Chapter 6: What's next on the resistance front? Conclusions, reflections, and future work

Antimalarial drug resistance came into the spotlight with the first documented reports of chloroquine resistance in the 1950s-1960s (Harinasuta et al., 1965, Payne, 1987, Talisuna et al., 2004, Wellems and Plowe, 2001). Since then, drug resistance has impeded malaria prevention, control, and elimination efforts—with no signs of leaving the world's stage. The most recent development in this saga is the failure of dihydroartemisinin-piperaquine (DHA-PPQ), one of the recommended frontline artemisinin combination therapies (ACT) in Southeast Asia (SEA) (World Health Organization, 2018, van der Pluijm et al., 2019). By combining functional molecular approaches with genomic studies, this dissertation has investigated the molecular mechanisms of piperaquine resistance in *P. falciparum* malaria.

In the first research chapter (chapter 3), I developed copy number assays for detecting the *plasmepsin 2* and *plasmepsin 3* (*PM2-3*) copy number variation (CNV) associated with piperaquine resistance using genomic DNA from patient blood or dried blood spots. In order to mitigate the effects and spread of resistance, it is critical to have reliable methods to detect and monitor potential markers of drug resistance. The breakpoint PCR and qPCR-based assays for determining *PM2-3* copy numbers developed by this study are robust, timely, and cost-efficient methods for measuring *PM2-3* copy numbers in comparison to whole genome sequencing (WGS) techniques. Additionally, these assays can be performed in the field as long as a PCR or qPCR machine is present. While these new methods have many advantages, one limitation of the breakpoint PCR is its specificity for the predominant *PM2-3* breakpoints observed in Cambodian isolates (Amaratunga et al., 2016, Amato et al., 2017). The PCR primers designed for the breakpoint PCR would fail if used for samples with a different breakpoint. Cambodian isolates that have different *PM2-3* breakpoints were reported by Witkowski *et al*. (Witkowski et al., 2017) in 2017, and additional analyses of the *PM2-3* breakpoints in isolates from 2017 to the present day have not been reported. Further research will be needed to determine if the *PM2-3* breakpoints have remained the same or if they have changed in the last couple of years.

It will also be important to track if the *PM2-3* amplification will persist in parasite populations as the frontline treatments shift from DHA-PPQ to artesunate-mefloquine (AS-MQ) and triple ACTs (TACTs) with DHA-PPQ plus MQ. The majority of contemporary

isolates from SEA have increased *PM2-3* copies and single *pfmdr1* copies. As AS-MQ is reintroduced in the region, samples should be monitored for increased copies of *pfmdr1*, which was found to be the molecular marker and mechanism of mefloquine resistance (Price et al., 2004). Since piperaquine is a component of the TACT regimens, it is possible that the *PM2-3* amplification will remain or that copies may increase, as seen in samples from 2014-2015 with increased numbers of parasites with three *PM2-3* copies (Jacob et al., 2019). However, the role this amplification plays in antimalarial drug resistance remains to be elucidated. Will the *pfmdr1* copy number increase in the presence of increased *PM2-3* copy numbers? Can parasites become resistant to both mefloquine and piperaquine? Would the genetic alterations cause fitness disadvantages? Continued surveillance of parasite populations for these markers is necessary and it is crucial to understand what functional role (if any) the *PM2-3* CNV serves.

Although the *PM2-3* CNV is currently used as a molecular marker of piperaquine resistance, it has yet to be validated as a causal genetic determinant of piperaquine resistance. Initial attempts by this dissertation to evaluate the functional role of the *PM2-3* amplification have found that overexpression of *PM2*-only in a Cambodian field isolate (163-KH3-005) does not have any effect on parasite response to piperaquine. This study has also synthesized a *PM2-2A-PM3* plasmid for overexpressing stoichiometric amounts of *PM2-3* in transfected parasites to more closely mimic the CNV observed in the field. I confirmed that the plasmid was transfected into a piperaquine-sensitive field isolate from Cambodia (163-KH3-005) and Dd2, but similar to the *PM2*-only transfectants, it is necessary to confirm RNA and protein levels in the transfected parasites compared to the parent lines. Once overexpression of both the *PM2* and *PM3* genes is confirmed, I will be able to perform functional assays on these parasites to determine if the *PM2-3* amplification results in increased survival or plays a more compensatory role in fitness. One drawback of this study was the lack of successful transfections in isolates with kelch13 (K13) mutations, one of the molecular markers of artemisinin resistance. Piperaquine resistance emerged on an artemisinin-tolerant background, thus it is hypothesized that the presence of K13 mutations or other molecular markers of artemisinin resistance and/or piperaquine resistance (PfCRT mutations) may be needed for the *PM2-3* amplification to demonstrate its role in parasite survival. Future work will be able to use the *PM2-2A-PM3* plasmid generated by this study to transfect isolates with different genetic backgrounds to evaluate if the *PM2-3* amplification has an effect on piperaquine susceptibility.

The importance of examining the role of the *PM2-3* amplification in piperaquine resistance has been further demonstrated by recent studies by Leroy *et al*. (Leroy et al., 2019) that have detected increased *PM2* copies in Burkina Faso and Uganda (>30% of samples in both countries) and in Mozambique and Gabon (12.5% and 11.3%, respectively). This was one of the first studies to report increased *PM2-3* copy numbers in Africa, but several earlier reports also reported low levels of increased *PM2* copy numbers in Mozambique (1.1% of isolates) (Gupta et al., 2018) and Mali (10.8% of isolates) (Inoue et al., 2018).

The discovery of increased *PM2* copy numbers in African countries suggests that this marker of piperaquine resistance may be the same across endemic countries, which supports a functional role for the variation. In the context of chloroquine, resistance independently emerged due to mutations in PfCRT throughout malaria endemic areas where the drug was used. It is possible that the *PM2-3* CNV will be found in other regions where piperaquine has been used as a partner drug. It is also possible that due to the different genetic backgrounds of parasites in South America, Africa, and other malaria endemic regions, mechanisms of piperaquine resistance will involve different genetic pathways, if resistance occurs. The environment that *P. falciparum* evolves in is very different throughout endemic regions that have various climates, *Anopheles* species, other *Plasmodium* species, insecticides, antimalarial drug use/history, transmission rates, and many other conditions. With so many different factors it is possible that piperaquine resistance may emerge differently in different areas. It is thus necessary to not only monitor for known markers of resistance, but to continue surveillance for other genetic markers of resistance throughout endemic regions.

In chapter 4, I evaluated the functional relevance of two additional candidate markers of piperaquine resistance: a putative exonuclease protein SNP, PF3D7_1362500 (*exo-E415G*), and a putative mitochondrial carrier protein SNP, PF3D7_1368700 (*mcp-N252D*). Although these non-synonymous SNPs were identified in a GWAS by Amato *et al.* (Amato et al., 2017) as the top signals associated with decreased piperaquine susceptibility *in vivo*, this dissertation found no difference in the responses of wildtype and mutant transgenic Dd2 parasites responses to piperaquine. This suggests the exo-415 and mcp-252 candidates are not causal determinants of piperaquine resistance. As discussed for the *PM2-3* overexpression plasmids, it is necessary to examine if the *exo-E415G* and *mcp-N252D* SNPs have any effect in the presence of other molecular markers of piperaquine resistance (PfCRT mutations or the *PM2-3* amplification) or markers of artemisinin resistance (K13).

Low transfection efficiency of field isolates in chapters 3 and 4 are limitations of these studies. Though multiple field isolates, different conditions, and different plasmids were tested, these variations did not have an effect on transfection efficiency. In future studies, it will be essential to survey more culture-adapted field isolates to test which isolates tolerate growth at very low parasitemia (as would be expected after transfection while on drug pressure and during cloning by limiting dilution). Another alternative would be to supress conversion of asexual parasites to gametocytes by use of the lipid compound, lysophosphatidylcholine or LysoPC. Studies in *P. falciparum* have found that a reduction in LysoPC triggers an environmental cue that causes asexual parasites to differentiate to gametocytes (Brancucci et al., 2017). This pathway could be used as a method to increase transfection efficiency by supplementing transfected cultures with LysoPC to prevent unwanted conversion of the parasites to gametocytes during the stress of transfection. Such modifications will be tested in future transfection attempts.

In chapter 5, I used a hypermutator *P. falciparum* parasite line to generate piperaquine-resistant parasites in a feasible experimental timeline of six months rather than years. The use of the hypermutator line allowed this dissertation to complement the reverse genetics studies in chapters 3 and 4 with a forward genetics approach. Rather than starting with candidate gene markers of resistance and performing functional studies to assess their potential role in resistance, I selected for a piperaquine-resistant phenotype *in vitro*. Initial WGS analyses of the piperaquine-pressured parasites identified mutations in PfCRT and PfMDR1, among others. As discussed, this is of particular relevance as mutations in PfCRT have been reported to confer piperaquine resistance *in vitro* (Eastman et al., 2011, Ross et al., 2018a, Dhingra et al., 2017) and are increasingly prevalent in field isolates. The WGS analysis is ongoing and additional gene candidates and variations present in the piperaquine-pressured parasites will be examined. It has not been determined if these parasites have any copy number variations, including the *PM2-3* CNV, but these parasites do not have mutations in the genes encoding the putative exonuclease (PF3D7_1362500) or mitochondrial carrier protein (PF3D7_1368700). Analysis of the WGS data will shape the course of continued studies with these parasite lines (competition assays, growth assays, gene-editing, and more).

Currently, DHA-PPQ treatment failures have been confined to SEA. If such treatment failures were to occur in African countries where malaria transmission is high, it would have devastating effects on the ability to cure malaria-infected patients. With only five recommended ACTs currently available (World Health Organization, 2018), it is crucial to

understand how drug resistance develops. Additionally, the partner drugs in use today are believed to have the same site of action in the digestive vacuole, so resistance to one drug could promote resistance to another. An understanding of the mechanism of resistance to antimalarial drugs will provide knowledge that can be used to prolong the lifespan of the limited drugs that are currently available. This should be prioritized, for the same drugs continued to be recycled and rotated in the face of treatment failures (AS-MQ to DHA-PPQ back to AS-MQ).

Advanced understanding of the mechanisms of drug resistance informs the development of new drugs. Recent studies have used knowledge of antimalarial drug resistance to select for new compounds that specifically select against known antimalarial drug resistance alleles (Lukens et al., 2014, Ross et al., 2018b). New antimalarial drugs are also being discovered through large high-throughput screens that select for compounds based on anti-*Plasmodial* properties and not on structural similarity to quinine or chloroquine, as done in the past (Fidock et al., 2004, Cowell et al., 2018). These studies also prioritize drugs that can target different stages of the parasite lifecycle (liver stage, sexual stage, and asexual stage), which will be the best way to fight malaria going forward. Drug discovery, development, and regulation takes time, however, several organizations in recent years have been created to discover and develop new antimalarial drugs. One example is the product development partnership (PDP), Medicines for Malaria Venture. Such organizations combine basic science, pharmaceutical industries, economic assets and more—multidisciplinary approaches—with the same purpose of discovering and producing new antimalarial drugs.

Before the World Summit on Malaria in April 2018, Bill Gates (who committed another $1 billion dollars to the fight against malaria in 2018 through the Bill & Melinda Gates Foundation) said that there is no standing idle with malaria. “If we stand still, the insecticides we use stop working, the drugs stop working because the parasite itself evolves around that, so this is a game where you are either falling behind or getting ahead (BBC, 2018).” Malaria eradication is possible. Understanding how resistance develops is one facet of the problem that will aid in defeating the disease, and this doctoral dissertation has shed light on drug resistance to one commonly used antimalarial drug. In the battle against malaria, there must be new innovations from all sides. Basic science can provide much insight through understanding parasite and mosquito biology and through the development of new drugs and vaccines. In addition to this, there must be innovative solutions to the social, economic, environmental, and operational challenges that are present in malaria endemic regions.

Through these combined efforts, the slogan “eradication within a generation (Feachem et al., 2019)” could come to fruition. To stay in the game, the path to elimination and eradication must be holistic and it must be global.