Ten best papers of the candidate, highlighting the important discoveries/contributions in the order of importance.

Work related to malaria parasite heme synthesis

1) Malaria parasite heme biosynthesis promotes and griseofulvin protects against cerebral malaria in mice (2022). Nature Communications, 13, 4028.

Discoveries/contributions/importance: My earlier study has shown that *de novo* heme biosynthetic pathway of malaria parasite is non-essential for RBC stages, but essential for the sporozoite formation in mosquitoes and exo-erythrocytic stages in liver. Interestingly, despite having access to heme derived from host hemoglobin, porphyrin intermediates and host enzymes, blood stage parasites synthesize heme. The present study offers new functional insights on the "dispensable" heme pathway of RBC stages and explains the dichotomy of parasite heme pathway and host heme acquisition pathways in RBC stages by showing the association of parasite heme pathway with disease virulence and pathogenesis. The answer to the role of *de novo* heme in the blood stages of malaria parasite that remained as an enigma for the last two decades is provided. The unexpected role of *de novo* heme in influencing the detoxification of imported hemoglobin-heme into hemozoin by modulating food vacuole integrity in the asexual stages has been demonstrated. Hemozoin is a key malarial PAMP that is associated with inflammation, aberrant host-immune responses, disease severity and cerebral pathogenesis. Heme pathway knockout *P. berghei* (the widely used rodent parasite model for human malaria) parasites synthesize less hemozoin and the mice infected with knockout parasites are completely protected from cerebral and severe malaria. *P. berghei* (or *P.yoelii*) is the only *in vivo* model available at present for disease severity and cerebral pathogenesis especially in the context of existing difficulties in performing research using non-human primate models. A unique approach to mitigate parasite virulence by targeting hemozoin through dispensable *de novo* heme pathway that may exert less selection pressure on the parasites has been identified. The translational potential of an already existing FDA-approved drug - griseofulvin that can be repurposed to tackle parasite virulence and disease severity along with the existing ACTs for preventing malaria mortality has been demonstrated. Griseofulvin inhibits parasite heme synthesis and prevents cerebral malaria in animal models. These findings have been taken forward for preclinical and clinical trials in collaboration with

Prado Pvt Ltd., Pune, and Ipca Laboratories Ltd., Mumbai. Since this work has been proposed for claiming this award, it is discussed in detail under **section d**.

2) Insights on heme synthesis in the malaria parasite (2017). Trends in Parasitology, 33(8), 583-586.

Discoveries/contributions/importance: In this opinion article, a number of outstanding questions on parasite heme synthesis in RBC stages have been put forth. Otherwise, the heme pathway in RBC stages has been written off by rest of the research community mentioning that it is non-esssential for RBC stages. The heme dynamics in the malaria parasite has been highlighted and the following questions that remain to be addressed are deliberated. Why do blood-stage parasites synthesize a non-essential heme and express heme pathway enzymes? Why do blood-stage parasites have multiple heme-acquisition pathways? Do blood-stage parasites import intermediates other than heme and PPIX? What are all the mechanisms involved in the uptake of heme/porphyrin intermediates from the host red cell? Do host enzymes contribute to heme synthesis in the parasite? In the context of *P. falciparum* predominantly invading RBCs, and *P. vivax* invading reticulocytes, is there a difference in parasite heme synthesis with respect to the host red cell? What are the contributions of endogenous glycine and exogenous ALA in parasite-infected red cells as starting metabolites in parasite heme synthesis in the blood stages? What are all the transporters involved in the shuttling of heme pathway intermediates across different organelles in the parasite? Based on these questions, the work published in Nature Communications 2022 submitted for this award has been carried out and the findings highlighted the role of "non-essential" heme in regulating the food vacuole integrity and hemozoin formation in the blood stage parasites, and its requirement for parasite virulence and cerebral pathogenesis.

3) Malaria parasite-synthesized heme is essential in the mosquito and liver stages and complements host heme in the blood stages of infection (2013). PLoS Pathogens, 9(8), e1003522.

Discoveries/contributions/importance: In this seminal study, I have shown for the first time that the *de novo* heme pathway of malaria parasite is dispensable for the development of blood stage parasites. However, it is absolutely essential for the formation of sporozoites in the salivary glands of the mosquitoes and malaria transmission. Similarly, *de novo* heme is also essential for the development of exo-erythrocytic stages in the liver. These *in vivo* studies have been carried out with *P. berghei* heme pathway knockout parasites, and to the best of my

knowledge these are the first transgenic knockout parasites developed in India without any collaboration in abroad. It has also been shown that the knockout blood stage parasites can utilize host heme for their mitochondrial cytochrome biogenesis that is essential for parasite survival, mitochondrial membrane potential and pyrimidine biosynthesis catalyzed by dihydroorotate biogenesis. Further, aminolevulinic acid (ALA) supplementation has been performed to show the sporozoite and liver stage rescue in the ALA synthase (first enzyme) knockout parasites. More importantly, these findings highlighted the important role played by parasite *de novo* heme in malaria transmission. This work has been recommended in F1000 Microbiology for new finding and novel drug target.

4) Localisation of *Plasmodium falciparum* uroporphyrinogen III decarboxylase of the heme-biosynthetic pathway in the apicoplast and characterisation of its catalytic properties (2009). International Journal for Parasitology, 39(5), 559-568.

Discoveries/contributions/importance: My earlier research involved the characterization of unique features of parasite heme pathway enzymes, their localization and expression in RBC stages and their involvement in *de novo* heme synthesis through an unusual hybrid pathway spanning across mitochondrion, apicoplast (redundant chloroplast) and cytosol. Uroporphyrinogen decarboxylase (fourth enzyme of parasite pathway) is a key enzyme in the heme-biosynthetic pathway and it occupies a strategic position in the unusual hybrid pathway for heme involving shuttling of intermediates between different subcellular compartments in the parasite. In this study, I have shown that the parasite enzyme is active and it is catalytically less efficient compared with the host counterpart. Molecular modeling suggested that the protein manifests a distorted triose phosphate isomerase (TIM) barrel fold and shares all the conserved or invariant amino acid residues at the active and substrate binding sites. Nevertheless, it is rich in lysine residues compared with the host enzyme. Mutation of specific lysine residues corresponding to residues at the dimer interface in human enzyme enhanced the catalytic efficiency of the enzyme and dimer stability indicating that the lysine rich nature and weak dimer interface are responsible for its low catalytic efficiency.

5) Unique properties of *Plasmodium falciparum* porphobilinogen deaminase (2008). Journal of Biological Chemistry, 283(1), 437-444.

Discoveries/contributions/importance: In this study, I have shown that the porphobilinogen deaminase (PBGD; third enzyme) encoded by the parasite genome has several unique biochemical properties. In particular, the apicoplast-localized PBGD catalyzes the conversion

of porphobilinogen to uroporphyrinogen III, indicating that it also possesses uroporphyrinogen III synthase (UROS) activity, catalyzing the next step. This in turn has provided an explanation for why the malaria parasite genome lacks the gene for UROS. Interestingly, PBGD gives rise to uroporphyrinogen III even after heat treatment, although UROS from other sources is known to be heat-sensitive. Based on the analysis of active site residues, a L116K mutant enzyme is generated and its specific activity is 5-fold higher than wildtype. More interestingly, L116K catalyzes the formation of uroporphyrinogen I in addition to uroporphyrinogen III indicating that with increased PBGD activity, the UROS activity of PBGD perhaps become rate-limiting, thus leading to non-enzymatic cyclization of preuroporphyrinogen to uroporphyrinogen I.

I have also performed the characterization of the other three enzymes present in the parasite cytosol (coproporphyrinogen oxidase) and mitochondrion (protoporphyrinogen oxidase and ferrochelatase) and these are included in the publications. Overall, it is an indigenous effort of two decades that has led to deciphering the unique features of parasite *de novo* heme pathway, its role in the entire life cycle of malaria parasite and its association with parasite virulence and disease pathogenesis. There is an unmet need of adjunct drug to prevent cerebral and severe malaria mortality that happens in significant number of children and adults despite treating them with antimalarials. As discussed under **section d**, I could succeed in identifying a FDA-approved antifungal drug, griseofulvin, that inhibits parasite heme synthesis and prevents cerebral and severe malaria. At present, these findings have been taken forward to preclinical and clinical trials. More importantly, the functional importance of *de novo* heme seems to be never ending with other interesting leads!

<u>Work related to asparagine requirement in the malaria parasite</u>

6) Distinct evolution of type I glutamine synthetase in *Plasmodium* and its species-specific requirement (2023). *Nature Communications*, *14*, 4216.

Discoveries/contributions/importance: *P. falciparum* is the deadliest parasite responsible for more than 90% of malaria infections and deaths. The emerging *P. falciparum* resistance against artemisinin and its partner drugs, and the recent evidences of artemisinin-resistant malaria in African countries together with the ongoing trials on triple combination therapies underscore the need of identifying new drug targets. In this study, it has been shown that the malaria parasite glutamine synthetase (GS) has distinctly evolved as an unusual type I enzyme with unique structural and regulatory features that are absent in other organisms. Human and plant enzymes belong to type II that differ in terms of structure and sequence from type I enzyme.

Parasite type I GS cannot be classified under α and β, and it defies the classical regulatory mechanisms such as feedback inhibition and allosteric regulation. It is also more (~20-50 times) sensitive to the classical GS inhibitors methionine sulfoximine (MSO) and phosphinothricin (PPT) than the type II GS of humans. The distinct evolution of parasite GS seems to support the adaptation to asexual niche wherein, the blood stage parasites are exposed to febrile temperatures, massive amounts of hemoglobin degradation releasing millimolar concentrations of amino acids and undergo minimal oxidative phosphorylation. More interestingly, GS provides glutamine for the synthesis of asparagine to support the asparagine-rich proteome displaying a species-specific essentiality in *P. falciparum*, but not in *P. berghei* or *P. vivax*. Further, GS and glutamine play a very important role in the survival of artemisinin-resistant parasites upon artemisinin exposure. Targeting GS inhibits the growth of artemisinin-resistant parasites. Hence, GS and asparagine requirement can serve as a new target for *P. falciparum* malaria and to combat artemisinin resistance. This has been proposed as another work for claiming this award and it is discussed in detail under **section d**.

7) Asparagine requirement in *Plasmodium berghei* as a target to prevent malaria transmission and liver infections (2015). *Nature Communications*, *6*(1), 8775.

Discoveries/contributions/importance: In this study, I have shown that the parasite asparagine synthetase (AS) is glutamine-dependent and it is dispensable for the entire life cycle of *P. berghei*. Nevertheless, the deletion of AS delays the asexual- and liver-stage development of *P. berghei* with substantial reduction in the formation of ookinetes, oocysts and sporozoites in mosquitoes. In the absence of asparagine synthesis, extracellular asparagine supports the suboptimal survival of sexual and liver stage AS knockout parasites. Depletion of blood asparagine levels by treating the knockout parasite-infected mice with asparaginase used in the treatment of acute lymphoblastic leukemia completely prevents the development of liver stages, exflagellation of male gametocytes and the subsequent formation of sexual stages. *In vivo* supplementation of asparagine in mice restores the exflagellation of the knockout parasites. These findings highlighted the potential of targeting asparagine requirement and extracellular asparagine for transmission. Further, amino acid requirements in the malaria parasite have been hitherto considered only in the blood stages where hemoglobin serves a major reservoir of amino acids. This is the first report on targeting amino acid requirement in the sexual and liver stages. Since malaria parasites are auxotrophic to most of their amino acids,

depleting their extracellular sources would effectively interfere with the development of sexual stages in mosquitoes and liver stages in vertebrate hosts.

Work related to the use of curcumin as a nutraceutical for parasite recrudescence and cerebral malaria

8) Nanocurcumin is superior to native curcumin in preventing degenerative changes in Experimental Cerebral Malaria (2017). Scientific Reports, 7(1), 10062.

Discoveries/contributions/importance: Curcumin has many pharmacological activities despite its poor bioavailability and *in vivo* stability. It acts as an immunomodulator and therefore, has the potential to serve as a nutraceutical for treating various ailments. In this study, it has been shown that the nanoformulated curcumin (PLGA-curcumin) has better therapeutic index than native curcumin in preventing the onset of neurological symptoms and delaying the death of mice in experimental cerebral malaria. Oral PLGA-curcumin is as effective as native curcumin at a 15-fold lower concentration in preventing the breakdown of blood-brain barrier and inhibition of brain mRNAs for inflammatory cytokines, chemokine receptors and ligands. A single oral dose of PLGA-curcumin is more effective and superior to native curcumin. PLGA-curcumin has potential to serve as a nutraceutical to prevent recrudescence and cerebral malaria.

9) Curcumin-arteether combination therapy of *Plasmodium berghei*-infected mice prevents recrudescence through immunomodulation (2012). PloS One, 7(1), e29442.

Discoveries/contributions/importance: In this study, the recrudescence model of *P. berghei* infection in mice has been used to show that a single dose of α,β-arteether (ART) with three oral doses of curcumin can prevent recrudescence. In case of ART treatment, the blood parasitemia becomes high during the recrudescence phase, leading to the death of mice. However, in ART and curcumin treatment, there is only a transient increase leading to protection and reversal of splenomegaly with a striking increase in TLR2, IL-10 and IgG-subclass antibodies The protection against recrudescence does not occur in TLR2 and IL-10 knockout mice. IL-10 injection to AE-treated wild type mice and AC-treated TLR2(-/-) mice was able to prolong survival. The blood samples from the recrudescence phase of ART and curcumin treated mice do not cause reinfections in naïve mice, and sera from the recrudescence phase reacted with several parasite proteins. The activation of TLR2-mediated innate immune response leading to enhanced IL-10 production and generation of anti-parasite antibodies seem

to contribute to this protective immunity observed in ART and curcumin treated mice. These results suggest the potential of curcumin as a nutraceutical for prevention of recrudescence in falciparum and relapse in vivax malaria.

10) Curcumin-artemisinin combination therapy for malaria (2006). Antimicrobial Agents and Chemotherapy, 50(5), 1859-1860.

Discoveries/contributions/importance: This is the first study to show that curcumin exhibits synergy with artemisinin to kill the parasites in *in vitro P. falciparum* cultures. Evaluation of the *in vivo* efficacy of α,β-arteether and curcumin combination in mice has shown that a single dose of α,β-arteether with three oral doses of curcumin could lead to a complete protection whereas, α,β-arteether alone could not. The combination could also prevent the recrudescence. Based on the findings mentioned in 8-10, pre-clinical toxicity studies and phase I human trial to test the safety of the combination in normal human volunteers have been completed. A double-blind Phase IIa clinical trial on evaluating the safety and efficacy of curcumin (Biocurcumax capsule) in combination with standard therapy [(Artesunate) + (Sulfadoxine-Pyrimethamine) tablet] for treatment of uncomplicated P. falciparum malaria is going to be initiated by NIMR, New Delhi, in collaboration with ILS, Bhubaneswar; Ipca Laboratories Ltd., Mumbai,; Arjuna Naturals Pvt Ltd., Aluva; Ispat General Hospital, Rourkela; and Shaheed Hospital, Dalli Rajhara. There has been a delay in the initiation of this clinical trial due to COVID-19 pandemic and subsequent lapse of the validity of DCGI approval.