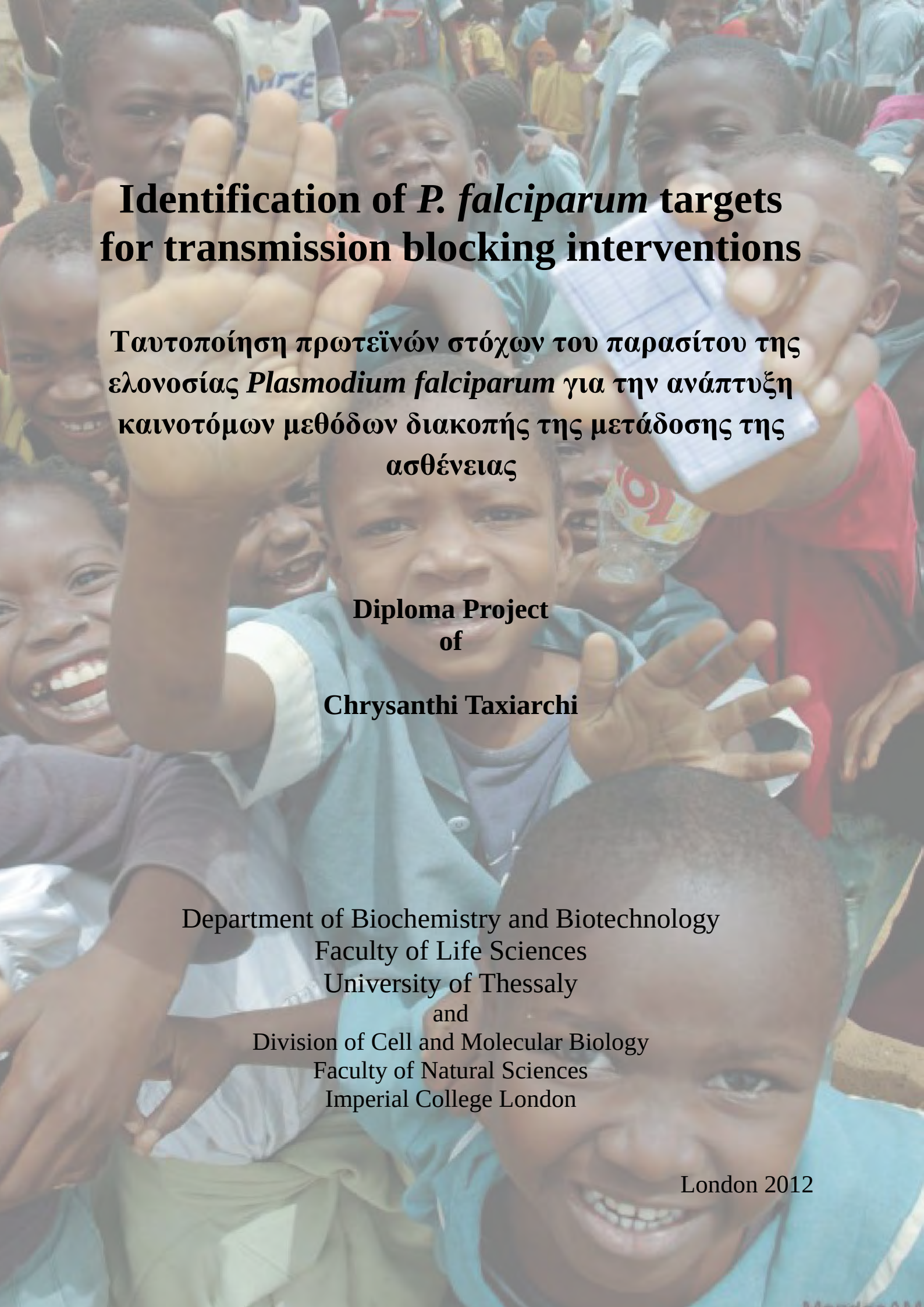

Identification of *P. falciparum* targets for transmission blocking interventions

Ταυτοποίηση πρωτεϊνών στόχων του παρασίτου της ελονοσίας *Plasmodium falciparum* για την ανάπτυξη καινοτόμων μεθόδων διακοπής της μετάδοσης της ασθένειας

Diploma Project
of

Chrysanthi Taxiarchi

Department of Biochemistry and Biotechnology
Faculty of Life Sciences
University of Thessaly
and
Division of Cell and Molecular Biology
Faculty of Natural Sciences
Imperial College London

London 2012

Three-member Board	Signature
Vlachou Dina - PhD Principal Investigator, Senior Research Fellow Division of Cell and Molecular Biology Department of Life Sciences Imperial College London	
Mathiopoulos Konstantinos – Associate Professor, Molecular Biology Department of Biochemistry and Biotechnology, University of Thessaly	
Mossialos Dimitrios - Assistant Professor, Microbial Biotechnology Department of Biochemistry and Biotechnology, University of Thessaly	

ABSTRACT

Plasmodium parasite, the causative agent of one of the deadliest diseases worldwide, malaria, must complete a successful developmental migration through *Anopheles* mosquitoes before transmission to human hosts. The current thesis demonstrates the identification and characterisation of four novel *P. falciparum* genes, *Pf_c01*, *Pf_c53*, *Pf_c02* and *Pf_c57*, which have crucial roles in the sporogonic and sexual development of *Plasmodium*. The identified genes are differentially regulated during the developmental stages of midgut invasion and ookinete-to-oocyst transition, which represent a critical bottleneck in *Plasmodium*'s lifecycle. Detailed *in vivo* and *in vitro* phenotypic analysis, following disruption of each of the four genes, revealed a severe reduction of the mosquito infection intensities. Interestingly, gene deletion does not affect the sexual development from gametogenesis and fertilization until ookinete (the motile invasive zygote) formation. *Pf_c02* and *Pf_c57* products are putative membrane proteins and are likely to interact with mosquito midgut molecules, facilitating parasite's traverse through the mosquito midgut. This study identified four novel key regulators of the parasite's sexual and sporogonic development contributing in the elucidation of fundamental questions about the parasite's development inside its mosquito vector and concurrently providing two potential targets for transmission blocking interventions.

ΠΕΡΙΛΗΨΗ

Το παράσιτο Πλασμώδιο (*Plasmodium*) αποτελεί τον αιτιολογικό παράγοντα μιας εκ των πιο θανατηφόρων νόσων παγκοσμίως, της ελονοσίας. Το Πλασμώδιο αναπαράγεται και πολλαπλασιάζεται υποχρεωτικά μέσα στον φορέα του, Ανωφελές (Anopheles) θηλυκό κουνούπι, πριν τη μετάδοση στον ανθρώπινο ξενιστή. Η παρούσα διπλωματική εργασία παρουσιάζει την ταυτοποίηση και το χαρακτηρισμό τεσσάρων νέων γονιδίων του *P. falciparum*, τα *Pf_c01*, *Pf_c53*, *Pf_c02* και *Pf_c57*, τα οποία έχουν καίριο ρόλο στη σπορογονική και σεξουαλική ανάπτυξη του Πλασμωδίου. Τα συγκεκριμένα γονίδια παρουσιάζουν διαφορική ρύθμιση της έκφρασής τους κατά τα αναπτυξιακά στάδια της διαπέρασης του μεσεντέρου και της μετατροπής του ωοκινέτη σε ωοκύστη, στάδια που αποτελούν κρίσιμο στενωπό στον κύκλο ζωής του Πλασμωδίου. Λεπτομερής *in vivo* και *in vitro* φαινοτυπική ανάλυση, που ακολούθησε τη στοχευμένη απαλοιφή καθενός εκ των τεσσάρων γονιδίων, αποκάλυψε μια σφοδρή μείωση στην ένταση των μολύνσεων των κουνουπιών. Είναι ιδιαίτερα ενδιαφέρον ότι η γονιδιακή απαλοιφή δεν επηρεάζει τη σεξουαλική ανάπτυξη από το στάδιο της γαμετογένεσης και της γονιμοποίησης μέχρι το στάδιο του σχηματισμού του ωοκινέτη (η κινητή και διεισδυτική μορφή ζυγωτού). Τα προϊόντα των γονιδίων *Pf_c02* και *Pf_c57* αποτελούν πιθανές μεμβρανικές πρωτεΐνες και ενδεχομένως αλληλεπιδρούν με μόρια του κουνουπιού, διευκολύνοντας τη διάσχιση του μεσεντέρου του κουνουπιού από το παράσιτο. Στη συγκεκριμένη μελέτη ταυτοποιήθηκαν τέσσερις ρυθμιστές «κλειδιά» της σεξουαλικής και σπορογονικής ανάπτυξης του παρασίτου, συμβάλλοντας στη διαλεύκανση θεμελιωδών ερωτημάτων σχετικά με την ανάπτυξη του παρασίτου μέσα στο κουνούπι-φορέα και ταυτόχρονα παρέχοντας δύο πιθανούς στόχους για την ανάπτυξη καινοτόμων μεθόδων διακοπής της μετάδοσης της ασθένειας.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Malaria: a contemporary scourge dating back to antiquity

1.1.1 Historical overview of malaria

For more than four millenniums, malaria or a disease that had the same characteristics with malaria has had a strong influence on humanity. References to what was almost certainly malaria occur date back to 2700 BC in China, when Nei Ching documented malaria symptoms in his medical writings: The Canon of Medicine. In the 4th century BCE, one of the most outstanding figures in the history of medicine, Hippocrates, based on studies in Egypt, was first to make connection between nearness of stagnant bodies of water and occurrence of fevers in local population, noting the principle symptoms of malaria.

The discovery of the causative agents of malaria was made in 1880 by Charles Louis Alphonse Laveran, a French physician, after microscopic detection of parasites a blood smear, taken from a patient who had just died of malaria, at a military hospital in Algeria (Bruce-Chwatt, 1987). Although Laveran did not achieve to determine the introduction of these protozoans into the human body, he was awarded with Nobel Prize for Physiology or Medicine in 1907, for the first discoveries of protozoan diseases and is credited with describing the etiologic agent of malaria.

Since antiquity, it was observed that malarial symptoms occurred frequently in close proximity to stagnant water and marshes, leading the Romans to pioneer efforts to drain swamps, in order to restrict the spread of what was called “Roman fever”, and the Italians to name this disease “mal’ aria”, which means “bad air” in Latin (Sallares R.,2002). Despite this significant datum, the mode of transmission of the disease was not determined until 1897, when Ronald Ross, a British military doctor, noticed the malarial parasite in the gastrointestinal tract of mosquitoes, while working in India. This observation led Ross to demonstrate that the parasite transmission between its vertebrate hosts is facilitated by mosquitoes. For this breakthrough discovery, he received the Nobel Prize for Physiology or Medicine in 1902.

The first demonstration of the transmission cycle of human malaria parasite in the mosquito was introduced by the Italian malariologists, Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, one year after the Ross’ discovery, in 1898. (http://www.cdc.gov/malaria/about/history/)

Human malaria is nowadays known that is caused by protozoan parasites belonging to the phylum *Apicomplexa* in the class of *Sporozoa*, order *Coccidiida*, suborder *Haemosporidiidae*, family *Plasmodiidae* and genus *Plasmodium* which infect two different species, *Anopheles* mosquito vectors and human hosts, at different stages of the parasite life cycle. Transmission of *Plasmodium* is mediated by the female *Anopheles* vector feeding on the blood from an uninfected human after having previously blood-fed on a malaria human carrier.

1.1.2 Diagnosis and symptoms

Figure 1.1. Blood sample collection for microscopic diagnosis

Diagnosis of malaria is comprised of the identification of malaria parasites or metabolic products in the blood of the patient. The mainstay method for diagnosis is the microscopic examination of thick and thin Giemsa stained blood smears for possible parasite presence. Thin films are preferred when species determination is required because the parasite morphology is well maintained using this method, but thick films allow the detection of lower parasitaemia. Although the usage of microscopy diagnosis is widespread, deficiency in microscopic facilities and experienced microscopists is usual in malaria endemic regions (Bell et al., 2006). Also, antigen-detecting Rapid Diagnostic Tests (RDT) are commercially available. RDTs are accurate in detecting malaria parasites but their threshold of detection is higher than that of traditional microscopy (Wilson, 2012). Although they do not require laboratory setting and staff, they are cost-effective, and, thus, they are not yet widespread applied in endemic regions. This lack of reliable diagnostic tools and specialised staff in the endemic areas usually leads to late and inaccurate diagnosis based on the non-specific symptoms of the disease (Bell et al., 2006).

The clinical manifestation of malaria varies, depending on a combination of parasite, host and socio geographical factors. The strain of the parasite, the age, pregnancy, genetic background, previous exposure etc. of the patient can affect the total clinical outcome and the host immune response (Miller et al., 2002; Greenwood et al., 1991). This is the reason why malaria develops into non-specific symptoms. Typically, the incubation period following infection lasts, approximately, 8-12 days for *falciparum* malaria but can be longer for other types of infections (Fairhust et al., 2010). The primary symptoms, shared by all types of malaria, are called “malaria argue” and overlap with the signs of flu (Bartoloni and Zammarchi, 2012). These symptoms occur cyclically every 2-3 days and are three stages of fever (paroxysm), beginning with shivering, then sudden rise of the temperature and, finally plenteous sweating usually accompanied fatigue, convulsions, emesis, cephalalgia, arthralgia, haemolytic anemia, haemoglobinuria and retinopathy (Beare et al., 2006). Depending on the associating symptoms, the progress of the disease and the type of infection, three types of malaria manifestations can be distinguished: severe malaria which progresses rapidly and usually leads to cerebral malaria or/and respiratory distress and, finally to death within days if not treated (mostly caused by *P. falciparum*); chronic malaria which presents clinical relapses months after the infection, arisen from dormant liver parasites (present in *P. vivax* and *P. ovale*); malaria during pregnancy which affects severely both mother and fetus (mostly caused by *P. falciparum*) (Breman et al., 2004).

1.1.3 Impact of the disease on Global health

Although malaria is an ancient disease, and, nowadays is preventable and treatable, it still remains a worldwide deadly scourge due to environmental disturbance, malnutrition and the failure of drugs previously used to combat the disease (http://www.malaria.org/lifehealth.html).

The latest World Malaria Report compiled by the World Health Organisation (WHO), includes 104 countries and territories in which malaria is classified as an endemic disease, 99 of which present ongoing malaria transmission and 5 are in prevention of reintroduction phase. In these countries, 3.3 billion people are estimated to have been at risk of malaria infection in 2011, while the highest risk of transmission is localised in sub-Saharan Africa (Figure 1.2). Although

there is a reduction in the numbers estimated in 2010, still, 26 million malaria cases leading to approximately a hundred thousand deaths, worldwide, were reported in 2011, maintaining malaria as one of the world's leading infectious killers.

The two most severely affected groups are young children and pregnant women. More specifically, the percentage of deaths caused by malaria in children under the age of five is estimated in 85% of the annual report, which corresponds to 20% of all childhood deaths in sub-Saharan Africa (Aregawi et al., 2008). Foetal and infant mortality, also, increases, due to malaria infection during pregnancy. Even when survival is succeeded, malaria can cause several health problems such as acute anaemia in women and children, low birth weight in infants and chronic neurological and physical disability.

Apart from the fact that malaria impacts individuals, it has, also, a huge burden in communities and the economy. At present, the overwhelming majority of the countries that face high rates of malaria endemism also face poverty. Malaria is judged as a disease of poverty, being a cause and a consequence of poverty at the same time (Gollin and Zimmermann, 2007). Malaria afflicting countries present 1.3% reduction in per capita income (Gallup and Sachs 2001) while they cannot afford the cost of preventing and controlling malaria and the education needed in order to succeed this aim (Chima,Goodman and Mills, 2003), resulting in a vicious cycle linking the disease to poverty and poverty to the disease.

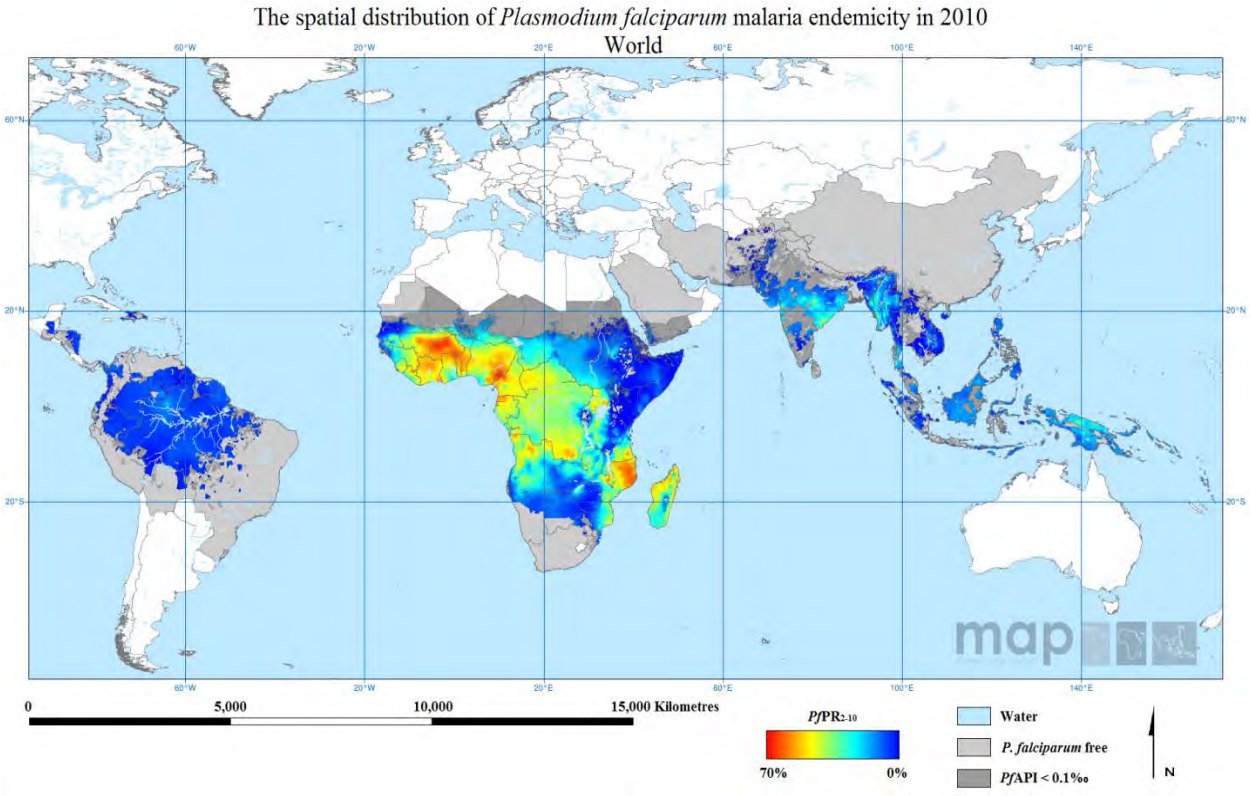

Figure 1.2. The spatial distribution of Plasmodium falciparum malaria endemicity in 2010. Model-based geostatistics (MBG) point estimates of the age-standardised annual mean *P. falciparum* parasite rate in 2-10 year olds ($PfPR_{2-10}$), for 2010, within the spatial limits of stable *P. falciparum* malaria transmission. Areas of no risk and unstable risk (*Pf*API < 0.1 ‰) are also shown in light and dark grey, respectively. Modified from (Gething, Patil and Smith et al., 2011)

1.2 Malaria cause and lifecycle

1.2.1 The etiologic agent of the disease: Plasmodium parasite

Plasmodium is a genus of Apicomplexan parasites, initially described by Ettore Marchiafava and Angelo Celli, in 1885. Up to date, over 200 species of *Plasmodium* genus are recognized while new species continue to be described (Perkins and Austin, 2008). Five *Plsamodium* species have long been recognised to naturally cause malaria in humans: *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax* and *P. knowlesi* (Mueller et al., 2007; Collins, 2012). Among these, the most widespread and deadliest is *P.falciparum* with a proportion of 75% of the total infections, worldwide (Nadjm and Behrens, 2012) and the predominant *Plasmodium* species in sub-Saharan Africa. Outside Africa, the species responsible for the majority of malaria cases is *P. vivax*. Generally, there are hundreds of types of malaria affecting non-human vertebrates, very few of which have been reported to affect humans as well, but with no significant public health imprint (*P. knowlesi* infections excluded) (Collins, 2012).

Plasmodia are unicellular obligate eukaryotic organisms which may display some structural differences during their similar lifecycle, between the different species. Characteristic organelles and structures, such as the apical prominence, rhoptries, polar rings and micronemes, apicoplasts etc. are present in all *Plasmodium* species as they are essential for all *Apicomplexa* during specific stages in their lifecycle (Figure 1.3).

The genomes of five *Plasmodium* species (*P. falciparum*, *P. knowlesi*, *P.vivax*, *P. berghei* and *P. yoelii*) have been sequenced, and others are currently underway, revealing a consistent distribution of the, approximately, 25 megabases genome across 14 chromosomes, the length of which varied between 500 kilobases and 3.5 megabases. This has opened the gate to promising and effective studies including comparative and functional genomics, at the level of transcriptome and proteome. Also, due to the high risk of working with the human pathogen, P. falciparum, and the lack of a non-primate animal model compatible with *P. falciparum* (Herrera et al., 2002), research has focused on the study of rodent model parasites, the most extensively characterised of which is *P. berghei*. *P. berghei*, the life-cycle progression of which presents high similarity with *P. falciparum*, constitutes an important model parasite for vaccine, pathogenesis, drug, transmission studies and studies on the parasite biology.

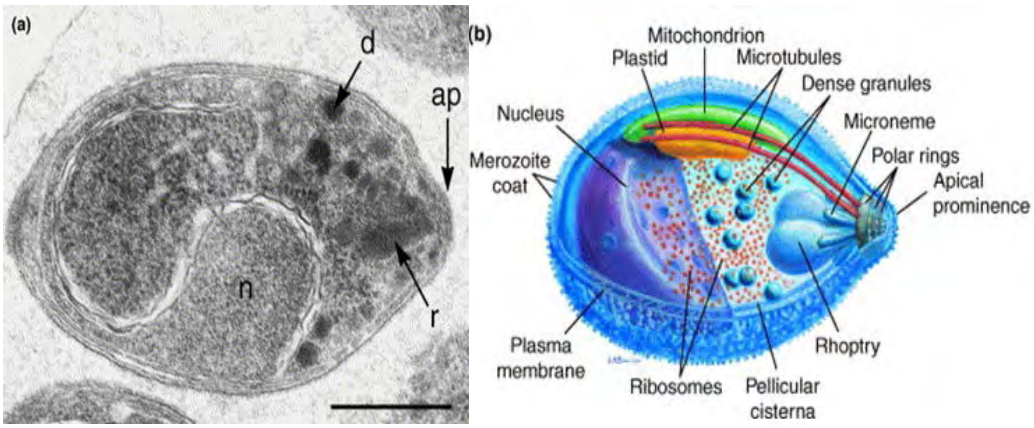

Figure 1.3. Characteristic morphology and distinct organelles of *P. falciparum* (merozoite stage).
a) Electron photograph (n=nucleus, ap=apical prominence, d=dense granule, r=rhoptry
b) Schematic representation
Adapted from (Kats et al., 2006)

1.2.2 The transmission vector: Anopheles mosquitoes

As malaria is naturally transmitted to humans by female mosquitoes of the genus *Anopheles*, the study of the *Anopheles* vectors, along with the study focusing on the parasite, is essential.

Anopheles is a genus of mosquito initially described and named by J. W. Meigen in 1818. Currently, approximately 460 species of mosquitoes belonging to the genus *Anopheles* are recognised, 30 to 40 of which commonly transmit effectively the five species of *Plasmodium* parasites that cause human malaria, depending on the environment of the endemic region. Among these, *Anopheles gambiae* with its sibling, *A. arabiensis*, wins the distinction for predominantly transmitting the deadliest parasite *P. falciparum*. Anophelines are found worldwide except Antarctica and, specifically, malaria transmitting species are also found in areas where malaria has been eliminated, raising the risk of reintroduction of the disease. (http://www.cdc.gov/malaria/about/biology/mosquitoes/).

Some species of *Anopheles* also can serve as vectors for the transmission of other diseases, apart from malaria and, also, the susceptibility of malaria infection varies between the different species of Anopheles (even within the same species). These facts are currently under investigation, as they could generate promising perspectives for eliminating malaria.

- *Life stages (Figure 1.4)*

Like all mosquitoes, Anophelines require a transition through four stages of development in their lifecycle: egg, larva, pupa, and imago (when it attains adultness). The pre-adult stages are aquatic and, depending on the species and the environmental temperature, they can last approximately 5 to 14 days. During the adult stage, only the female *Anopheles* mosquito is able to act as malaria vector. The maximum lifespan of adult females is a month (or more in captivity) but, natural conditions, usually, do not allow them to live more than 1-2 weeks.

Eggs: Adult females lay 50–200 eggs (dimensions about 0.5 × 0.2 nm) per opposition. Eggs are laid singly and directly on water where they are able to float on either side. They display no resistance to drying and hatch within 2–3 days (depending on the ambient temperature).

Larvae: *Anopheles* larvae prefer unpolluted water for their development, although they have the ability to grow in a broad range of habitats. They present a well-developed head, including mouth brushes useful for their feeding, a nine-segment abdomen and a broad thorax. *Anopheles* larvae position their bodies in parallel with the surface of the water due to their lack of respiratory siphon and the presence of spiracles located on the eighth abdominal segment. They feed on bacteria, algae, and other microorganisms and organic matter present in the surface microlayer. Larvae development undergoes four instars, before they metamorphose into pupae. At the end of each instar, when molting takes place, the larvae shed their exoskeletons, to allow for further growth.

Pupae: Pupa stage is a resting and non- feeding stage with duration of 2-3 days in temperate environment. Viewing form the side discloses a comma shape of pupae. The head and thorax are merged into a cephalothorax with the abdomen curving around underneath. Pupae's respiratory system consists of trumpets located on the cepahalothoraces which force them to come to the surface to breath. During the metamorphosis to adult, the dorsal surface of the cephalothorax ruptures and the adult mosquito emerges.

Adults: Like all mosquitoes, adult Anophelins' bodies comprise of three sections: head, thorax and abdomen. The head's exclusive functions are acquiring sensory information (eyes, antennae, maxillary pulps) and feeding (proboscis). The thorax is dedicated to locomotion, with three pairs of legs and a pair of wings assisting and the abdomen is specialized for food digestion and egg development (in females).

Anopheles mosquitoes can be distinguished from other mosquitoes by the palps, which are as long as the proboscis, and by the presence of discrete blocks of black and white scales on the wings. Adults can also be identified by their typical resting position: males and females rest with their abdomens sticking up in the air rather than parallel to the surface on which they are resting.

Male and female adults feed on nectar and other sources of glucose but only females require a blood meal following mating, which, after gradual digestion, will serve as a source of nutrients for the developing eggs. To which extend female of different *Anopheles* species prefer human blood (anthropophily) for their eggs production determines the efficiency of these species as human malaria vectors, making *A. gambiae* the dominant malaria vector in Africa.

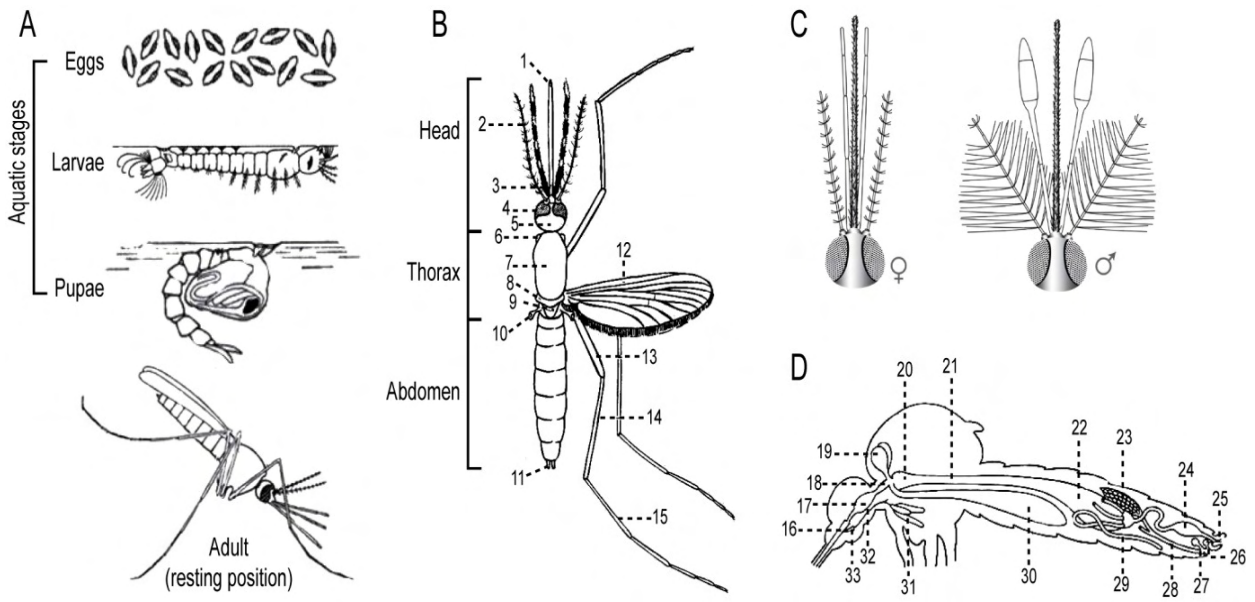

Figure 1.4 Schematic representation of *Anopheles* life cycle and anatomy
A. The four developmental stages of *Anopheles* mosquitoes.
B. External anatomy of a female *Anopheles* mosquito. (1. proboscis; 2. antenna; 3. Maxillary palpus; 4. eye; 5. occiput; 6. Anterior pronotal lobe; 7. scutum; 8. scutellum; 9. postnotum; 10. halter; 11. cercus; 12. wing; 13. femur; 14. tibia; 15. tarsus)
C. Structure of a male and female *Anopheles* mosquito head
D. Internal anatomy of a female Anopheles mosquito. (1. probocis; 2. Antenna; 3. Maxillary palpus; 4. Eye; 5. Occiput; 6. Anterior pronotal lobe;7. Mesonotum; 8. Scuttellum; 9. Postnotum; 10. Haltere; 11. Cerci; 12. Wing; 13. Femur; 14. Tibia; 15. Tarsus; 16. Pharynx; 17. Pharyngeal pump; 18. Oesophagus; 19. Dorsal diverticula; 20. Proventriculus; 21. Midgut; 22. Stomach; 23. Ovary; 24. Rectum; 25. Cerci; 26. atrium; 27. Spermatheca; 28. Oviduct; 29. malpighian tubules; 30. Ventral diverticulum; 31. Salivary glands; 32. Salivary duct; 33. Salivary pump.). Adapted from (Gilles et al. 1993)

1.2.3 Overview of the Plasmodium lifecycle

In short, *Plasmodium* parasite utilises a binary host system during its multi-stage lifecycle, consisting of a series of sequential intracellular proliferation and extracellular invasion stages (Figure 1.5). During its lifecycle, the parasite infects a vertebrate host and a mosquito vector, passing through two phases in the vertebrate: the pre-erythrocytic cycle and the erythrocytic cycle, and one in the mosquito: the sporogonic cycle.

When an infected female *Anopheles* mosquito feeds on a non-infected human, together with the saliva, *Plasmodium* parasites in the infectious form of sporozoites are injected into the human bloodstream and multiply within the hepatocytes as merozoites. Subsequently, the released merozoites invade the erythrocytes where they proliferate again resulting in more merozoites, capable of reinvasion of the red blood cells (asexual blood stages).

Some of the blood stage parasites develop into male and female gametocytes, the parasite forms that are infectious to mosquitoes. Consequently, on the next blood meal of a non-infected *Anopheles* mosquito, these gametocytes are transferred into the mosquito midgut, where they undergo a sexual stage of reproduction, concluding in sporozoites inside the salivary glands. These sporozoites are able to infect another human host during the next blood meal of the mosquito.

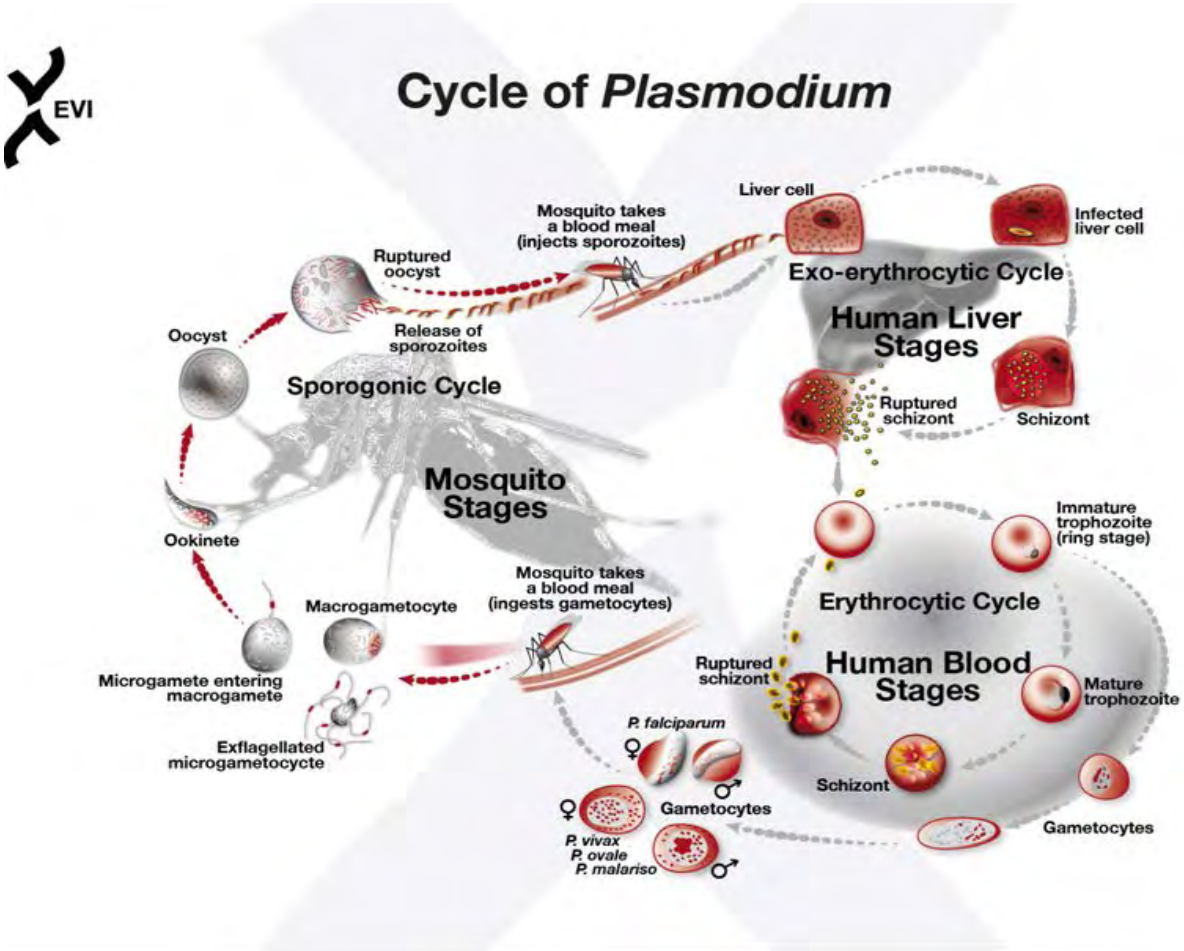

Figure 1.5. Distinct stages of the malaria parasite life cycle, divided between the mosquito vector where the sexual reproduction takes place and the human host where the asexual replication occurs. Detailed analysis in the two following sections. Source: http://www.euvaccine.eu/vaccines-diseases/diseases-poverty/malaria

1.3 A deep sight in the malaria life stages and the transmission chain

1.3.1 Malaria vertebrate lifecycle and pathogenesis

Plasmodium has a complex life cycle in humans, where the outcome is the malaria pathogenesis. During the uptake of a blood meal of a malaria infected female *Anopheline* on a non-infected human, infective sporozoites carried by the mosquito are in released in the bloodstream of the human with the insect's saliva. Within 30 minutes, sporozoites are transferred via the bloodstream to the liver where they invade the hepatocytes and initiate schizogony. During the next 5 to 16 days, referring as incubation period, the sporozoites undergo asexual reproduction inside the hepatocytes and develop into exoerythrocytic schizonts, structures that can contain thousands of merozoites (NIAID, 2002).

During the incubation period, the *Plasmodium* infection is completely asymptomatic. All the clinical effects of *Plasmodium* infection are caused after the rupture of mature schizonts and the release of the merozoites into the bloodstream, where they penetrate the red blood cells (RBCs) (Matuschewski, 2006).

After 1-2 minutes of their release, merozoites invade the RBCs where they consume haemoglobin as a source of energy to pass from the initial endoerythrocytic stage, termed as ring form, and mature into trophozoites. The trophozoite within a red blood cell grows and forms a blood-stage schizont which undergoes another round of asexual proliferation, producing 6 to 36 merozoites. Subsequently, the mature schizont ruptures destroying the erythrocyte and releasing the merozoites into the bloodstream, where they infect other RBCs. Apart from the burst of merozoites, the schizont rupture results, also, in the release of the metabolic products of the parasite into the patient's circulation, causing paroxysm, and hemozoin, a pigment produced by the digestion of haemoglobin which accumulates in the organs of the patient.

In *P. vivax* infections, some sporozoites do not immediately develop into schizonts, but instead form hypnozoites that remain dormant for several months to several years, until their reactivation leading to the generation of merozoites. Hypnozoites are responsible for long incubation and late relapses in *P. vivax* infections.

After some cycles of infection, multiplication and bursting, infection of new RBCs by some of the merozoites, do not result in another asexual production of merozoites but in sexual differentiation in microgametocytes and macrogametocytes (male and female gametocytes, respectively). It has been recently demonstrated, in *P. falciparum*, that the developmental switch from asexual replication to sexual development initiates at the ring stage and that all merozoites from that ring parasite and later schizont are committed to form gametocytes upon invasion of new erythrocytes (Dixon et al., 2008). The gametocytes can be ingested by a female mosquito during a blood meal leading to *Plasmodium* developement inside the vector and malaria transmission.

Out of the five species that cause human malaria, *P. falciparum* is the only one where severe and fatal pathogenesis frequently occurs (Pasvol, 2005). The reason for *P. falciparum*'s enhanced virulence, compared to other *Plasmodium* species, originates in the ability to invade not only reticulocytes (immature RBC) but also a large proportion of circulating RBCs and the great variety of the invasion pathways utilized for the penetration of RBCs. These privileges of *P. falciparum* make it capable of causing very high parasitaemia and reaching high multiplication rates and enhanced growth, compared to other species, resulting in a severe clinical outcome (Miller et al., 2002).

The ability of *P. falciparum* to sequester is another important factor affecting its enhanced pathogenicity. As endoerythrocytic P. falciparum parasites grow, they modify the infected RBC surface, by expressing adherent ligands which allow gametocytes, trophozoites

and schizonts to bind receptors expressed by endothelial cells in the deep vascular beds of the brain, the placenta and other organs (Figure 1.6). As a consequence, sequestration to brain and placenta is proposed to participate in the occurrence of cerebral and placental malaria (Weatherall et al., 2002).

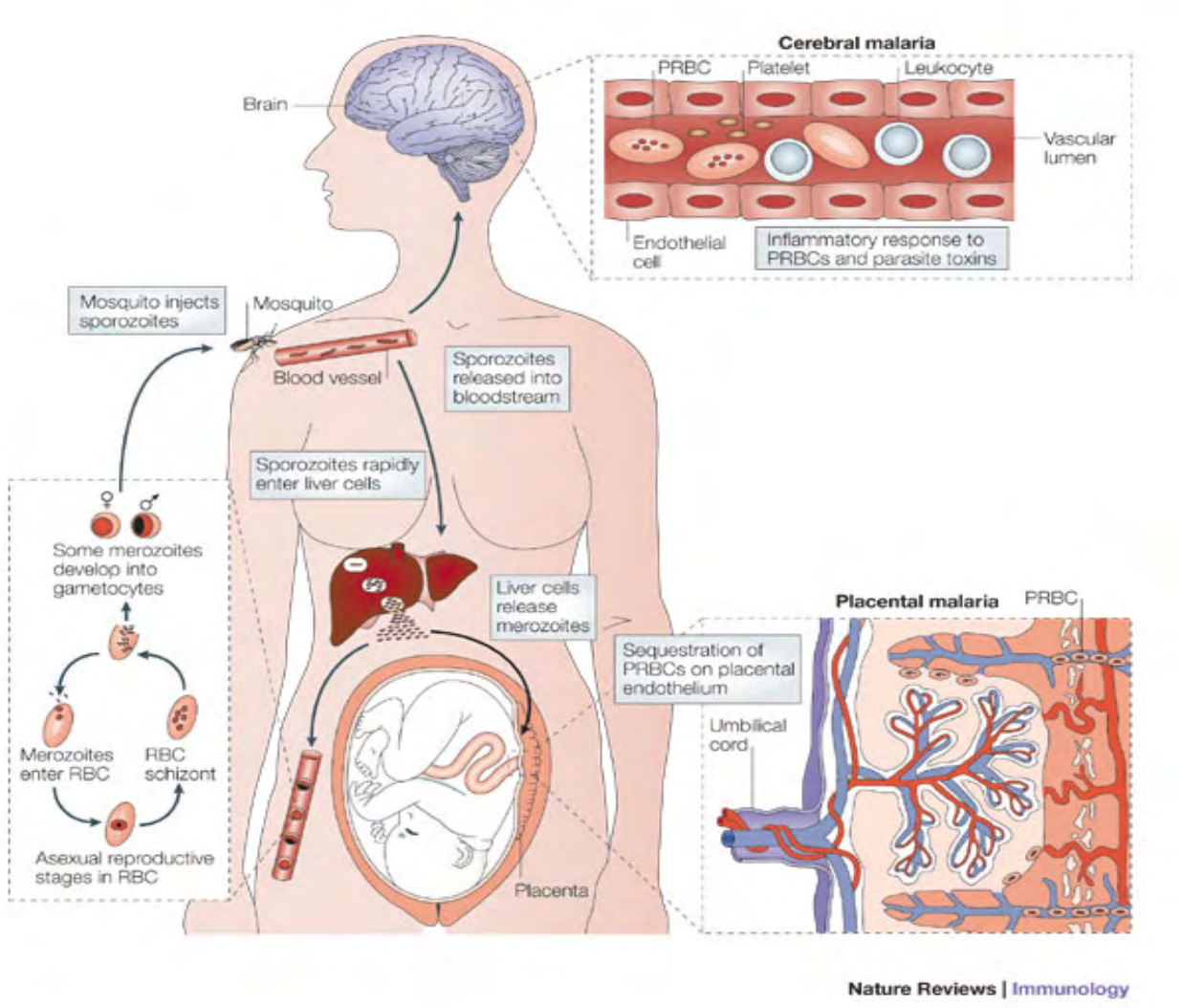

Figure 1.6. Overview of the *Plasmodium* development inside the human host. The malaria parasite enters a vertebrate host, during the blood meal of an infected female mosquito by injection of infectious sporozoites. Sporozoites travel to the liver, where they invade and replicate in liver cells, resulting in thousands of daughter merozoites released into the bloodstream. These merozoites initiate a cycle of RBC invasion and intracellular replication. The parasites circulating within RBCs, express adherent ligands that enable the maturing parasite to bind to the endothelium of organs such as the brain, lungs and placenta. The parasitized RBCs develop into schizonts, which rupture to release a new set of merozoites and parasite metabolic products into the bloodstream and the asexual cycle continues causing severe pathogenesis. A subset of trophozoites differentiates into sexual progenitor cells, the gametocytes, which are ingested by another feeding mosquito, and therefore follow the sexual cycle in the insect. Adapted from (Schofield and Grau, 2005).

1.3.2 Malaria transmission life cycle – mosquito stage development

As mentioned, gametocytes, the first sexually competent forms of *Plamsodium* development during its lifecycle, are essential for the transmission of malaria, as they are the springboard for the sporogonic cycle inside the *Anopheles* vector. Macrogametocytes and microgametocytes are produced inside the RBCs of the infected human host, by sexually commited trophozoites (Bruce et al., 1990; Mons B., 1986). Mature gametocytes (arrested in G_0 – inactivated) are transmitted from the human host to the female mosquitoes by an infected blood meal uptake, initialising the parasite journey in the mosquito. Inside the mosquito, the *Plasmodium* gametocytes undergo activation into gametes and fertilisation, and further transform to ookinetes, oocysts and finally sporozoites. The passing through these stages of sporogony comprises of processes characterised by differential gene transcription, protein expression, upregulation of biochemical pathways and, finally, morphological structure formation: gametogenesis, fertilisation and zygote formation, ookinete maturation and locomotion, midgut invasion, ookinete-to-oocyst transition, oocyst maturation and sporogony, sporozoite migration and salivary gland ivasion.

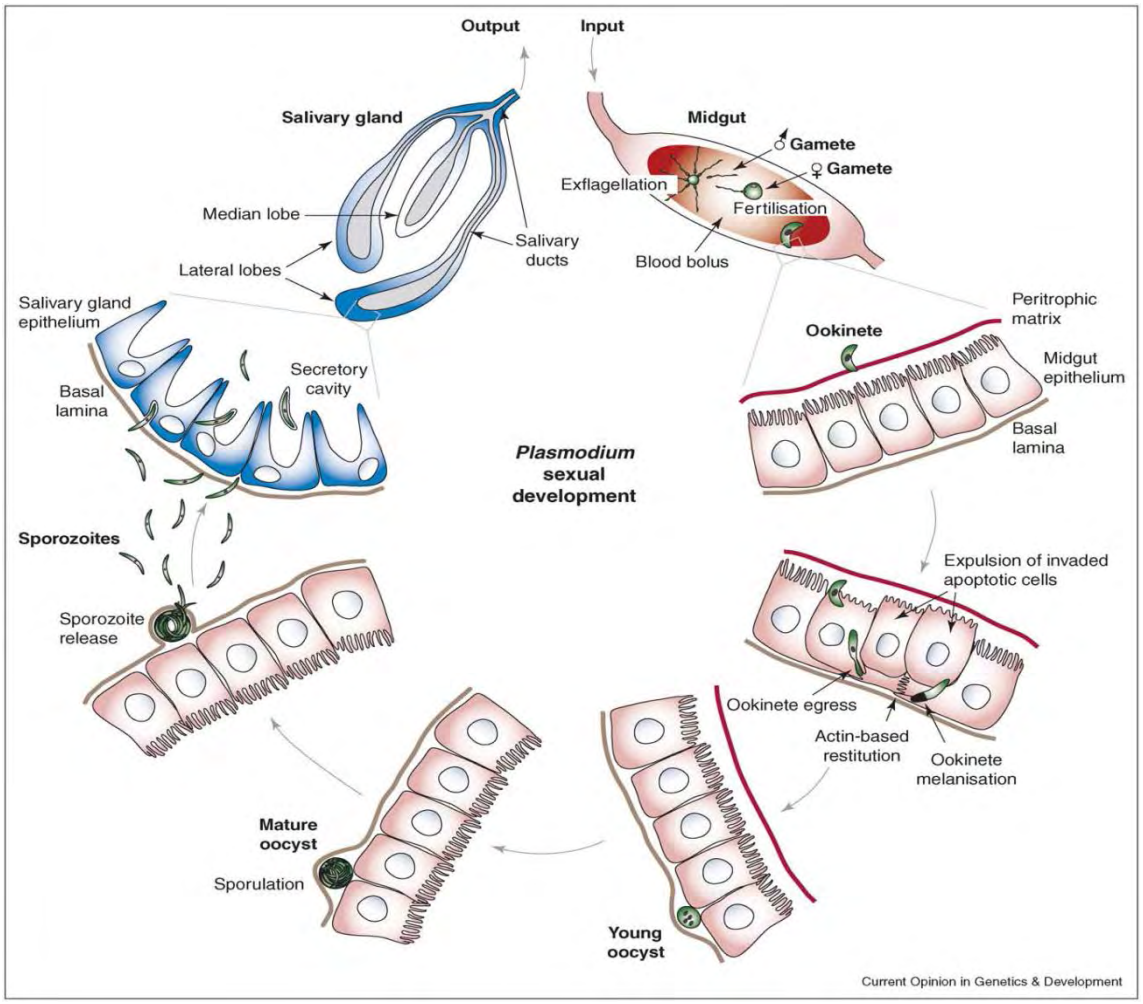

Figure 1.7. *Plasmodium* migration in the mosquito host. The developmental lifecycle of *Plasmodium* in the mosquito starts with a female mosquito bite and blood meal on a malaria-infected vertebrate host; it ends with a new bite approximately three weeks later. The processes followed during the development of the parasite inside the mosquito vector are described in the text. Adapted from (Vlachou et al., 2006).

1.3.2.1 Gametogenesis

Gametocytes ingested by a mosquito during a blood meal, are passively transferred into the mosquito midgut lumen where activation of the formation of gametes (gametogenesis) occurs, due to sensitivity of the gametocytes to environmental influence. The factors that initiate the gametogenesis are the presence of xanthurenic acid, a mosquito-derived molecule, in addition to drop in temperature and pH shift (Billker et al., 1997; Billker et al., 1998). Soon after the activation, an intracellular rise in Ca^{2+} concentration transduces the signal for the emergence of the gametes from the RBCs (McRobert et al., 2008). Following emergence, gametogenesis differentiates between male and female.

Male gametogenesis:

The mature male gametocyte includes few mitochondiria, apicoplasts and membrane bound vesicles, known as osmophillic bodies, while it lacks endoplasmic reticulum (ER). The main characteristic of it is an enlarged nucleus containing highly organised chromosomes, the kinetochores of which are attached to the Microtubule Organising Centre (MTOC) located on the cytoplasmic side of the nuclear envelope, via a nuclear pore (Sinden, 1998).

Upon activation, male gametocytes undergo three rounds of endomitosis, reaching an octoploid value and resulting, after karyokinesis and cell division, in the release of eight haploid and highly motile, flagellated microgametes (Alano, Billker and Sherman, 2005; Sinden, 1998). The success of the nuclear and cell division, during microgametogensis, is accomplished by the formation of a cytoskeletal core of the flagellum, called axoneme, which has the ability to lead the haploid genome into the developing microgamete (Sinden, 1998). In the rodent malaria parasite *P. berghei*, it is demonstrated that the initiation of DNA replication is regulated by the signalling response to the XA dependent rise of Ca^{2+} concentration, mediated by the calcium-dependent protein kinase 4 CDPK4 (Billker et al., 2004). Also, the stimulation of cytokinesis and release of microgametes depends on the function of mitogen-activated protein kinase 2, MAPK-2 (Rangarajan et al, 2005; Tewari et al., 2005), while in *P. falciparum* it is shown that MAPK-2 has no essential role in the mature male gamete development (Dorin-Semblat et al., 2007). This process, termed exflagellation, was observed by Laveran in 1880 and provided the first major clues that malaria was caused by a parasitic protozoan (Laveran, 1880).

Female gametogenesis:

In contrast to the male gametocyte, the mature female gametocyte has a small nucleus, a large number of osmophilic bodies and an extensive ER with increased ribosome production. Mitochondria, ribosomes, apicoplasts and mRNA are located in the cytoplasm (Sinden, 1998). The maturation process of the female gametocyte thus is a preparative stage for the extensive proteins synthesis associated with female gametocyte activation.

Female gametogenesis does not require genome replication, giving rise to a single, large round-shaped and motionless gamete. It is proposed that the presence of a large number of osmophilic bodies, the generation of which depends on the gametocyte specific expression of Pfg377 in *P. falciparum* (Alano et al., 1995), is responsible for the efficient process of macrogamete formation (Sinden, 1998).

1.3.2.2 Fertilisation and zygote formation

Fertilisation is dependent on the final stage of exflagellation, when exflagellation centres are generated. The emerging microgametes bind uninfected RBC, infected RBC and macrogametes, causing them to cluster around the exflagellating male gametocytes. The exact function of the exflagellation centres is not yet clear, but it is suggested that, due to their potential role in the enhancement of microgamete motility, they are important for the establishment of infection in the vector, (Eksi et al., 2006). During fertilisation, the microgametes bind on the surface of macrogametes, by expressing surface adherent proteins, *Pf*s230 (Eksi et al., 2006) and *P48/45* (van Dijk et al., 2001), as demonstrated in *P. falciparum*.

Fertilisation results in fusion of the plasmalemmas of the two gametes and entrance of the male nucleus and naked axoneme inside the female cytoplasm (Sinden et al., 1985) The two nuclei fuse, right after fertilization, in order to form a diploid spherical zygote (Aikawa et al., 1984), which inherits the maternal gamete plasma membrane, cytoplasm, and intracellular organelles. Nuclear fusion in the zygote is followed by DNA replication and meiosis without nuclear division or cytokinesis occurring (Sinden et al., 1985), resulting in a tetraploid genome (Janse et al., 1986). DNA replication during this meiotic event is regulated by the female-specific expression of two NIMA (never in mitosis/Aspergillus) related kinases, Nek2 and Nek4 (Reininger et al., 2005; Khan et al., 2005; Reininger et al., 2009).

1.3.2.3 Ookinete maturation and locomotion

Within 12-24 hours, the zygote gradually passes from spherical stage to elongated, retord stage, presenting a characteristic growing apical protrusion. The outcome of this transition is the first intravector invasive form of the Plasmodium, characterised by intense polarisation, a highly conserved subcellular organisationa and a unique form of locomotion, the mature crescent-shaped ookinete.

The development of the ookinete requires the generation of an extensive subpellicular microtubular network forming the associated MTOC, known as the apical polar ring, and the anterior secretory organelles, termed as micronemes (Canning and Sinden, 1973). Also, the successful ookinete maturation requires *de novo* protein synthesis and transfer via the micronemes. The transcripts of these proteins are not only ookinete specifically produced but also formerly translationally repressed, during the female gametocyte stage, as defined by gene deletion studies (Hall et al., 2005; Ekcer et al., 2008). Together these processes of ookinete maturation facilitate the motility and invasive ability required for the ookinete to escape the tightly packed blood bolus and traverse the midgut epithelium.

The mechanism that regulates the post-translational repression in female gametocytes of gene products essential for zygote formation and development, involves a DDX6-class RNA helicase named development of zygote inhibited (DOZI). The best characterised example is that of the *Plasmodium* surface proteins P28/25, between 368 other gene products (examined in *P. berghei*). DOZI co-localises with translationally repressed p25 and p28 transcripts, in cytoplasmic bodies in the female *P. berghei* gametocyte (Mair et al., 2006; Khan et al., 2005).

1.3.2.4 Midgut invasion

After maturation, the ookinete invades the midgut epithelium in order to reach the basal side of it, where the oocyst will be formed. This requires contractile, twisting and gliding motility of the ookinete and secretion of penetration mediating and protective proteins. Universally, the *Plasmodium* motility and invasion machinery is dependent on apical discharge of secretory vesicles.

In the beginning of the invasion, the ookinete crosses the chitinaceous peritrophic matrix, which covers the inner (apical) face of the midgut epithelium, a process facilitated by the secretion of a chitinase (CHIT1) (Huber et al., 1991; Dessens et al., 2001). Additionally, in *P. berghei*, a calcium-dependent protein kinase, CDPK3, probably involved in ookinete motility, has an important contribution to the early events of midgut invasion (Ishino et al., 2006; Siden and Kiamos et al., 2006).

The next barrier is the inner surface of the midgut epithelium, on which the ookinete attaches in order to penetrate the membrane and enter the lumen of more than one the epithelial cells in row (Vlachou et al. 2006). For this process, members of a conserved *Plasmodium* perforin like protein (PPLP) family are recruited. This family, characterised by a membrane-attack complex and and perforin (MACPF)-related domain, consists of five members. Their secretion during invasion is supported by the localisation of PLP1 and PLP3 identified in the micronemes (Kadota et al., 2004; Kaiser et al., 2004) and their essentiality to cell penetration is indicated by gene deletion experiments of PPLP3 and PPLP5.

Genes subject to DOZI-mediated translational repression are also known for their contribution to the invasion process. Three of these are the major *Plasmodium* ookinete surface proteins, P25 and P28 and von Willebrand factor A domain-related protein (WARP). Potentially mediating in intercellular interactions (encoding epidermal growth factor (EGF) domains), P25/28 proteins have been postulated to play a role both protective against proteases and essential to the development of ookinete and invasion (Tomas et al., 2001), while WARP localises in the micronemes only of mature ookinetes (Yuda et al., 2001).

Other (potentially) secreted proteins implicated in traversal of the midgut barrier are the secreted ookinete adhesive protein (SOAP) (Dessens et al., 2003) and putative secreted ookinete proteins (PSOPs), identified in *P. berghei* (Hall et al., 2005; Ecker et al., 2008).

1.3.2.5 Ookinete-to-oocyst transition

When the ookinete exits the midgut epithelial cell towards the basal side of the midgut, it confronts the hemolymph and the basal lamina. Interestingly, it has been proposed that this contact with the basal lamina induces the ookinete to oocyst transformation, without having elucidated the true role of it. This hypothesis was initially based on the observation that ookinetes injected into the mosquito haemocoel can transform into oocysts on the basal lamina of the malpighian tubules and the fat body, apart from that of the midgut (Weathersby, 1954).

Also, studies showing that KD of mosquito laminin-γ1 (*LANB2*), a basic component of basal lamina, results in a reduction in oocyst numbers support this theory (Arrighi et al., 2005). Additionally, it has been observed that P25 and P28 (Vlachou et al., 2001), the circumsporozoite and TRAP-related protein (CTRP) (Mahairaki et al., 2005), and SOAP (Dessens et al., 2003) bind mosquito laminin, assigning to these proteins a crucial participation in the developmental transition between ookinete and oocyst, apart to that in mediating invasion.

After ookinete is immobilised contacting the basal lamina, it begins the morphological transformation to round-shaped oocyst, by losing the subpellicular microtubules, apical complex and double-membrane pellicule but maintaining the tetraploid genome (Canning and Sinden, 1973). Finally, the apical and the posterior protrusion are absorbed and a single-membrane plasmalemma coats the new-born oocyst (Carter et al., 2007). A *P. berghei* nuclear formin-like protein, named MISFIT, is the first discovered protein with paternal effect on the development of the parasite. PbMISFIT is expressed in male gametocytes and localises to the nuclei of microgametes, zygotes and ookinetes. Gene deletion experiments have demonstrated that PbMISFIT has an essential male-inherited role in ookinete-to-oocyst transformation and that absence of expression leads to transmission blockade (Bushell et al., 2009).

1.3.2.6 Oocyst maturation and sporogony

During the development of the oocyst a proteinaceous capsule gradually surrounds the plasmalemma while the genome undergoes multiple rounds of replication (Sinden and Strong, 1978). During karyokinesis, the polyploid genome is apportioned in the sporoblasts, compartments formed by the plasmalemma invagination. The nucleus and subcellular organelles are distributed into the sporozoites budding off from the sporoblasts, ultimately resulting in the formation of the mature oocyst which contains thousands of haploid sporozoites, characterised as sporulated (Sinden and Strong, 1978). This is the only stage in the life cycle in which the parasite replicates extracellularly.

Despite the poor information about the molecular mechanisms regulating the oocyst maturation and sporogony, an essential for this process gene product has been characterised: the conserved among *Plasmodia* circumsporozoite protein (CSP). CSP is associated with the plasma membrane of both the maturing oocyst and the developing sporoblasts and becomes the main surface protein of oocyst and salivary gland sporozoites, through its glycosylphosphatidylinositol (GPI) anchor (Nagasawa et al., 1988). The essentiality of CSP in the sporogony is determined by *csp* gene deletion experiments in *P. berghei* revealing a cytokinesis blocking and impotence in sporozoite formation (Menard et al., 1997).

Also, members of the limulus clotting factor C, Coch-5b2 and LglI (LCCL)–lectin adhesive-like protein (LAP) family (Dessens et al., 2004; Trueman et al., 2004) are significantly involved in oocyst development and sporulation, as well as PSOP13 (Ecker et al., 2008).

Finally, a protein which is exclusively localised to the capsule of the developing oocyst, has been identified: CAP380. Absence of CAP380 results in gradual extermination of developing oocysts, suggesting a protective property of CAP380 during the maturation oocyst of the oocyst inside the immune-rich hostile environment (Srinivasan et al., 2008).

1.3.2.7 Sporozoite motility and salivary gland invasion

The next step of the parasite migration in the mosquito vector is the sporozoire egress from the oocyst and their journey to the salivary glands. The active process of sporozoite release is facilitated by the egression cysteine protease (ECP) (Aly and Matuschewski, 2005), which includes a region enriched in positively charged amino acids (Wang et al., 2005).

The sporozoite migration towards the salivary glands is not yet fully illuminated, thus there is a controversy between chemotactic and passive transfer through the hemocoel

circulation. It is clear, though, that sporozoite, as ookinete, motility is an absolute requirement for overcoming the challenges of salivary gland invasion.

Sporozoite surface proteins, TRAP, CSP (Tewari et al., 2002) and transmembrane protein S6, which is subject to post-translational repression during former stages, (Steinbuechel and Matuschewski, 2009) are essential for sporozoite motility and salivary gland invasion. Intriguingly, *trap* gene family includes *ctrp*, which is expressed during midgut and salivary gland invasion, as well as merozoite *trap* homologue, the expression of which is important for erythrocyte invasion (Kappe et al., 2004), suggesting that facing similar barriers leads the parasite to recruit similar molecular mechanisms both in the vertebrate and the mosquito host (Vlachou et al., 2004).

The force for *Plasmodium* gliding motility is generated by an actomyosin motor that is captured apically between the plasma membrane and the inner membrane complex (Kappe et al., 2004), through aldolase by transmembrane receptors of the TRAP family (Schuler and Matuschewski, 2005).

During the salivary gland invasion and in contrast to the midgut invasion, the sporozoite traverses the epithelium from the basal to the apical side, while homing in on the ducts of the glands, implying that not only similar but also different molecular mechanisms are inducing both invasions. A parasitophorous vacuole of host cell origin, absent during midgut invasion, is formed due to the interaction of the parasite with the basal plasmalemma, enveloping and feeding the parasite while leading it inside the epithelial cell (Pimenta et al., 1994). On the contrary, salivary gland and midgut peptide 1 (SM-1) inhibits both the midgut and the salivary gland invasion (Ghosh et al., 2001) at the initial binding and recognition of the epithelium, when merozoite apical erythrocyte-like protein (MAEBL) (Kariu et al., 2002) and CSP (Kappe et al., 2004) are recruited.

During the transfer to the secretory cavity of the glands, the parasite is coated by a second parasitophorous vacuole, which gradually disintegrates and releases the sporozoite. After egress, the sporozoites migrate to the ducts by glinding motility (Frischknecht, 2004), from where they are transmitted passively to a new vertebrate host via mosquito blood-feeding or remain in the secretory cavities for future transmission (Rodriguez and Hernandez-Hernandez Fde, 2004).

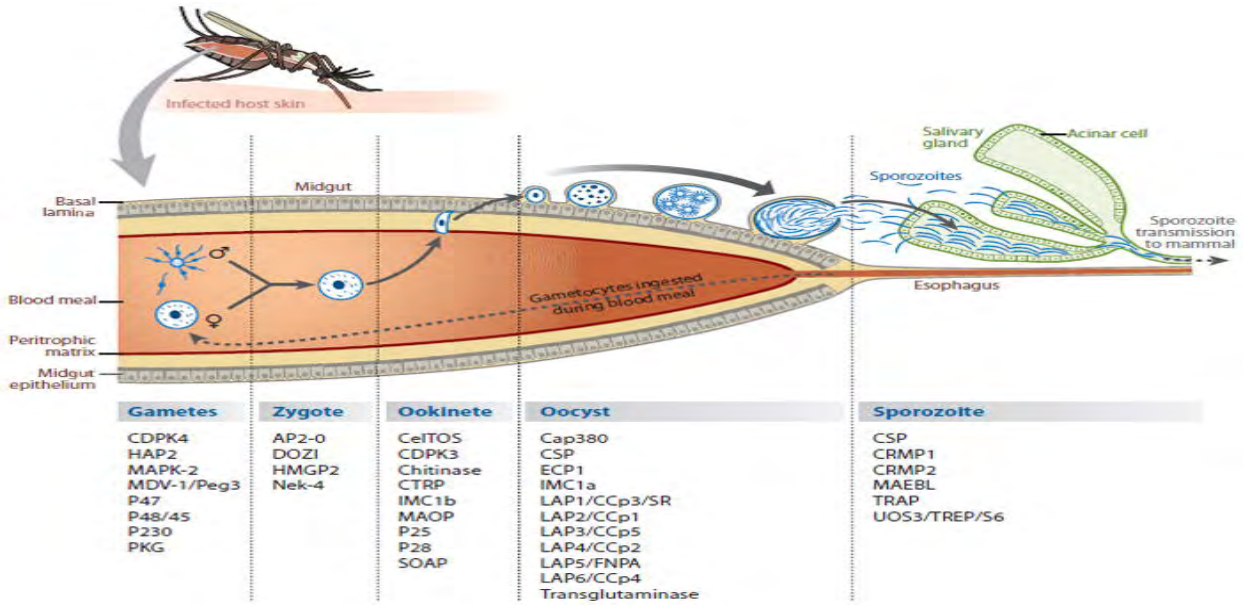

Figure 1.8. Overview of the identified *Plasmodium* genes, expressed during its intravector development. Adapted from (Ahmed et al., 2009).

1.3.3 Vector response – the transmission bottleneck

Plasmodium must complete a complex sexual development in the mosquito vector in order to achieve successful transmission to the vertebrate host. During its traverse through several mosquito tissue compartments, it encounters not only physical barriers but also host's defence responses. Due to parasite's impact on mosquito fitness, reproduction, and survival, mosquito's innate immunity is activated (Hogg & Hurt, 1991; Ferguson & Read, 2002; Ahmed and Hurt, 2006). The outcome of the finely balanced agonistic and antagonistic mosquito-parasite interactions developed during the parasite journey through the mosquito is either successful transmission, in *Plasmodium* susceptible *Anopheles* mosquitoes (with variant vectorial capacity) or destruction of the malaria parasite and transmission blockade, in refractory ones.

Developments in genome-wide approaches, genomics and proteomics, have shed significant light on the biology of the *Plasmodium* parasite during the malaria transmission cycle in the vector and the *Anopheles* immune response. Studies about the mosquito-parasite molecular interplay and the mosquito immune system have mainly targeted the major human malaria vector, *A. gambiae*, and include infections with the rodent model *Plasmodium berghei* (Christophides et al., 2004).

These studies have outstandingly proved that the parasite suffers substantial losses at different stages of the development inside the invertebrate host, due to mosquito factors that affect its development (Figure 1.9), not only in *Plasmodium* refractory but also in susceptible mosquitoes. Although the severest bottleneck of malaria transmission occurs during ookinete invasion of the midgut epithelium, the *Anopheles* defence reaction is not confined to this phase of the parasite migration. Antagonistic (and agonistic, as described earlier) interactions are also developed before midgut invasion in the midgut lumen, after the sporozoite release from the oocyst in the haemolymph and during the ivasion of the salivary glands (Vlachou et al., 2005).

The first significant reduction in the number of parasites is observed in the mosquito midgut lumen, during the parasite gametogenesis (Christophides et al., 2004). Although the initial mosquito response which includes identification of the parasite, generates a favourable environment for the process of gametogenesis (XA, low pH, optimum temperature), nonappearance of exflagellation has been observed in some mosquito species, suggesting the potential presence of inhibitors or absence of activators (Vlachou et al., 2005). The second critical stage in the parasite development that encounters an encumbrance by yet unknown factors of the mosquito is fertilisation, followed by zygote maturation inside the midgut lumen. It is suggested that digestive enzymes, such as trypsins, assault the parasites playing a notable role in the reduction of their number (Muller et al., 1993).

Another parasite limiting phase is observed after the release of sporozoites from the mature oocyst. The sporozoites must navigate the haemolymph which is enriched with abundant humoral and cellular immune factors that cause degradation of a respectable amount of sporozoites, in order to reach and invade the salivary glands. Thus, a rapid salivary gland invasion (>8 hours) is required in order to avoid the destruction by the mosquito immune reaction (Hillyer et al., 2007).

As outlined earlier, the most devastating for the parasite number stage is the invasion of the midgut, due to the extensive parasite-mosquito interactions that are developed. Based on this transmission bottleneck, several studies have examined targets potentially responsible for transmission blockade. Although the hypothesis that midgut invasion is receptor-mediated is not yet confirmed, it is shown that midgut expressed annexines which bind to the *P. berghei* invading ookinete are valuable to the process of invasion (Kotsyfakis et al., 2005).

The mosquito responses triggered by the parasite invasion of the midgut involve, as obviously suspected, the activation of innate immune reactions (which are proved to be fundamental in refractoriness (Habtewold et al., 2008)), of which the major ones are parasite lysis and melanisation, and local epithelial responses.

The molecular identification of potential immune factors against invading ookinetes was achieved through *Anopheles* genome sequencing and experimental studies (Meister and Koutsos, 2004; Christophides et al, 2004). The majority of the identified proteins that regulate the anti-*Plasmodium* immune responses are detected in the haemolymph, nominating the crucial role of this open circulatory system in the systemic defence against the parasite.

It is demonstrated that the obliteration of up to 80% of the *P. berghei* invading ookinetes is mediated by two *A. gambiae* gene products: thioester-containing protein 1 (TEP1), which is a complement-like protein, and leucine-rich repeat immune protein 1 (LRIM1). TEP1 and LRIM1 are found to be involved both in lysis of *Plasmodium* ookinetes and phagocytocis of bacteria (Blandin et al., 2004; Osta & Christophides et al., 2004) and to interact together with APL1C (a member of LRIM family) as they circulate inside the haemolymph (Povelones et al., 2009; Povelones et al., 2011).

A next barrier to the surviving ookinetes, raised by the mosquito immune system is melanisation, an important to arthropods humoral immune response (Cerenius et al., 2004). During this process, some ookinetes are destructed by toxic reactive oxygen intermediation or asphyxiation after being encased in melanine capsules. Melanine synthesis is catalised by active phenoloxidases (PO) which are generated by proteolytical cleavage of pro-phenoloxidases (PPO), a reaction regulated by an extracellular cascade of proteinases, including clip-domain serine proteases, inactive serine protease homologues that are missing catalytic triad residues, C-type lectins (CTLs), and serpin inhibitors (SRPNs). Recently, the first inhibitory serpin-serine proteinase pair in mosquitoes was identified, in which *A. gambiae* proteinase CLIPB9 is inhibited by SRPN2 (An et al., 2011).

Apart from the previous two main reactions, additional immune responses that assist the parasite killing before and during invasion have been indicated. Specifically, it is shown that antimicrobial peptides produced upon immune signalling mediate in the melanisation process (Meister et al., 2005).

A significant barrier to the *Plasmodium* ookinete is posed not only by the immune response of the mosquito but also by local epithelial reactions, activated upon intracellular invasion of the midgut epithelium. Apoptosis and actin cytoskeleton and microtubule remodelling of the invaded cell are triggered, resulting in the formation of an actin-based structure, known as the parasite hood, which enfolds the ookinete during its exit from the midgut. The hypothesis that the parasite hood is part of the a local epithelial immune response is supported by its resemblance of a phagocytic cup formed during bacteria phagocytosis and the documented mediation of TEP1 and LRIM1, two major anti-parasitic factors, in bacterial phagocytosis. Also, enhancement of this theory derives from RNAi-dependent gene silencing experiments of Wiskott–Aldrich syndrome protein (WASP), an activator of actin nucleation, and Apolipophorin II/I (ApoII/I), two major factors in mosquito's lipid transport. According to the obtained results, WASP is identified as an antagonist of parasite development while ApoII/I are involved in mosquito-egg development and ookinete survival (Vlachou et al., 2005b).

It is also suggested that, as in microbial pathogen infection, cytoskeleton remodelling of the midgut epithelial cells during ookinete invasion could be a result of either indirect stimulation (apoptosis) or direct induction by *Plasmodium* molecules, such as Subtilisin 2, a serine protease *Plasmodium* secrets inside the epithelial cells and is observed to be associated with actin filaments (Han et al., 2000).

Two other mechanisms recruited by the invaded cells, probably against parasites, are the reported enhancement of nitric oxide synthetase (NOS) activity and peroxidase activity which induce degeneration of the invaded cell by tyrosine nitration (Kumar et al., 2004). This was further supported by recent studies which illustrated that epithelial peroxidase/NOX5 (HPX2 peroxidase and NADPH oxidase 5) nitration and parasite lysis mediated by TEP1 are sequentially activated, pointing out a potential role of nitration to the mosquito complement activation against the parasites (Oliveira et al., 2012).

The nadir of the Plasmodium population is observed during this severe mosquito-parasite battle stage, with the parasite numbers reducing down to 1-10% of the initial ones (Christophides et al., 2004). Due to this reason, this stage presents the strongest bottleneck effect, with a critical result in the success of the parasite transmission (Figure 1.9). After the invasion of the midgut epithelium and the burst of only one mature oocyst, though, the sporozoite numbers can reach again a respectable value and, therefore be transmitted, impeding sterile immunity (in susceptible mosquitoes). An insight of the interplay between the parasite and the mosquito genes that significantly affect the parasite development especially in the transmission bottleneck stage could proffer new prospects in the development of transmission blocking interventions (Vlachou et al., 2005; Sinden et al., 2007).

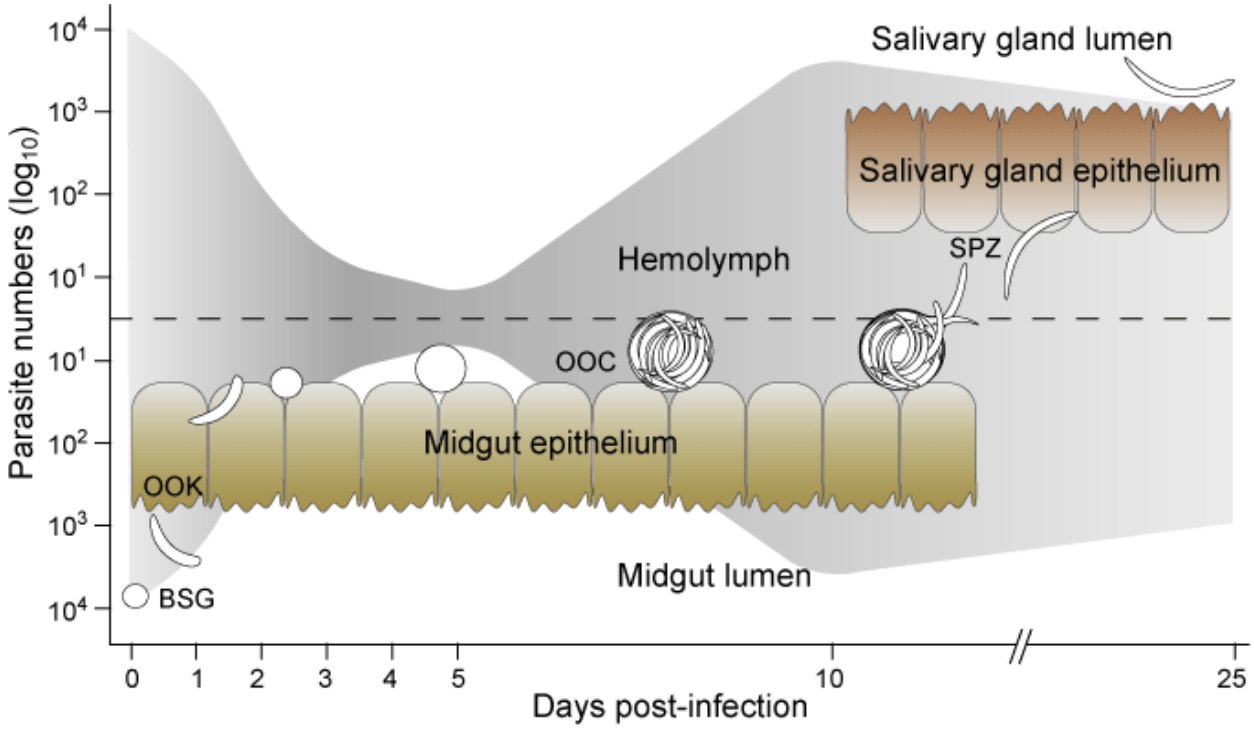

Figure 1.9. Graphic representation of the *Plasmodium* population bottleneck during its developmental migration in the mosquito vector, with the nadir in number recorded at the oocyst stage. Source: http://kafatos.openwetware.org/Research.html

1.4 Control measures and impediments to eradication

1.4.1 Mosquitoes

The primary goal of vector control is to reduce the vectorial capacity of local vector populations below the critical threshold in order to prevent endemic or epidemic transmission. Vectorial capacity (also mentioned earlier) refers to the efficiency of the prevailing mosquito populations to transmit malaria.

1.4.1.1 Limitation of the host-mosquito contact

Due to the nocturnal feeding pattern of most Anopheles vector species, a simple but intertemporal protection against malaria has been developed, aiming at the reduction of the contact between mosquitoes and humans: mosquito bednets. The modern advanced betnets are constructed with insecticide treated nets (ITN), which are impregnated with pyrethroids, reducing the female mosquito survival due to contact with lethal insecticides (Curtis et al., 2006). Also, long-lasting insecticide-treated nets (LLINs) have been developed, maintaining effective insecticide levels for at least three years. It is demonstrated that the outcome of ITN use is a significant reduction in the malaria related morbidity and mortality (Takken, 2002).

Figure 1.10. Insecticide treated mosquito net protecting a sleepy child.

Although, in endemic areas socioeconomic factors have raised barriers to the efficient use of ITN (Guyatt and Snow, 2002), a problem that national malaria control programmes and their partners are trying to overcome by ITN/LLINs free contribution (Guyatt and Snow, 2002; WHO Global Malaria Programme: Position Statement on ITNs). Nevertheless, genetic based and behavioural pyrethroid resistance of mosquitoes, related to ITN use is also an upcoming issue.

1.4.1.2 Destruction of vector mosquitoes and mosquito breeding sites (insecticidal and larvicidal methods)

Reduction or elimination of mosquito populations can be achieved via chemical, biological or genetic approaches directed towards adult or larval stages.

One of the most effective method that eradicated malaria in many temperate and tropical low-transmission regions was large scale indoor residual spraying (IRS) of insecticides. The principle of this method relies on the endophilic feeding pattern of many *Anopheline* females. After blood-feeding, the female mosquito rests on a nearby surface while digesting the blood-meal and, thus, insecticide sprayed walls can be an effective lethal trap, reducing the number of infected vectors, capable of transmission (Bruce-Chwatt, 1987).

The first and historically the most popular insecticide used for IRS is DDT, which in conjunction with chloroquine was the main control measure of WHO Global Malaria Eradication campaign launched in 1955. Although this large scale spraying eliminated malaria in many temperate and l temperate and tropical low-transmission areas (Sadasivaiah et al., 2007), it failed

to totally eradicate malaria in high-transmission endemic regions. This is attributed to the main problem that DDT-based IRS campaign and all forms of IRS confront: the insecticide resistance via evolution of mosquitoes.

Thus, the World Health Organization (WHO) currently advises the use of 12 different insecticides in IRS operations. These include small amounts of DDT and a series of alternative insecticides (such as the pyrethroids, deltamethrin and permethrin) to both, combat malaria in areas where mosquitoes are DDT-resistant, and to slow the evolution of resistance.

Another approach, which could be used in combination with IRS, uses spores of the *Beauveria bassiana*, an entomopathogenic fungus which kills mosquitoes. There is experimental evidence that contact with the fungi could yield up to 80% reduction of mosquito numbers and that fungi-infected mosquitoes decrease blood feeding frequency, resulting in a reduction of mosquito bites (Scholte & Ng'habi et al. 2005; Blanford & Chan et al. 2005; Scholte & Knols et al. 2006).

Biological agents can also be used against larval stages, which were restrained by environmental engineering methods, before the introduction of DTT. For example, commonly used biological control agents for larval stages include larvivorous fish and *Bacillus thuringiensis* (Bt), with Bt being a highly used control agent in Europe and the Americas (Mittal 2003).

More recently introduced strategies include sterile insect technique (SIT), release of other genetically modified mosquitoes and paratransgenesis. SIT comprises the sterilization of male mosquitoes (via irradiation or genetic means) prior to their release, in order for their offspring with wild female to be sterile. This technique, though, has a restricted scale application, as it results in a low mating competence of sterile males.

Several approaches have focused on the generation of genetically modified mosquitos which will cause suppression to the general mosquito population or replacement by malaria refractory strains. Genetically modified males the offspring of which is sexually restricted to male (Christophides, 2005), display robust refractoriness against *Plasmodium* or carry a dominant lethal gene (release of insects carrying a dominant lethal – RIDL) are already engineered, giving a boost to the promising application of these methods for malaria elimination.

The paratransgenesis approach relies on the large number of naturally symbiotic with the mosquito bacteria, which, after genetic modification, could have a lethal effect towards the mosquito or even the *Plasmodium* parasite (Riehle, Moreira et al. 2007; Wang et al., 2012).

1.4.2 Human (drugs/vaccines)

1.4.2.1 Anti-malarial drugs

Untreated malaria in a non-immune individual can readily progress within hours to life-threatening illness, thus anti-malarial drugs have currently wide-spread application, targeting both to treatment and prevention of the disease. Most of the anti-malarial drugs target the erythrocytic stage of *Plasmodium* infection in order to alleviate malaria symptoms. Specifically they target on four subcellular organelles and biochemical pathways of the parasite: food vacuole (haem-detoxification), cytoplasm (folate synthesis), mitochondrion (electron transport chain) and the apicoplast (protein synthesis), (Greenwood et al., 2008). Apart from short-term prophylaxis and treatment of the acute blood stage infection, terminal prophylaxis from the dormant liver stage hypnozoites (*P. vivax*) is necessary.

Currently, the drugs that have the broader use against malaria are quinine and its synthetic alternates, antifolates and artemisin and its derivatives. Quinine is a natural compound extracted from Cinchona tree, the anti-malarial effect of which concentrates on the mature trophozoites by inhibiting the parasite haem-detoxification process (food vacuole) (Egan, 2008; Hanscheid et al., 2007). On the other hand, artemisin, another natural compound extracted from Artemesia annua, displays efficacy against all sexual blood stages, by a mechanism yet unclear. Finally, the antifolates inhibit parasite nucleic acid synthesis by interfering with folate synthesis pathway (Hyde, 2007).

In order to reduce the risk of treatment failure and the side-effects, combination therapy comprises the contemporary practice in treating cases of malaria. Although combination therapy provides a decrease of this risk, still, drug resistance developed by the *Plasmodium* parasites poses the main barrier against malaria elimination, despite the broad use of anti-malarial drugs, as well as deficiency in new drug prospects.

1.4.2.2 Vaccine perspectives

Malaria vaccines are an area of excessive research. However, despite many years of effort, there has no effective vaccine been introduced into clinical practice, yet. As the use of an effective vaccine remains an elusive goal for every national malaria control programme, a great number of leads are emerging, with three vaccine strategies (Matuschewski, 2006):

1) Pre-erythrocytic, liver stage vaccines
2) Erythrocytic, blood-stage vaccines
3) Transmission blocking, mosquito stage

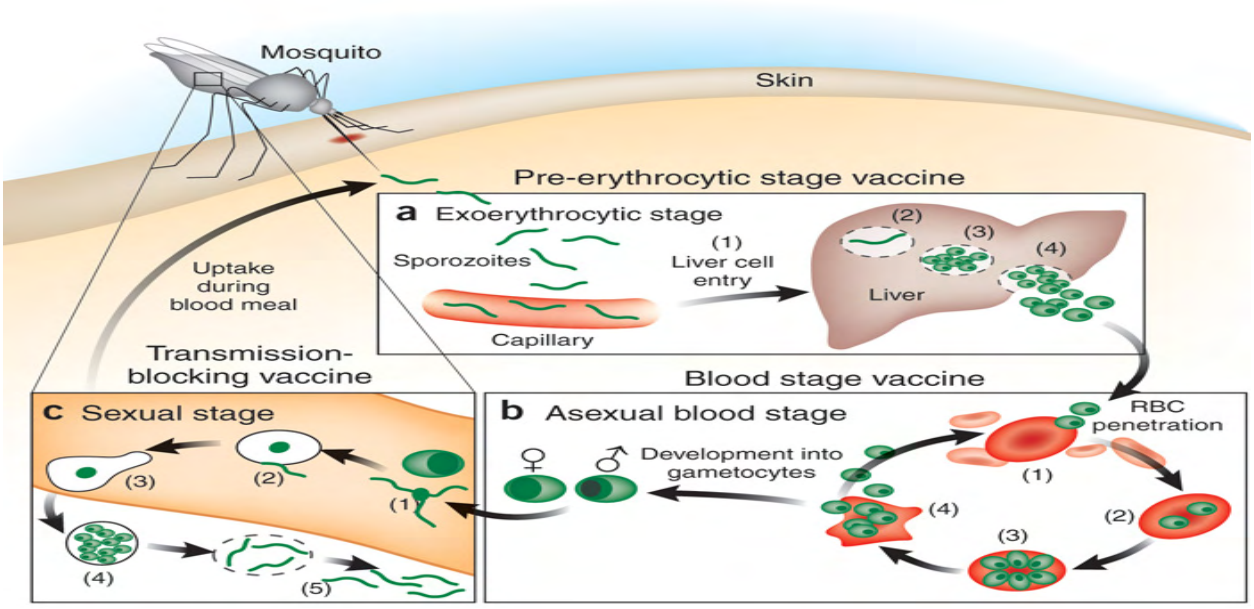

Figure 1.11. Sites in the malaria life cycle that could be interrupted by vaccines. a) Pre-erythrocytic stage vaccines target the invasion of the *Plasmodium* sporozoite and/or the infected liver cell (clinically silent stage). b) Blood stage vaccines target the eythrocytic asexual parasite stages (symptomatic stage). c) Transmission blocking vaccines target *Plasmodium*'s development inside the *Anopheles* vector, and consequently, malaria transmission to human.

The observation that low numbers of sporozoites invade liver, due to the natural bottleneck caused by epidermal injection, has proposed the targeting of liver stage sporozoites as an appealing approach. Pre-erythrocytic (liver stage) vaccines are designed to prevent the entry of sporozoites into hepatocytes or destroy infected hepatocytes (figure 1.11), by evoking protective immune responses that do not normally develop in natural infection, thereby preventing clinical disease.

This approach was initially applied in animal models of malaria by vaccination with radiation-attenuated sporozoites (Herrington et al., 1991; Hoffman et al., 2002) but the recent generation of genetically engineered, live attenuated sporozoites by a defined single gene knock-out is considered to be a safer for further application method (Mueller et al., 2005; van Dijk et al., 2005). A more cost-effective prospect, compared to that of a whole organism vaccine, is the development of a potential sub-unit vaccine. The most developed and promising subunit-vaccine candidate, targeting the liver stages of *Plasmodium* development, is RTS,S. RTS,S aims at protection achieved by immunization with the CS protein (CSP) (Casares et al., 2010) and is currently being evaluated in a phase III trial in Africa (Crawley, 2010).

Vaccines against the pathogenic asexual blood stages of *Plasmodium* parasites are designed with the aim of preventing clinical disease by destroying the merozoites (figure 1.11). Since protective semi-immunity to malaria is acquired after repeated *Plasmodium* infections, it may be possible to mimic and stimulate the acquisition of naturally acquired immunity by a vaccine (Crompton et al., 2010).

However, relatively few blood-stage antigens are in clinical development as vaccines, so far (Crompton et al., 2010). These include erythrocyte-binding antigen–175 (EBA-175) (Sahly et al., 2010), serine-repeat antigen 5 (SERA5) (Horii et al., 2010), apical membrane antigen 1 (AMA1) (Sagara et al., 2009), glutamate-rich protein (GLURP) (Essen et al., 2009; Hermsen et al., 2007) and merozoite surface protein 1 (MSP1) (Ogutu et al., 2009), MSP2 (Genton et al., 2003), MSP3 (Essen et al., 2009; Druilhe et al., 2005), all of which are highly expressed on the surface of the merozoite. Unfortunately, phase II trials of the most advanced blood-stage candidates, AMA1 and MSP1, that were recently conducted did not represent efficacy in African children (Sagara et al., 2009; Ogutu et al., 2009). Extensive *P. falciparum* polymorphism variation in parasite surface proteins, means that generation of an efficient blood-stage vaccine against this species is much more challenging (Matuschewski, 2006).

Transmission-blocking vaccines which target the intravector stages of *Plasmodium* development, preventing the parasites from infecting the mosquito vectors and thereafter the human hosts, are discussed in detail in the next section.

If malaria is to be eradicated a broader range of small molecule therapeutics able to target various stages in *Plasmodium*'s life cycle are required. Thus, interest in the development of a multi-antigen, multistage vaccine, is now increasing (Butler, 2009). This approach will possibly offer better protection than do single-antigen vaccines, and avoid vaccine failure caused by genetic diversity in parasite populations (Saul et al., 2007)

1.4.3 Impediments to eradication

Overall, although malaria is in essence a both preventable and curable disease, its eradication has not yet been possible. Despite substantial advances in treatment and prevention over the past decade, malaria still threatens the lives of millions of children in tropical countries. Although there has been an unprecedented surge in political commitment and international funding for malaria control (Babaeekho et al,. 2009), over the past decade, there are still negative parameters that reduce the efficiency of current control methods in both host and vector.

Two major hurdles threaten progress towards elimination: the increasing resistance of both parasite and mosquito to the chemical repertoire available and the limited availability of effective control methods (Egan and Kaschula, 2007). Specifically, drug treatment is continually undermined by the development of drug-resistant parasite strains and the insecticide resistance of the mosquito vector. (Engwerda and Good, 2008). Without the rapid engineer of effective prospects for treatment and prevention, that are able to circumvent resistance, malaria elimination will become increasingly intractable and eradication impossible. Furthermore, socioeconomic factors as well as and deficiency of essential facilities in endemic regions constitute part of the trammels of malaria elimination, setting the access to not only effective but also affordable treatment at community level as key challenge (Babaeekho et al,. 2009).

The numerous complicating factors which induce the continuing loss of almost a million human lives annually, underline the urgent need of new potential in malaria control.

1.5 Transmission blocking interventions

1.5.1 Transmission blocking vaccines: an ideal public good

Until recently, strategies for malaria vaccine development concentrated on the prevention or treatment of clinical disease. However, the considerable reduction in malaria burden reported in several endemic countries (WHO, World Malaria Report, 2012) and the goal of elimination and eradication propose that greater prominence should be given to the deployment of vaccines that interrupt transmission.

Highly effective pre-erythrocytic (and erythrocytic) stage vaccines can, in principle, reduce infection prevalence in the human host, including both the asexual stages and the gametocytes. Such vaccines can proffer protection against malaria and, coinstantaneously, contribute to the reduction of malaria transmission (The malERA Consultative Group on Vaccines, 2011). Nevertheless, decreasing the number of gametocytes potentially ingested by *Anophelines* does not guarantee the blockade of transmission.

Due to these reasons, a developing strategy aimed at breaking the cycle of malaria transmission is that of Transmission Blocking Vaccines (TBV). In contrast to the other two types of vaccine approaches that principally focus on direct human protection, the primary goal of TBV is to prevent or substantially reduce mosquito infectivity (Perlmann and Troye-Blomberg, 2002). The principle of TBV is the vaccination of individuals with mosquito-stage parasite proteins, immunising them in order to elicit antibodies that are subsequently taken up by the mosquito during a blood meal (Carter et al., 2000). The antibodies are designed so to bind parasite antigens within the mosquito midgut, where it is present extracellularly and in small numbers. Thereby, they assist parasite killing mediated by antibodies and/or complement, or alternatively, interrupt parasite protein function and, thus, development, consequently abolishing transmission (Saul, 2007).

TBVs are not designed so to directly protect the vaccinated individual from contracting malaria, but instead to indirectly protect individuals in the vaccinated community (herd immunity) by reducing the number of infection carrying vectors and circulating parasite population (Dinglasan and Jacobs-Lorena, 2008).

1.5.2 Transmission blocking targets

Research on transmission-blocking vaccines has traditionally targeted to *P. falciparum* and *P. vivax* surface antigens of mosquito stages (Carter, 2001). Specifically, there has been a large body of work focusing on four surface proteins exposed on gametes (pre-fertilisation) and ookinete surface proteins (post-fertilisation) (Saxena et al., 2007).

A successful transmission blockade has been demonstrated by the development of antibodies against two *P. falciparum* gametocyte expressed proteins Pfs48/45 and Pfs230 (present already within the vertebrate host). The parasite is killed upon entry into the midgut lumen when gametocyte activation occurs and, thereupon, the proteins are displayed on the gamete surface. Experiments with in vitro expressed Pfs48/45 antigen with the desirable conformation have resulted in successful animal immunogenicity with a transmission-reducing activity in the sera (Outchkourov et al., 2008; Chowdhury et al., 2009).

Two other TBV candidate proteins, Pfs25 and Pfs28 are only expressed and exposed on the parasite surface following entry into the mosquito midgut and are not present within the vertebrate. The limited polymorphism of these proteins, derived by the absence of adaptive immune pressure of the human host, constitutes an advantage over the erythrocytic vaccines (Saxena et al., 2007). A phase I trial of the P. falciparum and *P. vivax* proteins Pfs25 and Pvs25, respectively, formulated with Montanide ISA 51, demonstrated mild adverse events and functional transmission blocking immunity against the two *Plasmodium* species (anti-Pfs25 and anti-Pvs25 IgG response) (Malkin et al., 2005; Wu et al., 2008).

1.5.3 Transmission blocking vaccine strategies

Although the approach seems reasonable and promising, transmission-blocking vaccine development and implement encounters some difficulties. A concern associated with TBV is that the antigens are never naturally expressed in the human host, resulting in absence of natural boosting with antibody responses being, usually, short-lived. An additional issue is that the proportion of the gametocyte carriers immunised in a local transmission area could limit the efficacy of the vaccine. As a result, vaccination should be applied en masse in order for the vaccinated cohort to develop a robust immune response in order to achieve adequate transmission blocking levels (Saul, 2007; Carter, 2001). Finally, it may be difficult to widely implement a vaccine that has no direct benefit to the individual but, instead, offers protection to the vaccinated community (Crompton et al., 2010).

Nevertheless, several advantages of TBVs over pre- and erythrocytic vaccines have established a campaign towards their development. Since TBV mediated parasite killing is well defined (antibody or antibody and complement mediated killing), the efficacy of TBVs can be easily assayed *ex vivo* by membrane feeding upon gametocytes in the presence of immunised sera, without putting vaccinated individuals in the risk of infection. The localised nature of malaria transmission and the limited dispersal range of the mosquito vectors, within a few hundred to one or two kilometres, provide the advantage that vaccination of a relative small, localised group would cause a significant decrease in local transmission rates. Also, due to TBVs' attribute of preventing the escape of the parasites from the human host, if used in combination with pre-erythrocytic and blood stage vaccines, they could serve to obviate or reduce the spread of parasites with resistance developed against such vaccines and, thereby, prolong the effective lifespan of other malaria vaccines (Carter, 2001).

Under low endemic transmission conditions, a TBV program alone may be enough to eliminate malaria transmission within a locality. In high endemic areas, TBVs could make

eradication feasible with the concomitant implement of other interventions. The combination of a pre-erythrocytic and / or blood stage vaccine to prevent infection with a TBV would be an ideal vaccine strategy because it would reduce more effectively the rates of transmission and offer protection to vaccinated individuals (Carter, 2001). The establishment of a multi-target and multi-stage vaccine would relegate the low appeal to endemic communities arising from its altruistic concept (Saul, 2007).

Despite the theoretical problems cited above, and based on its promise for malaria elimination, preclinical and clinical development of transmission-blocking vaccines is underway. Thus, many more gametocyte or ookinete proteins are under investigation for their transmission blocking potential, currently (Pradel, 2007). In addition, mosquito proteins could be potential targets to raise transmission blocking antibodies, such as proteins directly binding to the parasite surface and mediate parasite killing, or alternatively, essential parasite-interaction partners that promote parasite development (Dinglasan and Jacobs-Lorena, 2008). The historical success of vector control underlines the importance of studying transmission and vector-host interactions in order to support the deployment of efficient malaria control methods.

CHAPTER 2: AIM OF THE PROJECT

CHAPTER 2: AIM OF THE PROJECT

As described previously, malaria is still a scourge for under-developed countries. Nevertheless, the understanding of the molecular processes during the parasite life cycle in both hosts increases. Consequently, there are promising perspectives of advances in the endeavour to the disease eradication.

The aim of this diploma project was to identify novel, sexually expressed *Plasmodium falciparum* genes that could regulate the parasite transmission. Genes of special concern were the ones indicating that play a significant role in interactions between the parasite and the vector or regulate the parasite development, during midgut invasion and ookinete-to-oocyst transition, when parasites encounter severe reduction in their numbers.

The project strategy consists of candidate gene selection mainly through the analysis of microarray transcriptional profiling, followed by functional characterisation of transgenic *P. berghei* parasites generated by targeted gene disruption (*P. falciparum* orthologs). The long-term goal of the project is the deepest understanding of the parasite behaviour during the mosquito midgut invasion and, hopefully, the identification of novel parasite targets for the generation of transmission blocking interventions.

CHAPTER 3: MATERIALS AND METHODS

CHAPTER 3: MATERIALS AND METHODS

3.1 Parasite strains, maintenance and manipulation

3.1.1 Parasite strains

For the generation of gene KO parasites by double homologous recombination, the parasite strain that was used was *P. berghei* ANKA clone 507 (referred to as *Pb*c507), (Janse et al., 2006a). The *Pb*c507 is a transgenic parasite line in which the expression of enhanced Green Fluorescent Protein (*e*GFP) is under control of native elongation factor-α loci promoter, resulting in constitutive expression of *e*GFP throughout the parasite lifecycle (Franke-Fayard et al., 2004). The 507 clone has no drug resistance marker (Janse et al., 2006a). This absence of resistance becomes an advantage in the selection of subsequent gene KO parasites by the use of the established *tgdhfr-ts* pyrimethamine selection system. As a consequence, it assists the phenotypic analysis of the generated transgenic parasite in a GFP background.

Genomic DNA from the *P. berghei* ANKA 2.34 (Wild-type, WT) clone was used for amplification of the upstream and downstream regions of target-genes and total RNA (converted to cDNA) from the same clone was used as template for RT-PCR.

3.1.2 Parasite maintenance

P. berghei parasites were maintained in Theiler's Original mice (TO) (Harlan, UK) as previously described (Sinden et al., 2002).

Intraperitoneal (IP) injection of 100-200 μL *P. berghei* infected blood was conducted for mice infection. Before transfection, reticulocyte proliferation was stimulated by IP injection of phenylhydrazinium HCl (PH); (6 mg/mL stock (Sigma) solution in Phosphate Buffer Saline (PBS); 10X stock solution of 0.2 M phosphate, 1.5 M NaCl, pH 7.4), in order for high parasitaemia / gametocytaemia to be induced.

P. berghei infected blood for passage, cryopreservation or experimental use was collected by cardiac puncture (using an 1ml syringe containing 0.05ml heparin (200 I.U./ml), Sigma), on mice under terminal anaesthesia (intramuscular injection (IM) of 0.05mL / 10g body weight of Rompun (2% stock solution, Bayer), Ketastet (100 mg / mL ketamine, Fort Dodge Animal Health Ltd.) and Phosphate Buffer Saline (PBS, Sigma) prepared to a ratio of 1:2:3), on day 3-4 of infection.

The course of parasitaemia was determined in Giemsa (Fluka) stained thin tail-blood smears, by counting the blood stage parasites at 100X magnification on a light microscope. The tail-blood films were air-dried, methanol fixed and Giemsa stained (1:5 dilution in Giemsa buffer (0.7% (w/v) anhydrous KH2PO4, 1% (w/v) anhydrous Na2HPO4)) for 20 min at room temperature.

All animal procedures were performed according to UK Home Office project license agreements by Valerie Chiamaka Ukekbu and Katarzyna Sala.

Cryopreservation

Parasite stocks were prepared at 1:2 ratio of infected heart-blood to freezing solution (90% v/v Alsever's solution (Sigma) and 10% v/v glycerol) and maintained at -80ºC. The stocks were thawed in room temperature, before immediate IP injection to mice.

3.1.3 Purification of mixed blood-stage parasites

Mixed blood-stage parasites were purified through lysis of red blood cells using ice-cold 0.17M NH4Cl (Sigma), followed by centrifugation at 200 g for 10 min. Parasite harvest was subsequently pelleted by 10 min centrifugation at 1500 g, supernatant removed and pellet washed in PBS.

3.1.4 Schizont cultivation

Blood was obtained from three anaesthetised mice infected with *P. berghei* ANKA clone 507 on day 1-2 of infection (parasitaemia ~5%), providing the blood-stage parasites for the transfections. The blood was immediately transferred to a tissue culture flask containing 100 mL of 75% (v/v) schizont culture medium RPMI1640 pH 7.3 (RPMI1640 with 25 mM HEPES, 2 mM L-glutamine, 24 mM NaHCO3 (Gibco)), 25% (v/v) foetal bovine/calf serum (FBS/FCS, Gibco) and 50 U / mL penicillin, 50 μg / mL streptomycin (PenStrep; Gibco). Then, the culture was gassed with CO_2 for 5 min and incubated overnight shaking gently (50-100 rpm) at 37°C.

3.1.5 Purification of viable schizonts

Overnight schizont culture was poured into 50 ml Falcon tubes and pelleted 15 min 180 g at room temperature. After removal of the majority of the supernatant, the pellet was resuspended in remaining supernatant and layered on top of a 15.2% (v/v) Nycodenz / PBS density gradient. Schizont-infected cells were separated from uninfected cells by 15.2% (v/v) Nycodenz / PBS density gradient centrifugation (30 min at 300 g). The resulting thin 'brown' layer including the mature schizonts and localizing at the interface between the two suspensions was collected, washed in 30 mL Schizont medium, pelleted at 300 g for 8 min and supernatant was discarded.

3.1.6 Ookinete in vitro cultivation

0.6-0.8 mL of infected mouse blood was collected from mice under terminal anaesthesia with a parasitaemia of >10 %. The blood was immediately transferred to a tissue culture flask containing 30 mL 80% (v/v) ookinete culture medium (RPMI1640 (Sigma), 25 mM Hepes (Gibco), 2 mM L-glutamine (Sigma), 0.2% (w/v) sodium bicarbonate (Sigma), 50 U / mL penicillin, 0.05 mg / mL streptomycin (PenStrep; Gibco), 50 mg / 77 mL hypoxanthine (Sigma), 100 μM xantheuric acid (pH 7.4, Aldridge) and 20% (v/v) FCS. Cultures were incubated at 19ºC for 24 hours for mature ookinete production.

3.2 Mosquito strains, maintenance, infections and tissue dissections

3.2.1 Mosquito strains and maintenance

A. gambiae N'gousho and *Anopheles stephensi* sda500 strains were cultivated according to standard methods (Sinden, 1997) and were reared and supplied by Tibebu Habtewold and Katarzyna Sala.

3.2.2 Mosquito infections

Blood feeding of mosquitoes on infected mice ensures natural infection. 3-7days post-emergence *A. gambiae* or *A. stephensi* females were infected with *P. berghei* after 3 hour or overnight starvation, respectively. Mosquitoes fed on anaesthetised mice with a parasitaemia of 2-10% and conspicuous gametocytaemia, while mice were mounted directly on the net-covered mosquito pots in the dark at 19ºC. After feeding was completed, mice were subjected to euthanasia by cervical dislocation while still sedated. Unfed females were removed 24 hours after the blood meal so to not interfere with the quality of infection and fed mosquitoes were maintained for 21-22 days p.i. feeding on solubilised fructose in in 19ºC, 70% humidity and 12 hours daylight cycle.

3.2.3 Mosquito midgut and salivary glands dissections

In order study the biology of mosquitoes and their responses to microorganisms, including the malaria parasites, it is required that the various tissues are dissected and examined in isolation.

Mosquito midguts were dissected in PBS under a dissection microscope, using dissecting forceps. The lower abdominal part of the mosquito was grasped and gently removed to reveal the midgut and separate it from malpighian tubules and foregut.

Salivary glands were dissected in the same conditions, by gently grasping the mosquito neck and carefully separating the head from the thorax. The salivary glands were eventually separated from the head.

3.3 Bacterial cultures

Bacterial culture aims at the massive production of genetically identical bacterial cells – clones, which thereafter will be used in specific biotechnological or molecular techniques. The provision of all the nutritional requirements of the cells is essential for the bacterial growth. The culture medium chosen for the culture must provide with carbon, nitrogen source, amino acids and several metal ions. Depending on the bacterial features and requirements, various time periods are appropriate for their growth and, also, different physical conditions, such as temperature, pH and oxygen, are required. Usually, the culture medium contains antibiotic in order to assist to the selection of the desirable clone.

The desired bacteria cells were plated onto Luria Broth (LB) agar (Merck) plates, including 50 μg/mL of appropriate antibiotic, under aseptic conditions (using a Bunsen burner).

A disposable inoculating loop was used for the bacteria spreading onto the petri dish. The plates were incubated at 37ºC overnight. Single colonies were collected and transferred in liquid LB medium (Merck) for overnight growth.

3.4 Nucleic acid isolation

3.4.1 Purification of mixed blood-stage parasites and parasite DNA extraction

P. berghei genomic DNA was extracted from infected RBCs, using Qiagen DNeasy Blood & Tissue Kit (Qiagen), following purification of RBCs from infected mouse blood. More specifically, ~200 μL of mouse blood collected by cardiac puncture were incubated in ice-cold 20 mL 1% RBC lysis buffer for 30 min. Parasites were subsequently pelleted by 10 min centrifugation at 200 g, supernatant removed and pellet washed in 200 μL PBS twice (centrifugation of 1 min at 13000 rpm). After washing, pelleted parasites were resuspended in 200 μL PBS and the protocol of Qiagen DNeasy Blood & Tissue Kit was followed. Briefly, 20 μL proteinase K and 200 μl Buffer AL (without added ethanol) was added and the sample was vortexed and incubated at 56°C for 10 min, prior to 200 μL ethanol (96–100%) adding and mixing by vortexing. Afterwards, the mixture was pipeted in 3 into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm. The DNeasy Mini spin column was placed in a new 2 ml collection tube (flow-through and collection tube were discarded) and 500 μL Buffer AW1 were added and centrifuged for 1 min at 8000 rpm. The DNeasy Mini spin column was placed in a new 2 ml collection tube (flow-through and collection tube were discarded), and 500 μl Buffer AW2 were added and centrifuged for 3 min at 14.000 rpm to dry the DNeasy membrane. The flow-through and collection tube were discarded once again and the DNeasy Mini spin column was placed in a clean 1.5 mL microcentrifuge tube, 50 μL Buffer AE were added directly onto the DNeasy membrane and incubated at room temperature for 1 min. A final centrifugation for 1 min at 8000 rpm was conducted to elute the parasite genomic DNA.

3.4.2 E. coli plasmid DNA extraction

Plasmid DNA was purified from the overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen) or the HiSpeed Plasmid Midi Kit (Qiagen), depending on the volume of the cultures, after centrifugation of the overnight cultures for 10 min at 3000 rpm.

3.4.2.1 QIAprep Spin Miniprep Kit Protocol using a microcentrifuge

Pelleted bacterial cells were resuspened in 250 μL Buffer P1, including RNase A, prior to 250 μL Buffer P2 adding and gentle mixing. This lysis reaction was incubated for 5 min at room temperature and, thereafter, 300 μL Buffer N3 were added and gently mixed. After centrifugation for 10 min at 13000 rpm the supernatant was transferred onto a QIAprep spin column for a second centrifugation for 1 min at 13000 rpm. The flow through was discarded and the column was washed by adding 750 μL Buffer PE, including ethanol. After disposal of the flow-through and an additional centrifugation for 1 min, elution of the plasmid DNA using 50 μL Buffer EB or dH_2O, at 13000 rpm was perfomed.

3.4.2.2 Qiagen HiSpeed Plasmid Midi Kit Protocol

Pelleted bacterial cells are resuspended in 6 ml Buffer P1, including RNase A, and 6 ml Buffer P2 are added and mixed thoroughly. After 5 min incubation at room temperature, 6 ml chilled Buffer P3 were added to the lysate and the sample was immediately and thoroughly mixed and poured into the barrel of a QIAfilter Cartridge. During 10 min incubation at room temperature, a HiSpeed Midi Tip was equilibrated by applying 4 ml Buffer QBT and allowing the column to empty by gravity flow. The QIAfilter outlet nozzle cap was removed and the plunger was inserted gently into the QIAfilter Midi Cartridge, in order for the cell lysate to be filtered into the previously equilibrated HiSpeed Tip. The cleared lysate was allowed to enter the resin by gravity flow. The HiSpeed Midi Tip was, subsequently, washed with 20 mL Buffer QC by gravity flow. DNA was eluted with 5 ml Buffer QF and, thereafter, precipitated by 5 min incubation with 3.5 mL room-temperature isopropanol. During the incubation the plunger from a 20 ml syringe was removed and the QIAprecipitator Midi Module was attached gently onto the outlet nozzle. The eluate/isopropanol mixture was transferred into the 20 ml syringe and filtered through the QIAprecipitator using the plunger, to a waste container. The QIAprecipitator and, then, the plunger were removed from the 20 ml syringe in order for the DNA to be washed with 2 ml 70% ethanol, following the same procedure as before. After two quick and forceful dryings of the membrane, the QIAprecipitator, was attached onto the outlet nozzle of a new 5 ml syringe and 500 μL of Buffer TE or dH_2O were used for the elution of the DNA through the membrane.

3.5 Nucleic acid manipulations

3.5.1 PCR purification

PCR products were purified using the QiaQuick PCR purification kit (Qiagen). Briefly, 5 volumes of Buffer PB to 1 volume of the PCR sample were added and mixed. The sample was applied onto a QIAquick spin column, in a 2 ml collection tube and centrifuged for 1 min at 13000 rpm. After the flow through discarding, the column was washed with 0.75 mL Buffer PE and dried at maximum speed. Finally, elution of DNA was ed using 30 μL Buffer EB (10 mM Tris·Cl, pH 8.5) or dH_2O by centrifugation for 1 min at 13000 rpm.

3.5.2 Ethanol precipitation of DNA (J. Sambrook, 2001)

1/10 volume of sodium acetate, pH 5.2, (final concentration of 0.3 M) and 2 volumes of cold 100% ethanol were added to the DNA sample. The sample was mixed well and incubated for 30 min on ice. Thereafter, the sample was centrifuged for 20 min at 14000 rpm and 4ºC and the supernatant was discarded. 400 μL ethanol 70% was added, the sample was centrifuged once more for 10 min at 14000 rpm and 4ºC and the supernatant was decanted carefully. The pellet was air dried and resuspended in the appropriate volume of dH_2O.

3.5.3 Photometric assessment of DNA concentration

Photometric assessment of nucleic acid concentration relies on the absorbance measurements made on a spectrophotometer. These measurements include the absorbance of all

molecules in the sample that absorb at the wavelength of interest. Since nucleic acids absorb at 260 nm, they will contribute to the total absorbance of the sample. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 – 2.0 is generally accepted as "pure" for DNA and RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

The concentration of DNA samples was determined in 1μL sample volume using the nucleic acid application of NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific).

3.5.4 Standard agarose gel electrophoresis

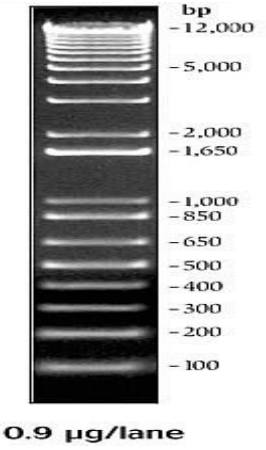

Figure 3.1. 1 Kb Plus DNA Ladder (Invitrogen)

Agarose gel electrophoresis is an easy and fast method for separation and visualisation of nucleic acid fragments, depending on their size. The principle of this method is based on the phenomenon of the migration of charged molecules into a field with an electric current towards the opposite charge. The phosphate molecules which compose the backbone of DNA molecules have a high negative charge. When DNA is placed on a field with an electric current, these negatively charged DNA molecules migrate towards the positive end of the field, which in this case is an agarose gel immersed in a buffer bath. The agarose gel is a cross-linked matrix which assists as a three-dimensional mesh causing a sieving action which allows separations of molecules based on molecular size. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the agarose gel. DNA is visualised in the gel by addition of special for DNA visualisation stains such as ethidium bromide. These stains bind strongly to DNA by intercalating between the bases and absorb UV light causing DNA fluorescence (Kakani, 2009).

DNA quality was assessed by standard agarose gel electrophoresis (J. Sambrook, 2001). 1% Agarose gel was prepared using a Tris / Borate / EDTA (TBE) buffer and 1X (final concentration) SYBR Safe DNA Gel Stain (Invitrogen), for DNA visualisation, and the ladder used for product length verification was 1 Kb Plus DNA ladder (Invitrogen) (Figure 3.1). For loading and tracking of DNA samples in the agarose gel, GelPilot DNA Loading Dye, 5X (Qiagen) was used.

3.5.5 Restriction digests

The natural role of restriction enzymes is to protect bacterial cells from invasion by foreign DNAs, especially infections by viruses. This is achieved by cutting double stranded DNA at specific 4 to 8 base pair inverted repeat recognition sequences within the target DNA. DNA cleavage by restriction enzymes yields 5'-phosphate and 3'-hydroxyl termini and the products are either blunt ended or contain 5' or 3' overhangs, depending on the restriction enzyme type.

In a 1.5 mL tube DNA, the appropriate buffer, BSA (if recommended by manufacturer), dH_2O up to total volume and, finally, the restriction enzyme(s) were added. Specifically, the final concentration of the reagents was 1X Buffer, 1X BSA, 5 units/μL enzyme and maximum 10

μg DNA. The sample was incubated at the appropriate temperature (25ºC or 37ºC depending on the restriction enzyme) for at least 1 hour.

3.5.6 DNA gel extraction

After electrophoresis, QIAquick Gel Extraction Kit (Qiagen) was used for the extraction of the desirable DNA molecules. Briefly, the desired gel piece was extracted using X-Tracta™ gel extraction tool and transferred into a 1.5 ml tube where 500 μL Buffer GQ was added and the sample was incubated at 50ºC for 5-10 min. The resulting mixture was applied to a column and centrifuged and the flow-through was discarded. The column was washed with 750 μL Buffer PE and dried by centrifugation. Finally, 30 μL Buffer EB was used for the elution of the DNA.

3.6 Plasmid cloning

Plasmids are circular, double-stranded DNA (dsDNA) molecules that are separate from a cell's chromosomal DNA. These extrachromosomal DNAs, which occur naturally in bacteria, yeast, and some higher eukaryotic cells, exist in a parasitic or symbiotic relationship with their host cell. The plasmids most commonly used in recombinant DNA technology replicate in *E. coli*. Generally, these plasmids have been engineered to optimise their use as vectors in DNA cloning. For instance, to simplify working with plasmids, their length is reduced; many plasmid vectors are only ≈3kb in length, which is much shorter than in naturally occurring *E. coli* plasmids. Most plasmid vectors contain little more than the essential nucleotide sequences required for their use in DNA cloning: a replication origin, a drug-resistance gene, and a region in which exogenous DNA fragments can be inserted (polylinker) (Lodish et al., 2000).

3.6.1 PCR-vector selection

Selection of the proper PCR-vector is based on the endings of the PCR product. More specifically, proof-reading polymerase creates blunt-ended PCR products in contrast to the products produced by Taq polymerase which have A (adenine) overhangs at their 3' ends. Therefore, blunt-ended PCR products can be ligated to linearised blunt-vector and Taq products can be ligated to linearised T-vector (carrying T overhangs at the 3' ends).

Herculase II Fusion (Agilent) PCR products, which are blunt-ended, were ligated to pCR-Blunt II-TOPO (Invitrogen) vector and GoTaq Green polymerase (Promega) products were ligated to pCR II-TOPO TA vector (Invitrogen).

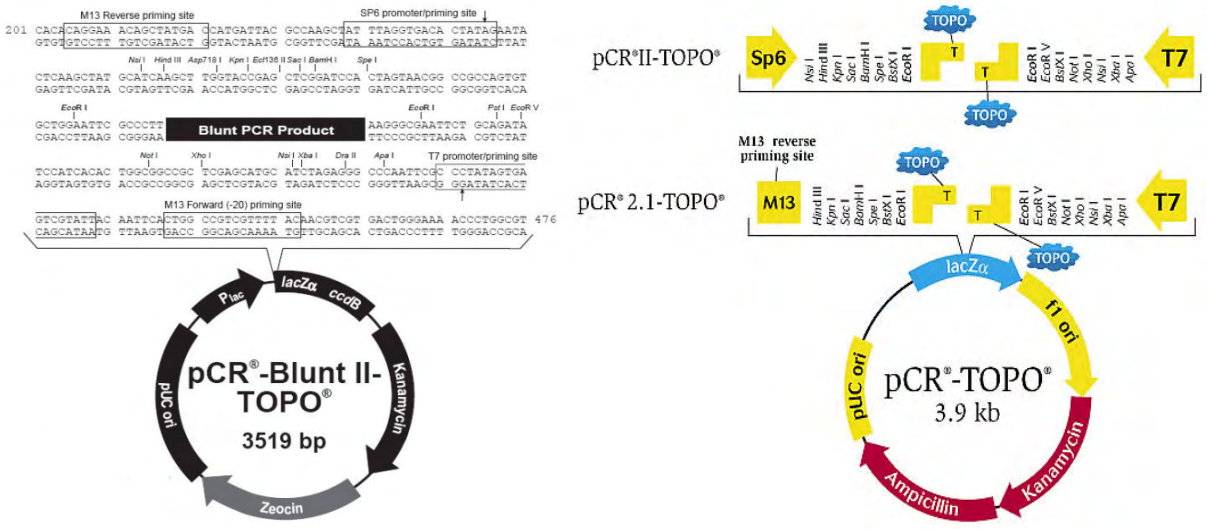

Figure 3.2. Maps of PCR-vectors used for plasmid cloning, indicating their components (drug resistance genes, ORI etc.) and highlighting the restriction sites.
Left map: pCR-Blunt II-TOPO vector (Invitrogen) used in ligation with blunt PCR products.
Right map: pCR II-TOPO TA vector (Invitrogen) used in ligation with Taq PCR products.

3.6.2 Ligations

The PCR products were cloned to the appropriate PCR-vectors by following the instructions of Zero Blunt PCR Cloning Kit (Invitrogen) or TOPO TA Cloning Kit (Invitrogen).

3.6.2.1 Zero Blunt PCR Cloning Kit protocol

5 μL of concentrated blunt PCR product were mixed with 1 μL pCR-Blunt II-TOPO vector (25 ng), 1 μL 10X Ligation Buffer (with ATP), 2 μL dH_2O (for a total volume of 9 μL) and, in the end, 1 μL T4 DNA Ligase (4 U/μL). The ligation reaction was incubated at 16°C for 1 hour and, then, ended on ice.

3.6.2.2 TOPO TA Cloning Kit protocol

4 μL of concentrated Taq PCR product were mixed with 1 μL pCR II-TOPO TA vector (10 ng), 1 μL salt solution (final volume 6 μL) and the ligation reaction was incubated for 5 min at room temperature (22–25°C).

3.6.3 Transformations

The transformation method which was followed was chemical transformation of DH5α Escherichia coli (Invitrogen) competent cells. Specifically, the vectors were transformed into DH5α cells, having kanamycin resistance, by heat shock methodology. Briefly, 50 μL of chemically competent DH5α cells were thawed on ice and 5 μL of the plasmid DNA were added.

The sample was incubated on ice for 30 min. The cells were heat shocked for 20 seconds in a 42°C water bath without shaking and, then, immediately placed on ice for 2 minutes. 250 μL S.O.C. medium (Invitrogen) were added to the cells and an incubation for 1 hour at 37°C and 225 rpm followed. Subsequently, the cells were plated onto 50 μg/mL kanamycin (Sigma) Luria Broth (LB) agar (Merck) plates and the plates were incubated at 37ºC overnight.

3.6.4 Identification of recombinant clones

The phenomenon of transformation permits plasmid vectors to be introduced into and expressed by *E. coli* cells. In order to be useful in DNA cloning, however, a plasmid vector must contain a selectable gene, most commonly a drug-resistance gene encoding an enzyme that inactivates a specific antibiotic. When the treated cells are plated on a petri dish of nutrient agar containing the antibiotic, only the rare transformed cells containing the antibiotic-resistance gene on the plasmid vector will survive. All the plasmids in such a colony of selected transformed cells are descended from the single plasmid taken up by the cell that established the colony.

The identification of transformed clones was assessed by the use of LB agar containing the antibiotic kanamycin (Sigma) 50 μg/mL.

3.7 Transfection vector cloning

3.7.1 Transfection vector

The plasmid vector used for parasite transfection was pBS/TgDHFR vector which contains:

1) Two polylinker sites, in which the homology regions, digested by the appropriate restriction enzymes, can be inserted

2) An antibiotic resistance marker (ampicillin resistance gene – AmpR) outside the replacement/marker cassette, assisting the selection of the transformed bacterial clones

3) A 5’ UTR and a 3’ UTR inside the replacement/marker cassette

4) A pyrimethamine resistance marker (*T. gondii* DHFR/TS gene) for the selection of the recombinant parasites.

The *Pbc*507 parasite line has no drug resistance marker, as mentioned earlier. This absence of resistance provides the opportunity to select subsequent gene KO parasites by the use of a drug selection system included in the integration construct. This drug selection system is provided by the pBS/TgDHFR vector.

DHFR/TS fusion gene encodes for the dihydrofolate reductase-thymidylate synthase, a bifunctional dimeric enzyme, essential for the proliferation and growth of bacteria, Plasmodia and normal and cancerous human cells. The role of dihydrofolate reductase is to regenerate folic acid into its reduced form tetrahydrofolate, and its inhibitors, such as pyrimethamine, have antibiotic, antimalarial and antineoplastic properties.

DHFR/TS gene derived from drug-resistant *Toxoplasma gondii* comprises a mutated around the active site of DHFR domain, confering resistance to pyrimethamine. Introduction of this gene into pyrimethamine sensitive *P. berghei* parasites results in a significant increase in

pyrimethamine resistance that allows for a relatively simple *in vivo* selection of transformed parasites procedure.

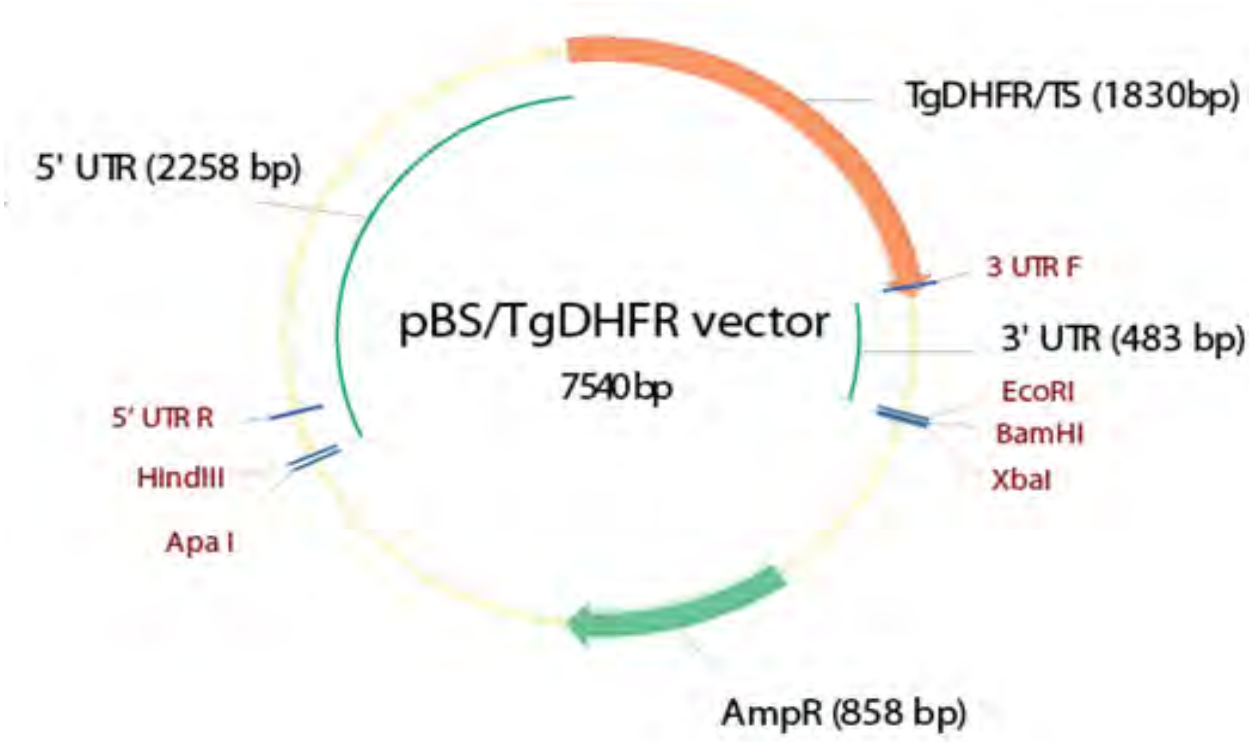

Figure 3.3. Map of pBS/TgDHFR vector indicating its components. The *T. gondii* DHFR/TS gene and the AmpR gene are highlighted in orange and green arrow, respectively and their length is shown in parenthesis. The restriction sites and enzymes and the primer sites and names are also illustrated. Also, the 5'UTR and the 3'UTR are represented by green lines and their length is shown in parenthesis.

3.7.2 Ligation

The targeting sequences and linearised transfection vectors were ligated using Quick Ligation Kit (New England Biolabs). Briefly, ~100 ng vector was combined with a 5-fold molar excess of insert and 10 µL 2X Quick Ligation Buffer, dH_2O to 19 µL final volume and, finally, 1 µL Quick T4 DNA Ligase were added. The ligation reaction was mixed thoroughly and incubated for 5 min at room temperature (22–25°C).

3.7.3 Transformation

The transformation method which was followed was chemical transformation of DH5α Escherichia coli (Invitrogen) competent cells, as described previously. The transformed cells were plated onto 50 µg/mL ampicillin (Sigma) Luria Broth (LB) agar (Merck) plates and the plates are incubated at 37ºC overnight.

3.7.4 Identification of recombinant clones

The identification of transformed clones was assessed by the use of LB agar containing the antibiotic ampicillin (Sigma) 50 μg/mL.

3.8 PCR

Polymerase Chain Reaction or PCR is an in vitro technique based on the principle of DNA polymerisation reaction that allows exponential amplification of a specific DNA fragment, resulting in millions of copies. It relies on thermal cycling, consisting of repeated cycles of heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA using thermostable DNA polymerase, primers (short DNA fragments complementary to target region), dNTPs, appropriate concentration of $MgCl_2$ (Mg^+ ions are essential for the polymerase operation) and a suitable buffer. It was invented by Kary Mullis in 1993 who was awarded the Nobel Prize in Chemistry for developing PCR.

"Beginning with a single molecule of the genetic material DNA, the PCR can generate 100 billion similar molecules, in an afternoon. The reaction is easy to execute. It requires no more than a test tube, a few simple reagents, and a source of heat. The reaction is easy to execute. It requires no more than a test tube, a few simple reagents, and a source of heat" (Mullis, 1990).

In a PCR tube, on ice, x ng DNA template (~30 ng plasmid DNA or ~300 ng genomic DNA), 10 μL 5X PCR Buffer (including Mg^{2+}), 0.5 or 1 μL dNTP mix 10 mM (10 mM each dATP, dTTP, dGTP, dCTP), 1.25 μL forward primer 10 μM, 1.25 μL reverse primer 10 μM, 1 μL polymerase 5u/μl and dH_2O to 50 μL final volume were mixed. Thorough mixing and brief centrifugation followed.

The standard PCR programme was comprised of: A denaturation step of 2 min at 95ºC followed by 30 cycles comprising of 30 seconds at 95ºC (denaturation), 45 seconds at, approximately, 5 ºC below the lowest Tm of the primers (primer annealing – approximately 5 ºC below the lowest Tm of the primer) and 1 min and 30 seconds at 72ºC (elongation) and concluded by a final elongation step of 3 min at 72ºC.

3.8.1 Primer sequences for PCR

PCR primers, generally, range in length from 15–30 bases and are designed to flank the region of interest. Primers should contain 40–60% (G + C), and care should be taken to avoid sequences that might produce internal secondary structure. The 3´-ends of the primers should not be complementary to avoid the production of primer-dimers, because primer-dimers unnecessarily delete primers from the reaction and result in an unwanted polymerase reaction that competes with the desired reaction. Ideally, both primers should have nearly identical melting temperatures (Tm); in this manner, the two primers should anneal at roughly the same temperature.

Following the restrictions mentioned, there was manual design of gene specific PCR and RT-PCR primers based on the available *P. berghei* genome sequence and annotation.

3.8.1.1 RT-PCR

The gene specific RT-PCR primers, following the cDNA synthesis, were designed to amplify the unique exons of the subject genes (**Table 3.1**). The Tm was 50.8-53.9ºC and the length of the products of, approximately, ~0.2-0.6 Kb.

Primer name	**Sequence (5′ to 3′)**
Pb_c57 F	GAAGATAGTGATAGAAACAGTAG
Pb_c57 R	GACTGAAGAGTTTTCTACATATG
Pb_c01 F	CATTTTCCAAAGTATTCATATAC
Pb_c01 R	CTATAACCGTCTATTATTATTTTTC

Table 3.1. Sequences of the primers used in RT-PCR. All primers are listed in a 5' to 3' direction.

3.8.1.2 Diagnostic PCR of transfectants

Diagnostic PCRs of transfectants were performed by using gene-specific forward (int fw) primers designed upstream of homology region A, in combination with the TgDHFR-TS 5'UTR reverse primer (**Table 3.2**). This primer positioning ensures that a product can only be obtained if integration taken place. Also, gene-specific reverse (wt r) primers were designed downstream of homology region A, within the coding region, which, in combination with the int fw primers, generate a product only if integration not taken place. Tm of the manually designed primers was 53.6-64.6 ºC and the resulting products had length of ~0.9 bp.

Primer name	**Sequence (5′ to 3′)**	**Description**
Pb_c57 int fw	GATGTCTAGCTAATTTGGGAATTAGTG	109 bp upstream of *Pb_c57* A
Pb_c57 wt r	GCATTATCTCTATCTTCATAATTTG	218 bp downstream of *Pb_c57* A
Pb_c01 int fw	GGATGTATTCCTATAACTATGTG	294 bp upstream of *Pb_c01* A
Pb_c01 wt r	CTCCTTGTATGTTGCTGCTGG	111 bp downstream of *Pb_c01* A
tgdhfr-ts 5'UTR R	GATGTGTTATGTGATTAATTCATACAC	200 bp into the 5'UTR of *tgdhfr-ts*

Table 3.2. Primers for diagnostic PCR of transfectants. The position of the primers is presented in the description column. All primers are listed in a 5' to 3' direction.

3.8.1.3 PCR amplification of homology regions and colony PCR of E. coli transformants

Gene specific primers with restriction enzyme site overhangs were manually designed with a Tm of 61.0-73.7ºC (**Table 3.3**). The homology regions corresponded to, approximately, 430-750 bp of the 5' UTR and 3'UTR of the target-genes or the most upstream and downstream coding sequences, depending on the available ORF.

Primer name	**Sequence (5′ to 3′)**	**Description and restriction enzyme**
Pb_c57 a	TTGGGCCCCTAATATATTATACATAGACAATTGCTC	Disruption upstream target (A) *ApaI*
Pb_c57 b	CCAAGCTTCATATATTGATATATGTATTTTAC	Disruption upstream target (A) *HindIII*
Pb_c57 c	TGAATTCGGTACCATATAACTATTCTGAAGCATATG	Disruption downstream target (B) *EcoRI*
Pb_c57 d	TTGGATCCGCTCATCATATAGAAAGTGGTATAATATATG	Disruption downstream target (B) *BamHI*
Pb_c01 a	TTGGGCCCCCAATACAATAGTATGATAC	Disruption upstream target (A) *ApaI*
Pb_c01 b	CCAAGCTTCCTCATTATTATAACTGGATGC	Disruption upstream target (A) *HindIII*
Pb_c01 c	TGAATTCGAAACATAGTTTATGCATATTTTATG	Disruption downstream target (B) *EcoRI*
Pb_c01 d	TTGGATCCGAAATTTAATCTTCCTCAATTAAG	Disruption downstream target (B) *BamHI*

Table 3.3. Primers for generation of *ΔPb_ c57* and *ΔPb_c01* transgenes. Where appropriate, target restriction sites are underlined. The appropriate restriction enzyme is presented in the description column. F, forward; R, reverse. All primers are listed in a 5′ to 3′ direction.

3.8.2 Reverse Transcription PCR transcriptional profiling

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of PCR commonly used in molecular biology, providing the possibility to assess gene transcription in cells or tissues. RT-PCR is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA complement through the use of reverse transcriptase. Subsequently, the newly synthesised cDNA is amplified using traditional PCR.

3.8.2.1 cDNA production

Total RNA extracted from *P. berghei* ANKA 2.34 (WT) parasites obtained 1, 6, 10 and 24 hours post-infection of *A. gambiae* N'gousho mosquito midguts (kind gift from Rajeev Rai, master student, Imperial College London) was used for generation of complementary double-stranded cDNA through reverse transcription. The cDNA synthesis was performed using SuperScript™ III Reverse Transcriptase (Invitrogen) and a combination of random hexamers and oligodT primers, according to manufacturer's instructions. Briefly, ~5 μg total RNA, primers, 1 μL dNTP Mix (10 mM) and dH_2O to 13 μL were mixed and heated at 65ºC for 5 min before adding 4 μL 5X First-Strand Buffer, 1μL 0.1 M DTT and 2μL SuperScript™ III Reverse Transcriptase and 60 min incubation at 50ºC. The reaction was heat inactivated at 70ºC for 15 min and the resulting cDNA served as template for RT-PCR.

3.8.2.2 Procedure of RT-PCR

GoTaq Green DNA polymerase (Promega) and gene specific primers were used to perform qualitative RT-PCR, at the proper annealing temperature.

3.8.3 PCR amplification of homology regions

PCR amplification of upstream (A) and downstream (B) homology regions for the introduction of targeted gene disruption through homologous recombination was accomplished using Herculase II Fusion polymerase (Agilent) or GoTaq Green DNA polymerase (Promega).

3.8.4 Colony PCR of transformed E. coli

Colony PCR is a quick method of screening for plasmid inserts directly from E. coli colonies. Also, it is useful in order to determine insert size. As a result, recombinant transformed *E. coli* colonies were identified through diagnostic colony PCR. GoTaq Green DNA polymerase (Promega) and the same gene specific primers which were used for the initial amplification of upstream (A) and downstream (B) homology regions were also used for the diagnostic colony PCR.

In a PCR tube, on ice, 5 μL of the colony were mixed with 5 μL 10X Buffer, 0.5 μL dNTP mix 10 mM (10 mM each dATP, dTTP, dGTP. dCTP), 1 μL forward primer 10 μM, 1 μL

reverse primer 10 μM, 12 μL dH_2O (for a total volume of 24.5 μL) and, in the end, 0.5 μL polymerase 5u/μl.

The PCR programme was as follows: An initial cell breakage and DNA denaturation step of 5 min at 95ºC followed by 30 cycles comprising of 30 seconds at 95ºC (denaturation), 45 seconds at, approximately, 5 ºC below the lowest Tm of the primers (primer annealing – approximately 5ºC below the lowest Tm of the primer) and 1 min and 30 seconds at 72ºC (elongation) and concluded by a final elongation step of 3 min at 72ºC.

3.8.5 Diagnostic PCRs of transfectants

GoTaq Green DNA polymerase (Promega) was used and PCR procedure was performed as previously described (at the appropriate annealing temperature).

3.9 Ttransfection procedure (Janse et al. (2006))

3.9.1 Preparation of schizonts

Prior to starting transfections, the parasite quality and quantity was verified by a Giemsa stained smear. Viable schizonts were purified from schizont overnight cultures, as described before. The pellet was re-suspended in schizont culture media, split into 1 mL aliquots and spun down for 1 min at 200 g.

3.9.2 AMAXA transfection procedure / electroporation

The supernatant from previous step was discarded and the pellet re-suspended in 100μL AMAXA supplemented nucleofector (AMAXA) containing the DNA which was to be transfected. Exactly 100 μL of the DNA / buffer / parasite solution were transferred to an electroporation cuvette (AMAXA) and electroporation was performed according to Protocol U33; AMAXA. The electroporated parasites were transferred to 150 μL of uninfected mouse blood (collected from a PH treated mouse on day 2-3 after treatment) and were incubated at 37ºC for 20 min to allow invasion of merozoites into erythrocytes before IP injection of the suspension into a mouse.

3.9.3 Pyrimethamine-based selection of transgenic parasites

Twenty-four to thirty hours after ransfection, 0.07 mg / pyrimethamine (Sigma) was administered orally with the drinking water of host mice in order to select the pyrimethamine-resistant (transgenic) parasites. The selection was performed in both of the mice used for the parasite production (one passage in between).

3.9.4 Limited dilution cloning of transgenic parasites

Clonal transgenic parasite populations (derived from one cell) were obtained by limited dilution cloning (Waters et al., 1997a). Infected tail blood was diluted in 10 mL schizont culture medium and blood-stage parasites were enumerated on a haemocytometer. The diluted sample was further diluted in schizont culture medium to a final concentration of 20 parasites / mL, prior to IP injection (200 μL) into ten TO mice.

3.10 Phenotypic analysis of mutants

3.10.1 In vitro phenotypic analysis

3.10.1.1 Exflagellation assay

Exflagellation was examined on day 4–5 post-infection. Exflagellation assays were conducted by transferring 4 μL of parasite infected tail blood in 150 μL ookinete culture medium. After 10 min of incubation at room temperature, exflagellation was observed by phase contrast microscopy at 40X magnification in a standard haemocytometer (Hausser Scientific, USA), where enumeration of exflagellation centres and RBCs (proportion of exflagellating male gametocytes) was done. In parallel, male gametocytaemia (total proportion of male gametocytes) was determined by Giemsa stained tail-blood smears. These two resulting proportions were compared and the percentage of male gametocytes that exflagellated was calculated.

3.10.1.2 Ookinete conversion assays

3.10.1.2.1 Ookinete conversion assay, method no1

The total female gametocytaemia (total proportion of female gametocytes) was determined in Giemsa stained thin tail-blood smears. The number of ookinetes was determined by counting the number of live ookinetes and number or RBC (proportion of ookinete converted gametocytes) on a standard haemocytometer at 40X magnification, 24h after *in vitro* cultivation of infected blood (described earlier), of the same mouse, in ookinete culture medium. These two proportions were directly compared and the conversion rate was resulted.

3.10.1.2.2 Ookinete conversion assay, method no2

After 24 hour ookinete cultivation, macrogamete to ookinete conversion assays were implemented using a haemocytometer in ookinete medium, using a Cy3 conjugated P28 (13.1) monoclonal antibody (mAb13.1), as previously described (Billker et al., 2004). After centrifugation at 500g for 5 min of 0.5 mL of the ookinete culture samples, the pellets were resuspended in 0.5 mL mAb13.1 diluted to 1:500 in ookinete medium and incubated on ice for at least 50 min. The total number of fluorescent ookinetes was divided by the total number of fluorescent macrogametes resulting in the conversion ratio.

3.10.2 In vivo phenotypic analysis

3.10.2.1 Mosquito midgut sample preparations for enumeration and imaging

Following dissection, midguts were immediately mounted in PBS on glass slides under cover-slips. Alternatively, midguts were fixed in 4% formaldehyde (v/v) (16% methanol free, ultra-pure stock diluted in PBS, Polysciences Inc.) for 20 min at room temperature before washing in cold PBS three times for ten minutes each. Afterwards, formaldehyde fixed midguts were placed in Vectashield Mounting Medium with or without DAPI (Vector Labs) on glass slides under sealed cover-slips and slides were stored in the dark at 4ºC until imaging. Oocyst counting was conducted with the aim of using a minimum of 50 infected midguts per parasite genotype and sporulating oocysts were imaged with the aim of using a minimum of 3 infected midguts per parasite genotype.

3.10.2.2 Sporozoite enumeration

Salivary gland sporozoite numbers were calculated from homogenated *P. berghei* infected *A. stephensi* or *A. gambiae* salivary glands, assayed in three batches of ten salivary glands on day 19-22 post-infection. Dissected salivary glands were immediately transferred to a 0.1mL glass tissue homogeniser (Jencons England, VWR), containing 100 µL of ice-cold PBS and mechanically homogenised to release the sporozoites. The final volume of the suspension was carefully measured using a Hamilton syringe (Hamilton Company Ltd.) and 10 µL were loaded onto a haemocytometer. The calculation of the total number of sporozoites per 10 mosquitoes was assisted by the final volume measurement.

3.10.2.3 Transmission to mice

Sporozoite infectivity was assayed by bite-back experiments, were *P. berghei* infected mosquitoes (day 18 and 21 post-infection) were allowed to feed on anaesthetised TO or C57BL/6 mice for 15 min. The mice were then allowed to recover and parasitaemia was monitored, by Giemsa stained tail-blood smears, on day 7-10 following the potential infection. Infected or uninfected mice were culled at the end of the experiment.

3.10.3 Microscopy (parasite enumeration and imaging)

After *P. berghei* infected midguts dissection, oocysts were enumerated at X10 or X20 magnification and the sporulating oocysts were imaged at X20 magnification (light and fluorescence microscopy; *Pbc507* GFP reference lines). Also, ookinetes and macrogametes rendered in the in vitro ookinete conversion assay were counted at X20 magnification (fluorescence microscopy; Cy3 monoclonal antibody). Parasites were imaged using a Zeiss AxioImager M2 (Carl Zeiss, Inc) microscope using fluorescence microscopy. Images captured using a Zeiss AxioCam HRc (Carl Zeiss, Inc) camera coupled to Zeiss Axiovision40 version 4.6.1.0 software.

3.11 Bioinformatics

3.11.1 Analysis of P. falciparum gene expression in A. gambiae and A. arabiensis

For the analysis and visualisation of microarray data of *P. falciparum* gene expression in *A. gambiae* and *A. arabiensis*, Cluster 3.0 and TreeView were used.

Cluster and TreeView is an integrated pair of programs for analysing and visualizing the results of complex microarray experiments. They are both developed by Michael Eisen while at Stanford Univeristy. Cluster offers a variety of types of cluster analysis and other types of processing on large microarray datasets and TreeView graphically browses results of clustering and other analyses from Cluster. Cluster 3.0 is an enhanced version of Cluster, developed by Michiel de Hoon of the University of Tokyo (de Hoon et al., 2004) (Saldanha, 2004).

Four distinct groups of parasite transcripts displaying significantly differential regulation upon timepoints (>0.7 in log2 scale) were generated, according to their developmental pattern with Self-Organizing Map clustering, via "euclidian distance" similarity metric method (initial tau value: 0.02) using Cluster 3.0. TreeView was utilised for the visualisation of the clustering results.

3.11.2 Candidate gene selection and in silico analysis

PlasmoDB is a functional genomic database for *Plasmodium spp*. that provides a resource for data analysis and visualisation in a gene-by-gene or genome-wide scale. PlasmoDB belongs to a family of genomic resources that are housed under the EuPathDB Bioinformatics Resource Center (BRC) umbrella. The latest release, PlasmoDB 5.5, contains numerous new data types from several broad categories annotated genomes, evidence of transcription, proteomics evidence, protein function evidence, population biology and evolution (Aurrecoechea et al., 2009). Sequence, general data and *Plasmodium* orthologs were queried by the use of gene IDs. (http://plasmodb.org/plasmo/).

Also, protein structure prediction was assayed by PROTEUS2, for further investigation for potential domains, such as secretory and/or transmembrane domains. PROTEUS2 is a web server designed to support comprehensive protein structure prediction and structure-based annotation. PROTEUS2 accepts either single sequences (for directed studies) or multiple sequences (for whole proteome annotation) and predicts the secondary and, if possible, tertiary structure of the query protein(s). Unlike most other tools or servers, PROTEUS2 bundles signal peptide identification, transmembrane helix prediction, transmembrane beta-strand prediction, secondary structure prediction (for soluble proteins) and homology modeling (i.e. 3D structure generation) into a single prediction pipeline (Montgomerie S. et al., 2008).

3.11.3 Statistical analysis

Basic statistic values (e.g. median, mean, standard deviation etc.) were calculated using Microsoft Excel, Microsoft.

Statistic tests, such as χ^2 test and U-test (Mann-Whitney), as well as graphing were performed by GraphPad Prism 5. GraphPad Prism 5 is a scientific 2D graphing and statistics software. It combines basic biostatistics, curve fitting and scientific graphing and is published by GraphPad Software, Inc.

CHAPTER 4: RESULTS

CHAPTER 4: RESULTS

4.1 Project strategy

Aiming at the identification of possible *P. falciparum* targets for the generation of transmission blocking interventions, *P. falciparum* transcriptome during the stages when transmission bottleneck occurs should be analysed. Thus, the first step of the project strategy includes the selection of candidate genes based on the *P. falciparum* genes expression upon mosquito infection, as illustrated by transcriptomic microarrays, and further in silico analysis. Subsequently, after the selection of the candidate genes, transgenic *P. berghei* parasites by targeted disruption of the homologous genes are generated in order to examine the impact of the gene deletion, to the parasite development and survival. *P. berghei* is used for this study due to the easy and safe manipulation of malaria infected mosquitoes (Sinden, 1996) and the facilitation to the experimental analysis of gene expression patterns, resulted from the unnaturally high infection levels in *P. berghei* compared to those in *P. falciparum* (Dong et al., 2006). Also, in contrast to *P. falciparum,* it is also possible to easily *in vitro* cultivate and purify different *P. berghei* vector stages (Janse and Waters, 1995). The phenotypic characterisation of the generated mutant parasites could provide information about the significance and the possible role of the knocked-out genes to the parasite development and/or parasite-mosquito interactions during the outlined stages.

During this project, four candidate genes were selected through *in silico* analysis but the gene disrupted mutant parasites of only two of them were generated. The other two lines of KO mutant parasites were generated by R. Rai. All four transgenic parasite lines were phenotypically analysed during this study.

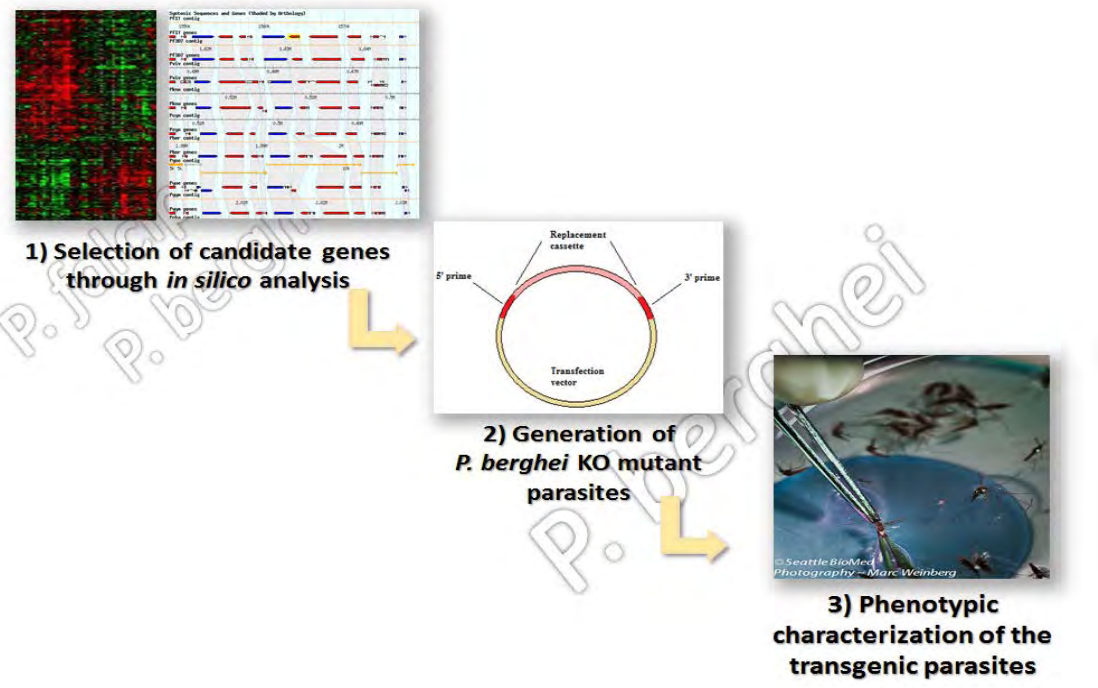

Figure 4.1. Illustration of the project strategy. 1) In silico analysis for the selection of the *P. falciparum* candidate genes for further phenotypic characterisation. 2) Generation of transgenic *P. berghei* parasites by targeted gene disruption (*P. falciparum* orthologs). 3) Phenotypic analysis of the generated KO parasites.

4.2 Selection of *P. falciparum* candidate genes

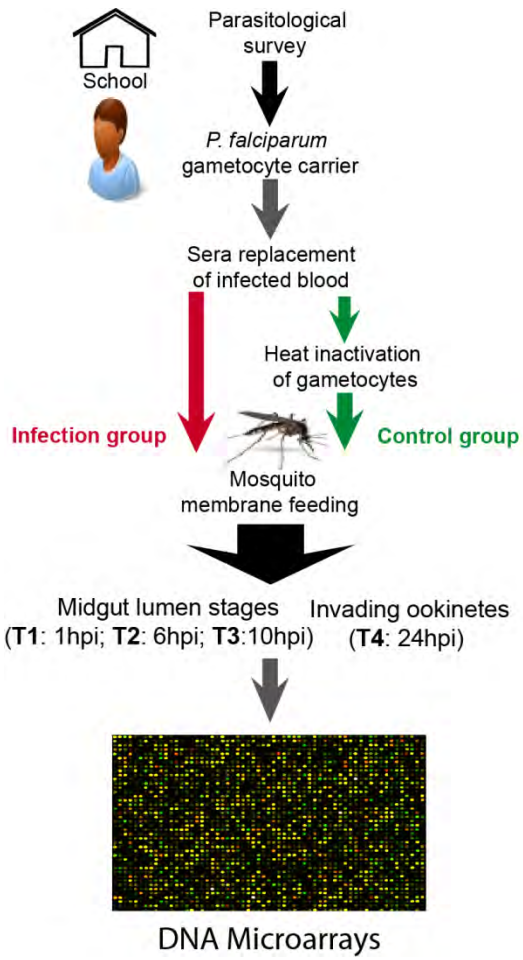

Figure 4.2. Experimental strategy of microarray assay design and. (Details in the main text).

The experimental strategy of the microarray assay (D. Vlachou, unpublished data), results of which are considered in this project, was initiated in Burkina Faso, by parasitological surveys for gametocyte carriers, in collaboration with local medical groups in primary schools. The collected blood samples, containing only one *Plasmodium* species, were subjected to serum replacement, with the aim of elimination of transmission blocking factors included in the blood plasma. The samples were divided in two experimental groups: the infection group, in which mosquitoes were infected with infectious parasites, and the control group, in which mosquitoes were infected with heat-inactivated (non-infectious) gametocytes, via membrane feeding in both cases. Infected mosquito midguts were dissected for total RNA extraction in four distinct time points, from both experimental groups. The four time points correspond to the midgut lumen stages of *Pasmodium* developement: gametogenesis and zygote formation (T1); zygote maturation and early motility (T2); ookinete locomotion (T3); midgut invasion and ookinete-to-oocyst transition (T4). This experimental assay was performed for both *A. gambiae* and *A. arabiensis* infections with sympatric *P. falciparum* populations. In order to identify the molecular interactions between mosquito and parasite, at the mentioned time points during these infections, a high-throughput screening approach was followed, using transcriptomic microarray technology.

Data extracted from analysis of the previously described microarrays (Nikolaos Trasanidis, final year project thesis, 2013), revealed *P. falciparum* transcript expression in the four distinct timepoints (1h, 4h, 10h and 24h) upon infection in the two major vectors *A. gambiae* and *A. arabiensis.*

SOM-based clustering performed in significantly differential regulated genes disclosed four major developmental patterns, similar in both infection types (Figure 4.3). Interestingly, two of the four clusters demonstrated a peak in specific developmental timepoint, implying a time-specific role of the genes involved. Specifically, transcripts included in C2 cluster display a developmental enhancement in 10h post-infection (ookinete stage prior to midgut invasion) and transcripts included in C4 cluster in 24h post-infection (ookinete stage during midgut invasion). Genes expressing the implying transcripts provide an intriguing pool for further functional characterisation, due to their potentially significant role to these stages of the parasite life cycle and/or the mosquito parasite interactions developed in the midgut.

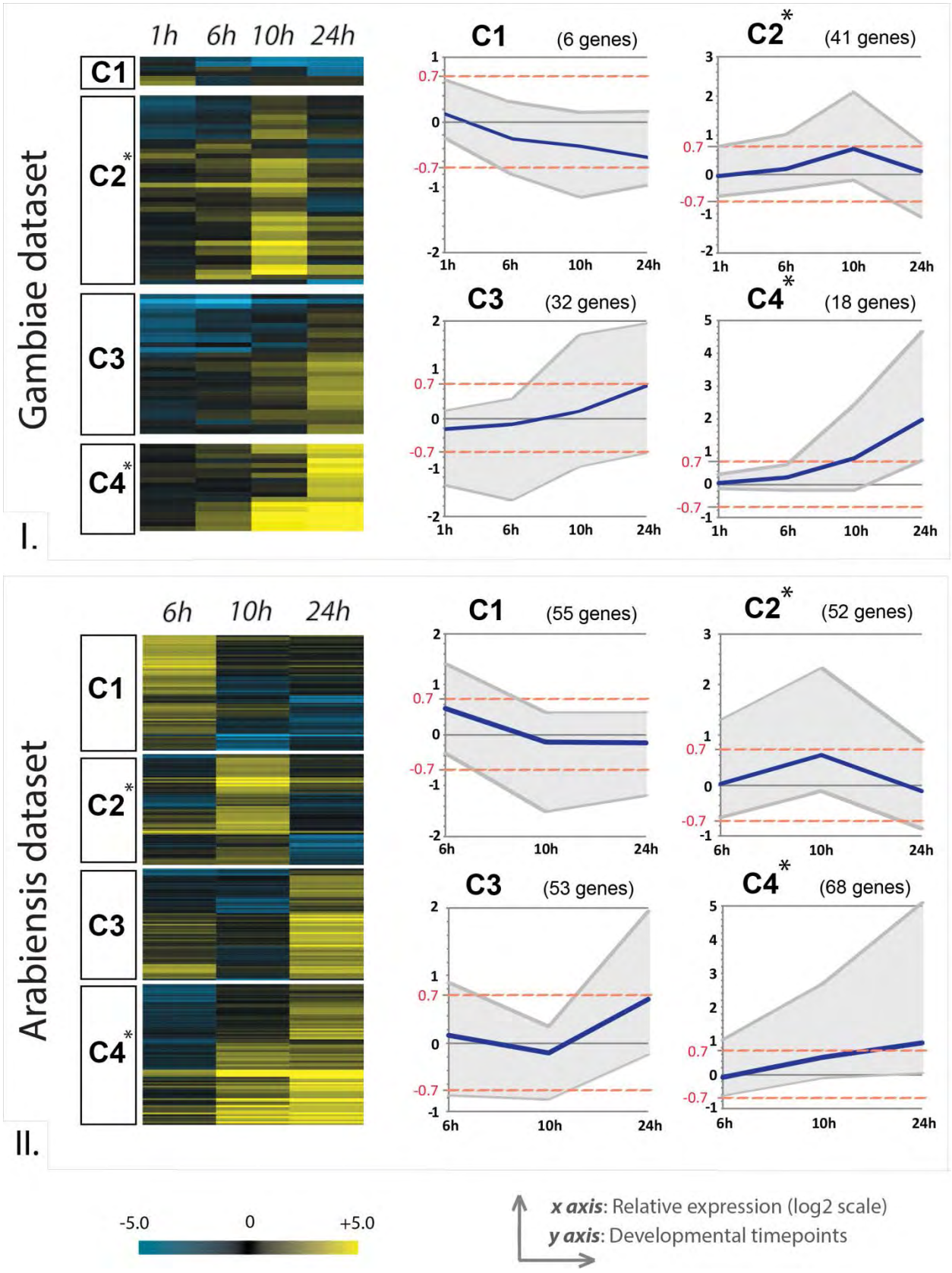

Figure 4.3. SOM-based clustering of significantly (0.7) differentially regulated Plasmodium genes. Stars indicate the gene clusters of high interest, from which the final candidate genes were selected. Left: Heatmap visualisation and SOM organisation. Right: Developmental trends of each cluster. The bold blue line represents the average expression of the genes included in the cluster, the gray area depicts the genes' range (Maximum to Minimum) of expression.

As mentioned earlier, although, the rodent parasites are not of direct practical concern to human, *P. berghei* is a practical model for the experimental study of mammalian malaria, due to their trend to be analogous to the human malarias in most essential aspects of structure, physiology and life cycle (Carter and Diggs, 1997). This is the reason why the existence of *P. berghei* orthologs of the candidate list genes has been an important criterion to the selection of the candidate genes. Specifically, information on the presence of *P. berghei* orthologs was provided by pre-computed BLAST results on PlasmoDB, assisting to the limitation of the number of the genes discriminated upon their expression profile and included in the previous pool.

Data, considering the *P. berghei* orthologs, from *P. berghei* (GFP_{CON}) infected *A. gambiae* (Yaounde) developmental arrays (D. Vlachou, unpublished data) were collected in order for investigation about the expression profile in *P. berghei* to be done. The microarrays were reflecting distinctive *P. berghei* developmental stages: mixed asexual and sexual bloodstages (1 hour PI), invading ookinete (24 hours PI), young oocysts (2 days PI), mid-oocyst (5 days PI), mature oocyst (10 days PI) and sporozoite release in the haemocoel (13-15 days PI).

Transcriptomic microarrays illustrating the expression profiles of *P. berghei* WT 2.34 strain and the 2.33 non-gametocyte producing parasite strain at 1 hour PI (mixed asexual and sexual bloodstages-gametocytes) of A. gambiae (Yaounde) (D. Vlachou, unpublished data), provided evidence about the expression of the genes in the first sexual stage of the parasite, the gametocytes.

Additionally, the expression profile of the genes was further examined considering published and unpublished transcriptome and proteome data about *P. falciparum* and *P. berghei* (Hall et al., 2005; Khan et al., 2005; Raibaud et al., 2006; Lasonder et al., 2002; Lasonder et al., 2008; Le Roch et al., 2003; Young et al., 2005). This provided further assistance to the selection of genes showing similar expression trends and regulation during the sexual development of the parasite.

After this investigation of the expression profile of the genes and considering all the information yielded, the list gene candidates for functional analysis by targeted gene disruption was finally restricted to four preponderant genes, *Pf_c57*, *Pf_c01*, *Pf_c53* and *Pf_c22*, which show significantly differential expression among the four examined time points and a developmental peak in one of these (Figure 4.4). Specially, of great consideration was a high expression in T3 or T4 time point, as described before, implying a potential significant role in these developmental parasite stages and/or the mosquito parasite interactions during these stages.

Pf_c53 and *Pf_c22* are differentially overexpressed at 10 hours post-infection, meaning in the ookintete maturation and locomotion stage, while *Pf_c57* and *Pf_c01* show a differential overexpression at 24 hours post-infection, in the midgut invasion and ookinete-to-oocyst transition stage (Figure 4.4).

Also, *Pb_c53* and *Pb_c22* show significantly high expression in the gametocyte stage, while no data were available for *Pb_c57* expression in gametocytes. According to the developmental arrays, *Pb_c53* is highly expressed in the first sexual stages, *Pb_c22* during the midgut invasion and the sporozoite release in the haemocoel stage and *Pb_c57* in the invading ookinete, while downragulated in other stages. No data about *Pb_c01* expression were available from these two microarray experiments.

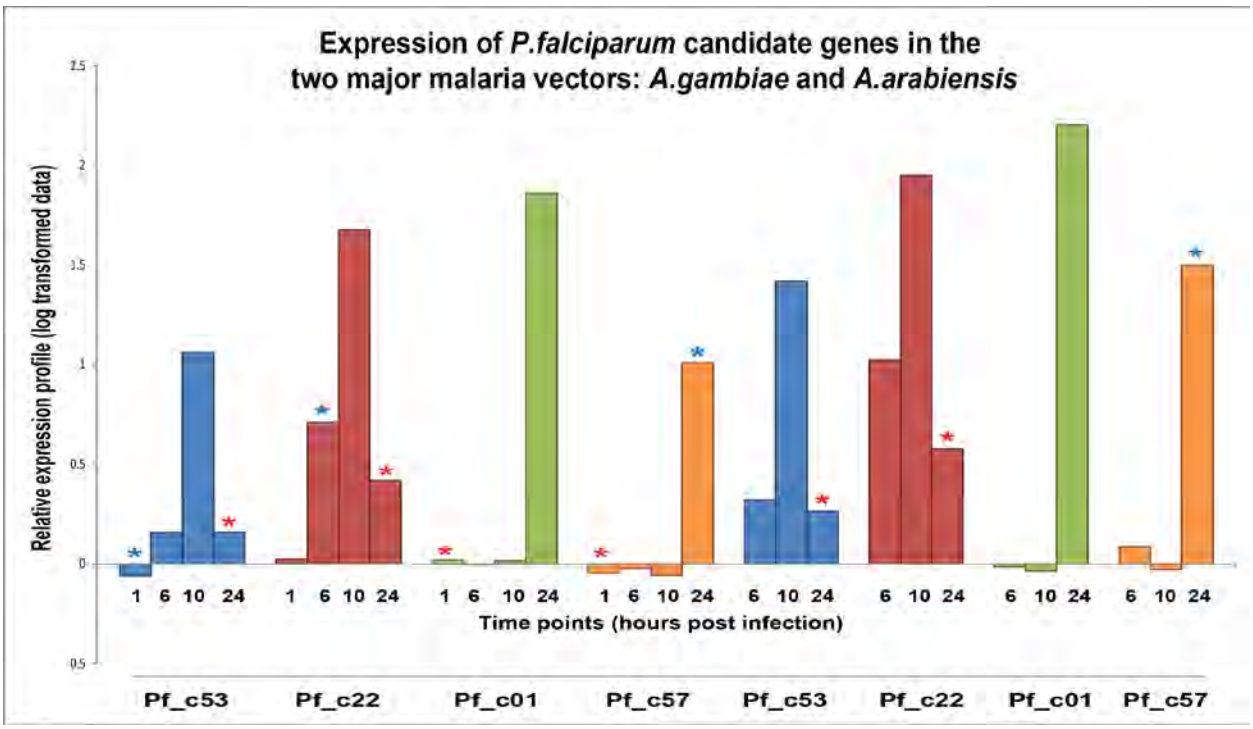

Figure 4.4.Transcriptional profiling of *P. falciparum candidate* genes in *A. gambiae* and *A. arabiensis*. Distinctive peaks of relative expression (log transformed data) of *Pf_c53* and *Pf_c22* at 10 hours post-infection, corresponding to ookinete pre-invasion stage and of *Pf_c57* and *Pf_c01* at 24 hours post-infection, corresponding to ookinete during the midgut invasion and ookinete-to-oocyst transition stage (data obtained from microarray analysis). The red stars indicate proteome derived data and the blue stars transcriptome derived data revealing expression of these genes in the stages highlighted. Vertical axis: relative expression in $\log_2$ scale.

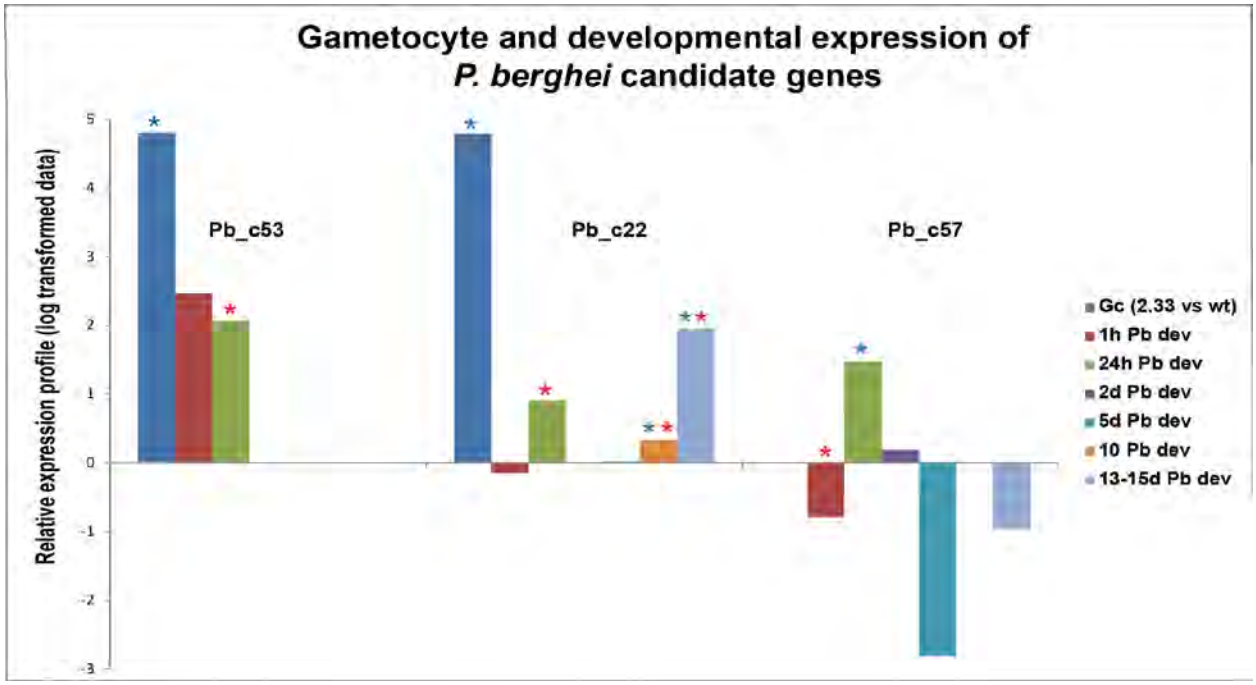

Figure 4.5. Graphical presentation of *P.berghei* orthologous candidate genes expression in *A. gambiae* and additional evidence of published transcriptome and proteome data about their expression in the highlighted stages, indicated by blue and red stars, respectively. Vertical axis: relative expression in $\log_2$ scale.

Finally, the selected *P. falciparum* gene candidates were subjected to further investigation about their functional domain predictions (Figure 4.6), apart from expression profiles indicated in microarrays and other published data (Figures 4.4 & 4.5). Homology with other eukaryotic genomes could provide an assignment of functional domains in the predicted proteins encoded by *Plasmodium*. An obstacle to this strategy is the extreme AT-richness of the *P. falciparum* and rodent Plasmodia genomes resulting in lack of any functional domain predictions of roughly 60% of predicted proteins. 31% of the predicted proteins possess one or more transmembrane domains while, approximately, 17% of the proteins have putative signal peptides or signal anchors (Gardner et al., 2002).

The presence of predicted transmembrane domains in the derived proteins of *Pf_c57* and *Pf_c01* genes (surface-located proteins) could suggest involvement in interactions between the parasite and the mosquito vector. Pf_c53 and Pf_c22 lacking secretory and transmembrane domains could play an important role in parasite development through the underlined stages. Corresponding domains were predicted in the *P. berghei* orthologs.

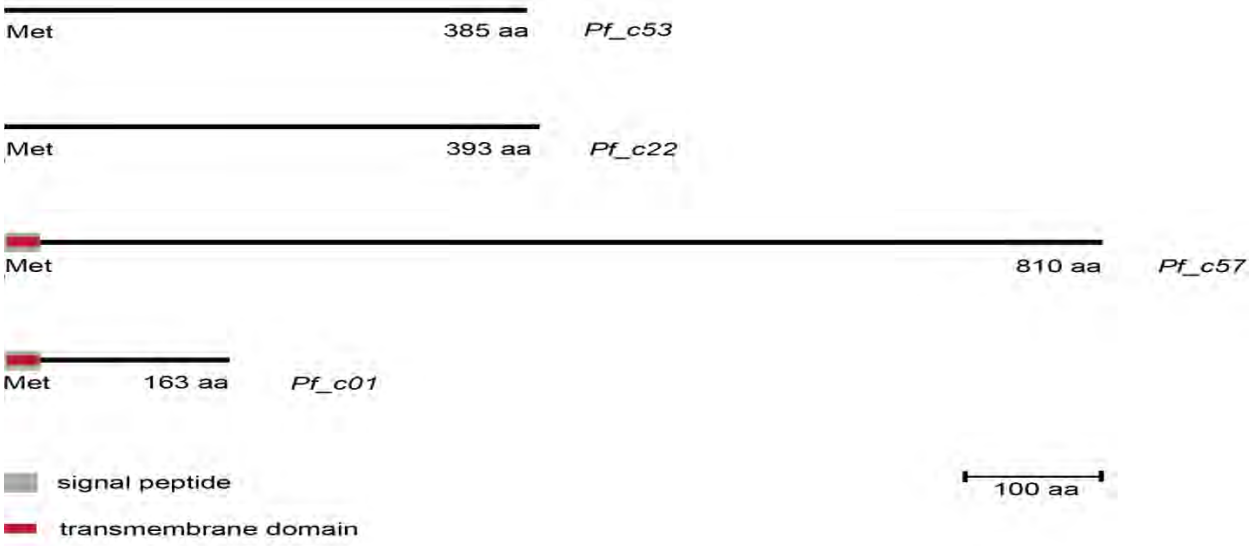

Figure 4.6. Schematic representation of protein features of Pf_c57, Pf_c01, Pf_c53 and Pf_c22. Signal peptide motifs and transmembrane domains were predicted for Pf_c57, Pf_c01 proteins. No functional domains were predicted for Pf_c53 and Pf_c22.

4.3 Generation of loss of function mutant parasites by targeted gene disruption

4.3.1 Experimental strategies

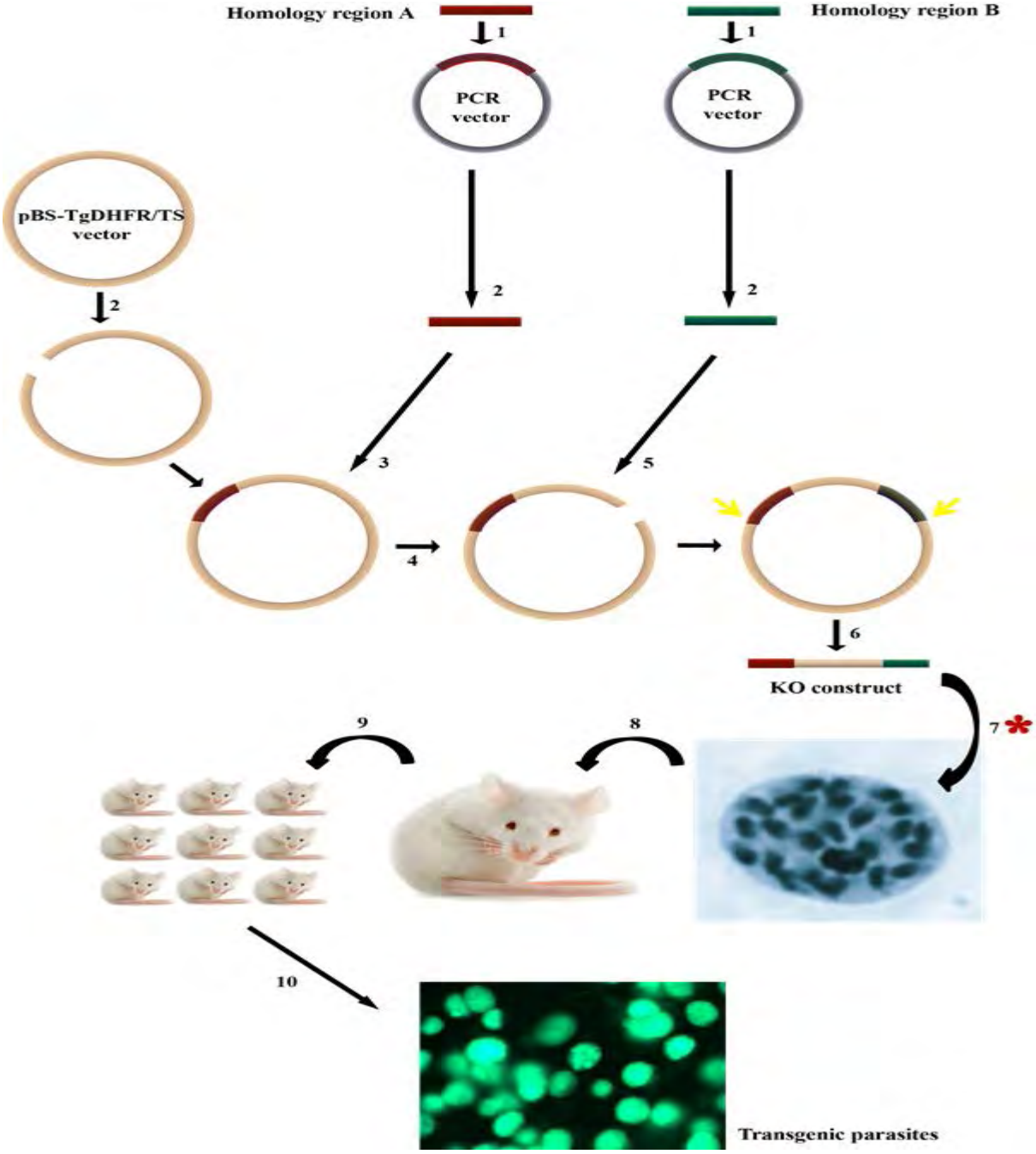

Figure 4.7. Schematic representation of the experimental strategies followed for the generation of each KO parasite line. After PCR amplification of target regions of homology and ligation to PCR vectors (1), the homology regions and the transfection vector (pBS/TgDHFR vector) are cut with the appropriate pairs of enzymes (2). The homology regions are ligated to the pBS/TgDHFR vector consecutively -a second restriction digest of the vector with the respective pair of enzymes mediating between the two ligations- (3, 4, 5). The resultant KO construct is linearized and used for transformation of parasite schizonts (6, 7). The parasites are cultivated and drug selected in mice hosts (8), before dilution cloning (9). Finally, a new KO parasite line is achieved after selection of the successfully transgenic parasite clones (10). The restriction sites used for vector linearization are indicated by yellow arrows. Homologous recombination, following transfection, is indicated by a red star and represented in the following figure.

4.3.2 Strategy for targeted gene disruption by homologous recombination

The generation of gene-specific targeting constructs is the most efficient strategy for introduction of targeted gene disruption by homologous recombination, in *P. berghei*. Included in these constructs, a TgDHFR pyrimethamine resistance cassette is flanked by homologous to the targeted gene downstream and upstream regions (van Dijk et al., 1996). Homologous recombination occurs after transfection leading to integration of the disruption construct into the target locus (Figure 4.8).

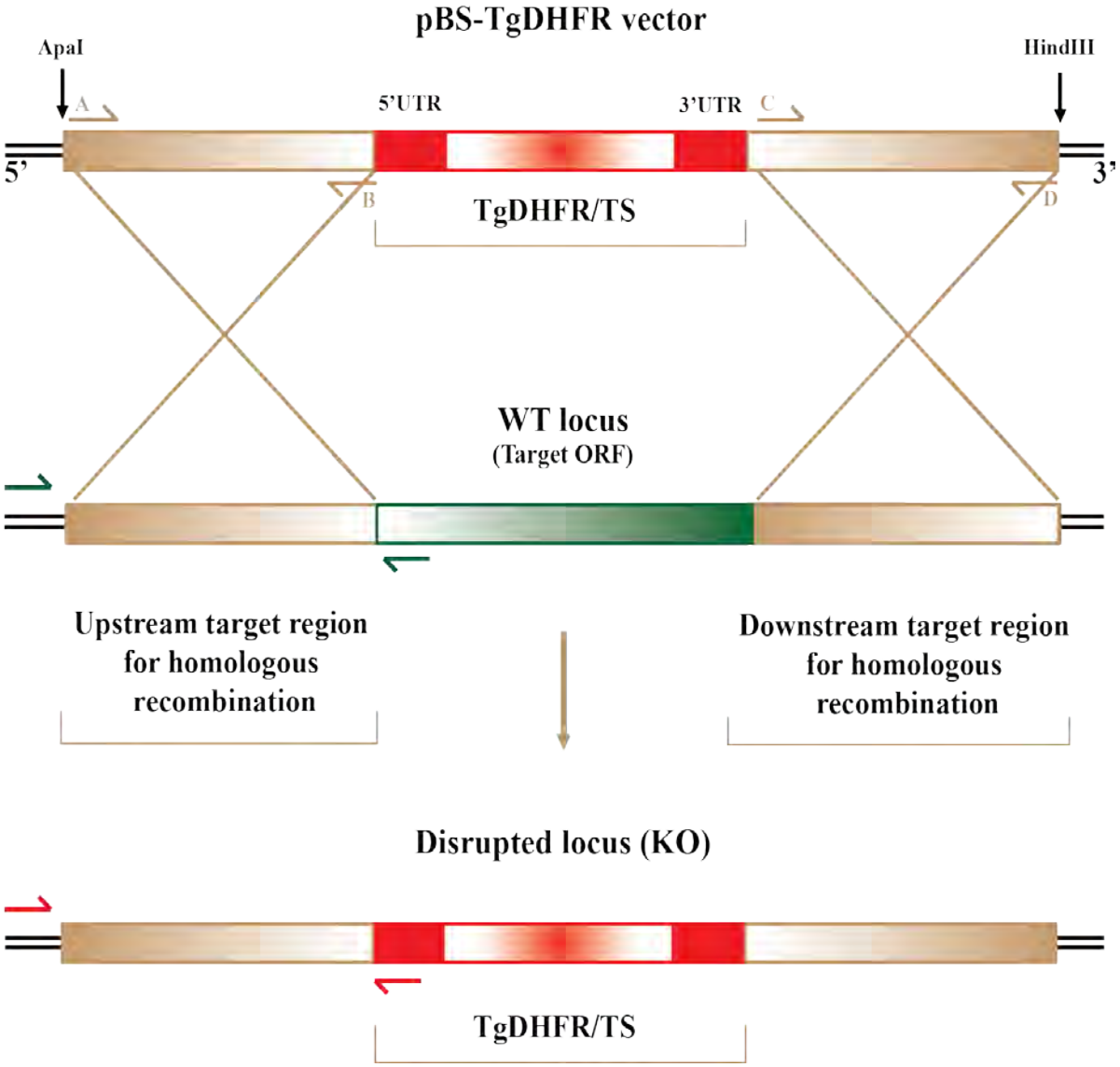

Figure 4.8. Representation of targeted gene disruption through homologous recombination in *P. berghei*. The regions for homologous recombination between the WT locus target and the corresponding fragments included in the disruption vector are indicated in beige. The TgDHFR cassette is shown in bright red with its 5' UTR promoter and 3' UTR regions indicated by red shading. Beige arrows show the position of the primers used for sub-cloning, the green arrows the assessment of wild-type (Int Fw and WT R primers) and the red arrows the detection of integration (Int Fw and 5' UTR R). The black arrows indicate the position of the restriction sites and the restriction enzymes used for linearization of the KO construct.

4.3.3 Qualitative confirmation of expression of P. berghei candidate genes

In order for the expression of the candidate genes to be experimentally examined, RT-PCR was conducted. *A. gambiae* N' gousho mosquitoes infected with *P. berghei* ANKA 2.34 (Wild-type) were used for the qualitative in vivo verification of the genes expression in four time-points. RNA was extracted from *A.gambiae/P.berghei* infected midguts (kind gift from R. Rai), dissected at 1, 6, 10 and 24 hours post-infection, and was used as template for the RT-PCR. The RT-PCR product of *Pb_c57* corresponded to 576 bp of the predicted RNA product, *Pb_c01* product to 252 bp, *Pb_c53* product to approximately 550 bp and *Pb_c22* product corresponded to approximately 780 bp. RT-PCR confirmed *in vivo Pb_c53* and *Pb_c22* expression at 10 hours post-infection and *Pb_c57* and *Pb_c01* 24 hours post-infection, and generally in the sexual stages (Figure 4.9).

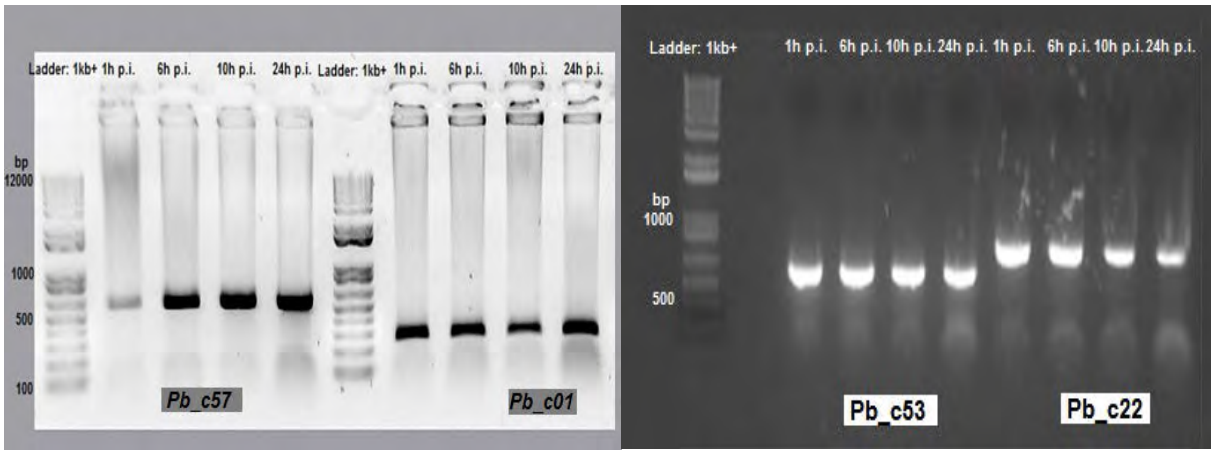

Figure 4.9. *In vivo Pb_c57, Pb_c01, Pb_c53* and *Pb_c22* expression in infected *A. gambiae* mosquitoes with *P.berghei*. RT-PCR performed using RNA extracted from *A.gambiae/P.berghei* infected midguts, dissected at 1, 6, 10 and 24 hours post-infection, as template. (RT-PCR of *Pb_c53* and *Pb_c22* was performed by R. Rai).

4.3.4 PCR amplification of homology regions

P. berghei genomic DNA was extracted from infected mouse blood, to serve as template for the amplification of the homology regions of genes *Pb_c57* and *Pb_c01*. The upstream target region of *Pb_c57* corresponded to 591 bp of the 5' UTR and the downstream target region to 685 bp of the most downstream coding sequence, inducing a partial disruption. The upstream homology region of *Pb_c01* corresponded to 430 bp of the 5' UTR and the most upstream coding sequence and the downstream homology region to 715 bp of the 3' UTR generating a partial knock-out, as well. After PCR amplification of these four regions, the quality of the products (product length, possible contamination) was verified using agarose gel electrophoresis (Figure 4.10) and the PCR products were purified. The concentration of purified PCR products was determined spectrophotometerically.

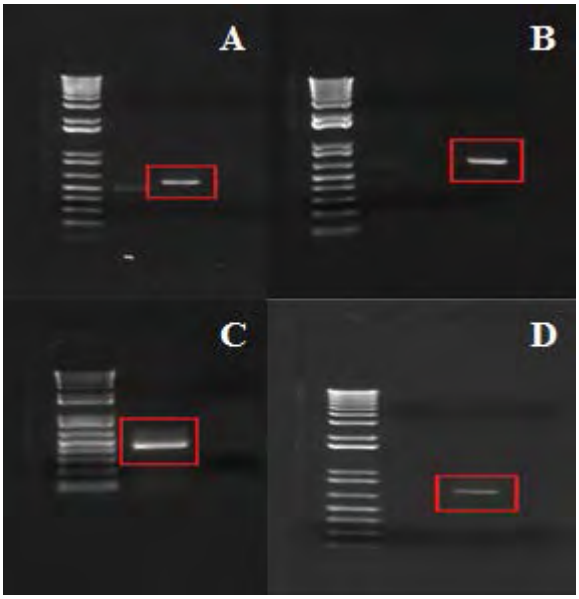

Figure 4.10. PCR products of amplified homology regions.
A. Herculase PCR product of homology region A (Pb_c57 a and Pb_c57 b primers) of *Pb_c57* gene.
B. PCR amplified homology region B (Pb_c57 c and Pb_c57 d primers) of *Pb_c57* gene, using Herculase.
C. PCR product of amplified homology region A of *Pb_c01* gene (Pb_c01 a and Pb_c01 b primers), using Go-Taq polymerase
D. Herculase PCR product of homology region B of of *Pb_c01* gene (Pb_c01 c and Pb_c01 d primers)

4.3.5 PCR-vector ligation and transformation

Each PCR purified product was concentrated to the desirable final volume and cloned to an appropriate PCR-vector. PCR products of homology regions A and B of *Pb_c57* and homology region B of *Pb_c01* were cloned to blunt vector and PCR product of homology region B of *Pb_c01* was cloned to T-vector. The resulting vectors were transformed into chemically competent Escherichia coli cells, having kanamycin resistance, by heat shock methodology. The cells were cultivated overnight LB agar plates containing kanamycin. Kanamycin assisted to the selection of transformed clones; only the transformed clones were resistant to kanamycin, due to the kanamycin resistance gene contained in the plasmid (PCR-vector).

Clones containing the recombinant plasmids were identified through diagnostic digestion with EcoRI. Both PCR vectors accommodate two EcoRI restriction sites which flank the insert (Figure 3.2). A restriction digest by EcoRI results in a product slightly larger than the original insert.

Overnight cultures in 50 μg /mL kanamycin LB medium were done after selection of the recombinant transformants in order for large quantity of recombinant cells, and therefore large quantity of plasmids, to be obtained. Plasmid DNA was extracted and purified from the overnight cultures.

4.3.6 First restriction digests

Purified plasmids, containing each homology region, were digested using the appropriate couple of restrictrion enzymes (Table 3.3). pBS/TgDHFR vector, in which polylinker sites flank a *tgdhfr-ts* pyrimethamine resistance cassette, was also cut by the same couple of restriction enzymes with the homology region which was going to be subcloned. The purpose of this restriction digest was to linearise the homology regions and the pBS/TgDHFR vector and generate complementary sticky ends for further subcloning. Preparative standard gel electrophoresis was used in order to distinguish and obtain the desirable digestion products

(Figures 4.11 & 4.12). After electrophoresis, the appropriate digestion products were extracted from the agarose gel. The concentration of purified digestion products was determined spectrophotometerically in order to calculate the vector/insert ratios for the ligation reactions.

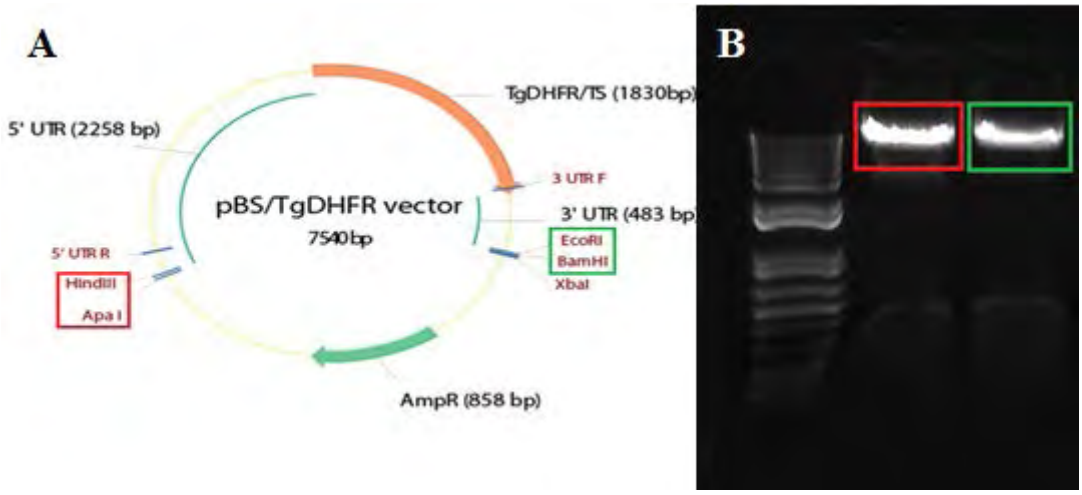

Figure 4.11. Restriction digest of pBS/TgDHFR vector.
A. map of pBS/TgDHFR vector. The couples of restriction enzymes used for each linearization aiming to further ligation are highlighted in red (HindIII, ApaI) and green (EcoRI, BamHI) squares.
B. pBS/TgDHFR vector cut with ApaI and HindIII (red square) and transfection vector digested with EcoRI and BamHI (green square).

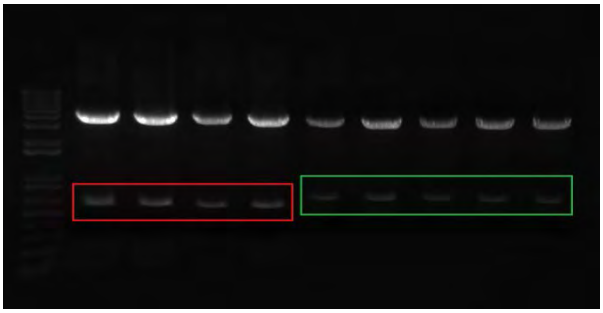

Figure 4.12. Preparative electrophoresis of double digested PCR vectors containing inserts. Red highlight: *Pb_c57* homology region A cut with ApaI and HindIII, green highlight: *Pb_c57* homology region B cut with EcoRI and BamHI. The upper bands represent the linearized PCR vector.

4.3.7 First ligation to transfection vector

Depending on the couple of restriction enzymes used in the digestion of the pBS/TgDHFR vector, the appropriate homology region (cut by the same couple of restriction enzymes) was firstly subcloned. For *Pb_c57* KO construct, homology region A was firstly subcloned to pBS/TgDHFR vector in contrast to *Pb_c01* KO construct for which homology region B was firstly subcloned to the vector. After the ligation of the first homology regions to the pBS/TgDHFR vector, followed by heat shock transformation of chemically competent *E. coli* cells, the resulting transformants were cultivated overnight in LB agar plates containing ampicillin. Ampicillin assisted to the selection of transformed clones, due to the presence of ampicillin resistance gene in the pBS/TgDHFR vector (Figure 3.3). Clones containing the recombinant plasmids were identified and selected through diagnostic colony PCR (Figure 4.13).

Overnight cultures in 50 μg /mL ampicillin LB medium were done after selection of the colonies including the recombinant pBS/TgDHFR vector. Plasmid DNA was extracted and purified from the overnight cultures.

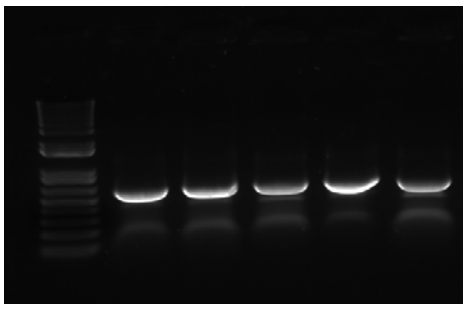

Figure 4.13. Diagnostic colony PCR of inserts within the pBS/TgDHFR vector. Colony PCR amplified homology region A of *Pb_c57* contained within the transfection vector, after subcloning.

4.3.8 Second restriction digestions, ligations and transformations

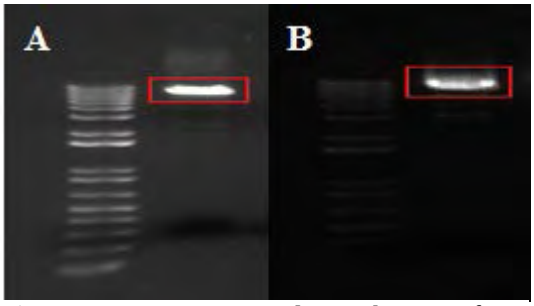

Figure 4.14. Preparative electrophoresis after second restriction digests.
A. transfection vector containing *Pb_c57* homology region A, cut with EcoRI and BamHI
B. transfection vector containing *Pb_c01* homology region B, cut with ApaI and HindIII.

Purified transfection vectors, including one homology region, were digested using the same couple of restriction enzymes used for the digestion of the second homology region (Table 3.3). Specifically, complementary overhangs were generated to *Pb_c57* homology region B and the pBS/TgDHFR vector containing the *Pb_c57* homology region A through double digestion with EcoRI and BamHI for further subcloning. *Pb_c01* homology region A and pBS/TgDHFR vector containing the *Pb_c01* homology region B were cut with ApaI and HindIII for the same purpose.

Preparative standard gel electrophoresis and gel extraction of the befitting products were performed before continuing to the ligation reactions of the second homology regions and the vectors.

The final ligations and transformations followed the same methodology as the initial ones. Overnight cultures of the selected colonies were performed in 200 ml ampicillin LB medium, providing a high concentration of final recombinant plasmids, following extraction, required for the transfection procedure.

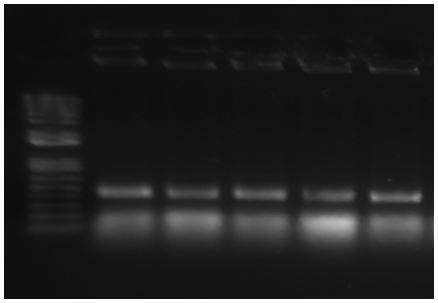

Figure 4.15. Diagnostic colony PCR of inserts within the pBS/TgDHFR vector. Colony PCR amplified homology region A of *Pb_c01* contained within the transfection vector including homology region B, after subcloning.

A diagnostic quadruple digestion of the purified plasmids, using all four restriction enzymes (ApaI, HindIII, EcoRI and BamHI), was performed before the final double digestion. This provided the verification of the double recombination of the pBS/TgDHFR vector, resulting to the desirable KO constructs. Also, before transfection, pBS/TgDHFR vectors carrying the homology regions were sent for sequencing to Beckman Coulter Genomics, UK, for the verification of successful generation of the KO constructs.

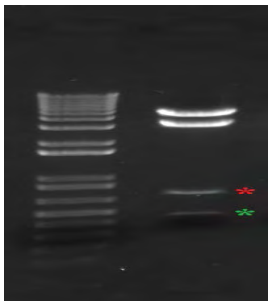

Figure 4.16. Diagnostic quadruple digestion before transfection. Digested regions of homology of *Pb_c01* gene and transfection vector. Red star: homology region B. Green star: homology region A. The two upper bands illustrate the two fragments of the digested pBS/TgDHFR vector.

4.3.9 Preparation of DNA for transfection

The constructs were linearised prior to transfection because linear DNA renders optimal integration into the *P. berghei* genome despite the fact that it results in lower DNA uptake by the cells relative to supercoiled DNA. Final overnight double digestion of the constructs was conducted using the ApaI and BamHI (ApaI-restriction site added in forward primers of homology region A and BamHI-restriction site added in reverse primers of homology region B), so to release the disruption cassette (Homology region A - *tg*DHFR-TS - Homology region B). The desirable fragment was gel extracted and subsequently purified by ethanol precipitation for high purity and quality of the DNA to be acquired.

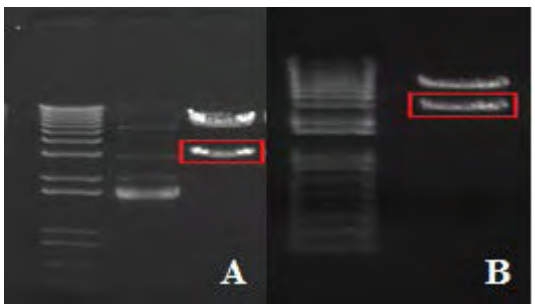

Figure 4.17. Final restriction digests of pBS/TgDHFR vectors carrying the homology regions, before transfection. **A**. construct for double homologous recombination of *Pb_c57* gene.
B. construct for double homologous recombination of *Pb_c01* gene.
Constructs participating in homologous recombination highlighted in red squares.

4.3.10 Transfection

After the transfection procedure outlined earlier, *ΔPb_c57* and *ΔPb_c01* transgenic parasites, selected in vivo by two pyremethamine treatments of infected mice were subjected to diagnostic PCR in order to assign whether surviving parasites carried integrated disruption constructs, and consequently a gene specific KO locus. Genomic DNA of *P. berghei* transfected parasites was extracted and subsequently used as a template for PCR amplification (Figure 4.18).

Integration is easily detected through PCR, using gene specific forward primers and a reverse primer located inside the 5'UTR region of the TgDHFR cassette (Figure 2.4). Also, detection of gene specific WT loci is facilitated by the use of the same gene specific forward primer and a reverse primer situated inside the target coding region. Each of these two loci can be detected either in clonal or non-clonal parasite populations (Figure 4.8).

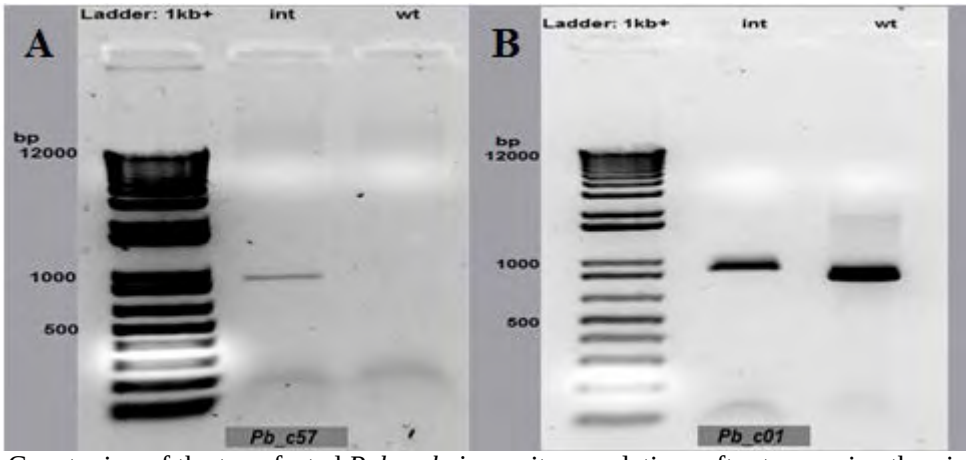

Figure 4.18. Genotyping of the transfected *P. berghei* parasite populations after two pyrimethamine selections.
A. Successful integration of *ΔPb_c57* parasite population displaying absence of WT parasites.
B. Presence of successful integration in *ΔPb_c57* parasite population.

Ten days after the limited dilution cloning, the presence of blood-stage parasites in the ten injected mice was examined by Giensa stained thin blood-smears. Blood from the infected mice was collected by cardiac puncture and 200 μL were used as template for diagnostic PCR while the remaining blood was preserved in glycerol stocks.

Limited dilution cloning of the trasfected population yielded one *ΔPb_c57* clone and five *ΔPb_c01* clones which demonstrated successful clonal integration. In these clones PCR detected only the disrupted locus, but not the WT locus (Figure 4.19).

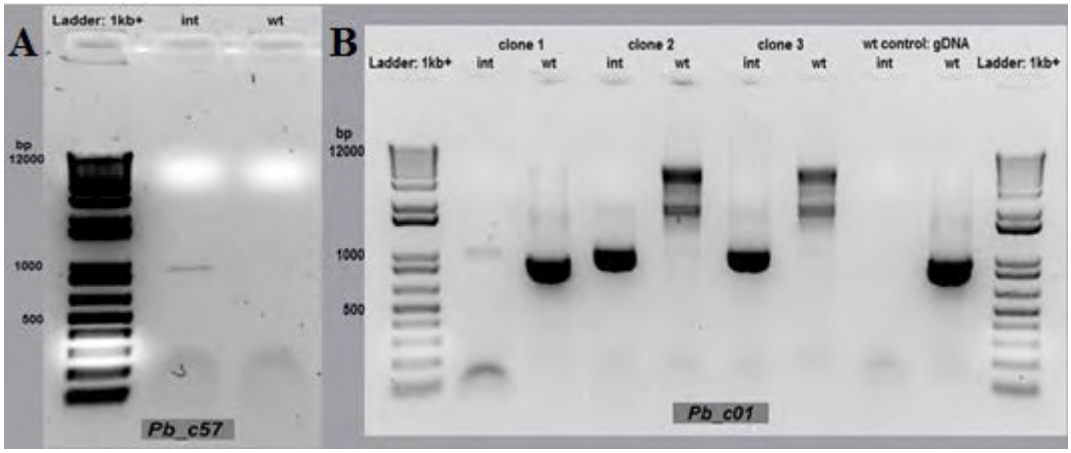

Figure 4.19. Genotypic analysis of clones after dilution cloning showing successful integration. Diagnostic PCR analysis of *ΔPb_c57* and *ΔPb_c01* clonal selection.
A. Successful integration in one clone of *ΔPb_c57*.
B. Successful integration in two *ΔPb_c01* clones (clone 2 and clone 3) and unsuccessful integration in *ΔPb_c01* clone no1. Extracted gDNA from *P. berghei* ANKA 2.34 parasite clone was used as template for negative control.

4.4 Phenotypic characterisation of transgenic parasites

After the generation of four transgenic KO parasite lines (*ΔPb_c57*, *ΔPb_c01*, *ΔPb_c53* and *ΔPb_c22*) they were subjected to *in vivo* and *in vitro* phenotypic analysis.

4.4.1 In vitro phenotypic analysis

On day 4–5 after the infection of PH treated mice with the transgenic parasite lines and WT *Pbc*507 parasites, assisting as biological control, exflagellation assay was performed. Blood was collected by cardiac puncture in order to be used for 24 hour ookinete cultivation, from the same mice used for the exflagellation assays. As a result, ookinete formation was assessed one day after the exflagellation assay. After confirmation of mature ookinete production through Giemsa stained smears, ookinete conversion assays were conducted. The biological replicates of in vitro assays were three for each transgenic parasite line.

Giemsa staining of male and female mutant gametocytes illustrated no different staining pattern than the typical for *P. berghei* gametocytes and a typical female to male sex ratio, as well. Also, Giemsa stained transgenic ookinetes, cultivated *in vitro*, revealed a morphologically normal crescent shape. Staining with anti-Pbs21-Cy3 demonstrated that the rate of in vitro formation of ookinetes from female macrogametes is comparable to that of the WT and exflagellation assay showed a typical rate of exflagellated mutant male gametocytes compared to the WT (χ^2 test).

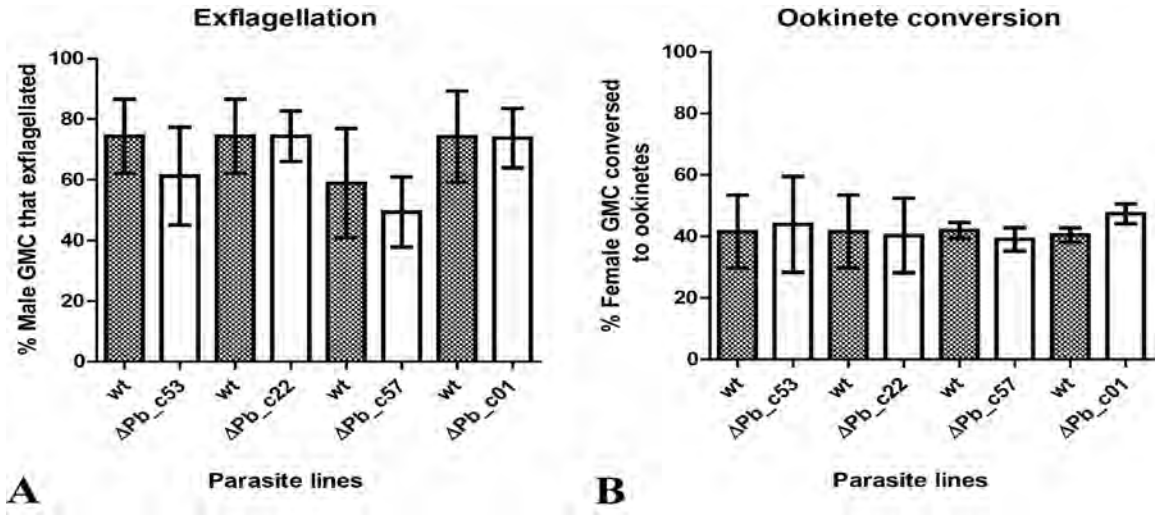

Figure 4.20. Graphical presentation of results of *in vitro* phenotypic assays. No significant difference was - observed in the exflagellation (**A**) or the macrogamete to ookinete conversion (**B**) percentage of the four KO mutants compared to the wt (χ^2 test: p value > 0.05). Error bars represent the standard error of the mean of the three biological replicates.

4.4.2 In vivo phenotypic analysis

In the beginning of *in vivo* phenotypic analysis, PH treated mice were infected with the transgenic parasite lines and *Pb*c507 wt line (biological control). When parasitaemia reached 2-10% (assayed by Giemsa stained smears), *A. gambiae* and *A. stephensi* starved female mosquitoes –approximately 150 female mosquitoes for each parasite line in each biological replicate– were allowed to feed on the anaesthetised mice. The day of *per os* infection corresponds to day 0 of the assay. After the removal of the unfed females, on day 1 p.i., mosquitoes were maintained for another maximum 22 days. On day 8-10 p.i. *P. berghei* infected *A. stephensi* or *A. gambiae* midguts were dissected for oocyst counting and imaging (minimum 30 total number of dissected midguts) and on day 15 for sporulating oocyst imaging. 4-7 days later (19-22 day p.i.) salivary glands were dissected for sporozoite enumeration. Sporozoites were enumerated in suspensions of 30 homogenised salivary glands, assayed in three batches of ten, for each parasite line (including WT). Bite-back experiment was performed on the same or next day of the salivary glands dissection in order to determine mutant sporozoite infectivity to mice. The presence of parasites in the mouse blood was assessed four to five days after the bite-back assay by Giemsa stained smears. Finally, the blood-stage parasites were purified and subjected to genotyping through PCR in order to define the purity of clonal WT/KO population.

Three biological replicates of *A. stephensi* infections were analysed for *ΔPb_c53*, *ΔPb_c22* and *ΔPb_c57* and two for *ΔPb_c01.* Three *A. gambiae* infections with *ΔPb_c53* and *ΔPb_c22* and one infection with *ΔPb_c57* and *ΔPb_c01* were analysed. *ΔPb_c53* and *ΔPb_c22* shared the same WT biological control in every biological replicate, both in *A. gambiae* and *A. stephensi* infections.

Mosquito infections revealed that at day 8-10 post-infection that oocyst numbers in *A. stephensi* infected midguts of all the generated mutants were significantly reduced ($P<0.05$) compared to that of the WT control, according to Mann-Whitney analysis. Compared to the WT, oocyst numbers of *ΔPb_c53*, *ΔPb_c01* and *ΔPb_c57* in infected midguts were lower ($P<0.001$) than those of *ΔPb_c22* ($P=0.012$). Specifically, in the absence of *Pb_c53* severe disruption of oocyst development was observed (-21.76 fold difference between medians) (Table 4.1 and Figures 4.21, 4.22 & 4.23).

A. gambiae infections could not provide results leading to safe conclusions about the phenotype of the mutant parasite lines due to low infection prevalence of the WT control or low parasitaemia of mice provided for infection (Tables 4.1 & 4.2, parasitaemia low in *ΔPb_c01* infection – data not provided). Only *ΔPb_c57* infection can provide evidence, consistent to the results of *A. stephensi* infections.

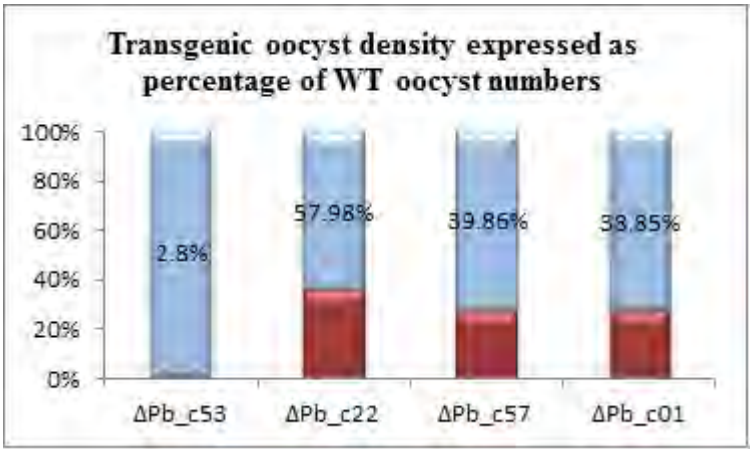

Figure 4.21. Transgenic parasite lines display a significant reduction in oocyst densities in *A. stephensi* / *P. berghei* infections. The transgenic oocyst numbers in *A. stephensi* expressed as a percentage of WT oocyst numbers. The percentages were calculated using medians.

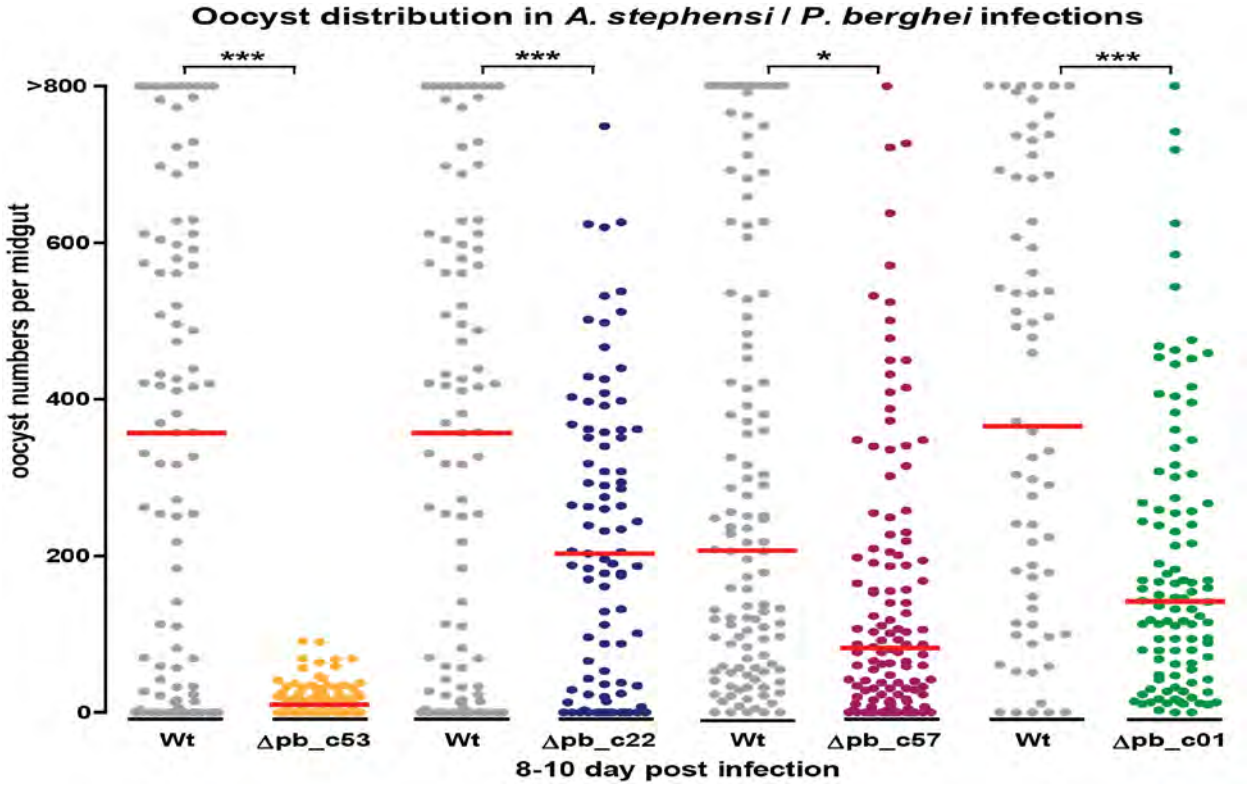

Figure 4.22. Distribution of the four KO mutant and WT oocyst numbers in midguts of *A. stephensi* mosquitoes, at day 15 p.i. The medians of oocyst numbers are represented by red lines. Highly significant reduction (Mann-Whitney, P<0.001, ***) of *ΔPb_c53*, *ΔPb_c22* and *ΔPb_c01* oocyst numbers compared to WT controls is detected. Significant reduction (Mann-Whitney, P=0.012, *) of *ΔPb_c57* oocyst numbers compared to WT control is also detected.

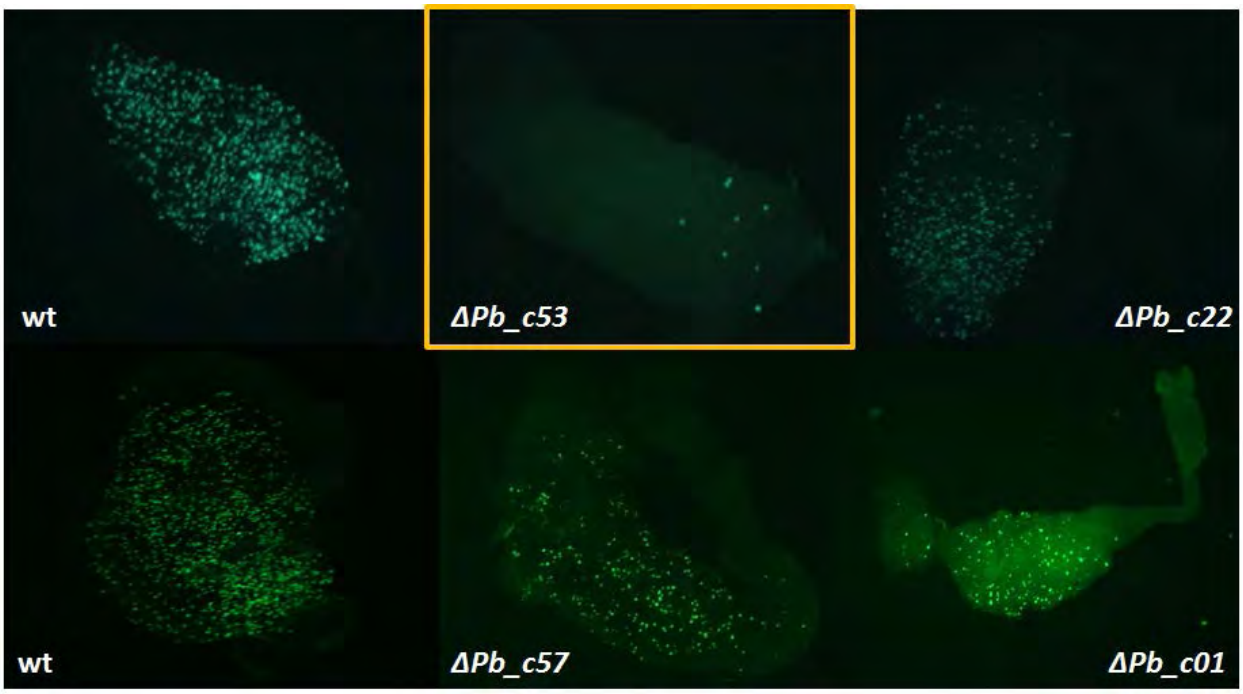

Figure 4.23. Fluorescent microscopy images of GFP expressing oocysts in *A.stephensi* infected midguts at 10X magnification (15 day p.i.). *A.stephensi* infected midguts with WT and transgenic parasites represent the

reduction in the oocyst numbers of the KO mutant parasites compared to the WT. Conspicuous reduction of oocyst density in *ΔPb_c53* parasite line can easily be detected (highlighted in yellow borders).

***A.stephensi/P.berghei* infections**									
					Parasite intensity			Mann Whitney Analysis	
Parasite line	# Repli-cates	Day p.i.	# midguts	Infection prevalence (%)	Arithme-tic Mean	Median	Parasite Range	P value	Fold Differ-rence
wt	3	8	93	82.8	358.57	357	0-1082		
Δpb_c53			105	78.3	16,457	10	0-91	**<0.0001**	**-21.76 (-35.7)**
Δpb_c22			91	85.7	226,308	203	0-837	**0.012**	**-1.58 (-1.76)**
wt	3	9	116	95.7	303	207	0-1250		
Δpb_c57			120	87.5	145.175	82.5	0-792	**<0.0001**	**-2.09 (-2.51)**
wt	2	8	66	92.4	413,348	365.5	0-1250		
Δpb_c01			106	98.1	192,472	142	0-880	**<0.0001**	**-2.15 (-2.57)**
***A.gambie/P.berghei* infections**									
wt	3	9	120	44.2	3.558	0	0-70		
Δpb_c53			142	0.7	0.007	0	0-1	-	(-508.29)
Δpb_c22			131	20.6	1.374	0	0-48	-	(-2.59)
wt	1	8	38	86.8	21.37	9	0-84		
Δpb_c57			49	65.3	6.1	3	0-38	0.002	-3 (-3.5)
Δpb_c01			32	2.4	0	0	0-1	-	-

Table 4.1.
This table reports results from *ΔPb_c57*, *ΔPb_c01*, *ΔPb_c53*, *ΔPb*_c22 and *Pbc507* WT parasite infections of *A. stephensi* (upper section) and *A. gambiae* (bottom section). Equal numbers of transgenic or WT infected midguts were pooled for each group (total number of dissected midguts is shown in the fourth column). Prevalence illustrates the percentage of midguts with at least one oocyst. Midguts with zero parasites were also considered for the calculation of the arithmetic mean and median of parasite densities (number per midgut). P value (Mann Whitney) for each transgenic vs. WT group of infections was calculated using medians when available. Fold differences between wt and mutant oocyst densities were computed using the medians and arithmetic means. Values of fold differences using arithmetic means are shown in parenthesis.

At day 15 post-infection, the oocyst imaging revealed successful sporulation of a proportion of oocysts for all parasite lines. Also, although the oocyst developmental rate is non-synchronous in *P. berghei* infections, resulting to heterogeneity even in the WT oocyst size, the average size of all mutant oocysts was comparable to the average size of WT oocysts (Figure 4.23).

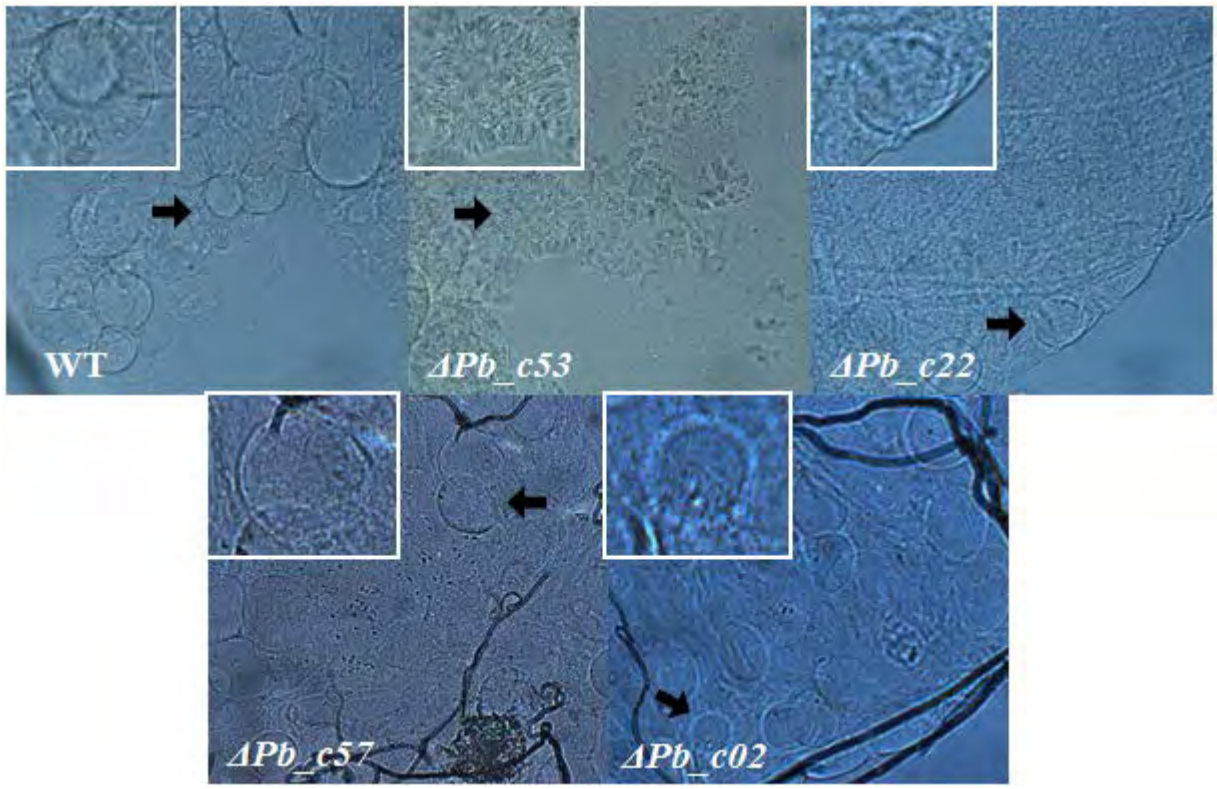

Figure 4.24. Microscopic imaging of oocysts in *A. stephensi* infected midguts, at the stage of sporulation, on day 15 p.i. As WT, all mutant oocysts display normal size and efficient sporulation. Sporulating oocysts are shown by arrows and are magnified in highlighted borders.

All *ΔPb_c53*, *ΔPb_c22* and *ΔPb_c57* biological replicates were finalised by enumeration of salivary gland sporozoites but none of the two replicates assayed for *ΔPb_c01*, in *A. stephensi* infections.

As all transgenic parasites displayed successfully sporulating oocusts, sporozoites were detected in the salivary glands of *A. stephensi* mosquitoes of all assayed infections. Salivary gland sporozoite numbers were significantly reduced (Mann-Whitney analysis, $P<0.05$) in *ΔPb_c53* and *ΔPb_c57* infected *A. stephensi* mosquitoes but not in *A. stephensi* infected by *ΔPb_c22*, compared to the WT (Table 4.2). Although the sporozoite numbers were reduced, the feedback of all the bite-back assays was efficient infectivity to mice, as revealed by Giemsa stained smears.

Also, although the infection prevalence of *A. gambiae* biological replicates was extremely low (Table 4.1), the presence of infective sporozoites in the salivary glands allowed successful transmission to mice, as confirmed by bite-back assays (Table 4.2).

***A.stephensi/P.berghei* infections**							
Parasite line	Day p.i.	Parasite intensity		SE	Mann Whitney Analysis		Bite-back assay
		# Replicates	Arithmetic Mean		P value	Fold difference	
wt	21-22	3	2,821.36	397.186			√
Δpb_c53			659.42	69.016	<0.0001	-4.28	√
Δpb_c22			2,031.57	252.089	0.2224	-1.39	√
wt	19	3	2,632.44	198.514			√
Δpb_c57			1,373.33	267.232	0.0028	-1.92	√
***A.gambiae/P.berghei* infections**							
wt	19-20	2	1,363.97	472.424			√
Δpb_c53			167.53	46.153	<0.0001	-8.14	√
Δpb_c22			665.33	230.033	0.2224	-2.05	√

Table 4.2.
The table outlines mean Pbc507 *wt* or transgenic sporozoite densities in *A. stephensi* and *A. gambiae* salivary glands given as arithmetic mean per mosquito. SE represents standard error. Infectivity to mice was assayed by allowing transgenic or *WT* infected mosquitoes to feed on one TO or C57BL/6 mouse (bite-back) at day 21-22 of infection. Mice were allowed to recover and parasitaemia was assessed at day 5-6 post-feeding.

CHAPTER 5: DISCUSSION

CHAPTER 5: DISCUSSION

As malaria is characterised as one of the major infectious diseases in the world, causing hundreds of thousands deaths annually, mostly among African children, it is well understood why effective control measures are urgently needed. In the battle against malaria, several strategies have been developed, including targeting the *Plasmodium* parasite inside both hosts and controlling mosquitoes. Transmission blocking interventions are attracting much of the attention as they target the parasite's development during transmission by the *Anopheles* vector, which represents the weakest link in malaria life cycle. The development of more effective approaches, though, requires the broadening of the existing knowledge about the parasite's biology and the parasite-mosquito interactions.

Although the sequencing of many *Plasmodium* spp, including the rodent model *P. berghei*, has been already completed, the limited knowledge about the functions of *Plasmodium*'s gene products comprises an obstacle to the development of successful transmission blocking interventions. This is the reason why a respectable proportion of the recent studies, including gene-candidate or high-throughput approaches, are focused on the understanding of *Plasmodium*'s biology gene-specific during sexual and sporogonic development and the molecular interactions between mosquitoes and malaria parasites.

The aim of this project is focused on the identification of novel *P. falciparum* molecules that are essential to parasite's sexual development, especially during the midgut invasion by the ookinete, and their products putatively interact with mosquito midgut molecules during the same period and their regulation has a prominent effect on the transmission capacity. The functional characterisation of these genes and their products could enforce not only the knowledge about parasite's sexual and sporogonic differentiation mechanisms but also the further deployment of transmission blocking approaches as they could constitute novel effective targets of transmission blocking interventions.

A genome wide transcriptional profiling of field isolated *P. falciparum* during infections in the two major African malaria vectors, *A. gambiae* and *A. arabiensis* (Host lab, unpublished data), revealed robust regulation of a group of *P. falciparum* genes in four distinct time points of the parasite's development inside both *Anopheles* vectors (Nikolaos Trasanidis, final year project). The working hypothesis is that genes specifically up- and down-regulated during *Plasmodium* sexual and sporogonic development are likely to be implicated in parasite differentiation and interaction with the mosquito. The expression levels of some of these genes may determine the outcome of the parasite's interaction with the mosquito immune and local epithelial response systems. As a result, the significantly regulated genes included in this analysis, and especially in the time points of interest, comprise intriguing targets for further investigation and functional characterisation (Figure 4.3).

Based on the evidence provided from the high-throughput screenings, four genes showing significantly differential expression in the sexual stages of *Plasmodium*'s lifecycle and a developmental peak in one of the distinct time points were selected as candidates for functional characterisation. The transcriptional peak of two of these genes was in the stage of ookinete maturation and locomotion (*Pf_c53* and *Pf_c22* in T3) and of the other two in the stage of midgut invasion and ookinete-to-oocyst transformation (*Pf_c01* and *Pf_c57* in T4) (Figures 4.4 and 4.5). These transcription profiles are of high interest because they could illustrate the production of molecules that possibly regulate the procedure of midgut invasion (peak in T4) but also molecules that contribute to the successful maturation, motility of the ookinete could consequently regulate the early midgut invasion (peak in T3).

Then, the orthologues of these *P. falciparum* genes were identified in the rodent parasite, *P. berghei* and their expression profile determined during infection of *A. gambiae*. RT-PCR analysis (Figure

4.9) together with high-throughput transcriptional data of *P. berghei* in the mosquito midgut (Host lab, unpublished) showed that *P. berghei* ortholoques of *c01, c57, c53 and c22* genes exhibiting a similar profile to their *P. falciparum* orthologues and thus they selected for further functional characterization.

Subsequently, the knock-out of these genes by homologous recombination in *P. berghei* was successfully conducted using strategies previously established, leading to the generation of four different transgenic parasite lines. These parasite clones were used during this study for the analysis of their resulting phenotype and can be further used for the completion of the phenotypic characterisation of the specific gene deletion.

Interestingly, as revealed by the *in vitro* phenotypic assays, the deletion of these genes has no significant effect on the gamete production rate or morphology; both male and female, and produce morphologically normal ookinetes (Figure 4.20). This indicates that the functional role of these genes is focused on later stages of the *Plasmodium* development.

Significant reduction in the oocyst numbers of the transgenic parasite lines compared to the wt was observed by the *in vivo* phenotypic analysis (Figures 4.21, 4.22 & 4.23). Specifically, on day 8-9 p.i. *ΔPb_c22* oocyst numbers correspond to 57.98% of that of WT oocysts, *ΔPb_c57* to 39.86%, *ΔPb_c01* to 38.85% and, finally and most importantly, *ΔPb_c53* oocyst density reaches only 2.8% of that of WT (Figure 4.23). This provides strong evidence of critical involvement of all four genes in the parasite survival through the midgut invasion and/or ookinete-to-oocyst transition stage.

All these results lead to the conclusion that the regulation of all the four examined genes has a semantic effect in the crucial stage of ookinete invasion and transformation to oocyst, when the major bottleneck in the parasite lifecycle occurs. The fact that there was no impact to the gametocyte production and fertilisation (ookinete conversion assay) or the sporozoite formation of the few oocysts, implements that the regulation of these four genes is restricted between the stages of ookinete maturation and motility, midgut invasion and transformation of the ookinete to oocyst. *Pb_c53* seems to have the most effective involvement during these stages, as its deletion has the most protrusive phenotype.

Although no reliable conclusions can be derived from the *A. gambiae* infections presented in this study, further experiments monitoring *P. berghei* development in *A. gambiae,* successfully conducted in the host lab (D. Vlachou, personal communication), disclosed a pungent effect of the deletion of these genes. Specifically, consistent with the results from the *A. stephensi* infections, all four transgenic lines presented severe reduction in the oocyst numbers compared to the WT, with *ΔPb_c53* presenting, again, the most outstanding phenotype.

Also, ookinete motility assay revealed that *Pb_c01* deletion has no effect in the ookinete motility and invasive ability, with the *ΔPb_c01* transgenic line producing normally motile ookinetes, compared to the WT (D. Vlachou, personal communication). This proposes that *Pb_c01* gene is most probably involved in the stage of ookinete-to-oocyst transformation, consistent with its *P. falciparum* ortholog's transcriptional peak in this stage (Figure 4.4).

Overall, the results derived from the continuation of the phenotypic analysis of these four transgenic lines in the host lab reinforce the major conclusion of this thesis that all four genes play a substantial role during the period of the severest bottleneck in the parasite's sexual development, the ookinete invasion and transformation to oocyst. Specifically, the *Pb_c53* and *Pb_c22* which have no predicted transmembrane domains or signal peptide motifs (allowing for no *in silico* based predictions about putative functions to be made) are most probably responsible for the successful development of *P. berghei* during the midgut invasion and ookinete-to-oocyst transition stages. On the other hand, the *Pb_c01* and *Pb_c57,* based on the predicted protein structures which include both signal peptide motif and a transmembrane domain, are most likely

to produce molecules that putatively interact with mosquito midgut molecules, possibly facilitating midgut invasion, and thus candidates for TBV interventions.

Undoubtedly, transcriptional regulation of these four genes plays a key role in the efficacy of *P. berghei* sexual development and transmission, suggesting that their *P. falciparum* orthologs are most likely to have a similar role, based on their expression profiles (Figure 4.4) and their predicted protein structures (Figure 4.6).

CHAPTER 6: CONCLUDING REMARKS AND FUTURE RESEARCH PERSPECTIVES

CHAPTER 6: CONCLUDING REMARKS AND FUTURE RESEARCH PERSPECTIVES

The successful targeted gene disruption of four *P. berghei* candidates and their detailed *in vitro* and *in vivo* phenotypic analysis identified four novel genes that have crucial roles, as demonstrated for the first time, during the stages between ookinete maturation and oocyst formation and are really important for rodent malaria transmission. This study has provided precious information that could be exploitable towards the elucidation of key gaps in the knowledge of the parasite mechanisms that are recruited during its development inside the mosquito vector and has, also, revealed two novel potential targets for the deployment of new transmission blocking interventions.

The extensive phenotypic analysis conducted during this study, identified key regulators of sexual development of the malaria parasite in the mosquito midgut and novel targets for TBV interventions. Future work will elucidate the exact function of these four novel genes. Future work will encompass the completion of this phenotypic analysis, including *in vivo* ookinete invasion assays for all the transgenic parasite lines in order to determine the transgenic ookinete motile behaviour and invasive ability (already performed for *Pb_c01* in the host lab). By these assays it will be possible to specify whether the gene products are involved in the development of these parasite abilities or they regulate later developmental stages.

Complementation experiments, including the replacement of *P. berghei* genes with the *P. falciparum* orthologs (knock in), could also be performed. By these means, the hypothesis that the orthologous genes have a conserved function at the same time point of the development of the two different parasite spp could be illuminated if there is rescue of KO the phenotype, or vice versa.

The research should be continued with the cloning of these *P. falciparum* genes in expression vectors for the production of the human malaria parasite proteins, a procedure already initiated in the host lab. These proteins can be used for the generation of native antibodies targeting the products of the identified genes, which will assist localisation experiments that will shed light on their subcellular localisation, and consequently their function, utilising confocal microscopy.

Apart from the utility of the native antibodies in the localisation experiments, they will, most importantly, be used in order to reach the long-term goal, the development of TBV. The products of *Pf_c01* and *Pf_c57*, which are putatively surface proteins of the parasite, comprise extremely intriguing targets for this approach. Antibody inhibition assays, performed in the field, will evaluate the capability of the generated antibodies to inhibit the sexual development of *Plasmodium* and the potential of these antigens as transmission blocking intervention targets.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere appreciation to Dr Dina Vlachou for kindly including me in her lab group and assigning an important project to me, building trust upon me. Except for offering her warm encouragement and thoughtful guidance, she has opened the gate for me to the fascinating world of malaria research. Also, I feel obliged to pay homage to Professor George Christophides for his generosity in accepting my Erasmus Training application and providing the opportunity to live a unique experience.

Deffinitely, I owe a very important debt to Professor Kostas Mathiopoulos who encouraged me to apply for the Erasmus scholarship, supported my work and gave his precious advice which led me to the right way. He has been a significant inspiration for me not only regarding my studies in Biochemistry and Biotechnology and my scientific career but also regarding my life in general, becoming a guiding light for me.

Special thanks to Valerie Chiamaka Ukekbu and Katarzyna Sala for the patient technical assistance and the emotional support. They managed to transform hard work into joy and fun.

Finally, I would like to extend my appreciation to Jee-Sun Cho and Tibebu Habtewold for the mosquito manipulation instructions, Michael Povelones, Zannatul Ferdus and Stavros Stathopoulos for the scientific advice and the whole immunogenomics lab group for the reassurance.

Last but not least, I would particularly like to thank Nikolaos Trasanidis, a respectable colleague but most importantly a precious friend, without whom this experience would never be as exhilarating as it was, and I.P.M. for altruistically contributing to the formation of my character.

REFERENCES

Abdulla, S., et al. "A research agenda for malaria eradication: vaccines." (2011).

Ahmed, Ashraf M., and Hilary Hurd. "Immune stimulation and malaria infection impose reproductive costs in *Anopheles gambiae* via follicular apoptosis." *Microbes and Infection* 8.2 (2006): 308-315.

Alano, P., O. Billker, and I. W. Sherman. "Gametocytes and gametes."*Molecular approaches to malaria* (2005): 191-219.

Alavi, Y., et al. "The dynamics of interactions between Plasmodium and the mosquito: a study of the infectivity of Plasmodium berghei and Plasmodium gallinaceum, and their transmission by Anopheles stephensi, Anopheles gambiae and Aedes aegypti." *International journal for parasitology* 33.9 (2003): 933-943.

Aly, Ahmed SI, and Kai Matuschewski. "A malarial cysteine protease is necessary for Plasmodium sporozoite egress from oocysts." *The Journal of experimental medicine* 202.2 (2005): 225-230.

Aly, Ahmed SI, Ashley M. Vaughan, and Stefan HI Kappe. "Malaria parasite development in the mosquito and infection of the mammalian host." *Annual review of microbiology* 63 (2009): 195.

An, Chunju, et al. "Characterization of a regulatory unit that controls melanization and affects longevity of mosquitoes." *Cellular and Molecular Life Sciences* 68.11 (2011): 1929-1939.

Aregawi, M., et al. "Programme, WGM (2008)." *World Malaria Report* (2008).

Aurrecoechea, Cristina, et al. "PlasmoDB: a functional genomic database for malaria parasites." *Nucleic acids research* 37.suppl 1 (2009): D539-D543.

Babaeekho, Laleh, Sedigheh Zakeri, and Navid Dinparast Djadid. "Genetic mapping of the duffy binding protein (DBP) ligand domain of Plasmodium vivax from unstable malaria region in the Middle East." *The American journal of tropical medicine and hygiene* 80.1 (2009): 112-118.

Bartoloni, Alessandro, and Lorenzo Zammarchi. "Clinical Aspects of Uncomplicated and Severe Malaria." *Mediterranean journal of hematology and infectious diseases* 4.1 (2012).

Beare, Nicholas AV, et al. "Malarial retinopathy: a newly established diagnostic sign in severe malaria." *The American journal of tropical medicine and hygiene*75.5 (2006): 790.

Bell, David, Chansuda Wongsrichanalai, and John W. Barnwell. "Ensuring quality and access for malaria diagnosis: how can it be achieved?." *Nature Reviews Microbiology* 4.9 (2006): 682-695.

Billker, O., et al. "Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito." *Nature* 392.6673 (1998): 289-292.

Billker, O., et al. "The roles of temperature, pH and mosquito factors as triggers of male and female gametogenesis of Plasmodium berghei in vitro."*Parasitology* 115.01 (1997): 1-7.

Billker, Oliver, et al. "Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite." *Cell* 117.4 (2004): 503-514.

Blandin, Stephanie, et al. "Complement-Like Protein TEP1 Is a Determinant of Vectorial Capacity in the Malaria Vector *Anopheles gambiae*." *Cell*116.5 (2004): 661-670.

Breman, J. G., et al. "Dichlorodiphenyltrichloroethane (DDT) for indoor residual spraying in Africa: how can it be used for malaria control?." (2007).

Breman, Joel G., et al. "Conquering the intolerable burden of malaria: what's new, what's needed: a summary." (2004).

Bruce-Chuvatt, L. J. "Alphonse Laveran's discovery 100 years ago and today's global fight against malaria." *Journal of the Royal Society of Medicine* 74.7 (1981): 531.

Bruce-Chwatt, L. J. "Malaria and its control: present situation and future prospects." *Annual review of public health* 8.1 (1987): 75-110.

Bushell, Ellen SC, et al. "Paternal effect of the nuclear formin-like protein MISFIT on Plasmodium development in the mosquito vector." *PLoS pathogens*5.8 (2009): e1000539.

Butler, Declan. "Initiative targets malaria eradication." *Nature* 462.7269 (2009): 19.

Canning, ELIZABETH U., and R. E. Sinden. "The organization of the ookinete and observations on nuclear division in oocysts of Plasmodium berghei."*Parasitology* 67.1 (1973): 29.

Carter, Richard, and Carter L. Diggs. "Plasmodia of rodents." *Parasitic protozoa*3 (1977): 359-451.

Carter, Richard, et al. "Malaria transmission-blocking vaccines—how can their development be supported?." (2000).

Carter, Richard. "Transmission blocking malaria vaccines." *Vaccine* 19.17 (2001): 2309-2314.

Carter, Victoria, et al. "Minimum requirements for ookinete to oocyst transformation in Plasmodium." *International journal for parasitology*37.11 (2007): 1221-1232.

Casares, Sofia, Teodor-Doru Brumeanu, and Thomas L. Richie. "The RTS, S malaria vaccine." *Vaccine* 28.31 (2010): 4880-4894.

Cerenius, Lage, Bok Luel Lee, and Kenneth Söderhäll. "The proPO-system: pros and cons for its role in invertebrate immunity." *Trends in immunology* 29.6 (2008): 263-271.

Chima, Reginald Ikechukwu, Catherine A. Goodman, and Anne Mills. "The economic impact of malaria in Africa: a critical review of the evidence." *Health policy* 63.1 (2003): 17-36.

Chowdhury, Debabani Roy, et al. "A potent malaria transmission blocking vaccine based on codon harmonized full length Pfs48/45 expressed in Escherichia coli." *PloS one* 4.7 (2009): e6352.

Christophides, George K., Dina Vlachou, and Fotis C. Kafatos. "Comparative and functional genomics of the innate immune system in the malaria vector Anopheles gambiae." *Immunological reviews* 198.1 (2004): 127-148.

Christophides, George K., Dina Vlachou, and Fotis C. Kafatos. "Comparative and functional genomics of the innate immune system in the malaria vector Anopheles gambiae." *Immunological reviews* 198.1 (2004): 127-148.

Collins, William E. "Plasmodium knowlesi: A Malaria Parasite of Monkeys and Humans*." *Annual review of entomology* 57 (2012): 107-121.

Crawley, Jane, et al. "Malaria in children." *The Lancet* 375.9724 (2010): 1468-1481.

Crompton, Peter D., Susan K. Pierce, and Louis H. Miller. "Advances and challenges in malaria vaccine development." *The Journal of clinical investigation* 120.12 (2010): 4168.

de Hoon, Michiel JL, et al. "Open source clustering software." *Bioinformatics* 20.9 (2004): 1453-1454.

Dearsly, A. L., R. E. Sinden, and I. A. Self. "Sexual development in malarial parasites: gametocyte production, fertility and infectivity to the mosquito vector." *Parasitology* 100.Part 3 (1990): 359-368.

Dessens, Johannes T., et al. "CTRP is essential for mosquito infection by malaria ookinetes." *The EMBO journal* 18.22 (1999): 6221-6227.

Dessens, Johannes T., et al. "SOAP, a novel malaria ookinete protein involved in mosquito midgut invasion and oocyst development." *Molecular microbiology*49.2 (2003): 319-329.

Dessens, Johannes T., Robert E. Sinden, and Charles Claudianos. "LCCL proteins of apicomplexan parasites." *Trends in parasitology* 20.3 (2004): 102-108.

Dinglasan, Rhoel R., and Marcelo Jacobs-Lorena. "Flipping the paradigm on malaria transmission-blocking vaccines." *Trends in parasitology* 24.8 (2008): 364-370.

Dixon, M. W., et al. "Sex in Plasmodium: a sign of commitment." *Trends in parasitology* 24.4 (2008): 168.

Dong, Yuemei, et al. "Anopheles gambiae immune responses to human and rodent Plasmodium parasite species." *PLoS pathogens* 2.6 (2006): e52.

Dorin-Semblat, Dominique, et al. "Functional characterization of both MAP kinases of the human malaria parasite Plasmodium falciparum by reverse genetics." *Molecular microbiology* 65.5 (2007): 1170-1180.

Druilhe, Pierre, et al. "A malaria vaccine that elicits in humans antibodies able to kill Plasmodium falciparum." *PLoS medicine* 2.11 (2005): e344.

Ecker, Andrea, et al. "Reverse genetics screen identifies six proteins important for malaria development in the mosquito." *Molecular microbiology* 70.1 (2008): 209-220.

Egan, Timothy J., and Catherine H. Kaschula. "Strategies to reverse drug resistance in malaria." *Current opinion in infectious diseases* 20.6 (2007): 598-604.

El Sahly, H. M., et al. "Safety and immunogenicity of a recombinant nonglycosylated erythrocyte binding antigen 175 Region II malaria vaccine in healthy adults living in an area where malaria is not endemic." *Clinical and Vaccine Immunology* 17.10 (2010): 1552-1559.

Engwerda, Christian R., and Michael F. Good. "Disarming the malaria parasite."*Nature medicine* 14.9 (2008): 912-913.

Esen, Meral, et al. "Safety and immunogenicity of GMZ2—a MSP3–GLURP fusion protein malaria vaccine candidate." *Vaccine* 27.49 (2009): 6862-6868.

Ferguson, Heather M., and Andrew F. Read. "Why is the effect of malaria parasites on mosquito survival still unresolved?." *Trends in parasitology* 18.6 (2002): 256-261.

Frischknecht, Friedrich, et al. "Imaging movement of malaria parasites during transmission by Anopheles mosquitoes." *Cellular microbiology* 6.7 (2004): 687-694.

Gardner, Malcolm J., et al. "Genome sequence of the human malaria parasite Plasmodium falciparum." *Nature* 419.6906 (2002): 498-511.

Genton, Blaise, et al. "A recombinant blood-stage malaria vaccine reduces Plasmodium falciparum density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea." *Journal of Infectious Diseases* 185.6 (2002): 820-827.

Gething, Peter W., et al. "A new world malaria map: Plasmodium falciparum endemicity in 2010." *Malar J* 10.378 (2011): 1475-2875.

Gilles, Herbert M. "Epidemiology of malaria." *Bruce-Chwatt's essential malariology* (1993): 124-163.

Ghosh, Anil K., Paulo EM Ribolla, and Marcelo Jacobs-Lorena. "Targeting Plasmodium ligands on mosquito salivary glands and midgut with a phage display peptide library." *Proceedings of the National Academy of Sciences*98.23 (2001): 13278-13281.

Gollin, Douglas, and Christian Zimmermann. "Malaria: Disease impacts and long-run income differences." (2007).

Greenwood, B., K. Marsh, and R. Snow. "Why do some African children develop severe malaria?." *Parasitology today* 7.10 (1991): 277-281.

Greenwood, Brian M., et al. "Malaria: progress, perils, and prospects for eradication." *The Journal of clinical investigation* 118.4 (2008): 1266.

Guyatt, Helen L., and Robert W. Snow. "The cost of not treating bednets."*Trends in parasitology* 18.1 (2002): 12.

Habtewold, Tibebu, et al. "Transmission blocking immunity in the malaria non-vector mosquito Anopheles quadriannulatus species A." *PLoS pathogens* 4.5 (2008): e1000070.

Hall, Neil, et al. "A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses." *Science* 307.5706 (2005): 82-86.

Han, Yeon Soo, et al. "Molecular interactions between Anopheles stephensi midgut cells and Plasmodium berghei: the time bomb theory of ookinete invasion of mosquitoes." *The EMBO journal* 19.22 (2000): 6030-6040.

Hänscheid, Thomas, Timothy J. Egan, and Martin P. Grobusch. "Haemozoin: from melatonin pigment to drug target, diagnostic tool, and immune modulator."*The Lancet infectious diseases* 7.10 (2007): 675-685.

Hermsen, Cornelus C., et al. "Glutamate-rich protein (GLURP) induces antibodies that inhibit in vitro growth of Plasmodium falciparum in a phase 1 malaria vaccine trial." *Vaccine* 25.15 (2007): 2930-2940.

Herrera, Sócrates, et al. " Aotus monkeys: their great value for anti-malaria vaccines and drug testing." *International journal for parasitology* 32.13 (2002): 1625-1635.

Herrington, Deirdre, et al. "Successful immunization of humans with irradiated malaria sporozoites: humoral and cellular responses of the protected individuals." *The American journal of tropical medicine and hygiene* 45.5 (1991): 539.

Hillyer, Julián F., Catherine Barreau, and Kenneth D. Vernick. "Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito haemocoel." *International journal for parasitology*37.6 (2007): 673-681.

Hoffman, Stephen L., et al. "Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites."*Journal of Infectious Diseases* 185.8 (2002): 1155-1164.

Hogg, J. C., and H. Hurd. "The effects of natural Plasmodium falciparum infection on the fecundity and mortality of Anopheles gambiae sl in north east Tanzania." *Parasitology* 114.04 (1997): 325-331.

Huber, Marcel, Enrico Cabib, and Louis H. Miller. "Malaria parasite chitinase and penetration of the mosquito peritrophic membrane." *Proceedings of the National Academy of Sciences* 88.7 (1991): 2807-2810.

Hyde, John E. "Drug-resistant malaria− an insight." *Febs Journal* 274.18 (2007): 4688-4698.

Ishino, Tomoko, et al. "A calcium-dependent protein kinase regulates Plasmodium ookinete access to the midgut epithelial cell." *Molecular microbiology* 59.4 (2006): 1175-1184.

Janse, Chris J., et al. "DNA synthesis in *Plasmodium berghei* during asexual and sexual development." *Molecular and biochemical parasitology* 20.2 (1986): 173-182.

Kadota, Kimie, et al. "Essential role of membrane-attack protein in malarial transmission to mosquito host." *Proceedings of the National Academy of Sciences of the United States of America* 101.46 (2004): 16310-16315.

Kaiser, Karine, et al. "A member of a conserved Plasmodium protein family with membrane-attack complex/perforin (MACPF)-like domains localizes to the micronemes of sporozoites." *Molecular and biochemical parasitology* 133.1 (2004): 15.

Kakani, E. "Molecular analysis of genetic loci involved in the development of insecticide resistance mechanisms of the most important olive fruit pest, the olive fly Bactrocera oleae." *Unpublished Doctoral Dissertation*, (2009).University of Thessaly. Larisa, Greece.

Kappe, Stefan HI, Carlos A. Buscaglia, and Victor Nussenzweig. "Plasmodium sporozoite molecular cell biology." *Annu. Rev. Cell Dev. Biol.* 20 (2004): 29-59.

Kariu, Tohru, et al. "MAEBL is essential for malarial sporozoite infection of the mosquito salivary gland." *The Journal of experimental medicine* 195.10 (2002): 1317-1323.

Kats, Lev M., et al. "Plasmodium rhoptries: how things went pear-shaped." *Trends in parasitology* 22.6 (2006): 269-276.

Khan, Shahid M., et al. "Proteome Analysis of Separated Male and Female Gametocytes Reveals Novel Sex-Specific Plasmodium Biology." *Cell*121.5 (2005): 675-687.

Kotsyfakis, Michalis, et al. "Plasmodium berghei ookinetes bind to Anopheles gambiae and Drosophila melanogaster annexins." *Molecular microbiology* 57.1 (2005): 171-179.

Kumar, Nirbhay, et al. "Molecular complexity of sexual development and gene regulation in Plasmodium falciparum." *International journal for parasitology*34.13-14 (2004): 1451.

Lasonder, Edwin, et al. "Analysis of the Plasmodium falciparum proteome by high-accuracy mass spectrometry." *Nature* 419.6906 (2002): 537-542.

Lasonder, Edwin, et al. "Proteomic profiling of Plasmodium sporozoite maturation identifies new proteins essential for parasite development and infectivity." *PLoS pathogens* 4.10 (2008): e1000195.

Laveran, A. "A new parasite found in the blood of malarial patients. Parasitic origin of malarial attacks." *Bull. Mem. Soc. Med. Hosp. Paris* 17 (1880): 158-164.

Le Roch, Karine G., et al. "Discovery of gene function by expression profiling of the malaria parasite life cycle." *Science* 301.5639 (2003): 1503-1508.

Lodish, Harvey, et al. "Molecular cell biology." *New York* (2000).

Mair, Gunnar R., et al. "Regulation of sexual development of Plasmodium by translational repression." *Science* 313.5787 (2006): 667-669.

Malkin, Elissa M., et al. "Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for Plasmodium vivax malaria." *Vaccine* 23.24 (2005): 3131-3138.

Mandell, Gerald L. "Mandell, Douglas, and Bennett's principles and practice of infectious diseases; Chapter 275. Plasmodium species (malaria)" (2005).

McRobert, Louisa, et al. "Gametogenesis in malaria parasites is mediated by the cGMP-dependent protein kinase." *PLoS biology* 6.6 (2008): e139.

Meissner, Markus, et al. "Tetracycline analogue-regulated transgene expression in Plasmodium falciparum blood stages using Toxoplasma gondii transactivators." *Proceedings of the National Academy of Sciences of the United States of America* 102.8 (2005): 2980-2985.

Meister, S., A. C. Koutsos, and G. K. Christophides. "The Plasmodium parasite—a 'new'challenge for insect innate immunity." *International journal for parasitology* 34.13 (2004): 1473-1482.

Ménard, Robert, et al. "Circumsporozoite protein is required for development of malaria sporozoites in mosquitoes." (1997): 336-340.

Miller, Louis H., et al. "The pathogenic basis of malaria." *Nature* 415.6872 (2002): 673-679.

Mons, Barend. "Intra erythrocytic differentiation of Plasmodium berghei." *Acta Leidensia* 54 (1986): 1.

Montgomerie, Scott, et al. "PROTEUS2: a web server for comprehensive protein structure prediction and structure-based annotation." *Nucleic acids research*36.suppl 2 (2008): W202-W209.

Mueller, Ann-Kristin, et al. "Plasmodium liver stage developmental arrest by depletion of a protein at the parasite–host interface." *Proceedings of the National Academy of Sciences of the United States of America* 102.8 (2005): 3022-3027.

Mueller, Ivo, Peter A. Zimmerman, and John C. Reeder. "Plasmodium malariae and Plasmodium ovale–the 'bashful'malaria parasites."*Trends in parasitology* 23.6 (2007): 278-283.

Müller, H. M., et al. "Members of a trypsin gene family in Anopheles gambiae are induced in the gut by blood meal." *The EMBO journal* 12.7 (1993): 2891.

Mullis, Kary B. "The unusual origin of the polymerase chain reaction." *Scientific American* 262.4 (1990): 56-61.

Nadjm, Behzad, and Ron H. Behrens. "Malaria: an update for physicians."*Infectious disease clinics of North America* 26.2 (2012): 243.

Nagasawa, Hideyuki, et al. "*Plasmodium malariae*: Distribution of circumsporozoite protein in midgut oocysts and salivary gland sporozoites."*Experimental parasitology* 66.1 (1988): 27-34.

Ogutu, Bernhards R., et al. "Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya." *PLoS One* 4.3 (2009): e4708.

Oliveira, Giselle de Almeida, Joshua Lieberman, and Carolina Barillas-Mury. "Epithelial nitration by a peroxidase/NOX5 system mediates mosquito antiplasmodial immunity." *Science Signaling* 335.6070 (2012): 856.

Osta, Mike A., George K. Christophides, and Fotis C. Kafatos. "Effects of mosquito genes on Plasmodium development." *science* 303.5666 (2004): 2030-2032.

Outchkourov, Nikolay S., et al. "Correctly folded Pfs48/45 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in mice." *Proceedings of the National Academy of Sciences* 105.11 (2008): 4301-4305.

Pasvol, Geoffrey. "The treatment of complicated and severe malaria." *British medical bulletin* 75.1 (2005): 29-47.

Perkins, Susan L., and Christopher C. Austin. "Four new species of Plasmodium from New Guinea lizards: integrating morphology and molecules."*Journal of Parasitology* 95.2 (2009): 424-433.

Perlmann, Peter, and Marita Troye-Blomberg. "Malaria and the immune system in humans." (2004): 229-242.

Peter W., et al. "A new world malaria map: Plasmodium falciparum endemicity in 2010." *Malar J* 10.378 (2011): 1475-2875.

Pimenta, Paulo F., Musa Touray, and Louis Miller. "The journey of malaria sporozoites in the mosquito salivary gland." *Journal of Eukaryotic Microbiology* 41.6 (1994): 608-624.

Povelones, Michael, et al. "Leucine-rich repeat protein complex activates mosquito complement in defense against Plasmodium parasites." *Science Signaling* 324.5924 (2009): 258.

Povelones, Michael, et al. "Structure-function analysis of the Anopheles gambiae LRIM1/APL1C complex and its interaction with complement C3-like protein TEP1." *PLoS pathogens* 7.4 (2011): e1002023.

Pradel, G. "Proteins of the malaria parasite sexual stages: expression, function and potential for transmission blocking strategies." *Parasitology* 134.14 (2007): 1911-1929.

Raibaud, Anna, et al. "Differential gene expression in the ookinete stage of the malaria parasite Plasmodium berghei." *Molecular and biochemical parasitology* 150.1 (2006): 107-113.

Rangarajan, Radha, et al. "A mitogen-activated protein kinase regulates male gametogenesis and transmission of the malaria parasite Plasmodium berghei."*EMBO reports* 6.5 (2005): 464-469.

Reininger, Luc, et al. "A NIMA-related protein kinase is essential for completion of the sexual cycle of malaria parasites." *Journal of Biological Chemistry*280.36 (2005): 31957-31964.

Reininger, Luc, et al. "An essential role for the Plasmodium Nek-2 Nima-related protein kinase in the sexual development of malaria parasites." *Journal of Biological Chemistry* 284.31 (2009): 20858-20868.

Rodriguez, Mario H., and Fidel de la C. Hernández-Hernández. "Insect–malaria parasites interactions: the salivary gland." *Insect Biochemistry and Molecular Biology* 34.7 (2004): 615-624.

Sagara, Issaka, et al. "A randomized controlled phase 2 trial of the blood stage AMA1-C1/Alhydrogel malaria vaccine in children in Mali." *Vaccine* 27.23 (2009): 3090-3098.

Saldanha, Alok J. "Java Treeview—extensible visualization of microarray data." *Bioinformatics* 20.17 (2004): 3246-3248.

Sallares, Robert. *Malaria and Rome: a history of malaria in ancient Italy*. Oxford: Oxford University Press, 2002.

Saul, Allan, and Michael P. Fay. "Human immunity and the design of multi-component, single target vaccines." *PLoS One* 2.9 (2007): e850.

Saxena, Ajay K., Yimin Wu, and David N. Garboczi. "Plasmodium p25 and p28 surface proteins: potential transmission-blocking vaccines." *Eukaryotic cell* 6.8 (2007): 1260-1265.

Schüler, Herwig, and Kai Matuschewski. " Plasmodium motility: actin not actin'like actin." *Trends in parasitology* 22.4 (2006): 146-147.

Sherman, Irwin W. *Molecular approaches to malaria*. ASM Press, 2005.

Schofield, Louis, and Georges E. Grau. "Immunological processes in malaria pathogenesis." *Nature Reviews Immunology* 5.9 (2005): 722-735.

Siden-Kiamos, Inga, et al. "Distinct roles for pbs21 and pbs25 in the in vitro ookinete to oocyst transformation of Plasmodium berghei." *Journal of cell science* 113.19 (2000): 3419-3426.

Sinden, R. E. "Molecular interactions between Plasmodium and its insect vectors." *Cellular microbiology* 4.11 (2002): 713-724.

Sinden, R. E., and Karen Strong. "An ultrastructural study of the sporogonic development of *Plasmodium falciparum* in *Anopheles gambiae*."*Transactions of the Royal Society of Tropical medicine and Hygiene* 72.5 (1978): 477-491.

Sinden, Robert E. "Gametocytes and sexual development." *Malaria: Parasite biology, pathogenesis, and protection* (1998): 25-48.

Sinden, Robert E., et al. "Progression of Plasmodium berghei through Anopheles stephensi is density-dependent." *PLoS pathogens* 3.12 (2007): e195.

Srinivasan, Prakash, Hisashi Fujioka, and Marcelo Jacobs-Lorena. "PbCap380, a novel oocyst capsule protein, is essential for malaria parasite survival in the mosquito." *Cellular microbiology* 10.6 (2008): 1304-1312.

Takken, Willem. "Do insecticide-treated bednets have an effect on malaria vectors?." *Tropical Medicine & International Health* 7.12 (2002): 1022-1030.

Tewari, Rita, et al. "An atypical mitogen-activated protein kinase controls cytokinesis and flagellar motility during male gamete formation in a malaria parasite." *Molecular microbiology* 58.5 (2005): 1253-1263.

Tewari, Rita, et al. "Function of Region I and II Adhesive Motifs ofPlasmodium falciparum Circumsporozoite Protein in Sporozoite Motility and Infectivity."*Journal of Biological Chemistry* 277.49 (2002): 47613-47618.

Tomas, Ana M., et al. "P25 and P28 proteins of the malaria ookinete surface have multiple and partially redundant functions." *The EMBO journal* 20.15 (2001): 3975-3983.

Trueman, Holly E., et al. "Functional characterization of an LCCL-lectin domain containing protein family in Plasmodium berghei." *Journal of Parasitology* 90.5 (2004): 1062-1071.

Trueman, Holly E., et al. "Functional characterization of an LCCL-lectin domain containing protein family in Plasmodium berghei." *Journal of Parasitology* 90.5 (2004): 1062-1071.

Vlachou, Dina, et al. "Real-time, in vivo analysis of malaria ookinete locomotion and mosquito midgut invasion." *Cellular Microbiology* 6.7 (2004): 671-685.

Vlachou, Dina, et al. "The developmental migration of *Plasmodium* in mosquitoes." *Current opinion in genetics & development* 16.4 (2006): 384-391

Wang, Qian, Hisashi Fujioka, and Victor Nussenzweig. "Exit of Plasmodium sporozoites from oocysts is an active process that involves the circumsporozoite protein." *PLoS pathogens* 1.1 (2005): e9.

Weatherall, David J., et al. "Malaria and the red cell." *ASH Education Program Book* 2002.1 (2002): 35-57.

Weathersby, A. Burns. "The ectopic development of malarial oocysts."*Experimental parasitology* 3.6 (1954): 538.

Wilson, Michael L. "Malaria rapid diagnostic tests." *Clinical infectious diseases* 54.11 (2012): 1637-1641.

Wu, Yimin, et al. "Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51." *PloS one* 3.7 (2008): e2636.

Yuda, Masao, et al. "von Willebrand Factor A Domain-related Protein, a novel microneme protein of the malaria ookinete highly conserved throughout *Plasmodium* parasites." *Molecular and biochemical parasitology* 116.1 (2001): 65-72.

ABBREVIATIONS

AMA	Apical Membrane Antigen
ANKA	Anvers/Kasapa
APL	*Anopheles Plasmodium*-responsive Leucine-rich repeat
Apo	Apolipophorin
AT	Adenine-Thymine
ATP	Adenosine TriPhosphate
BC	Before Christ
bp	base pair
BSA	Bovine Serum Albumin
C57BL/6	C57 Black 6
CAP	Capsule Protein
CDPK	Calcium Dependent Protein Kinase
cDNA	complementary DNA
CHIT	Chitinase
CSP	CircumSporozoite Protein
CTL	C-Type Lectin
CTP	Citidine TriPhosphate
CTRP	Circumsporozoite and TRAP Related Protein
DAPI	4',6-diamidino-2-phenylindole
DDT	DichloroDiphenylTrichloroethane
DHFR-TS	DiHydroFolate Reductase-Thymidilate Synthase
DNA	DeoxyriboNucleic Acid
DOZI	Development Of Zygote Inhibited
ECP	Egress Cysteine Protease
EDTA	Ethylene Diamine Tetraacetic Acid
EGF	epidermal growth factor
eGFP	enhanced Green Fluorescent Protein
ER	Endoplasmic reticulum
F	Forward
FCS	Foetal Calf Serum
FBS	Foetal Bovine Serum
GPI	Glycosylphosphatidylinositol
GTP	Guanosine TriPhosphate
Hepes	4-(2-HydroxyEthyl)-1-Piperazine Ethane Sulfonic acid
IM	Intra-Muscular
INT	Integration
IP	Intraperitoneal
RNAi	RNA interference
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Nets

KD	Knock-Down
KO	Knock-Out
LAN	Laminin
LAP	LCCL/lectin adhesive- like protein
LB	Luria broth
LCCL	Limulus Clotting factor C, Coch-5b2 and Lgl1
LRIM	Leucine Rich-Repeat Immune gene
mAb	monoclonal Antibody
MACPF	Membrane Attack Complex Perforin
MAEBL	Merozoite AMA1/Erythrocyte Binding Ligand-like protein
MAPK	Mitogen-Activated Protein Kinase
Mg	Milligram
µg	microgram
MISFIT	Male-Inherited Sporulation Factor Important for Transmission
mL	milliLiter
µL	microLiter
mRNA	messenger RNA
MSP	Merozoite Surface Protein
MTOC	MicroTubule Organising Centre
ng	nanogram
Nek	NIMA related kinase
NIAID	National Institute of Allergy and Infectious Diseases
NIMA	Never-in-mitosis/Aspergillus
NOS	Nitric Oxide Synthase
ORF	Open Reading Frame
PBS	Phosphate-Buffered Saline
pBS	pBluescript
PCR	Polymerase Chain Reaction
PH	PhenylHydrazinium chloride
P.I.	Post infection
(P)PO	(Pro)PhenolOxidase
PPLP	*Plasmodium* Perforin-Like Protein
PSOP	Putative Secreted Ookinete Proteins
PV	Parasitophorous vacuole
R	Reverse
RBC	Red Blood Cell
RDT	Rapid Diagnostic Tests
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse Transcriptase PCR
SE	Standard Error
Serpin	Serine protease inhibitor
SOAP	Secreted Ookinete Adhesive Protein
Spp	Species (plural)
SRPN	Serpin

TBE	Tris-Borate-EDTA
TEP	Thioester-containing protein
TBE	Tris-borate-EDTA
TBV	Transmission blocking Vaccine
*Tg*DHFR-TS	*Toxoplasma gondii* DiHydroFolate Reductase-Thymidilate Synthase
Tm	Template Melting temperature
TO	Theiler's Original
TRAP	Thrombospondin-related adhesive protein
TTP	Thymidine TriPhosphate
UTR	UnTranslated Region
WARP	von Willebrand Factor A domain-Related Protein
WASP	Wiskott–Aldrich Syndrome Protein
WHO	World Health Organisation
WT	Wild Type
XA	Xanthurenic Acid