Chapter 5: Conclusion and future work

Advances in understanding population dynamics, drug resistance, gene function and molecular evolution of malaria parasites has been greatly enhanced by population and genetic and genomics studies (Su *et al,* 2019). Identification of drug development and diagnostics targets is strengthened by the availability of the *P. falciparum* genome sequence that underpins our understanding of the complex biology of *Plasmodium* parasites (Govindarajalu, *et al* 2019). *In vitro* resistance can be generated for most *de novo* candidates of antimalarials that have been placed in the drug development pipeline suggesting the continuous restoration of the antimalarial pipeline with novel targets (Medicines for Malaria Venture, 2020; Antonova-Koch *et al,* 2018; Flannery *et al,* 2013).

Rapid emergence of resistance to antimalarials, including previous and current drugs, remains a major problem in malaria chemotherapeutics. There are increasing reports of resistance to the frontline antimalarial artemisinin and its derivative, and partner drugs in ACTs, mostly in Southeast Asia and recently in other regions including Africa (Uwimana *et al,* 2020; Rosenthal, 2018). Mutations in *Pfkelch13* have been implicated in artemisinin resistance with some polymorphisms such as the C580Y allele dominant over other associated mutations in Southeast Asia. This dominance has been linked to enhanced fitness conferred by resistance associated mutations. Inherent fitness differences across different strains has also been observed in strains of different origin *in vitro* (Goel *et al,* 2021; Ménard and Fidock, 2019). Gene editing tools such as CRISPR/Cas9 and modern sequencing methods such as NGS have immensely accelerated advances made in unravelling these essential aspects of the complex biology of *P. falciparum* and identification of markers associated with resistance, drug targets and development. Developments in the CRISPR/Cas9 system

allows the advanced genetic manipulation of *P. falciparum* using multiple plasmids in a transfection (Carrasquilla *et al,* 2020). I deconvoluted a complex pool of CRISPR/Cas9 plasmids to isolate individual viable donors of *Pfkelch13* mutations at position 580 and made up a new sub-pool. This plasmid sub-pool was generated with the aim of enhancing transfection efficiency and was used to transfect strains of different genetic backgrounds in order to address the question of how genetic background impacts parasite fitness with different *Pfkelch13* alleles. This would build on previous work from the lab that showed that different *Pfkelch13* alleles were returned by different genetic backgrounds when CRISPR-edited parasites were offered a range of potential donors. A major limitation of this work was the discontinuation of the experiments from the closure of the laboratories. However, this can be done in the next steps with a broader selection of parasites of varying origin, as these viable plasmids are readily available. I tested two lines of African origin that did not return transfected parasites with the control plasmid, in contrast to the well-established Dd2 line indicating some parasites are more easily transfectable than others. Thus, additional work will also require identification of strains that are more readily transfected and cultured.

In this study I was able to determine the resistance phenotypes and fitness of different *P. falciparum* strains of different genetic background in the absence and presence of drugs using barcode sequencing revealing the fitness disadvantage of resistant strains in the absence of drugs. Although the specific drug resistance phenotypes of these lines were already known by conventional drug assays, this work was to demonstrate proof-of-principle that this barcode sequencing methodology could be applied to mixed parasite pools to accurately reveal differences in drug susceptibility. In future work, this can be applied in larger scale settings to allow

comparison of lines from different epidemiological regions, to understand resistance associated mechanism in genetic background and fitness in resistant parasites by using the BarSeq approach to tag parasites of the same genetic background with different mutations in genes involved in these mechanisms. Significance of the barcode readout for the growth assays was analysed using a one-way ANOVA test combined with a Tukey post-hoc test to identify differences between lines. However, analysis was performed on the change in proportion rather than the absolute proportion that is plotted in figures 3.5-3.7 because the starting proportion of the individual lines was not exactly equal. In some cases, apparently large differences in the behaviour of the lines was not significant (e.g. 10nM mefloquine, Fig. 3.6); this likely results from variability in particular from the V1/S lines, which were at low abundance in the pool by the time the assay was initiated due to losses during synchronisation. Thus, relatively small fluctuations in absolute proportion of these lines yielded large apparent changes in proportion, contributing to variability. Future assays would aim to initiate the experiment with more equal representation of each line, and would avoid synchronisation post mixing. An application of this BarSeq approach can be used to test the effects of different alleles of a particular gene in parasites of different genetic background. This could be an essential approach in assessing the major alleles of *Pfkelch13* in different genetic backgrounds particularly C580Y, R539T, Y493H and I543T alleles that have been genetically confirmed to confer high level of resistance *in vitro* in Southeast Asia where artemisinin resistance and chloroquine emerged. Understanding the basis of the dominance of the C580Y major resistance allele will provide insight into whether this form of the protein might be a viable drug development target. But more importantly, understanding the spread of C580Y would help dissect the contribution of both resistance and fitness of different Pfkelch13

alleles, and help address the challenges of preventing spread or emergence of artemisinin resistant parasites on different genetic backgrounds and to other regions where artemisinin resistance is not prevalent. Determining the effect of these mutations in parasites from other regions where artemisinin resistance linked with these major *Pfkelch13* alleles has not been established can be achieved with this BarSeq approach. Additionally, the barcoding approach can be utilised to tag progeny from genetic crosses, in order to allow mixing and competition among the progeny to understand the effect of different loci in specific traits in the progeny, as well as the parents.

Resistance studies included in the early stages of the development process of *de novo* antimalarial candidates are important in establishing molecular mechanisms involved in increased parasite tolerance to drugs and progressive targeted drug discovery efforts (Schlott *et al,* 2019). One area of interest is the identification of antimalarials that antagonise resistance by displaying greater potency against specific resistance alleles, a processed termed collateral sensitivity. As examples, compounds that are more potent against resistant versions of dihydroorotate dehydrogenase and chloroquine-resistant *pfcrt* have been identified (Ross *et al,* 2018; Lukens *et al,* 2014). One potentially important discovery would be compounds that are more effective against mutant *Pfkelch13*, in order to protect artemisinin combinations. I therefore tested human KEAP1 kelch inhibiting compounds for antimalarial activity using wild type and mutant lines and established the efficacy of these compounds on mutant lines harbouring artemisinin resistance-associated mutations. Two of the KEAP1 kelch inhibiting compounds I tested were observed to demonstrate moderate potency against artemisinin resistant parasites suggesting that further target-based chemistry programmes might yield more specific compounds with higher potency against

parasites resistant to current antimalarials. This highlights the importance of developing antimalarial candidates of different classes to the current ones. As these assays were done in duplicate, I aim to expand this work to perform additional biological replicates of these assays to measure the statistical significance of these results.

Continuous surveillance of drug resistance markers and mechanisms is required to tackle the potential spread of artemisinin to other endemic regions such as Africa where reports of artemisinin resistance lines are now emerging. Moreover, genomic studies looking at resistance markers, as well as parasites of the different endemic regions in joint studies making use of gene editing and sequencing as highlighted in this project should be applied more. This will greatly accelerate progress in malaria control and treatment efforts due the great potential insights that can be achieved with the approaches applied in this project.