

3. Immunology and Vaccine Development

3.01 New and convergent chemical synthesis of full-length GPI-anchor of *Plasmodium falciparum*

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Since the discovery of glycosylphosphatidylinositol (GPI) molecules as a novel and alternative mode of membrane-anchoring of cell-surface proteins, the biology of these complex glycolipids has remained in focus, and several GPI-anchors and protein-free GPIs have been isolated all across the eukaryotic species. However, the GPIs are expressed in much higher abundance by protozoan parasites (Malaria, Trypanosoma, Leishmania) and are essential virulence-factors that allow these parasites to infect, proliferate and subvert the host immune system. Marked differences in the GPIs from the parasites and human cells have been identified providing valuable new targets for drug and vaccine design. Even among the parasites, various species express GPIs with subtle structural differences that manifest in remarkable and, at times, opposing biological functions. Malarial GPIs, also known as malaria-toxins, contribute to the pathology and mortality due to *P. falciparum* infection and it has been shown in rodent model that they can serve as effective antitoxin vaccine. Malarial GPIs are the most complex class of glycoconjugates as they include lipid, carbohydrate, phosphate and peptide moieties, and their isolation from parasite culture is extremely difficult. Therefore, to address questions related to their biosynthesis, structure-activity relationship and vaccine-design, the chemical synthesis is a viable option to get these remarkable GPIs in sufficient quantity. Despite the concerted efforts of several leading groups, total synthesis of full-length GPIs remains complicated due to (a) structural and functional differences among the species and (b) significant micro-heterogeneity in the lipid and glycan domains. In our ongoing efforts on chemistry and biology of parasitic GPIs, we have designed a new and efficient approach for the synthesis of the full-length GPI-anchor of *P. falciparum*, and have used it for the preparation of novel fluorescent and [4-deoxy-Man-III]-GPI mimics as probes to address specific questions pertaining to the last two steps of GPI-biosynthesis and the topology of the GPIs during their assembly in the ER. This new approach for the synthesis of full-length GPI-anchor of *P. falciparum* will be presented.

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3.02 Innate immune responses in the malaria vector, *Anopheles gambiae*

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Anopheles gambiae mounts active immune responses against *Plasmodium* parasites. Previous results suggested that *Anopheles* responded to different parasite species differently at genetic and molecular levels. Recently genetic mapping showed that different genetic loci were involved in melanotic encapsulation when different monkey parasite species infected *A. gambiae*. Thus, the innate immune system of *A. gambiae* is capable of differentiating closely related pathogens. We are also examining the functions of the Imd-Rel2 pathways in innate immunity in *An. gambiae*. Results showed that Rel2 is required for immunity against both Gram (+) and Gram (-) bacteria, and against *Plasmodium* parasites. We will discuss how the Imd-Rel2 signalling pathway is activated during *Plasmodium* infection in mosquitoes.

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3.03 Both malaria parasite and mosquito vector do not distinguish between friends and foe why should people? Making a case for malaria transmission blocking vaccination

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Mosquitoes can transmit malaria only if they feed on an infected person who is carrying sexual stages (both male and female gametocytes) of *Plasmodium* spp. Asexual to sexual transformation occurring in the infected individual and subsequent sexual development of the parasite in the mosquito vector present numerous antigenic targets in various stages for immune intervention. Antibodies recognizing surface proteins in the sexual stages, if present in the blood meal, can effectively reduce or even completely block sexual development of the parasite in the mosquito. Malaria transmission blocking vaccines under development aim to achieve such a goal, i.e. immunize people so that a mosquito is rendered non-infectious and thus incapable of transmitting malaria to others in the community. Studies have provided a solid proof-of-concept, however, there are numerous scientific and technical challenges that lie ahead, whether it is for recombinant protein based vaccine development or for vaccines based on naked DNA. In the end, just like malaria transmission can not occur without mosquitoes, no transmission will occur without successful development of parasites in the mosquitoes. Transmission blocking vaccines thus have the potential to do real good for public health.

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3.04 Of mice and men: practical approaches to malaria vaccine discovery and development

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The initial screen employed to decide of the vaccine potential of a given malaria protein may well constitute the original sin in a development process which extends for some of the candidates (e.g. MSP1, CS) over several decades, a few dozen million dollars and a substantial number of clinical trials (yet without necessarily leading to a firm conclusion).

Rodent models have played a key role in the initial discovery of several of the current candidates. They are likely to continue to do so for digging in the remaining 99.5 % of the genome data and this more for practical than scientific reasons. Mouse mAbs, i.e. essentially the immune response of Balb/C mice to *P.falciparum* Ags, have been for instance, in large part responsible for the selection in invasion-inhibition (GIA) assays of major candidates such as MSP1, MSP2, AMA-1, etc. That Abs with similar characteristics to those induced in mice or rabbits, can be elicited in humans, has yet to be demonstrated. A similar path was used for so-called major pre-erythrocytic molecules, such as CS. Mice also played a key role in the design of either delivery platforms, e.g. Prime-boost regimens, or Ag combinations, eg. CS-TRAP, which potential, failed to be confirmed by clinical trials.

Parasites are strictly adapted to a given host, ie. die in an abnormal host. Though little studied, this strict adaptation has obviously a molecular basis and hence molecular implications. Through random mutations and recombinations, which are many for *P.falciparum*, proteins have been selected over ages, fitted to adapt best to the usual host i.e. induce little effective immune responses. When introduced in an abnormal host, this exquisite molecular fitness with humans is lost and a given molecule can become more immunogenic, targetted by a larger variety of immune effectors, CS being a demonstrative example. For vaccine development this situation has very significant consequences: it implies that a number of molecules, which can induce Abs with anti-parasite activity in animals, have far less chances to do so in humans.

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Contd.

In this context, we have made the choice to base our research orientations on clinical observations, as they constitute the sole reliable information, and to define a number of guidelines, which have been sometimes difficult to maintain in the past, since they were going frequently against the tide: eg. the importance of "functional immunity assays", i.e. surrogate markers or mechanisms mediating clinical protection, as a main means to provide a rational guide to the identification of molecules with vaccine potential and to their development.

For instance, MSP-3 was identified by an approach where the protection which could be passively transferred by Immune African IgG into naive infected subjects, was used to identify a novel mechanism of defence (not demonstrated in models). The latter, called ADCI, was used to screen a *P. falciparum* genomic expression library and to identify a novel merozoite antigen, MSP-3, as being the target of antibodies with protective effect in human beings. In a similar manner LSA3 was selected by differential display of immune responses from volunteers either protected or not upon challenge by *P.falciparum*, both similarly immunized by irradiated sporozoites.

Further down the line, the design of the various LSA3 and MSP3 vaccine constructs have also been based on studies in humans. For MSP3, this led to identify 4 Th1-cell epitopes and 3 dominant B-cell epitopes targeted by cytophilic antibodies that are both significantly associated with acquired protection and can mediate a strong ADCI effect *in vitro*. When injected into human volunteers with simple adjuvants, low doses of the MSP3 construct readily induced antibodies of cytophilic classes, directed to fully conserved epitopes, that were long lasting and with strong biological activity against *P.falciparum* erythrocytic stages. Thus, results available to-date would support this immuno-clinical strategy for Ag discovery, vaccine design and clinical development.

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3.05 Biology of the Apical Membrane Antigen-1 of *Plasmodium* and strain specificity of immune responses against this important malaria vaccine candidate

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Apical Membrane Antigen-1 (AMA-1) is present on invasive stages of *Plasmodia* and its expression is essential for host cell invasion by apicomplexans. Antibodies to *Plasmodium falciparum* AMA-1 inhibit merozoite invasion and AMA-1 vaccination induces a protective response in animal models. As a step towards testing the efficacy of an AMA-1 based vaccine, we produced GMP grade AMA-1 ectodomain of two *P. falciparum* strains, the 3D7 and FVO. The two proteins differ at 24 amino acid positions within the ectodomain. The 3D7 AMA-1 was tested in a Phase I clinical trial at WRAIR. This vaccine was found to be safe, immunogenic and induced invasion inhibitory antibodies. The role of AMA-1 during merozoite invasion remains a subject of intense investigation. Current hypotheses of AMA-1 function include its suspected role in merozoite reorientation and interaction with a RBC receptor. The RBC binding activity of AMA-1 protein is debatable and its presence on sporozoites is puzzling. One cellular process which incontrovertibly involves AMA-1 and occurs during invasion is a two step-proteolytic processing. A primary pro-sequence cleavage and a secondary ectodomain shedding is accompanied by translocation of AMA-1 from micronemes to the merozoite surface. Antibodies that inhibit invasion also affect AMA-1 processing. Experimental data will be presented supporting our view that processing of AMA-1 is critical for invasion and that biological models of AMA-1 function need to accommodate the observed processing of AMA-1 at specific cleavage sites. A cause of concern to malaria researchers has been the strain-specificity of antibodies against key vaccine targets and AMA-1 is no exception. Strain-specificity of AMA-1 antibodies can potentially neutralize the efficacy of a future malaria vaccine. Current views on the extent of AMA-1 variability and its effect on parasite invasion will be presented. Future strategies to overcome strain-specificity and producing a pan-reactive AMA-1 vaccine will be discussed.

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3.06 *P. falciparum* Enolase

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P. falciparum is the causative agent of most fatal forms of malaria. Asexual blood stages of this parasite which are responsible for clinical symptoms of the disease, are bereft of active mitochondria and hence lack Krebs cycle. In these stages, parasite solely relies on glycolysis for its energy needs. Thus, inhibition of any glycolytic enzyme, can result in control of parasite growth. In this context, it is an attractive proposition to examine the structural and functional properties of glycolytic enzymes from the parasite.

Enolase (2-Phospho-D-glycerate hydrolase; EC 4.2.1.11) is one of the glycolytic enzymes, whose levels are elevated by ~15 fold in infected cells. Further, in several other organisms, it is reported to have diverse (non glycolytic) biological functions. In our lab, we have cloned, over expressed and purified *P. falciparum* enolase (Pfen). We have raised polyclonal and monoclonal antibodies against this protein. Using these antibodies, we have examined the sub-cellular localization of this protein by immuno staining as well as sub-cellular fractionation followed by Western analysis. Results indicate diverse sub-cellular localization of enolase in *Plasmodium*. There appears to be 4-5 post-translationally modified forms of enolase present in the parasite. Sequence comparison of Pfen with other enolases indicate, Pfen to be more homologous to plant enolases as compared to mammalian ones. Deletion of a pentapeptide insert, -EWGWS- (characteristic plant sequence) in Pfen, results in decrease in activity and dissociation of dimer into monomers. Immunization of mice with Pfen was found to confer partial protection against malaria.

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3.07 Cross-stage immunity induced by vaccination with blood parasite under chloroquine treatment

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In this study, we present the first systematic analysis of the immunity induced by normal blood stage parasites in mice against liver and blood stages. Immunization with stage parasites, which was carried out under chloroquine treatment in order to minimize the influence of blood stage parasites, induced a strong protection against a subsequent sporozoite, and to a lesser extent against infected red blood cells challenges. Protection induced by blood stages parasites was not found to be mediated by the antibodies elicited against pre-erythrocytic and blood stage parasites as demonstrated by inhibition assays of sporozoite penetration or development *in vitro*, and *in vivo* assays of sporozoite infectivity or blood stage parasite development. CD4⁺ and CD8⁺ T cells were however, responsible for the protection through the induction of interferon- γ and nitric oxide.

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3.08 Production and immune analysis of Domain I+II of AMA-1 ectodomain from Indian *P. falciparum* isolates

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Plasmodium falciparum apical membrane antigen (AMA-1) is one of the most promising erythrocyte stage malaria vaccine candidates. AMA-1, an 83 kDa precursor localized in the micronemes of the apical complex of merozoite or sporozoite surface, is an integral membrane protein shown to be present in all species of plasmodia. AMA-1 is of particular interest as it has been implicated in critical steps of invasion of human erythrocytes by merozoites and sporozoite. The ectodomain of AMA-1 contains 16 cysteine residues forming 8 intra-molecular disulphide bonds. It has been shown that the disulphide bond stabilized structure is important for providing protection. The structure elucidation of AMA-1 suggested a three domain structure, namely domains 1, 2 and 3, for the ectodomain. Our previous study has indicated that the construct encompassing domains 1 and 2 accounts for generating most of the inhibitory antibodies. Though the overall structure of AMA-1 appears to be conserved as compared to other surface proteins, numerous amino acid substitutions have been identified among different *P. falciparum* isolates. In order to examine the strain specificity of antibodies elicited to AMA-1, we have cloned and expressed two diverse allelic variants of domain I+II of AMA-1 ectodomain from Indian *P. falciparum* isolates in *E. coli*. Analyses have shown that majority of the expressed proteins have aggregated into inclusion body. For the first step purification, Ni-NTA affinity chromatography was used. Refolding of the partially purified product was done using a rapid dilution method and refolded proteins were further purified using an ion-exchange chromatographic method. The purified proteins were analysed by a conformation specific monoclonal antibody and hyper immune sera and further characterized.

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3.09 Production of tumor necrosis factor- α , nitric oxide, hydrogen peroxide and the involvement of immune complexes in the pathogenesis of complicated malaria

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Severe anemia cases produced highest levels of TNF- α (pg/mL) in Western Kenya on admission than their gender and age matched symptomatic controls ($p < 0.05$). Severe anemia cases produced highest concentrations of TNF- α compared with controls on admission ($p < 0.01$). On the second visit, after treatment, there was no significant difference in TNF- α production between severe anemia cases and controls ($p > 0.05$). Similarly, we found higher concentrations of TNF- α in severe anemia than in cerebral malaria cases on first presentation ($p = 0.04$). With respect to NO, we measured higher NO (mM) concentrations on the first visit than on the second visit after one month of treatment ($p < 0.05$). Cerebral malaria patients produced higher levels of NO than severe anemia cases on first presentation ($p < 0.05$). We also found that immune complexes (IC) stimulated human leucocytes *in vitro* to produce high levels of NO in cerebral malaria cases compared with the unstimulated leucocytes (*ex vivo*) of the cases ($p < 0.05$). With regard to Hydrogen peroxide (H_2O_2), severe anemia cases showed depressed H_2O_2 (mM) production compared with symptomatic controls on the first visit ($p < 0.001$). On the second visit, there was no significant difference in the level of H_2O_2 production ($p > 0.05$) between the severe anemia (SA) cases and symptomatic (SC). In conclusion, NO and TNF- α probably in concert play a pivotal role in the pathophysiology of both severe anemia (SA) and cerebral malaria (CM). TNF- α seem to play a more critical role in SA and NO is clearly pivotal in the pathogenesis of CM. Moreover, our findings strongly support the suggestion that leucocytes are one of the most important and probably the prodigious source of the TNF- α , NO, and ROI in severe anemia and cerebral malaria.

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3.10 Polymorphism studies and structural analysis of variation in AMA1 from field isolates in India

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Parasites causing malaria exhibit extensive sequence diversity for a number of stage specific antigens. Apical membrane antigen 1 (AMA 1) is one of the most promising erythrocytic stage vaccine target under investigation. A number of experimental findings suggest that AMA-1 elicits a protective immune response against infections by the parasite in various animal models. Despite the fact that the apical membrane antigen is a very important vaccine candidate and the AMA1 allelic diversity is an effect of immune pressure, the degree of polymorphism of this antigen is little investigated in an endemic country like India. In this study, we have investigated the sequence diversity of the apical membrane antigen of *Plasmodium falciparum* in field isolates from different parts of India. Structural studies of these sequences reveals considerable variation in all the three domains of AMA 1 and in all the reported B and T cell epitopes as compared to reported sequences from other parts of the world. This could be of significance in vaccine design.

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3.11 Pre-erythrocytic immunity in Primates: CD8+ T cells play a prominent role in protecting rhesus monkeys against infection by *Plasmodium knowlesi* sporozoites

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Pre-erythrocytic malaria vaccines are being developed for humans but there is little experimental evidence on which immune responses can kill liver stage malaria parasites. In rodents, CD8+ T cells are important in protection against malaria liver stages. To examine the role of CD8+ T cells in a primate model, we immunized nine rhesus monkeys (*Macaca mulatta*) with irradiated sporozoites from *Plasmodium knowlesi* (Pk). Five of nine immunized animals did not develop blood stage infection after challenge with live Pk sporozoites. Three of the five protected animals were treated with a monoclonal antibody to the CD8 molecule which transiently reduced levels of CD8+ T cells in the peripheral blood to near zero. These three monkeys were challenged second time with Pk sporozoites while their CD8+ T cells were depleted and two of three developed blood stage infection. When CD8+ T cells re-populated these two animals, they regained immunity and were protected against a third Pk sporozoite challenge. We conclude that CD8+ T cells are important mediators of pre-erythrocytic malaria immunity in primates, as they are in rodents, and are likely important for pre-erythrocytic immunity in humans as well.

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3.12 Prediction of promiscuous MHC binding antigenic peptides of some malarial proteins

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In the light of development of resistance to many antimalarial drugs by *Plasmodium* and to pesticides by Anopheles, the development of vaccine for malaria is necessitated. We have predicted promiscuous MHC binding antigenic peptides of some malarial proteins to develop a multiple-subunit vaccine for malaria. Plasma membrane proteins (with sequences) of *Plasmodium falciparum* were selected from the PlasmoDB, a genome database of *Plasmodium* based upon the evidence code of the Gene Ontology cellular component assignment, mass spectrometric data available for the proteins and the stage in which they were expressed. The sequence of Apical membrane antigen 1 of *P.vivax* was obtained from the protein sequence database of NCBI. It was confirmed that these proteins do not have human counterparts by carrying out a tBLASTn search against the human genome database. The proteins thus selected included cytoadherence linked asexual protein of *P. falciparum*, transmission blocking target antigen precursor of *P. falciparum*, circumsporozoite related antigen of *P. falciparum*, apical membrane antigen 1 of *P.falciparum* and apical membrane antigen 1 of *P.vivax*. MHC Class I and Class II binding antigenic peptides of these proteins were predicted using computational tools viz., nHLAPred and ProPred respectively. Ninety one MHC alleles were considered for prediction of promiscuous MHC Class I binding antigenic peptides and fifty one HLA-DR alleles were considered for prediction of MHC Class II binding peptides. A total of twenty seven peptides were predicted which cover all these alleles and target all the stages of life cycle of *Plasmodium*. These peptides could be used to develop an ideal multiple-subunit vaccine for malaria.

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3.13 Purified parasite antigens of *Plasmodium berghei* protect against severe rodent malaria

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Even after more than a century of fight to eradicate or control it, malaria still remains one of the major killer diseases affecting nearly 50% of the world's population. Field and experimental observations have shown that protection against blood-stage infection is largely mediated by antibodies as immunity can be passively transferred from immune to nonimmune individuals. Antisera from mice immunized with cytosolic fraction of *Plasmodium berghei* were used to purify 35% ammonium sulphate precipitated parasite proteins by immuno-affinity adsorption. Proteins of molecular weights 38KDa, 54KDa, 59KDa and 66KDa were isolated from asexual blood-stages of *P. berghei*. These proteins when used for immunization of mice on a regular schedule showed antibody titre of 1:2048 and on subsequent challenge with 1×10^5 *P. berghei*-infected erythrocytes showed protection against severe rodent malaria. Of the 4 mice challenged, the maximum parasitaemia in protected mice were 2.16% and 8.2%, which subsequently decreased and the mice finally recovered from infection.

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3.14 *Plasmodium falciparum* merozoite surface protein 1₁₉ (MSP-1₁₉) - humoral responses to allele specific variants from India

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Malaria parasites exhibit sequence diversity for a number of stage specific antigens. The Merozoite Surface Protein-1 (MSP-1) is a 195-kDa protein present on the surface of the merozoite. Several studies have proved that Merozoite Surface Protein-1 (MSP-1) is an effective target eliciting a protective immune response. The MSP-1₄₂ region comprising of two EGF like domains is involved in generating protective immune response in humans and other experimental animals.

We have evaluated the antibody profile in naturally infected population from different regions of India. Peptides delineating linear epitopes from four different reported MSP-1₁₉ alleles were used for this study. Of these, E-KYG-F is a novel variant being reported for the first time. All the peptides responded to the circulating antibodies with varying intensities. These peptides were further characterized by immunizing mice and challenging with *P.berghei*. Our initial findings suggest there is a cross reactivity among different alleles in humans and mice. The peptide from EGF 1 appears to be immunodominant. Mice immunized with different allelic peptides, when challenged with *P.berghei* showed delayed and low level parasitemia. Also, suppressed and persistent low level parasitemia was significant, when mice were passively immunized with pooled hyper immune sera. This data could be of use in designing effective MSP-1₁₉ peptide based vaccine against malaria.

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3.15 A simple and sensitive ELISA method for determining malaria endemicity

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Usually malaria incidence measured by microscopy, which is a tedious method and largely used by malaria programmer in India. At present different immunological and chromatographic kits are available. But they are costly in comparison to microscopy. When the sample size reaches more than lakhs, the test based on the recent techniques are quite expensive. Moreover this method only indicates point prevalence. It can not provide the past experiences of malaria in the community. We are estimating incidence of malaria transmission dynamics. It involves a peptide ELISA. The peptide used, is a synthetic nona peptide which can be synthesized by solid phase, formula is EENVEHDACY, a cystiene molecule has been attached at the carboxy end to enhance the binding efficiency in the ELISA system. We have compared different peptides such as from liver stage, merozoite, sporozoite, gametocyte and ring stages. Among all these, AR1 peptide which is a repeat unit of octapeptides in RESA antigen, found to be the best. It can stratify various malariogenic areas. Longitudinal study in a malaria endemic village showed antibody present in infected adult stays for 10-12 months. In children peak antibody reaches after 2-3 months of infection. Antibody remains for 10-12 months and then gradually declines to a base line. This peptide is excellent for estimating malaria transmission of the previous year of sample collection. Our hypothesis: AR1 ELISA OD can stratify malaria incidences, such as < 0.3 OD presents low, 0.4-0.7 OD indicates moderate and >0.7 OD indicates high endemicity. We have also developed a formula which can supplement Annual Parasite Index (API) which is estimate of malaria experienced in a community throughout the year, at a unit of one thousand population per year. A formula Equivalent Transmission Index (ETI) has been derived from API and AR1 ELISA OD Values. ELISA based ETI is a simpler and cheaper alternative of establishing prevalence, studying transmission and evaluating efficacy of control measures. Reliability of the method was examined under different malariogenic situations.

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3.16 From field to lab: Blocking malaria transmission by targeting *Anopheles* molecules

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Diverse approaches are currently developed to try and limit malaria transmission mainly by controlling vector populations and implementing the systematic use of bed nets to reduce contact between mosquitoes and the human. An efficient transmission blocking vaccine could also be beneficial for reducing malaria transmission. Such an approach has been developed using *Plasmodium falciparum* ookinete surface protein Pfs25, as a vaccine component. Using field-infected *An. gambiae*, we explore whether *Anopheles* molecules expressed in the mosquito midgut could also constitute a target for blocking the transmission of *P. falciparum*. Our analysis led to the identification of *Anopheles* carboxypeptidases as candidate molecules for a transmission-blocking vaccine. Interestingly, antibody-blockade of carboxypeptidase's activity in the mosquito midgut also reduces significantly the fecundity of *Anopheles* females and therefore should contribute more efficiently at reducing malaria transmission by acting on both parasite development and mosquito population size.



The military hospital in Constantine, Algeria, where Laveran discovered the malaria parasite in 1880