6 DISCUSSION

6.1 Key remaining challenges in *P. vivax* research field

P. vivax malaria remains a risk for over a quarter of the world's population and a significant global morbidity burden despite the gains in controlling malaria achieved over the last decade. Research into *P. vivax* lags significantly behind that of its deadlier cousin, *P. falciparum*, not least in the area of vaccine research and development. A number of studies suggest that the burden of *P. vivax* is significantly underestimated and a comprehensive strategy to combat both species will be required to achieve the ambitious goal of a malaria-free world.

6.1.1 *In vitro* culture

Despite extensive efforts by many groups, *P. vivax* still lacks a reliable and widely usable *in vitro* blood stage culture system [reviewed in (Noulin et al., 2013)]. This remains the largest barrier to the study of fundamental *P. vivax* biology and the search for better vaccine candidates, at least for erythrocytic stages. While this challenge was not the focus of this work, it remains the continued focus of several other groups using either reticulocytes enriched from cord blood, whole blood or derived from hematopoietic stem-cells (Roobsoong et al., 2015) (Panichakul et al., 2007). In the absence of a robust and widely useable culture system for *P. vivax* itself, there is promising potential for harnessing the more closely-related *P. knowlesi*, which was recently adapted for *in vitro*

culture in human RBCs (Moon et al., 2013, Lim et al., 2013) and for which early transfection work shows positive results. It may be possible to study *P. vivax* genes *in vitro* through allelic replacement into the *P. knowlesi* parasites. *P. knowlesi* shares 4732/5188 (91%) one-to-one orthologous genes with *P.* vivax [(Pain et al., 2008) and OrthoMCL], and the two species are much more phylogenetically closely related to each other than to *P. falciparum* (Carlton et al., 2008). Like *P. vivax*, *P. knowlesi* uses DARC as a receptor for human RBC invasion (Horuk et al., 1993, Singh et al., 2002). It also appears to prefer invading younger RBCs in *in vitro* cultures, though the adaptation to continuous culture has resulted in invasion of a wider range of erythrocyte ages (Lim et al., 2013, Moon et al., 2013, Gruring et al., 2014). This adaptation to erythrocytes of all ages may explain the dangerously high parasitemias observed in some human infections (Lim et al., 2013). Thus there is potential for *P. knowlesi* to serve as an *in vitro* model for *P. vivax* asexual stages, though with potentially divergent and/or adaptable invasion pathways between the species in addition to a 24-hour replication cycle compared to the 48-hour replication cycle in *P. vivax*.

6.1.2 Hypnozoites

P. vivax transmission remains stubbornly entrenched in areas even as prevalence rates of *P. falciparum* decline. This persistence has been linked to a variety of factors including the early appearance and high infectivity of *P. vivax* gametocytes and the biting habits of common *P. vivax* vectors (Mueller et al., 2009a). However, the presence of dormant hypnozoites which leads to relapsing *P. vivax* infections is likely a major cause of continuing transmission (White and Imwong, 2012) with some estimates suggesting that over 50% of infections result from emerging hypnozoites (Betuela et al., 2012). This stage represents a major and so far relatively unexplored roadblock to *P. vivax* elimination. There is only one currently approved drug, primaquine, to remove or kill hypnozoites; its 14-day treatment regimen and contraindication for pregnant women and/or those with G6PD deficiency make it inconvenient at best and unsafe at worst in many of the populations where *P. vivax* is common. A new drug aiming to replace primaquine, tafenoquine, is of the same drug class, will reduce the treatment regimen to a single dose and is currently in Phase III clinical trials (Llanos-Cuentas et al., 2014). If tafenoquine makes it to the clinic, the reduced dose will be a significant improvement for patient compliance, but the drug is still contraindicated for patients with G6PD

deficiency. Lacking a cost-effective, point-of-care test for G6PD deficiency means that neither drug is likely to be used in many transmission regions, such as Southeast Asia where G6PD deficiency is common (Nkhoma et al., 2009). The cellular, molecular and biochemical features of hypnozoites, such as how dormancy is maintained, how infected cells remain silent to the immune system, and the cues which induce activation of hypnozites are all mysteries. A much more detailed understanding of the hypnozoite life-stage is essential to allow the targeted development of drugs aimed at radical cure of *P. vivax*. Recent work with human-liver chimeric mice showed successful infection with *P. falciparum* sporozoites and subsequent maturation in infected hepatocytes (Sacci et al., 2006, Vaughan et al., 2012). The recent adaptation of this model for *P. vivax* may give a significant boost to the study of hypnozoites, radical cure drug development and vaccines aimed at the pre-erythrocytic stages (Mikolajczak et al., 2015).

6.1.3 *P. vivax* invasion

As discussed throughout this work, the understanding of the interactions required for *P. vivax* invasion lags significantly behind that of *P. falciparum*. While one interaction is known, that between *P. vivax* DBP and human erythrocyte receptor DARC, the human receptor for reticulocyte tropism and any other interactions involved in the invasion cascade are still unknown. The discovery of such ligand-receptor interactions would most certainly widen the field of vaccine candidates to consider for *P. vivax*, for which PvDBP remains the strongest asexual-stage candidate.

The RBPs, specifically PvRBP1b and PvRBP2c, have been shown to have reticulocyte-binding capabilities but their binding partners have yet to be elucidated (Galinski et al., 1992). Whether *P. vivax* RBPs perform similarly redundant roles to the *P. falciparum* EBAs and RHs is also unknown but would not be surprising from the 9 to 10-member group (Carlton et al., 2008). The crystal structure of the erythrocyte binding domain from PvRBP2a (the first RBP structure reported) displays structural similarity to PfRH5, suggesting that the main structure for erythrocyte binding is conserved across species (Gruszczyk et al., 2016). However, the erythrocyte-binding domain was highly polymorphic among field isolates and other surface properties were distinct between PfRH5 and PvRBP2a suggesting that these differences are specific for different ligand-receptor interactions between species (Gruszczyk et al., 2016).

The PfRH5-basigin interaction in *P. falciparum* is of great interest as it appears to represent an essential interaction required to complete erythrocyte invasion, and PfRH5 is under active development as a vaccine candidate (Crosnier et al., 2011, Reddy et al., 2014, Douglas et al., 2015). PfRH5 has been found to form a complex with PfRIPR and PfCyRPA, both of which have one-to-one orthologs in *P. vivax* (Reddy et al., 2015). This leads to the question of whether such a complex also exists in *P. vivax*, and if so, which protein is performing the function of PfRH5. It is interesting to note that PfRH5 and PfRIPR appear to have lower (40-50%) seropositivity rates than most other merozoite surface/invasion proteins (>75%) potentially indicating they are exposed to the immune system for a relatively limited time, or are fundamentally less immunogenic (Richards et al., 2013). This is in contrast to PvCyRPA and PvRIPR, which were among the most immunogenic antigens in several of the screens performed in this work, with 57-79% and 92-96% of patient samples recognizing these antigens respectively (Table 5.3, Table 5.4). This may indicate more exposure to the immune system during *P. vivax* infection, thus suggesting a potential different function of these proteins in the *P. vivax* invasion process.

6.1.3.1 New methods for detecting *P. vivax* erythrocyte interactions

A major goal of this work was aimed at expanding the understanding of proteins involved in *P. vivax* reticulocyte invasion. At the start of this work, the AVEXIS assay had recently uncovered the PfRH5-basigin interaction (Crosnier et al., 2011), and appeared to have great potential for uncovering ligand-receptor interactions in *P. vivax*. A definite strength of AVEXIS in the detection of cell-surface interactions is that the assay produces very few false positives, which is useful for limiting the wasting of resources for downstream functional work. However, it is also known to produce false negatives potentially due to ectodomain misfolding or incorrect glycosylation, and the fact that it can only be applied to surface proteins with extensive ectodomains, meaning some classes such as multi-transmembrane proteins, are omitted from AVEXIS screens.

Several strategies can be used to overcome these limitations. One previously applied strategy is pull-down experiments, where receptors are enriched by immunoprecipitating their binding partner, although this approach, with its multiple wash steps, is ill suited for potentially low-affinity cell-surface interactions. Newer high throughput protein interaction screening strategies are under active development in the Wright and Rayner laboratories as well as other external groups. One promising new approach involves

binding recombinant proteins to cancer cell lines, which often up-regulate a number of surface proteins, including erythroid specific ones. Once binding to a cell line has been identified for a given ligand, CRISPR is then used to create a knock-out library of cells within that line. Sorting by binding and flow cytometry identifies cells that have lost the ability to bind the *Plasmodium* ligand, and gRNA sequencing from the sorted cells identifies which genes have been been targeted in the cells which do not bind. This approach has already recapitulated the RH5-basigin interaction (S. Sharma, data not yet published) and may lead to the uncovering of additional parasite ligand-receptor binding events. An alternative approach is under way to express hundreds of receptors in arrays on slides to then screen with recombinant proteins thus overcoming the constraint of AVEXIS to only include single ectodomains in screening. This approach was recently applied to identify endothelial protein C receptor (EPCR) as the receptor for several PfEMP1 subtypes associated with severe childhood *P. falciparum* malaria (Turner et al., 2013). These two examples demonstrate that high-throughput protein interaction screening methods are under active development and will hopefully bridge the "invasion" knowledge gap in the *P. vivax* research field as well as continuing to expand the known ligand-receptor list in *P. falciparum*, all of which may lead to stronger vaccine candidates than are currently in the development pipeline.

6.1.4 *P. vivax* infections of Duffy-negative individuals

Individuals with the Duffy-negative phenotype were thought to be completely protected from infection with *P. vivax* due to the essential interaction between *P. vivax* DBP and DARC on human erythrocytes. Numerous publications over the last ten years have challenged this assumption with reports of *P. vivax* isolates which are capable of infecting Duffy-negative people throughout Africa (Menard et al., 2010, Mendes et al., 2011, Ngassa Mbenda and Das, 2014, Ryan et al., 2006, Woldearegai et al., 2013). It is not known whether these isolates represent a new and emerging adaptation or a minor but always present capability that had previously been overlooked. If the former is true, the phenomenon may have huge implications for control throughout Africa, where *P. vivax* became absent with the spread of the Duffy-negative phenotype. Understanding the genetic and/or transcriptional profile of such parasites through DNA and RNA sequencing will be essential to addressing these questions. While clinical isolates are still necessary for such studies, improvements in next-generation sequencing technology,

make using patient samples (even with very low parasite densities) more feasible. Studies, like ours, aimed at better understanding the transcription prior to invasion and the similarities and differences driving variation between *P. vivax* isolates, particularly those leading to severe disease, are likely to be on the rise over the next few years. We plan several long-term collaborations for analysing the genomes and transcription of field isolates from Ethiopia, where recent *P. vivax* infection in Duffy-negative individuals has been detected (Woldearegai et al., 2013).

6.1.5 *P. vivax* vaccine development

As discussed throughout this work *P. vivax* is both difficult to study and frequently underestimated in terms of global morbidity, with numerous global reports of severe disease (Price et al., 2007). Both factors have led to the skewed research funding vs the more deadly *P. falciparum* and a relative paucity of active vaccine candidates. A recent review of leading *P. vivax* vaccine candidates shows that while pre-erythrocytic stages, asexual and transmission-blocking vaccine candidates are all under active development, no candidate has progressed further than the Phase I/IIa clinical trial for a PvCSP-based candidate which provided no sterile protection [reviewed in (Mueller et al., 2015)]. The leading asexual candidate in pre-clinical development phase remains *P. vivax* DBP. DBP contains conserved erythrocyte-binding epitopes and has demonstrated antibody-associated protection from clinical disease, both of which support its continued development as a vaccine target. However, the reliance on a single antigen may have drawbacks including limited efficacy (as has been shown in the single-antigen PfCSP-based vaccine) and the potential for being ineffective against parasites which infect Duffy-negative individuals, although as noted above it remains to be seen whether *P. vivax* infection of Duffy-negative individuals are mediated through alternative invasion pathways or perhaps still utilize DBP binding to alternative receptors. Overall, as suggested by Mueller *et al.*, a widely effective *P. vivax* vaccine may require the targeting of multiple critical *P. vivax* antigens from both asexual, sexual and pre-erythrocytic stages (Mueller et al., 2015). The initial RNA-Seq data generated in this work hinted at variable expression for a number of host-interaction genes, including the RBPs, and expanding the number of samples will enable us to establish whether this pattern is the norm. This may provide insight into strategies of the parasite for evading the host-immune response and potentially impact vaccine design, as genes with variable

expression may represent some functional redundancy and should possibly be avoided as vaccine candidates.

6.1.5.1 High-throughput immunoepidemiological studies

Given the challenges of *P. vivax* lab–based research, immunoepidemiological studies remain a key tool for identifying targets of naturally-acquired immunity (NAI) and hence prioritization of vaccine candidates. Screening individual proteins against individual sera samples by ELISA is a reliable and well-understood assay and has formed the basis of most immunoepidemiological studies. However, the process remains labor intensive, which frequently limits the scale of proteins or sera samples for testing. Most seroreactivity studies use purified proteins for screening, adding an additional limitation. We overcame this limitation by producing biotinylated proteins, which could effectively be purified from complex mixtures by coating on streptavidin plates prior to screening with sera samples, and this enabled the simultaneous screening of a panel of 34 recombinant *P. vivax* proteins against plasma samples from Cambodia and SI. This method was also used successfully for studying the development of NAI to *P. falciparum* (Osier et al., 2014). We expect the use of this library of *P. vivax* recombinant proteins, without the need for prior rigorous purification, will continue to be a benefit for drawing systematic conclusions in immunoepidemiological studies in other *P. vivax*-endemic settings outside of Southeast Asia and Oceania, such as South America and India, where *P. vivax* malaria remains a significant public health concern.

The screening technology itself is also changing, and immunoepidemiological studies using protein arrays are beginning to increase in number and usefulness. Protocols for array production are in development in the Wright laboratory, with spotting/arraying of individually expressed proteins on glass slides allowing for systematic screening of many proteins in parallel and the potential for long term, stable storage (in glycerol). This may enable the repeated screening of identical sets of proteins. Currently for *P. vivax*, 3 protein array studies have been used for assessing reactivity (Chen et al., 2010, Lu et al., 2014, Molina et al., 2012). An *E. coli*-expression based array was also recently used to study reactivity in both symptomatic and asymptomatic *P. vivax* infections, showing that symptomatic children carried fewer antibodies (Finney et al., 2014).

Another higher throughput alternative is the Luminex assay, which while requiring purified proteins, enables the screening of hundreds of sera samples in a flow cytometry-

based assay. Because individual sera samples can be screened with several proteins simultaneously, the assay requires less serum volume and has the potential for extending the number of studies accomplishable with finite and precious serum collections. In the context of this work, increasing the number of purified proteins screened by Luminex in the PNG longitudinal cohort study would provide further insight into the development of NAI in a high-transmission *P. vivax* setting, and potentially expand the list of antigens to consider as vaccine candidates. It would also help to further address the hypothesis that the breadth of response is a crucial element of protection from clinical *P. vivax* disease as has been found to be the case for immunity to *P. falciparum* (Osier et al., 2014), and was indicated by screening of only 6 *P. vivax* antigens in this work (Franca et al., 2016).

Cross-protection (or lack thereof) between species will also be important to evaluate in a more systematic way; this can be facilitated by both the *P. vivax* and *P. falciparum* recombinant protein libraries assembled in this and prior work in the Wright and Rayner laboratories. Studies in neurosyphilis patients and the lack of cross-protection between *P. vivax* and *P. falciparum* in PNG suggest that while the development of immunity can be strain transcending, it does not appear to be species transcending (Doolan et al., 2009, Franca et al., 2016). However, more systematic screening using both *P. falciparum* and *P. vivax* merozoite antigens would be useful for fully evaluating this. Any antigens correlating with cross-protection would be prime candidates as vaccine targets, as any mass-administered vaccine must ultimately include efficacious components for both *P. falciparum* and *P. vivax*. Otherwise, we run the risk of reducing *P. falciparum* only to have *P. vivax* continue to persist in causing millions of cases per year and significant global morbidity.

6.1.5.2 Functional validation of *P. vivax* vaccine candidates

The functional and immunoepidemiological studies in Chapters 4 and 5 identified several parasite protein-protein interactions, several highly immunogenic proteins and IgG responses to 3 proteins that correlated with protection. However, the full potential of these candidates as vaccine targets will require additional functional studies. Planning for this work is already underway, and we have generated polyclonal antibodies against several recombinant *P. vivax* proteins, including those associated with protection. We will first confirm that antibodies to the recombinant merozoite proteins react to the corresponding native *P. vivax* merozoite proteins through western/immunoblotting

techniques. Immunofluorescence microscopy will be used to confirm that antibodies bind to expected locations in the merozoite (i.e., surface, apical, microneme, etc.), using *P. vivax*-infected reticulocytes collected from volunteers in the field. This will provide evidence as to whether interaction pairs detected *in vitro* (by AVEXIS and SPR) potentially interact *in vivo* by co-localizing in the parasite.

We also plan to make use of a published *P. vivax* invasion-blocking assay (Russell et al., 2011) to test whether antibodies directed toward the *P. vivax* merozoite antigens significantly inhibit *P. vivax* invasion of reticulocytes. The polyclonal antibodies can be used to potentially disrupt the merozoite protein-protein interactions detected and/or any association with as yet undetected reticulocyte receptors. We will additionally use the candidate recombinant proteins to compete for reticulocyte receptors involved in merozoite invasion. We will attempt to block invasion by targeting single proteins and combinations, as efficient inhibition of *P. vivax* invasion may require a multi-target approach.

6.2 *P. vivax* research summary

This work was specifically aimed at addressing the remaining knowledge gap in understanding the proteins involved in the *P. vivax* merozoite invasion of erythrocytes through the use of transcriptomics, recombinant protein expression and immunoepidemiological studies (Figure 6.1). The ultimate goal of the project was to develop a prioritized list of *P. vivax* proteins for future study as vaccine candidates.

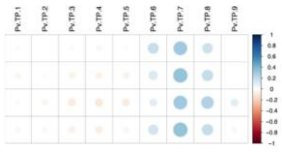
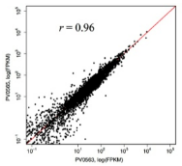
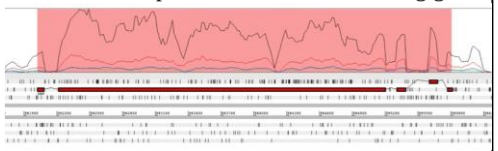
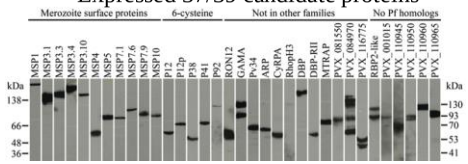
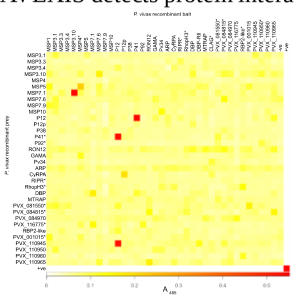
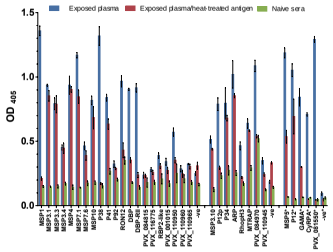
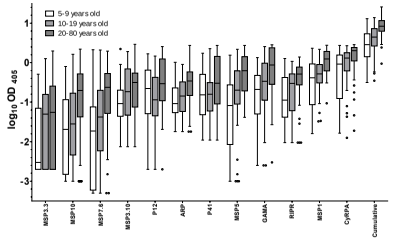
Chapter 3 *P. vivax* transcriptomics	**Chapter 4** *P. vivax* protein expression	**Chapter 5** *P. vivax* immunoepidemiology
Aim: Study transcription in schizont-stage *P. vivax* parasites **Approaches:** • Short term *ex-vivo* culture of *P. vivax* clinical isolates • Use RNA-seq to produce unbiased transcript abundance data **Results:** • Extracted high quality and quantity RNA from 4 schizont –enriched *P. vivax* clinical isolates • Generated over 150x average coverage using strand-specific Illumina Hi-Seq libraries • Corrected over 300 gene models, uncovered 20 novel transcripts and expression data for over 400 additional genes • Schizont-stage expression  • Expression highly correlated  • Variable expression for host-interacting genes 	**Aim:** Produce a library of *P. vivax* merozoite proteins and investigate function **Approaches:** • Combine RNA-seq, microarray data and homology with *P. falciparum* • Utilize mammalian expression system • Assess erythrocyte binding by flow cytometry • High-throughput interaction screens (AVEXIS) **Results:** • Detected parasite protein-protein interactions: predicted (P12-P41), novel (MSP7.1-MSP3.10, P12-PVX_110945) • Confirmed interactions using SPR • Expressed 37/39 candidate proteins  • AVEXIS detects protein interactions 	**Aim:** Investigate *P. vivax* merozoite protein immunoreactivity **Approaches:** • Assess IgG responses from patient plasma to recombinant *P. vivax* proteins using ELISA and Luminex **Results:** • *P. vivax* recombinant proteins reactive in three *P. vivax*-endemic settings • IgG responses to P12, P41, PVX_081550 correlated with protection in PNG • 27/34 proteins reactive in Cambodia  • Age-associated IgG increases for 12 proteins in SI 

Figure 6.1: Overview of each experimental chapter, with summarized aims, approaches, and results.

At the initiation of this project in late 2011, the *P. vivax* Sal 1 reference genome had recently been published (Carlton et al., 2008), as well as the first large-scale studies of transcription using microarray technology (Bozdech et al., 2008, Westenberger et al., 2010). While the first *P. falciparum* RNA-Seq study had been published (Otto et al., 2010), the prospects for similar *P. vivax* RNA-Seq studies were limited, due to the difficulty in obtaining RNA from clinical isolates of sufficiently high quality and quantity. There were few vaccine candidates under consideration partially due to the limited availability of recombinant *P. vivax* proteins. Most blood-stage protein studies relied on one or several protein fragments often produced in different expression systems (with the exception of a single panel of *P. vivax* protein fragments produced in the wheat-germ cell-free system). This significantly limited any ability to make systematic comparisons between proteins (for instance for immunoepidemiological studies), and no studies of panel of proteins for cross-sectional or longitudinal cohorts of *P. vivax*-endemic populations existed at the time.

This project aimed to broaden the potential field of vaccine candidates in a more comprehensive way. This first involved determining the largest pool of potential candidates through identification of proteins upregulated in the schizont stage of asexual blood-stage parasites. The Bozdech *et al*. microarray dataset provided a solid basis for this but lacked a complete set of probes, and we endeavoured to expand on this by producing RNA-Seq data using *P. vivax* clinical isolates from Cambodia. This involved the testing of RNA extraction methods and the bespoke production of Illumina strand-specific libraries, which led to over 150x average coverage for 4 isolates. The data provided schizont-stage transcription data (including transcription data for over 400 additional genes not included in the original microarray dataset), enabled the correction of hundreds of gene models, as well as uncovering novel transcripts. It also opened a window into potential differences between clinical isolates. Overall, it provided a basis from which to begin to build a list of proteins with potential involvement in *P. vivax* merozoite invasion of erythrocytes.

The next phase of the project involved the assembling of a list of *P. vivax* candidates for protein expression and downstream functional and immunoepidemiological screening. Utilizing the available RNA-Seq and published microarray data as well as homology comparisons with *P. falciparum* invasion antigens, we developed a list of 39 *P. vivax* candidates, 37 of which were successfully expressed in the mammalian HEK293E

system. I screened this protein panel in several large-scale functional assays including flow cytometry-based erythrocyte binding assays and 2 AVEXIS assays, which identified both predicted and novel parasite protein interactions. The erythrocyte-binding assays confirmed the binding of *P. falciparum* EBA175 to both erythrocytes and hematopoietic stem cell-derived reticulocytes and potentially the binding of *P. vivax* MTRAP to erythrocytes and reticulocytes, though with the need for additional confirmatory experiments. AVEXIS and subsequent SPR experiments confirmed for the first time that the *P. falciparum* P12-P41 interaction is conserved in *P. vivax* and has a much higher binding affinity. AVEXIS and SPR also supported 2 novel interactions between *P. vivax* MSP3.10 (MSP3α) and MSP7.1, and between *P. vivax* P12 and PVX_110945, which open the door to further functional studies with field isolates.

The protein library enabled the study of antibody responses directed against the proteins from 3 *P. vivax*-endemic countries (Cambodia, SI, PNG), enabling one of the first screens of a panel of *P. vivax* proteins in reactivity, cross-sectional and longitudinal cohort studies. The vast majority of the protein library was immunogenic in all 3 settings. Several proteins were reactive in over 90% of patient samples in both Cambodia and the SI including MSP1, GAMA, and P12. A factorial screening of 144 patient samples in SI found stronger age-associated increases for IgG responses to 12 proteins compared to infection-associated increases, potentially pointing to the acquisition of long-lived, stable antibody responses to many *P. vivax* antigens. Responses to 3 proteins were additionally found to be associated with protection from clinical disease including P12, P41, and PVX_081550, the latter of which is a hypothetical protein for which little is known.

Overall, the project largely accomplished the primary goal of expanding the number of *P. vivax* antigens for further study as vaccine candidates and sets the stage for a number of follow-up studies in the years to come.

6.3 Conclusion

P. vivax research continues to lag significantly behind *P. falciparum*. However, steady improvements, often facilitated by changing technologies, are making several studies possible that were not feasible when this project began. One of the biggest improvements has been in the field of genomics as reductions in the quantity of DNA needed for making Illumina libraries had made the sequencing of field isolates feasible. A single *P. vivax*

isolate (the reference genome *P. vivax* Sal 1) had been sequenced at the start of this work; in the intervening years next generation sequencing technology has enabled the sequencing and analysis of hundreds of *P. vivax* isolates (Hupalo et al., 2016, Pearson et al., 2016). Genomics research for *P. vivax* may have caught up with *P. falciparum*, but *P. vivax* transcriptome studies, particularly those using RNA-Seq [for which only a single recent study exists (Zhu et al., 2016)], are still far behind.

While this project did not attempt to address the challenges of *P. vivax in vitro* blood stage culture, we did aim to expand the study of the proteins potentially involved in *P. vivax* merozoite invasion of reticulocytes. The availability of proteins to study in functional and immunoepidemiological assays remains a significant barrier for many groups, and we hope this will be somewhat eased by the deposition of our complete library of *P. vivax* vectors at the non-profit plasmid repository, Addgene.org. Several other groups are also assembling either full-length or subdomain *P. vivax* protein libraries (Finney et al., 2014, Lu et al., 2014), primarily for use in immunoreactivity studies. Collectively these protein panels will be important for enabling systematic comparisons between many proteins in the search for markers of exposure and IgG response profiles which correlate with protection, as these remain a potentially critical method for prioritizing candidates to develop as vaccine targets.

In conclusion, the work presented in this thesis identified several predicted and novel parasite protein-protein interactions as well as IgG responses directed against proteins, which were stably acquired with age and/or correlated with protection. All of these proteins are potential *P. vivax* vaccine candidates that we plan to investigate in further functional studies.