all known mutations associated with antimalarial drug resistance in parasites from PNG children, adults and new infections occurring after radical cure. Twenty-five known SNPs from four *P. falciparum* genes associated with drug resistance were analysed using DNA chip technology.

Haplotypes were found to differ between subsets of the host population. A seven fold mutated haplotype was significantly reduced in adults compared with children and new infections, whereas parasites harbouring fewer mutations were more frequent in adults. We concluded that the reduced frequency of highly mutated parasites in chronic infections in adults is likely a result of fitness costs of drug resistance, which increases with number of mutations and is responsible for reduced survival of mutant parasites.

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### 1.6 Analysis of SNPs in human genes involved in pharmacogenetics and pharmacokinetics

Parasite resistance to antimalarial drugs is indeed only one of many factors that contribute to treatment effectiveness; pre-existing immunity and adherence to treatment are other key players. In addition, human genetics certainly plays a role. Interindividual variability in drug response is partly attributable to differences caused by single-point mutations in drug-metabolising enzymes, particularly in members of the cytochrome P450 superfamily and in transporters. We have further developed our SNP analysis system to estimate the contribution of pharmacogenetics in overall malaria treatment effectiveness. We are currently comparing pharmacogenetic profiles of genes that encode proteins relevant for the metabolism of the main antimalarials currently available in different malaria endemic zones. These comprise several cytochrome P450 genes but also N-acetyltransferase genes. We are currently conducting community-based cross-sectional surveys in PNG, Tanzania and Cambodia to assess human polymorphisms in these genes, as well as population pharmacokinetic studies carried out in

nearby health facilities in Cambodia and Tanzania. In collaboration with the University of Lausanne we have been able to compare pharmacokinetics with the pharmacogenetics characteristic of people in Cambodia and Tanzania. We should therefore be in a position to estimate for the first time the role of pharmacogenetics in the overall efficacy of malaria treatment, taking into account the other major factors identified so far. Ultimately, the work should serve to deliver population-specific data for use in policy-making for malaria in endemic areas.

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Funding: SNSF



Staff of the Molecular Parasitology-Epidemiology and Molecular Diagnostic units. (Photo S. Schlumpf)

## Publications

See section 2, page 18.

## Glossary

3D7/FC27	strain names of <i>P. falciparum</i> and references for <i>msp2</i> allelic families.
ComB	experimental vaccine against <i>P. falciparum</i> malaria containing three nearly full-length or partial recombinant <i>P. falciparum</i> antigens [namely, ring-infected erythrocyte surface antigen (RESA), merozoite surface antigen 1 and 2 (MSP1, MSP2)].

CRT	chloroquine resistance transporter: involved in resistance to chloroquine.
DBL domain	Duffy binding-like domains: domains found within PfEMP1 which show similarities to domains found in the Duffy binding protein of <i>P. vivax</i> .
DHFR	dihydrofolate reductase: metabolic enzyme in the folate pathway; target enzyme for pyrimethamine.
DHPS	dihydropterate synthase: metabolic enzyme in the folate pathway; target enzyme for sulphadoxine.
GFP	green fluorescent protein: often used as a chimeric protein for trafficking and localisation of proteins under live conditions.
IFA	immunofluorescence assay: technique for visualising certain proteins with specific antibodies and fluorochrome-labelled secondary antibodies.
IHRDC	Ifakara Health Research and Development Centre.
kb	kilobase.
MAHRP1/2	membrane-associated histidine-rich proteins 1 and 2 of <i>P. falciparum</i> .
Maurer's clefts	parasite-derived membranous structures found in the cytosol of infected erythrocytes.
MDR	multidrug resistance: glycoprotein involved in resistance to aminoquinolines.
MOI	multiplicity of infection.
MSP2	merozoite surface protein 2; highly polymorphic antigen on <i>P. falciparum</i> merozoites that occurs in two allelic forms (3D7, FC27).
PCR	polymerase chain reaction: specific logarithmic enzymatic amplification of DNA fragments.
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism: DNA amplification with subsequent restriction digest and analysis of resulting fragments.
PfEMP1	<i>P. falciparum</i> erythrocyte membrane protein 1: large parasite-derived protein located in the infected erythrocyte membrane. PfEMP1 shows antigenic variation (see <i>var</i> genes) and is considered to be the major virulence factor of <i>P. falciparum</i> due to its ability to confer adherence of infected erythrocytes to host endothelia.
PNG IMR	Papua New Guinea Institute of Medical Research.
PV, PVM	parasitophorous vacuole, parasitophorous vacuole membrane: <i>P. falciparum</i> resides in the host erythrocyte within a parasite-derived membranous vacuole.
qPCR	quantitative PCR: polymerase chain reaction that incorporates fluorochromes and quantifies by measuring the fluorescence uptake.
Rosetting	Rosetting is an aggregation of uninfected erythrocytes around infected erythrocytes and is thought to contribute significantly to malaria pathology.
RT-PCR	reverse transcriptase-polymerase chain reaction: amplification of reverse-transcribed cDNA (DNA copied from mRNA).
SNP	single-nucleotide polymorphism: exchanges of DNA nucleotides at certain positions.
Stable episomal transfection	technique to introduce plasmids into the parasite nucleus and to maintain the plasmid under selection.
<i>var</i> genes	genes that code for the antigenic variable protein PfEMP1 (see above) of <i>P. falciparum</i> . Each parasite harbours approximately 60 <i>var</i> genes, but expresses only one at a time.