Conclusion

In this study, the role of parasite-synthesized heme in all stages of malaria parasite growth has been assessed. \textit{P. berghei} ALAS and FC gene knockout parasites were generated. The Knockout parasites were used to track the parasite-synthetized heme and host haemoglobin heme during the intraerythrocytic stages of the parasite development. The knockout parasites injected intraperitoneally to mice showed no growth defects in the intraerythrocytic stages and all the infected animals died within 10 to 12 days, when parasitemia reached around 60%. The synthesis of mitochondrial cytochromes is essential for parasite survival, so the results would mean that the \textit{PbKO} parasites used hemoglobin-heme to synthesize cytochromes during the intraerythrocytic stages. This was demonstrated by radiolabeling hemoglobin-heme with [4-\textsuperscript{14}C]ALA in short-term mouse reticulocyte cultures. Radiolabelled hemozoin and mitochondrial cytochromes were found in reticulocytes infected with \textit{PbWT}, \textit{PbALASKO}, and \textit{PbFCKO} parasites. It was not possible to assess the contribution of hemoglobin-heme and parasite synthesized-heme in short term \textit{in vitro} cultures, because the use of [4-\textsuperscript{14}C]ALA to radiolabel heme would bypass the potential ALASKO block. At the same time, the \textit{PbFCKO} parasites would not be able to incorporate [4-\textsuperscript{14}C]ALA into heme. Previous study has shown that \textit{P. berghei} imports host ALAD as well as host FC (Bonday \textit{et al.}, 1997). Therefore, the possibility that the parasite used FC imported from the host to synthesize heme cannot be ruled out. This was addressed using \textit{P. falciparum} cultured in human RBC, as the western analysis indicated that the human RBCs used to culture \textit{P. falciparum} did not contain detectable levels of ALAS and FC. Further, the human RBCs did not incorporate [4-\textsuperscript{14}C]ALA into heme. So, all the radiolabeled heme in \textit{P. falciparum} was synthesized \textit{de novo} by the parasite.

Earlier studies have shown that high concentration of Succinyl Acetone (1-2 mM) (Surolia and Padmanaban, 1992) inhibited both heme synthesis and parasite growth. However, 50 \textmu M SA was found to completely inhibit heme synthesis in \textit{P. falciparum}, the concentration much lower than required to inhibit parasite growth, which means that \textit{P. falciparum} can use haemoglobin-heme to sustain growth under these conditions indicating that \textit{de novo} heme synthesis is not essential for \textit{P. falciparum} growth in culture. Similarly, 50 \textmu M SA completely inhibited heme synthesis in \textit{P. berghei}-infected reticulocytes as well but, did not affect \textit{P. berghei} growth in short-term cultures. The use of specific gene knockouts in the pathway has re-evaluated the earlier reports correlating the growth of the parasite with inhibition of heme synthesis or host enzyme import (Surolia and Padmanaban, 1992),
Since the parasite can survive in the absence of de novo heme synthesis, it may appear that the parasite heme-biosynthetic pathway has no role in the intraerythrocytic stages. However, it has been shown for the first time that *P. falciparum* growing in human RBCs incorporated parasite-synthesized heme radiolabeled with [4-14C]ALA into hemozoin as well as into mitochondrial cytochromes. Hemoglobin-heme in the RBCs was not radiolabeled; so the heme in the parasite hemozoin and mitochondrial cytochromes was synthesized de novo by the parasite. It has long been assumed that only hemoglobin-heme is converted into hemozoin in the parasite food vacuole. But now, it is clear that parasite-synthesized heme can also give rise to hemozoin in the food vacuole. It is possible that haemoglobin-heme and parasite synthesized heme serve as back up mechanisms for each other and the relative contributions of each has to be assessed under different environmental conditions. Since hemoglobin transport into the food vacuole involves cytostomes and other vesicle-mediated transformations (Elliott *et al*., 2008), it is not clear at this stage how the parasite-synthesized heme made in the mitochondrion finds its way to the food vacuole. The results from the study also emphasize the fact that hemozoin is, perhaps, the only mechanism for heme detoxification in the parasite. A recent study showed that the malaria parasite lacks the canonical heme oxygenase pathway for heme degradation and relies on hemozoin formation to detoxify heme (Sigala *et al*., 2012), although an earlier study suggested the possible presence of heme oxygenase in the apicoplast [van Dooren *et al*., 2012; Okada K, 2009]. It appears that the parasite mitochondrion would need a two-way transporter for heme: one to incorporate hemoglobin-heme into the mitochondrion and another to transport mitochondrial heme into the pathway leading to hemozoin formation in the food vacuole. Free heme was also detected in the erythrocyte at a concentration around 1 µM (Liu *et al*., 1988) and the parasite may be able to scavenge this heme directly [van Dooren *et al*., 2012]. It was also suggested that ferriprotoporphyrin could leach from the food vacuole into the parasite cytosol (Campanale *et al*., 2003). We found that SA inhibited the radiolabeling of hemozoin and of mitochondrial cytochromes in PbFCKO parasites. But, CQ inhibited the radiolabeling of hemozoin but not of mitochondrial cytochromes. These results suggest that hemoglobin-heme may be incorporated into mitochondrial cytochromes and into hemozoin through independent processes.

The growth pattern of the Knockout parasites in the mosquito stages was striking. While ookinetes formed, oocysts formation decreased substantially, and no sporozoites appeared in the salivary glands. Furthermore, when these mosquitoes fed on mice, we found no
intraerythrocytic-stage parasites in the blood of the mice. ALA supplement to the mosquitoes enabled \( PbALASKO \) to form oocysts and sporozoites. This is clear proof that de novo parasite heme synthesis is required for parasite development in mosquitoes. Hence, inhibitors of heme and porphyrin synthesis, such as diphenyl ether herbicides, can be explored to prevent parasite development in mosquitoes (Jacobs et al., 1992). Equally striking was the growth pattern of \( PbALASKO \) parasites in the liver stage. The sporozoites formed in the mosquitoes with ALA supplement could infect mice only when the mice received ALA supplement. This again shows that parasite de novo heme synthesis is required for development in the liver stage. The liver stage is a major focus of malaria interventions and the role of parasite heme synthesis in liver-stage development needs to be investigated in more detail. Inhibitors of parasite heme synthesis offer newer drug candidates for blocking infection and transmission, since the parasite enzymes involved have unique properties (Nagaraj et al., 2008; 2009; 2010; Padmanaban et al., 2013). Irradiated sporozoites serve as a malaria vaccine candidate (Vanderberg, 2009). There are several current efforts to design and stabilize irradiated sporozoites for large-scale clinical trials (Vanbuskirk et al., 2009; Roestenberg et al., 2013). Based on the results obtained for \( PbALASKO(Mq^{AL}) \) sporozoite infections in mice, \( PbALASKO \) sporozoites were tested for its potential as genetically attenuated sporozoite vaccine. The immunization experiments and the subsequent challenging with WT sporozoites showed that 7 out of 9 immunized mice were protected due to an increase in the levels of memory CD8\(^+\) T cells among immunized mice. These results suggest that \( PbALASKO(Mq^{AL}) \) sporozoite could serve as a genetically attenuated sporozoite vaccine in animal model.

The biology of parasite heme synthesis may change drastically between the intraerythrocytic stages and the mosquito and liver stages. The malaria parasite essentially depends on glycolysis to generate ATP in the intraerythrocytic stages. Hemoglobin is available as a heme source in addition to parasite-synthesized heme. In the mosquito and liver stages, the parasite depends entirely on its own biosynthetic machinery to provide heme. It is possible that the de novo heme-biosynthetic pathway of the parasite is augmented during the mosquito and liver stages. The ATP synthesized by the Electron transport chain may be necessary to provide the energy needed for ookinetes in the mosquito midgut to develop into sporozoites in the mosquito salivary glands. The energy provided by the Electron transport chain may also be necessary for the sporozoites to explore the mammalian host from the skin to liver and give rise to merozoites in the hepatocytes.