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Molecular Approaches to Malaria 2020

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Introduction

Just 25 days before Australian borders were closed due to the Covid-19 global pandemic, malaria researchers from around the world assembled in Lorne, outside of Melbourne Australia, from February 23-27th, for the sixth Molecular Approaches to Malaria (MAM) Conference, a meeting held once every four years. This was the largest MAM conference to date and included 454 attendees from 31 countries. This fortunate and remarkable achievement is a testament to the prior successes of MAM in establishing the conference as an important international meeting and demonstrates the expansion of researchers utilizing molecular techniques to understand the biology of *Plasmodium* parasites that cause malaria. There has also been an expansion in molecular epidemiology and genetic studies aimed at elucidating how *Plasmodium* parasites have developed resistance to front-line antimalarials and their spread to other nations. Combined, this knowledge has led to the identification of novel drug targets and development of new therapeutic approaches to help combat *Plasmodium* spp. Below we summarise the key findings and progress made in each of these areas that were presented at MAM2020 and the common themes emerging from the conference are represented in Figure 1.

In addition to putting together a high caliber scientific program, we wanted the conference to be highly inclusive and diverse. To that end, a 50:50 gender balance of speakers (both invited and chosen

from abstracts) was achieved and >30% of oral presentations were by postdoctoral scientists and students. Generous support from funding bodies enabled 36 travel grants to be awarded to researchers from malaria endemic countries so they could attend the meeting. Three poster sessions (showcasing >270 posters) and five workshops rounded out the program, the latter especially catering to students and early career researchers. These workshops were diverse in nature, providing insight and tips into how to publish a manuscript, how to attain gender equity and diversity in the workplace, interviewing for a job in industry, using CRISPR/Cas9 to elucidate parasite biology and molecular mechanisms of drug resistance and different methodologies that can be used to analyse bioimages.

The 6th MAM conference was also the first to be certified Carbon Neutral by the Australian Government and to incorporate live streaming of presentations from Africa and the USA. The latter was indeed successful, revealing the potential of live streaming at conferences as one mechanism to reduce the carbon footprint of conferences whilst providing access to people who otherwise could not attend. Already we have had numerous enquiries from attendees who are currently organizing other conferences as to how to achieve carbon neutrality beyond carbon offsetting. We recommend that live streaming be considered at other conferences as it may provide a valuable opportunity for researchers who are unable to attend (eg. due to work or parental responsibilities) to still share their research. It is ironic, therefore, that immediately after MAM2020, conferences were forced to run solely via live-streaming because of Covid-19 restricting travel.

Scientific sessions

MAM2020 commenced with a warm and vibrant Welcome to Country by the local Eastern Marr Aboriginal Corporation followed by a moving tribute by Professor Andy Waters to the life of Associate Professor Shahid Kahn who passed away in October 2019, and who dedicated his career to the pursuit of excellence in malaria parasite research.

Prof Jane Carlton, from New York University, then opened the scientific proceedings with a Plenary highlighting her multi-faceted approach to comprehensively understand and target the “changing face” of malaria in India. As the leader of the Center for the Study of Complex Malaria in India, she is bringing together scientists, clinicians and public health researchers to undertake studies on malaria epidemiology, transmission, pathogenesis, and genomics studies across India as it transitions through an ambitious ten-year elimination program using vector and person-focused interventions. She captivated the audience relaying her experience evolving from a laboratory-based geneticist to establishing and leading a comprehensive field-based malaria research center. She outlined the considerable work that underpins establishing multi-disciplinary malaria research programs, from developing infrastructure and transferring technology, to engaging with stakeholders. An important culmination of her extensive program was building research capacity in India and emphasised that all of us have a role to play in increasing research capacity in malaria endemic settings.

For the second Plenary talk, the CSO of Medicines for Malaria Venture (MMV), **Dr Timothy Wells**, gave a holistic overview of the current MMV drug development portfolio (Medicines for Malaria Venture,

2020), acknowledging the high attrition rate of compounds as they move through the development pipeline. He urged the international audience for new molecular tools required to reach malaria elimination by 2050, and which will help better understand parasite resistance, particularly to artemisinin and its combination therapies. The independent emergence of drug resistance in different endemic regions is threatening the gains made over the past decade and underscores the need for novel antimalarial drugs with new mechanisms of action and a more thorough understanding of resistance in the laboratory. A clear understanding of the impact of current resistance concerns for ACT requires molecular analysis of the activity of the partner drugs. A molecular 'wish list' was outlined at the end of the presentation – asking collaborators for any information on lumefantrine or pyronaridine resistant clones, with validated molecular markers, and also clones of lines with artesunate failure (defined as an MIC shift > 3-fold or parasite reduction half-life of 20h). Finally, the key to success would be having new molecular targets with inhibitor starting points where no resistance can be generated in the laboratory *in vitro* against 10^9 parasites.

For the next four days, a diverse line up of speakers presented their latest and often unpublished research on a range of themes from understanding the molecular biology of *Plasmodium* spp. across the life-cycle and its pathogenesis, advancing new interventions, such as drugs and vaccines, and the application of molecular approaches to understand and track emerging drug resistance and malaria outbreaks. Underpinning these themes were the application of emerging technologies to advance the malaria research agenda, which was also the focus of the final session on frontier technologies.

Strategies *Plasmodium* parasites employ to invade, remodel and egress from erythrocytes and contribution of the host cell

In the host-parasite interactions session, which was chaired by Asst Prof Alexis Kaushansky (Seattle Children's Hospital) and Prof Jake Baum (Imperial College London), the core themes centred around understanding how *Plasmodium* parasites invade and remodel their new RBC and then finally egress from their host cell to propagate the infection. In the case of *P. vivax*, the Duffy antigen on human red blood cells (both normocytes and reticulocytes) serves as a receptor for *P. vivax* entry. Invited speaker **Assoc Prof Wai-Hong Tham** from The Walter and Eliza Hall Institute, Australia, presented recent work that revealed how entry of *P. vivax* into reticulocytes is governed by intimate connections between the parasite protein Reticulocyte Binding Protein 2b (PvRBP2b), Transferrin Receptor 1 (TfR1) and Transferrin (Tf) at the reticulocyte membrane, such that knockdown of TfR1 leads to reduced *P. vivax* invasion (Gruszczyk *et al.*, 2018b). Interestingly, the apical binding site of TrR1 also governs interactions with other pathogens such as the New World arenaviruses. Cryo-electron microscopy analysis of complexes containing RBP2b, TfR1, human Tf and iron enabled identification of interactive sites between these proteins (Gruszczyk *et al.*, 2018a). Polymorphism studies revealed that whilst PvRBP2b displays high diversity under balancing selection, this is not at the TfR1/Tf binding sites. Moreover, mapping of naturally occurring antibodies demonstrated invasion-blocking antibodies inhibit PvRBP2b binding to TfR1/Tf, while antibodies incapable of blocking invasion target non-binding interfaces. As PvRBP2b and the essential *P. falciparum* invasion ligand PfRh5 contain regions of

overlap, Prof Tham's research revealed it may be possible to identify species-transcendent invasion blocking antibodies.

The mechanism of *P. falciparum* invasion is complex, and the parasite utilizes several alternative pathways to invade human RBC. CD44, a membrane glycoprotein with a transmembrane domain and single ectodomain that is expressed at low levels on RBCs, was previously shown to serve as a host factor for invasion through an shRNA-based forward genetic screen in red blood cells derived from primary human hematopoietic stem cells. CRISPR/Cas9 knockout of CD44 in the immortal JK-1 erythroleukemia line lead to strain-transcendent reduction in invasion (Kanjee *et al.*, 2017). Using a flow-based assay **Dr Elizabeth Egan** from Stanford University, USA, could show that free merozoites can bind to recombinant CD44. Via immunoprecipitation, CD44 was found to interact with EBA175 and EBA140. As these two proteins interact with glycophorin A (GlyA) and glycophorin C (GlyC), respectively, human derived erythroid progenitor cells were used to create combinatorial deletions of CD44, GlyA/GlyC. Sequential deletion experiments showed that CD44 potentially serves as a co-receptor for GlyA/GlyC mediated merozoite invasion and could have a role in signaling to the host cytoskeleton for successful parasite invasion.

So what role, if any, does the RBC play in *Plasmodium* invasion? **Dr Viola Introini** from Cambridge University, UK, used Dantu RBC to address exactly this question (Kariuki *et al.*, 2020). The Dantu blood group variant arises from a rearrangement of Glycophorin A and B, which gives homozygotes individuals up to 70% protection from severe malaria. Here, Dr Introini used live imaging to study the ability of five different *P. falciparum* strains to invade Dantu RBC and identified that while merozoites attempted cell deformation, all strains showed drastic reduction in invasion potential. Biomechanical studies of Dantu RBC revealed they have an elevated tension when compared to non-Dantu RBC. Above a particular tension threshold, the ability of these RBC to warp around the merozoite was affected, leading to failed invasion. As proof of principle, non-Dantu RBC were treated with glutaraldehyde to mimic the membrane tension of Dantu RBC and this also resulted in weak deformation of the RBCs and parasite wrapping and consequently the RBC were refractory to parasite invasion.

Once *Plasmodium* parasites have successfully invaded RBC, they then drastically remodel their new host cell by exporting hundreds of their proteins into the RBC. Many of these exported proteins have been linked to pathogenicity and evasion of the host immune response. *P. falciparum* encodes 19 serine/threonine kinases termed FIKK kinases, 18 of which are putatively exported. To determine the role of these proteins in host cell remodeling, **Mr Hugo Belda** from The Crick Institute, UK, undertook quantitative phosphoproteomics of RBC infected with *P. falciparum* and *P. knowlesi*, the latter of which does not export any FIKKs (Davies *et al.*, 2020). In *P. falciparum*-infected RBC, an increase in phosphorylation rates, particularly of RBC cytoskeleton proteins, was observed. The DiCre/LoxP conditional gene knockout system, in conjunction with quantitative phosphoproteomics, was then applied to determine the function and phosphosubstrates of each FIKK. None of the FIKKs examined were found to be essential for parasite growth, despite there being a non-redundant phosphorylation fingerprint for each FIKK and different preferred substrates. Detailed analysis of FIKK4.1 revealed it has multiple phosphosites on RBC cytoskeletal proteins and other exported proteins, indicating

FIKK4.1 may regulate host cell rigidity or cytoadhesion. Indeed, a FIKK4.1 knockout line showed reduced PfEMP1 surface translocation and infected RBC exhibited impaired cytoadhesion and rigidity. The work is now being expanded to develop a pan-FIKK inhibitor.

Two talks in this session were also focused on how *Plasmodium* parasites egress from the host cell. Invited speaker **Prof Mike Blackman** from The Francis Crick Institute, UK examined the timing and kinetics of egress using conditional gene knockouts as well as Compound 2, a selective inhibitor of the cGMP dependent Protein Kinase G (PKG) to regulate egress, and an inhibitor of the subtilisin-like protease 1 (SUB1) (Hale *et al.*, 2017; Thomas *et al.*, 2018). Schizont egress was shown to occur very rapidly from the inside out, with the PVM initially breaking down, followed by poration of the RBC membrane. PKG is a critical player in the egress cascade as PKG activation, which is caused by an increase in cGMP levels, leads to the discharge of SUB1 into the PV, resulting in cleavage of SERA6 and breakdown of the PVM. SERA6 is rapidly processed in two steps - the first cleavage event is mediated by SUB1, which results in the interaction of SERA6 with two non-contiguous fragments of a novel PV-located protein, termed S6PP. This interaction is essential for the second autocatalytic processing of SERA6 that gives rise to mature active SERA6. SERA6 and S6PP aggregate into peripheral foci and cleave beta-spectrin, triggering breakdown of the RBC cytoskeleton. So does that make SERA6 a druggable target? Compound E64 blocks egress by blocking the second autocatalytic step of SERA6 and preventing beta-spectrin cleavage, however, this compound is very broad spectrum. It will be interesting to see if more potent and selective inhibitors that block SERA6 and which could potentially be combined with compounds that block other steps in the egress or invasion pathway can be identified.

The mechanism that triggers the cGMP signaling cascade that leads to PKG activation is not known. **Assoc Professor Vasant Muralidharan** from The University of Georgia, USA, presented work which demonstrated the Endoplasmic Reticulum-resident Calcium-binding protein (PfERC) plays a critical role in regulating the egress proteolytic cascade (Fierro *et al.*, 2020). A conditional knockdown (cKD) of PfERC using the GlmS riboswitch system showed the PVM fails to rupture and parasites cannot egress, leading to parasite death. The phenotype was not due to a failure in parasite calcium signalling as originally hypothesised, nor was there an impact on apical organelle biogenesis, protein trafficking to these organelles or secretion of Apical Membrane Antigen 1 (AMA1) from the micronemes. Rather, the PfERC cKD inhibited AMA1 and MSP1 processing, which was found to be due to a failure of proteolytic maturation of Plasmepsin X. This protease cleaves SUB1, which in turn cleaves many substrates, including MSP1. Hence PfERC cKD led to the failure in PVM rupture because the processing of substrates downstream of the proteolytic cascade was inhibited, indicating that PfERC is a key regulator of the egress proteolytic cascade.

Novel tools for advancing our understanding of emerging drug resistance, parasite diversity, vaccine escape and relapse

The molecular epidemiology session, chaired by Prof Carol Sibley (University of Washington, USA) and Dr Sarah Auburn (Menzies School of Health Research, Australia) showcased molecular data from

diverse geographical regions (sub-Saharan Africa, South America and New Guinea) as well as studies using the exceptional global genomic repositories generated by the malaria Genomic Epidemiology Network (malariaGEN). Most studies implemented Illumina whole genome sequencing data, which appears to remain the favoured approach for high-throughput molecular epidemiology studies of malaria and, aside from one *P. vivax* presentation, all studies focused on *P. falciparum*, which remains the priority species for containment in many malaria-endemic regions. The talks addressed a range of topics, with some recurrent themes including assessment of the rise and spread of artemisinin resistance outside of the Greater Mekong Subregion (the epicentre of artemisinin resistance)(Figure 2), methods for barcoding malaria parasites, and the utility of identity by descent (IBD)-based methods for improving measures of parasite relatedness and gene flow.

Firstly, **Assoc Prof Abdoulaye**, an invited speaker from the University of Science, Techniques and Technologies of Bamako, Mali, presented on the hot topic of “Is artemisinin resistance coming to Sub-Saharan Africa?” In order to identify any early evidence of delayed *P. falciparum* parasite clearance after artesunate treatment in Mali he undertook two therapeutic efficacy studies at two sites (Kone *et al.*, 2020). The clinical results indicated that artesunate remains efficacious in Mali. However, there was heterogeneity in parasite clearance rates between sites that could not be explained by K13 mutations, and other factors such as host immunity may play a role in observed differences in parasite clearance. In addition, longer parasite clearance times were observed with quantitative PCR (qPCR), relative to microscopy, which may indicate early signs of artemisinin resistance development. Prof Djimde advocated for the wider use of qPCR as a more sensitive approach to detect early warning signals of artemisinin resistance in Africa and other at-risk regions.

Complementing this talk, **Assoc Prof Alyssa Barry** from Deakin University and Burnet Institute, Australia, gave an important update on the emergence of artemisinin-resistance on the island of New Guinea. Assoc Prof Barry presented data derived from two large multicentre collaborations. Using Illumina data generated from *P. falciparum* isolates sourced from cross-sectional surveys undertaken in Wewak Province, PNG, in 2016 and 2017, 3 (of 239) infections were revealed to carry kelch13 C580Y mutations. Analysis of the genomic background of these 3 infections demonstrated that were unlikely to have been imported from the Greater Mekong Subregion; rather, they shared close genetic relatedness to Papua Indonesian isolates and thus, may have arisen by *de novo* mutation. However, more extensive molecular and clinical surveillance between 2015-18 conducted at 8 sites in PNG has only detected one infection with the C580Y *kelch13* mutation, and not found any evidence of the spread of the C580Y mutation locally. Nonetheless, ongoing surveillance through the STRIVE PNG program is underway to identify any changes to this concerning situation.

Understanding parasite diversity is key to understanding gene flow, population structure and identifying importations on parasite. Invited speaker **Prof Karen Day** (AM) from the University of Melbourne, Australia, described unpublished new research describing the utility of the highly diverse *var* genes to uniquely barcode, or “varcode”, distinct *P. falciparum* isolates enabling high-resolution characterisation of individual infection dynamics and population-level diversity and gene flow. Using the *var* Duffy binding-like domain (*var* DBLalpha), parasite varcodes can be derived using a single PCR and hundreds of parasites can be typed in parallel using platforms such as Illumina. To

give an idea of the diversity, Prof Day described some results from the high transmission setting of Ghana, where a staggering 42,399 distinct varcodes were identified amongst 1,099 patients revealing that ~80% of infections were polyclonal. In broader studies, the single var DBLalpha marker has also been shown to accurately characterise the population structure in African populations. An unexpected finding was that there were few recombinants, and the var genes had a very non-random population structure. Network analysis revealed that immune selection is shaping this structure to maximise the opportunities for parasites to repeatedly infect the same hosts (Pilosof *et al.*, 2019). The varcode was also highly informative in a study site in Ecuador where it was used to characterise the dynamics and source as well as evolution of a malaria outbreak clone. The utilities of var DBLalpha justify inclusion of this marker in broader *P. falciparum* barcodes.

Molecular methods for outbreak investigations was also the topic of **Dr Manuela Carrasquilla** from Harvard University who presented unpublished research on the molecular epidemiology of *P. falciparum* infections from a recent outbreak linked to illegal gold mining in the Pacific Coast region of Colombia. IBD analysis of genomic data (Illumina) generated on 151 *P. falciparum* isolates revealed highly related clonal clusters, reflecting the history of selection with antimalarials in the region. Several suspected importations from Venezuela were also identified in this analysis highlighting the risk of resistance dissemination due to importations, which is of particular concern in Colombia due to the recent de novo emergence of kelch13 C580Y mutations in neighbouring Guyana. More comprehensive molecular surveillance should facilitate tracking and containment of infections, including importations, helping to prevent future outbreaks and widespread dissemination of drug resistance.

Understanding the global antigenic diversity of *P. falciparum* is essential for the development of broadly effective malaria vaccines. **Mr Myo Naung**, a PhD candidate at the Walter and Eliza Hall Institute of Medical Research, Australia, presented unpublished analyses on the largest and most comprehensive dataset used to explore antigenic diversity in *P. falciparum* to date (dataset included the extensive repository of >2.5 thousand *P. falciparum* genomes from >15 countries across the globe in the malariaGEN Pf3K dataset, in addition to 156 newly generated genomes from PNG. One of the challenges of the study was in handling the short reads generated by Illumina, which can be notoriously difficult to analyse in diverse regions such as many antigen-encoding genes. Mr Naung described a novel data analysis approach incorporating the development of a new R package, VaxPack, providing accurate haplotype reconstruction and variant calling of antigen genes in single clone infections. Mr Naung also described an innovative new algorithm for analysing spatial averaging of selection pressure on 3D protein structures. One of the insights from the analysis was the revelation that current vaccines are based on relatively rare antigen variants, potentially constraining their efficacy. The information on local and global patterns of diversity, and predictions of immune targets, provided by this analysis pipeline will be informative for identifying more effective new vaccine candidates for *P. falciparum* and other species.

Lastly, **Dr Sasha Siegel** from the Wellcome Sanger Institute, UK, presented on an innovative new approach for estimating the probable cause of *P. vivax* recurrent infections with lineage-informative

microhaplotypes. This unpublished collaborative study with Menzies School of Health Research focuses on developing high-throughput genotyping tools to determine IBD between pairs of *P. vivax* infections to help to identify relapses. The rationale is that pairs of infections that are genetically different, but which have high IBD due to recent shared parents (such as pairs of siblings), are more likely to have come from the same mosquito inoculation and therefore to reflect a relapse than a reinfection event. IBD is usually determined from whole genome data, but this can be difficult to derive from low density *P. vivax* infections. Using a unique repository of ~1700 *P. vivax* genomes from >20 countries generated by malariaGEN and vivaxGEN, Dr Siegel has characterised the *P. vivax* “heterozygome”; identifying regions across the accessible portion of the genome where multiple high diversity SNPs constituting microhaplotypes reside within a short distance (<200bp). A panel of 100 microhaplotypes with high diversity in different endemic regions and uniform distancing across the genome, have been selected to characterise the IBD between pairs of infections using amplicon-based sequencing data. These tools will greatly enhance our understanding of the epidemiology of *P. vivax* relapses, which account for 80% of infections in some endemic areas.

Identifying factors that contribute to developmental progression of sexual stages and strategies to block parasite transmission

Understanding sexual commitment, transmission and development was the focus of the sex and transmission session, chaired by Dr Franziska Hentzschel (Glasgow University, UK) and Prof Geoffrey McFadden (University of Melbourne, Australia). Invited speaker Dr Mara Lawniczak from Wellcome Sanger Institute, UK commenced the session with an exciting presentation on the Malaria Cell Atlas, a study of single parasite transcriptomes across the lifecycle to examine individual variability in gene expression during lifecycle progression of different *Plasmodium* spp (Howick *et al.*, 2019). Whilst the number of genes detected varied greatly according to parasite life stage, developmental progression could clearly be identified, including the branch point for male and female gametocytes, providing a resource that will accelerate the understanding of biology for multiple *Plasmodium* species.

Invited speaker **Prof Matthias Marti** from Glasgow University, UK described a powerful genetic analysis of sexual commitment, using samples from patients with uncomplicated malaria. Using the transcriptomes and matched genomes of these samples, and structuring sexual conversion based on expression of the master regulator Api2g, target loci of Api2g were identified. Non-coding RNA in the intergenic regions between known gametocyte genes were identified to be linked to sexual conversion, suggesting they play a role in regulation of Api2g expression and sexual differentiation. This will help to understand how and when parasites convert from asexual to sexual stages for transmission and how this changes in different endemic regions.

Dr Claire Sayers from Umea University presented the development of an elegant screen to identify genes required for parasite fertility. Lines that produce only fertile male or female gametocytes were crossed and subsequent oocyst phenotypes examined to identify fertility genes. Many male and female sex specific genes were identified, validating the screen and providing an exciting outlook for the discovery of as yet unidentified genes required for parasite transmission.

Prof Andy Waters from Glasgow University discussed that *Plasmodium* sex determination occurs before sexual commitment and is subject to translational regulation. He described the role in this process of an RNA/DNA binding protein called ALBA3 that partly colocalize and are functionally essential for transmission by influencing the parasite cell's ability to prepare itself for gametocyte conversion. This work thus identifies important upstream players in sexual differentiation of asexual parasites to gametocytes for transmission.

Whilst understanding parasite biology may identify ways to block parasite transmission, other approaches include targeting the definitive host, the mosquito. **Dr Sofia Tapanelli** from Imperial College, UK, described the development of transgenic *Anopheles gambiae* mosquitoes that express antiparasitic peptides from different loci. Using standard membrane feeding assays with *P. falciparum*, a reduction of oocysts was reported for one of the modified mosquito strains, paving the way to future experiments that will test whether wild-type mosquitoes could be replaced with transmission-blocking mosquito strains in the field.

Dr Robert Shaw from Harvard University completed the session by discussing how parasite development within the mosquito is regulated by mosquito metabolism, which affects the rate of transmission. This was elegantly demonstrated through numerous mechanisms, including providing a second mosquito blood meal, which accelerated the development of *P. falciparum* oocysts and infectious sporozoites, thereby reducing the age of infectious mosquitoes. Transmission by younger mosquitoes demonstrates the plasticity of transmission, with important implications for malaria control.

Drug Development – compound screening, target identification and deciphering modes of action

The drug development session chaired by Prof Susan Charman (Monash Institute for Pharmaceutical Sciences, Australia) and Prof Dennis Kyle (University of Georgia, USA), showcased screening approaches to identify new potential antimalaria drugs, the methodologies used to characterise new and well as existing anti-malaria compounds in an endeavour to understand their mode of action, as well as exploration of off-the shelf insect control agents to assess whether they could be repurposed to block parasite transmission.

Invited speaker **Prof Elizabeth Winzeler** from the University of California, USA, spoke about her experiences conducting phenotypic drug screens, including their potential limitations, including the fact that they can be costly and be limited by chemical diversity. Moreover, there are risks associated with not understanding toxicity or how resistance could develop. Using P218, a dihydrofolate reductase inhibitor as an example, Prof Winzeler demonstrated how it is possible to design active molecules against good targets that have become compromised by drug resistance alleles. She also spoke about the malaria drug accelerated program, MalDA, and the benefits of collaborative teams that enable a wide variety of chemogenomic methods to be deployed to identify the targets for a wide variety of compounds with whole cell activity and their mode of action. In addition, she revealed that when the types of evaluation methods for a screen were altered (eg. short 48 h and long 96 h periods

of incubation with compound compared to the typical 72 h screen), new compounds and targets started coming out of the screen (Abraham *et al.*, 2020). She also highlighted that compound scaffolding structuring to group compounds based on structural similarity, showed that compounds with similar structures have similar activities across the lifecycle. Finally, given the length of time it can take to generate drug resistance in *P. falciparum* to identify the drug target, Prof Winzeler showed how a 'Green Monster' strain of *Saccharomyces cerevisiae* lacking efflux transporters can be used to rapidly create drug-resistance to compounds that are also active in yeast as a way to identify targets more quickly. However, whilst targets tend to be conserved across species, the mechanisms of resistance may not. Interestingly, some intergenic mutations were responsible for resistance phenotypes, highlighting that mutations in these regions should not be ignored.

Invited speaker **Dr David Olsen** from Merck and Co. Inc and invited speaker **Prof Alan Cowman** from The Walter and Eliza Hall Institute, Australia, then gave back-to-back presentations on their collaborative program to develop Plasmeprin inhibitors and understand their modes of action (Favuzza *et al.*, 2020). Dr Olsen showed that a representative subset (1000) of Merck's aspartyl protease inhibitors were screened against *P. falciparum* asexual stages at The Walter and Eliza Hall Institute. Several scaffolds were potently active and through subsequent medicinal chemistry, compounds WM4 and WM382 were developed, which had a very potent IC₅₀ of 8 and 0.5 nM, respectively. Resistance to WM382 could not be generated, but in WM4-resistant parasites the Plasmeprin X gene was amplified leading to its overexpression. Click chemistry pull-down revealed both WM4 and WM382 could pull down Plasmeprin X. Moreover, a FRET-based assay revealed both compounds inhibited recombinantly expressed Plasmeprin X. Compound WM382 was also shown to hit Plasmeprin IX, which was confirmed by inducible knockdown and targeted cellular thermal shift assays (CETSA). Compound 382 exhibited >1,000-fold selectivity over mammalian targets and demonstrated good *in vivo* activity against *P. berghei* and *P. falciparum* models in mouse and humanised mouse models, respectively. Crystal structures of Plasmeprin X revealed this protein has a conserved active site and the dual activity of compound WM382 against Plasmeprin IX and X may explain the increased barrier to generation of resistance. Importantly, it also provides coverage of additional lifecycle stages: blocking transmission to mosquitoes and rendering liver merozoites non-infectious. The mechanisms of action of WM4 and WM382 were also discussed. Both compounds were shown to block asexual blood stage parasite growth at the schizont stage and inhibit erythrocyte egress and merozoite invasion. The compounds also blocked transmission of *P. falciparum* and *P. berghei* gametocytes to mosquitoes. Liver stage killing was also examined and while there was no activity against asexual exoerythrocytic growth in the liver, fewer merozoites were seen and no parasites were observed in the blood, indicating liver merozoites are non-infectious. WM382 also blocks transmission of *P. falciparum* gametocytes to mosquitoes by membrane feeding assays, whereas WM4 did not. This suggests that Plasmeprin IX, but not Plasmeprin X plays a role in gametocyte development. That compound WM4 hits Plasmeprin X whilst WM382 hits both Plasmeprins has enabled the substrates of these two proteases to be teased out by FRET-based peptide cleavage assays. These studies have revealed there is a significant substrate overlap between the two proteases but their different localisation in merozoites probably drives their selectivity and that they are exciting targets for drug development.

In the following talk, **Dr Christina Spry** from the Australian National University, Canberra, presented her research on a commercially available insect control agent. The agent was found to inhibit blood-stage *Plasmodium falciparum* growth quickly and irreversibly *in vitro*. Furthermore, it was shown to act directly on the parasite and to deplete ATP in the parasite rapidly via a yet-to-be-determined mechanism. It was also shown to have mosquitocidal activity against *Anopheles stephensi* when the mosquitoes fed on a blood meal spiked with it. Although oral administration with a single dose of the agent failed to inhibit *Plasmodium berghei* proliferation in mice, mosquitoes that fed on treated mice were killed, even if the feeding occurred days later. This demonstrates its long-lasting activity and potential as a transmission-blocking endectocide.

Prof Katherine Andrews from the Griffith Institute for Drug Discovery, Australia, presented her research on new insights into the mode of action of proguanil (PG). The *in vivo* activity of PG has been attributed to its cyclisation product cycloguanil, a DHFR inhibitor. PG is also thought to potentiate atovaquone activity but was considered to have weak intrinsic activity against *P. falciparum*. Professor Andrews and colleagues have discovered that PG has more potent activity than previously thought and that this activity is via a slow acting mechanism different to the classic delayed death phenotype observed for antibiotics. Thus Malarone, which comprises atovaquone and PG, may be a triple drug combination that includes antagonistic partners. Prof Andrews and her collaborators are investigating whether a cyclisation blocked proguanil (tBuPG) may be an alternative to PG (Skinner-Adams *et al.*, 2019). Like PG, tBuPG has slow-acting activity, synergises with atovaquone and appears to have different mechanism of action to cycloguanil and delayed death inhibitors. tBuPG shows similar activity to proguanil *in vivo* in a murine malaria model. Professor Andrews and her colleagues have generated PG and tBuPG resistant parasites and analysis of the SNPs revealed by whole genome sequencing is now underway to identify the mechanism of action of PG and tBuPG.

The final talk of the session was given by **Prof Andrew Tobin** from the University of Glasgow about their target-directed discovery program that was successfully used to find inhibitors that target the cyclin-dependent-like kinase, PfCLK3 (Alam *et al.*, 2019). This is an essential kinase that plays a key role in pre-RNA splicing and processing. By screening the Tres Cantos Anti-Malaria Set compound library, compound TCMDC-135051 was found to kill parasites across the lifecycle very rapidly. By generating resistance to this compound, WGS revealed a mutation in PfCLK3 outside the kinase domain that increased kinase activity 3-fold. Phosphorylation of the target protein was tested in parasites to confirm on-target activity and that inhibiting PfCLK3 disrupts splicing. Prof Tobin also presented a model of the structure of CLK3 bound to the compound which is providing insight into how the compound inhibits CLK3 function.

Harnessing our understanding of natural and vaccine-induced immunity to improve vaccine immunogenicity

The immunity and vaccines session was chaired by Prof Denise Doolan (Australian Institute of Tropical Health and Medicine, Australia) and Prof Michael Good (Australian Institute of Tropical Health and

Medicine. Understanding immune tolerance in the development of natural and vaccine induced malarial immunity in epidemiological and human challenge models was a key theme as well as understanding the immune response which interrupts malaria transmission. Overall findings highlighted potential strategies to improve vaccine immunogenicity as well as the potential for whole parasite sporozoite and blood-stage vaccines.

In high transmission areas many infants are born to mothers who have had malaria in pregnancy and these infants may have altered susceptibility to infection in infancy. While true congenital infection is rare, malaria antigens access fetal circulation immune response to malaria *in utero* but potential immunomodulatory mechanisms induced by prior malaria exposure are unclear. Invited speaker **Dr Margaret Feeney**, from the University of California, USA, provided valuable new data on the impact of *in utero* malaria antigen exposure on fetal T cell differentiation and function, using antenatal samples from Uganda. She demonstrated that immune complexes containing malaria blood-stage antigens may traverse across the placenta to the infant via FcRN antigens transferred by Fcγ receptors in immune complexes, in a 'low dose' fashion. Neonates born to mothers with active placental malaria had higher frequencies of effector-memory CD4⁺ T cells as well as CD4⁺ and CD8⁺ T cells that produced inflammatory cytokines and exhibited greater *in vitro* proliferation to malaria antigens. Foetal T cells with greater proliferation were associated with protection from malaria during childhood suggesting that malaria exposure *in utero* can prime highly functional malaria-specific T cells and may influence postnatal susceptibility to malaria (Odorizzi *et al.*, 2018). FOXP3⁺ CD4 cells (Tregs) were observed in infants whose mother were infected with malaria early in pregnancy. However, overall there was no difference in the frequency of Tregs in women with or without placental malaria. Infants exposed to placental malaria also had higher T effector memory cells and ki67 CD4 cells, which are indicative of *in vivo* proliferation, suggesting that infants may have been exposed to malarial antigens *in utero*. When correlated with protection against malaria, infants with CD4⁺ T cells with strong *in vivo* proliferation in response to malaria antigens were the most protected against malaria in infancy. Understanding the priming of fetal malaria-specific T-cells by antigen exposure *in utero* will be essential for implementing current intervention strategies and optimizing vaccine immunogenicity in infants.

Tolerance was also the theme of **Ms Diana Muñoz Sandoval's** (University of Edinburgh) presentation, which showed that host control of inflammation can establish disease tolerance in malaria. Using a human malaria re-challenge model, she investigated the epidemiological observation that immunity to severe malaria can be acquired after the first few infections of life without a reduction in parasite density. To reveal the mechanisms that underpin this phenomenon, healthy volunteers were infected with *P. falciparum* three times over a 12-month period and changes in the immune response were tracked through time. Diana first showed that volunteers did not develop any immunity against infection, with parasite growth rates remaining the same during each homologous challenge. In contrast, she used a systems immunology approach (including RNA sequencing and plasma multi-analyte profiling) to demonstrate that the human immune response to *P. falciparum* changes dramatically after just one preceding infection. Notably, the massive polyclonal activation of T cells observed in naive hosts is silenced during re-infection and instead activated memory T cells become functionally specialised to prevent collateral tissue damage. Importantly, these changes all take place

independently of pathogen load. The data from this re-challenge model therefore indicate that human T cells quickly adapt to malaria to reduce inflammation and protect host tissues, and this study supports the epidemiological evidence that disease tolerance can be acquired after the first few infections of life.

The development of immunity to *P. falciparum* was also investigated by **Dr Xi Zen Yap** from the Radboud University Medical Centre, in trials of chemoprophylaxis with *P. falciparum* sporozoite (CSP) immunisation, whereby naïve volunteers are exposed to bites of infected mosquitoes under chloroquine prophylaxis. CSP immunisation induces highly effective sterile homologous protection in naïve volunteers. However, some volunteers are protected after only one immunisation (fast responders), whereas others require two or more immunisations to be fully protected (slow responders). Using samples from CSP immunization trials, Dr Yap investigated whether CD4⁺ T-cell expression of checkpoint inhibitors (regulatory molecules which inhibit immune responses and regulate acquisition of adaptive immunity) could explain these observations. Fast responders have lower CD4⁺ T cell inhibitory scores prior to the first immunization, based on a cumulative score using expression levels of the checkpoint inhibitors TIM-3 and CTLA-4. Fast responders are also almost twice as likely to be sterilely protected and have lower parasitaemia during the challenge infection. Lower inhibitory scores correlate with higher *in vitro* production of effector granulation markers Granzyme B, IFN- γ and CD107a in subsequent immunizations, as well as lower peak parasitaemia and delayed onset of parasitaemia during challenge. These novel data inform next-generation vaccine development, providing insights into how immune pathways in slow responders may be modified so as to become a fast responder who achieves sterile immunity after one vaccination.

Whilst many malaria vaccines are in development in the clinic and field, it is critical to understand how vaccines work in order to design more effective immunogens. As part of their “vaccine to interrupt malaria transmission” program, invited speaker **Professor Patrick Duffy** from National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health USA, aims to combine vaccines that prevent infection with ones that block transmission. In vaccination studies, they and others have found that the whole sporozoite Sanaria PfSPZ Vaccine provided high protection against homologous controlled human malaria infection and reduced natural infections in Mali. After vaccination, V δ 2 $\gamma\delta$ T cell numbers expanded and individuals with higher V δ 2 expansion never became infected with *P. falciparum* in comparison to individuals without an expansion who were not protected. Complementary studies in mice found that $\gamma\delta$ T cells during vaccination were essential for sterile protection. However, these cells were not directly mediating protection, rather they were required for the induction of protective CD8⁺ T cell responses. Data suggested that $\gamma\delta$ T cells might be the first player with sporozoites entering the liver, resulting in an expansion of DCs, with the potential that DCs and/or V δ 2 may present antigen to CD8⁺ T cells and that these resident T cells may confer protection against infection. As part of their transmission blocking vaccine program, investigations focus primarily on antibodies. To examine transmission blocking antibodies, the NIAID group focused on conjugate nanoparticles whereby antigens were conjugated to carrier proteins. Sera from vaccinees with two doses of Pfs230 with Alhydrogel saw a dramatic reduction in the number of mosquito oocysts, that was not seen with Pfs25. Generated human monoclonal antibodies varied in their transmission

reducing activity and were found to be epitope specific and complement-dependent in parasite lysis assays and standard membrane feeding assays. These studies provide important data to improve the design and monitor the development of vaccines which aim to interrupt malaria transmission.

The immune response to, and vaccine strategies against, sporozoite stages were further revealed by **Prof William Heath**, University of Melbourne, Australia, who sought to identify antigens targeted by liver tissue resident memory T cells that could mediate immunity against malaria. Liver-resident memory CD8 T cells are critical for fighting infection and move around the liver sinusoids looking for infections. Recently they were shown to mediate efficient protection against *Plasmodium* sporozoite infection, prompting development of immunization strategies such as “prime-and-trap” to favour the generation of these cells for anti-malarial immunity (Fernandez-Ruiz *et al.*, 2019). Prof Heath identified a novel epitope from a putative 60S ribosomal protein L6 (RPL6) of *P. berghei* ANKA, as an ideally suited antigen for liver tissue resident memory T cells generation and protection against malaria (Valencia-Hernandez *et al.*, 2020). A single dose prime-and-trap vaccination that targets RPL6 was shown to provide highly effective and prolonged sterilizing immunity against large sporozoite challenges in 100% of mice. Interestingly, RPL6 is expressed in both liver and blood-stages, across *Plasmodium* species, and has minimal polymorphisms in *P. falciparum*, suggesting favourable translation potential as a vaccine candidate. The therapeutic potential of vaccination approaches that induce liver tissue resident memory T cells and provide an optimal, broadly conserved liver stage *Plasmodium* target antigen need to be further explored.

While sub-unit vaccines are the predominant approach, vaccines comprising whole parasites have the advantage in that delivery to proper organs for immune priming occurs and correct pattern recognition signals are exposed to the immune system. **Prof James McCarthy**, QIMR Berghofer Medical Research Institute, Australia, presented new data on the development of a genetically attenuated parasite (GAP) blood-stage vaccine and its infectivity and immunogenicity in healthy volunteers. A KAHRP knockout (KO) was chosen because KAHRP KO parasites have lost the ability to cytoadhere and so end up in the spleen to generate robust immunity. Furthermore, monkeys infected with KAHRP KO parasites do not develop clinical malaria and are also protected when challenged. The GAP vaccine was produced by targeted deletion of *kahrp* from *P. falciparum* 3D7 under GMP conditions, released, and tested in a phase 1 clinical trial. In the first in-human dose escalation trial, none of the subjects administered with either low or medium doses of parasites developed parasitaemia, whereas the majority of subjects administered the higher dose of parasites developed parasitaemia. All GAP parasitaemia induced MSP-2 antibody responses similar to previous challenge studies and natural infection in individuals from Papua New Guinea. Data presented by Prof McCarthy demonstrated that while a GAP blood-stage vaccine is immunogenic and can be successfully tested in vivo, it can still lead to patent parasitemia at high doses.

Targeting the liver stage: contribution of parasite genes, host cell preference and host immune response to pre-erythrocytic infection and subsequent pathology

Before infecting erythrocytes, *Plasmodium* sporozoites injected into the vertebrate host during a blood meal of an infected female mosquito infect the liver, with each exo-erythrocytic form ultimately generating tens of thousands of merozoites by exo-erythrocytic schizogony (Figure 3). The mechanisms of hepatocyte invasion differ between *Plasmodium* spp and are not well understood. In this pre-erythrocytic biology session, chaired by Dr Erika Flannery from Novartis Institute for Tropical Diseases, USA and Asst Prof Ashley Vaughan from Seattle Children's Hospital, USA, invited speaker **Dr Olivier Silvie** from Sorbonne University, France reported the identification of a cysteine-rich sporozoite protein related to the blood-stage invasion protein CyRPA that is required for productive invasion of hepatocytes by *P. berghei*. This suggests that protein modules involved in invasion may be conserved between zoites.

Once the hepatocyte is infected, what parasite genes are expressed? **Ms Maria Toro-Moreno** from Duke University, USA, described the *P. berghei* transcriptome during early liver infection (Toro-Moreno *et al.*, 2020). By focusing on early infection, she showed that parasites derepress translation and then differentially express genes as early as 2 hours post infection. Whilst genes involved in invasion are downregulated, upregulated genes included those involved in remodelling the parasitophorous vacuole and membrane. Interestingly, 87% of genes upregulated 24 hours post-infection were already upregulated at 12 hours, demonstrating the importance of early gene expression changes during liver infection.

Dr Annie Yang from Radboud University Medical Center, The Netherlands, addressed whether specific hepatocyte cells or parasite isolates are preferential for liver infection. She described three types of hepatocyte cells in liver based on zones and showed that *P. falciparum* sporozoites have a preference for cells in zone 3, which express a unique enzyme. She also identified transmissible *P. falciparum* strains from Cambodia and Nigeria that are more tractable for transfection and liver infection than current strains.

Liver stage parasites grow at a rapid pace. **Dr Magali Roques** from the University of Bern, Switzerland, described the use of the PlasmoGEM resource to conduct a knockout phenotyping screen of liver stages (Toro-Moreno *et al.*, 2020). The screen revealed that significant reprogramming of parasite metabolism occurs to achieve rapid growth in the liver. Seven different metabolic subsystems were shown to be essential during liver infection compared with blood stages: type II fatty acid synthesis and elongation (FAE), tricarboxylic acid, amino sugar, heme, lipoate, and shikimate metabolism, providing a deeper understanding of how liver stages grow so quickly.

Given that *Plasmodium* liver infection is asymptomatic, does it influence malaria pathology? Invited speaker **Prof Maria Mota** from the Institute of Molecular Medicine, Portugal, provided a tantalizing answer to this question through the demonstration that exposure of mice to multiple, closely spaced sporozoite challenges protected them against subsequent blood stage disease. Interestingly, there was no delay to the onset of blood stage infection or reduction in the parasitemia, indicating the host response was altered. Therefore, the liver phase of *Plasmodium* infection can precondition the host response to malaria disease during the blood stage of infection and the underlying molecular mechanisms for this novel discovery were discussed.

Prof Dennis Kyle from University of Georgia, USA, revealed a large-scale drug screening platform for screening of compounds that target *P. vivax* liver stages. Excitingly, the team identified several new compound classes with activity against *P. vivax* liver stages that could offer uses in prophylaxis or radical cure whilst others cleared parasites by acting on host cell targets. This work provides new hope for novel therapeutics targeting recrudescence malaria through the identification of important new scaffolds for advancing through drug development pipelines in the future.

Dissecting pathways that are fundamental to asexual stage progression to understand mechanisms of drug resistance and to develop new diagnostic tools

The asexual biology session was chaired by Dr Julie Healer (The Walter and Eliza Hall Institute) and Dr Moritz Treeck (Crick Institute). Talks provided new insight into *Plasmodium* invasion, parasite development, replication and egress, and demonstrated how understanding the fundamental biology of the parasite can lead to new approaches to diagnose malaria infection or determining how the parasite has developed resistance to drugs. Specifically, **Dr Tobias Spielmann** from the Bernhard Nocht Institute for Tropical Medicine, Germany, described the function and contribution of the Kelch13 (K13) protein in artemisinin (ART) resistance (Birnbbaum *et al.*, 2020). Using the BioID system (a proximity labelling approach), the K13-interactome was identified, and modification of a selection of these K13-interacting partners conferred resistance to ART. K13 was found to localize to a novel compartment in close proximity to hemoglobin (Hb)-filled vesicles that also contains proteins involved in endocytosis of Hb from the host cells. It is known that ART needs to be activated by heme and that resistance in the parasite correlates with reduced Hb digestion. Indeed, conditional inactivation of K13 impaired hemoglobin endocytosis. ART-resistance conferring mutations reduced K13 protein levels; increasing K13 levels using the resistant version on an ART resistant background (ie. parasites have only mutated K13 but more or it) restored ART sensitivity. Hence the amount of K13 seems to explain resistance and there likely is no qualitative change in the mutated version. Taken altogether, the K13 complex defines an endocytosis pathway that controls hemoglobin availability and in turn drug activity levels. Importantly these findings provide new markers for the surveillance of ART resistance, but also points to potential new avenues to circumvent it.

Invited speaker **Dr Audrey Odom** John from the Children's Hospital of Philadelphia, USA, spoke about the potential of targeting metabolism of asexual parasites to diagnose malaria. A clinical study of pediatric patients in Malawi presenting with acute febrile illness revealed that uncomplicated *P. falciparum* infection leads to changes in volatile compounds, including in the exhaled breath of the children (Schaber *et al.*, 2018). Specific breath volatiles were identified that represent candidate breath biomarkers, offering the potential of developing a breathalyzer test for these biomarkers. Validation of these breath biomarkers in additional populations is currently underway.

Invited speaker **Prof Manoj Duraisingh** from the Harvard T. H. Chan School of Public Health, USA, demonstrated the power of forward genetic screening to identify the molecular determinants required by *Plasmodium* spp to successfully invade and support growth inside RBCs. The analysis revealed strain-specific as well as strain-transcendent determinants of *P. falciparum* invasion. Several

essential RBC molecules required for *P. falciparum* invasion were identified; in contrast, *P. vivax* was found to invade reticulocytes using a distinct but overlapping set of host cell receptors. The *P. vivax* reticulocyte tropism has a profound effect on its development and osmotic stability. This knowledge may facilitate methodologies for *in vitro* continuous culture of *P. vivax*.

Dr Maryse Lebrun from the University of Montpellier, France, took a further afield approach to understand the mechanism the drives rhoptry secretion. By looking at regulated secretion systems in organisms phylogenetically close to Apicomplexa, she identified in *Toxoplasma* and *Plasmodium* a protein that is homologous to a protein in the exocytic machinery of their free-living ciliate relatives, and which contributes to the formation of an apical rosette of particles embedded in the plasma membrane. Using conditional strategy in *T. gondii* and *P. falciparum*, she linked the formation of a similar rosette structure in apicomplexans with rhoptry exocytosis and invasion.

Assoc Prof Jeffrey Dvorin from Harvard Medical School, USA, coupled the high resolution of electron microscopy with automated serial sectioning and presented the resulting 3D reconstructions of developing *P. falciparum* schizonts, giving the audience a merozoite-eye view of its nuclear, organellar and membrane arrangements in a temporally sequential manner (Rudlaff, Kraemer, Marshman & Dvorin, 2020). This revealed that the final nuclear division is likely asynchronous between nuclei within a single schizont and that the final round of nuclear division is also asynchronous with cytokinesis.

Dr Margarida Ressurreição from the London School of Hygiene and Tropical Medicine, UK, reported an unusual example of an essential, non-exported PEXEL-containing protein that is cleaved by Plasmepsin V but which is primarily retained in the PV. This protein is a putative lipid transfer protein, and parasites lacking it have an altered shape and smaller PVM and subsequently fail to develop soon after invasion, indicating the protein plays a role in PVM formation. The charge at the P4 position of the PEXEL motif was suggested to be an important determinant of PV localization. Interestingly, while some *T. gondii* proteins that are cleaved by the orthologue of Plasmepsin V, called ASP5, are exported to the host cell and nucleus, some matured substrates are not exported but rather function in the PV and PVM, thus this study confirms other reports that this also occurs in *Plasmodium* spp.

Regulation of gene expression and DNA replication and the power of genetic crosses

The genetics and epigenetics session was chaired by Dr Sophie Adjalley (Wellcome Sanger Institute, UK) and Dr Michael Duffy (University of Melbourne, Australia). The talks in this session described a broad range of elegant and innovative experimental techniques being used to inform on a range of mechanisms pertaining to the regulation of gene expression and the cell cycle in *P. falciparum* parasites.

The first presentation by invited speaker **Assoc Prof Till Voss**, from the Swiss Tropical and Public Health Institute, Switzerland, described an intricate series of experiments to try to dissect the function of a novel member of the ApiAP2 family of putative transcription factors in *Plasmodium falciparum*, as part of a broader goal to enhance the general understanding of the mechanisms involved in establishing, maintaining and regulating heterochromatin and downstream gene expression. Heterochromatin

protein 1 (HP1), which silences more than 400 *P. falciparum* genes, and is essential for asexual proliferation, virulence gene silencing and repression of sexual commitment; however, the mechanisms of heterochromatin regulation remain poorly understood. Co-immunoprecipitation experiments with HP1 identified a novel ApiAP2 factor in the complex. This factor, termed AP2-HC, was found to be expressed in the parasite nucleus, localising exclusively to heterochromatin. However, AP2-HC did not appear to bind its cognate DNA motif, or other chromosomal DNA *in vivo*, nor have a role in regulating gene expression or be required for heterochromatin formation. With these properties, AP2-HC is an unusual ApiAP2 factor. While its role in parasite biology remains a mystery, the results highlighted the functional diversity among the members of this important family of nuclear proteins. Prof Voss called for further research into this unusual and potentially important factor.

The second invited speaker was **Assoc Prof Catherine Merrick** from the University of Cambridge, UK, who described an innovative new methodology developed to monitor the patterns of DNA replication within the cell cycles in *P. falciparum*; a process that is poorly understood in Apicomplexan parasites. In model organisms, nascent DNA replication has been labelled with modified nucleotides to visualise what DNA is made and when. However, *Plasmodium* parasites cannot metabolise modified nucleotides. Prof Merrick devised the novel approach of adding thymidine kinase to allow visualization of DNA replication in *P. falciparum* to track spatio-temporal patterns at the cellular, single molecule, and omic level, and to view how it changes in mutants. This is an elegant method that doesn't need much material but does need long strands of DNA. Insights included the revelation that replication forks move relatively slowly in *P. falciparum* compared to other model organisms and that the origins of DNA replication are closely spaced in this species. Using this method in RecQ helicase mutant lines, Prof Merrick's lab showed that the mutants had slower replication forks and appeared to fire more replication forks closely in time in a possible effort to overcome the slower replication. She also showed that parasites without the RecQ helicase WRN have comparatively unstable genomes, with large accumulation of recombinants and deletions owing to stalling of replication forks. Unpublished research at the omic level revealed that *P. falciparum* replication origins are non-random and focused in AT-rich regions, but it remains unclear if there is a sequence motif or if origin activity maps with gene density, chromatin or epigenetic features. Experiments in non-erythrocytic stages, specifically gametocytes, showed that gametogenesis is very fast in *P. falciparum*; with the genome being replicated approximately 10x faster than in *S. cerevisiae*. It remains unclear if this is due to a lack of checkpoints. Further work is planned to compare different species and to see what happens under stress, for example, in the presence of antimalarial drugs.

Dr Sachel Mok from Columbia University Medical Center, US, presented on a topic of important public health relevance, namely understanding the mechanisms of resistance to the artemisinin combination partner drug, piperaquine (PPQ), which is spreading through the Greater Mekong Subregion. Genome-wide association studies have identified Plasmepsin II (*pfpm2*) gene amplification as a molecular marker of resistance in patient isolates (Amato *et al.*, 2017; Mukherjee, Gagnon, Wirth & Richard, 2018) but *in vitro* studies suggest that other determinants including *pfcr* are also involved (Ross *et al.*, 2018). Dr Mok described the use of *P. falciparum* genetic crosses in humanized mice and QTL linkage mapping to try to identify new determinants of PPQ resistance. A PPQ-resistant parasite

line with KEL1-PLA1 haplotype from Western Cambodia (RF7) was crossed with a PPQ-sensitive line from Africa (NF54). The cross generated 186 progeny and Illumina whole-genome sequencing identified 67 genetic recombinants comprising 14 distinct haplotypes at 16,332 polymorphic loci. The haplotypes were phenotyped *in vitro* to determine the IC₅₀ against a range of compounds including PPQ, lumefantrine, mefloquine, tafenoquine, and a new MMV drug (MMV675939), and genetic association analyses are underway to identify genetic determinants underpinning resistance to each of these antimalarials. Early insights suggest that PPQ-R may be multigenic, with a *pfpm2* copy number effect augmenting mutant *pfcr1*-driven resistance.

Assistant Prof. Richard Bartfai, from Radboud University, The Netherlands, gave a talk centred on recently published work by his lab that elucidated how *P. falciparum* histone tail modifications (PTMs) are read and interpreted by the parasite in the process of epigenetic regulation (Hoeijmakers *et al.*, 2019). A novel histone peptide pull-down method coupled with quantitative proteomics by mass spectrometry was employed to "fish" for reader proteins such as heterochromatin protein 1 (HP1) and bromodomain proteins 1 and 2 (BDP1 and 2) as well as associated proteins. Approximately 50 proteins were identified, including 14 new and putative reader proteins in blood stage parasites. Most of these proteins were proven to be organized into five main protein complexes with a high degree of connectivity. Other insights were that the BDP1 and 2-containing complex is recruited in an acetylation-dependent manner, while a PhD-finger protein mediates the crosstalk between H3K4me2/3 and H3K9ac marks. As well as providing new insights into epigenetic regulation in *P. falciparum*, the study has generated a detailed list of PTM reader interactions and composition of epigenetic complexes.

Dr Emma McHugh, from the University of Melbourne, Australia, presented on unpublished new results giving insights into regulation of splicing in *P. falciparum*. Alternative splicing is widespread in the *P. falciparum* genome. Dr McHugh's research is investigating the contribution of alternative splicing to the differentiation of parasites during their lifecycle. In the first stage Dr McHugh has focused on dissecting the mechanisms of SR proteins (RNA-binding proteins) and SR protein kinases that are putative mediators of alternative splicing in *P. falciparum*. A CRISPR/Cas9 approach was used to generate epitope-tagged inducible knockdown parasites for 6 SR proteins and 3 SR protein kinases. This approach enabled localisation of SRs to the nucleus and cytosol in varying degrees and showed that SR knockdowns have attenuated growth. RNA-seq analysis revealed that the knockdowns do not have significantly different mRNA levels relative to the wildtype lines, but analysis with the JunctionSeq Bioconductor package for R demonstrated alternative splicing. This is a promising approach to provide further insights into the regulation of alternative splicing in *Plasmodium*.

The last speaker of the session was **Prof Karine Le Roch**, University of California, US, presenting work aimed at elucidating the role of long non-coding RNAs (lncRNAs) in regulating chromatin structure and downstream gene expression in *P. falciparum*. Using Illumina sequencing and nascent RNA expression profiling, her team have identified more than 2,500 lncRNAs; 58% of which are novel. Most of the lncRNAs localised to the nucleus, but some were observed in the nucleus and cytoplasm. The lncRNAs were generally shorter than protein coding RNAs (pcRNAs) and were quite AT-rich. They also exhibited

much lower expression levels and were more unstable than pcRNAs. RNA-FISH was used to validate the localisation of the lncRNAs, confirming their presence in different life-cycle stages of the parasite. Chromatin Isolation by RNA Purification (ChIRP) was also used to assess the genomic localisation/interaction of several candidate lncRNAs at different life-cycle stages. This revealed focal, sequence-specific localisation in the genome. Several lncRNAs localised with promoters of several parasite-specific gene families, including the *vars*. Stage-specific pattern were also observed, such as specific expression of lncRNAs in gametocytes. In summary, these intricate experiments reveal stage specific RNA-chromatin interactions and emphasize the role of lncRNA in chromatin structure and gene regulation.

Mechanisms that contribute to the development of severe malaria disease

In the pathogenesis session, chaired by Prof Terrie Taylor (Michigan State University, USA) and Prof Alister Craig (Liverpool School of Tropical Medicine, UK) talks focused on the mechanisms that impact an individual's ability to succumb to severe disease. Invited speaker **Assoc Prof Bridget Barber** from QIMR Berghofer Medical Research Institute, Australia, spoke about the risk factors and mechanisms that lead to severe disease from studies conducted on Malaysian patients infected with *P. knowlesi*. Risk factors for severe *P. knowlesi* infection include a high parasitemia and older age. The characteristics of *knowlesi* and *falciparum* severe disease were found to be similar with the exception that *P. knowlesi* does not cause cerebral malaria. Features shared between severe *knowlesi* and *falciparum* malaria that contribute to adverse outcomes include endothelial activation, impaired microvascular reactivity and reduced RBC deformability (Barber *et al.*, 2018). Another key feature of severe *knowlesi* malaria is intravascular hemolysis, which is associated with acute kidney injury, and it was reported that this could be attenuated by regular paracetamol administration (Cooper *et al.*, 2018). Patients with severe *knowlesi* malaria also generate increased glycocalyx breakdown products such as urinary glycosaminoglycans and plasma syndecan-1. The presence of plasma syndecan-1 correlates with markers of endothelial activation and endothelial cell adhesion molecules, as well as impaired microvascular reactivity and lactate, suggesting plasma syndecan-1 plays a role in impaired tissue perfusion. Moreover, plasma syndecan-1 was found to be an independent risk factor for severe *knowlesi* disease. Thus, breakdown of the endothelial glycocalyx may represent a target for adjunctive treatment for the prevention of complication of severe malaria.

Mr Debanjan Mukherjee from Instituto de Medicina Molecular, Portugal, presented findings from experiments using specific pathogen-free (SPF) and germ-free (GF) mice infected with either *P. berghei* K173 (which gives rise to respiratory distress) or *P. berghei* ANKA (which causes cerebral malaria) to reveal how the host microbiota dictates one severe pathology over another. *Plasmodium* K173 parasites caused an increase in bacterial load in the lungs of mice that died of malaria-associated respiratory distress syndrome (MA-ARDS), with 16s rRNA sequencing demonstrating marked changes in the microbiome. The increased bacterial load in the lung was dependent on parasite sequestration in the lung. Host factors were also responsible for lung microbiota dysbiosis, being dependent on the production of IL-10 by CD4⁺ and CD8⁺ T cells; administration of IL-

10 receptor blocking antibody protected GF mice from developing lung pathology after infection with *P. berghei* K173 parasites. Infection of germ-free versus specific pathogen-free mice resulted in the majority of germ-free mice dying later of hyperparasitemia, confirming that microbiota contribute to the outcome of *Plasmodium* infections. Treatment with the antibiotic linezolid reduced the bacterial load in the lungs and prevented mice from developing MA-ARDS, although it did not affect parasitemia, suggesting the host microbiota is critical for the onset of MA-ARDS. Hence it may be possible to introduce antibiotic interventions to prevent MA-ARDS.

In order to better understand the pathophysiology of cerebral malaria caused by *P. falciparum* **Dr Maria Bernabeu** from EMBL Barcelona, Spain, spoke about two new *in vitro* 3D microvascular models she has engineered to better understand parasite-host cells interactions (Bernabeu *et al.*, 2019). The first model presented was a 3D brain microvessel model that displays vessels of 100 μm of diameter, which generates a wide range of wall shear stress within the same device. Using this system, *P. falciparum* clonal lines expressing different PfEMP1 antigens were found to respond differently to endothelium activation. In the second model, endothelialised capillary-size vessels (5 – 10 μm) were utilised. These mimic the flow dynamics of blood transition from arterioles to capillaries to venules allowing spatiotemporal sequestration kinetics of wild-type parasites to those lacking PfEMP1 or knobs to be compared. Knobless infected RBCs were found to sequester in the transition of capillaries to venules, where they are exposed to flow deceleration, whereas PfEMP1-negative infected RBCs and uninfected RBC did not cytoadhere to any region. These systems will provide a new platform to study the biomechanical and biological determinants of sequestration of infected RBCs.

The quality of circulating RBC, including *Plasmodium* infected RBC is controlled by the spleen. Innate clearing of a proportion of infected RBC blocked upstream from narrow slits in the red pulp help to slow the pace at which the parasite population grows before the adaptive immune response or anti-malaria therapy comes into play. In his talk, invited speaker **Prof Pierre Buffet** from Paris Descartes University, France, outlined how RBC clearance by the spleen can potentially be pathogenic as it can lead to anemia. In addition, because the spleen harbours live parasites in asymptomatic subjects, it may also serve as a reservoir for parasite transmission. The spleen can also remove dead parasites from within their host RBC and release them into the circulation. This process is known as pitting and is artesunate dependent, leading to the genesis of post-artesunate delayed hemolysis. This side effect is observed in 5-40% of patients with severe malaria who have been treated with artemisinins. Pitted RBCs contain HRP2 as in imprint of their former parasite infection, explaining the week-long positivity of HRP2-rapid diagnostic tests following treatment with artemisinin combination therapies (ACTs). That the spleen can clear stiff RBC from the circulation may enable novel therapies that cause drug-induced alteration of mature gametocytes and rapid elimination from the spleen, thereby leading to a block in parasite transmission (Henry *et al.*, 2020).

Dr Arlene Dent from Case Western Reserve University, USA, examined the inflammatory and metabolic profiles in the plasma and peripheral blood mononuclear cells of children from Papua New Guinea aged 2-10 years of age that had acute febrile malaria (either *P. falciparum* or *P. vivax*) and compared these to children with acute non-malaria febrile illness caused by bacterial infection.

Convalescent samples after nine weeks of anti-malaria or anti-bacterial treatment were also collected. Enriched metabolites were analysed by advanced mass spectrometry technology. Significant differences in the metabolome of acute *falciparum* malaria compared to acute *vivax* malaria were observed in four pathways pyrimidine-uracil, sphingomyelin, lysophospholipid, and fibrinogen cleavage peptide metabolism. A range of metabolic pathways were able to distinguish between children with acute malaria and acute non-malaria febrile pathways. These included diacylglycerol, dipeptides, fatty acids monohydroxy, sphingomyelins, ceramides, lysophospholipids, fatty acid metabolism acyl choline, glutathione and fibrinogen cleavage peptides. However, a greater contrast was observed between metabolites/pathways (95 pathways in total) in convalescence plasma from malaria cases when compared to non-malaria cases, suggesting that children with acute malaria had persistent immune metabolic perturbations relative to children with non-malaria febrile illness who had more rapid recovery of metabolic homeostasis.

Another study conducted in PNG by PhD Student **Ms Eliza Davidson** from the Burnet Institute, Australia, looked at the interactions between iron deficiency, anemia and malaria during pregnancy and at the 6 month and 12-month postpartum period. Both malaria and iron deficiency are risk factors for anemia and pregnant women are susceptible to all three. Iron deficiency (defined as ferritin <15 $\mu\text{g/l}$) was the key determinant of anaemia during pregnancy and postpartum *Plasmodium* species infection at enrolment (as determined by PCR) was associated with significantly lower levels of haemoglobin at enrolment and delivery but detected in only 12% of women. There was a significant interaction between *Plasmodium* sp. infection and ferritin but not iron deficiency, suggesting this is due to an acute phase response and raised ferritin in infected individuals. The studies supported the use of iron supplementation in pregnancy and postpartum.

Finally, **Dr Gavin Wright** from the Wellcome Sanger Institute, UK, presented research that points to how *P. falciparum* is restricted to cause infections in humans, despite being zoonotically transferred from gorillas (Galaway *et al.*, 2019). Genome comparisons revealed an introgressed sequence in the ancestor of *P. falciparum* containing RH5, which likely allowed the ancestral parasites to infect both gorilla and human erythrocytes. RH5 is an invasion ligand that is essential for *P. falciparum* to invade RBCs. Using quantitative protein interaction assays, the ancestral introgressed RH5 sequence could bind the cognate basigin receptor from both humans and gorillas. This promiscuous receptor binding phenotype of RH5 was shared with the parasite clade that transferred its genome segment to the ancestor of *P. falciparum*, whereas the other lineages exhibited host-specific receptor binding. Since its transfer to humans, *P. falciparum* and the RH5 ligand have evolved a strong human-specificity which is attributable to just a single amino acid mutation in the RH5 sequence. These findings reveal a molecular pathway for the origin and evolution of human *P. falciparum* malaria, which may inform molecular surveillance in the prediction of future zoonoses.

Dissecting the mechanisms associated with resistance to anti-malaria drugs

Overcoming the emergence of drug resistance has been one of the greatest challenges to achieving global malaria control and elimination over the past few decades. Forty years ago resistance to

chloroquine (CQ) emerged in the Greater Mekong Subregion and spread globally. In the past decade, we have seen the emergence of reduced efficacy to current first line antimalarial, artemisinin derivatives, and in the past few years to the first-line combination drug piperaquine (PPQ) also in the Greater Mekong Subregion, but resistance has the potential to emerge outside SE Asia (see molecular epidemiology session). In this session chaired by Prof Leann Tilley (Bio21 Institute, Australia) and Assoc Prof Darren Creek (Monash Institute of Pharmaceutical Sciences, Australia) a diverse array of novel molecular approaches that are required to understand and measure the extent of, and the mechanisms behind, drug resistance were showcased with the ultimate aim of developing new strategies to detect, treat and eliminate resistant infections.

The alarming recent emergence of clinical resistance to the partner drug PPQ (with DHA-PPQ treatment failures now exceeding 50% of cases across the Greater Mekong Subregion) led invited speaker **Prof David Fidock**, Columbia University Irving Medical Center, USA, to investigate parallels with resistance to CQ, a chemically related 4-aminoquinoline and previous first-line antimalarial. Resistance to CQ and PPQ has been associated with distinct sets of point mutations in the *P. falciparum* CQ resistance transporter PfCRT. Genome-wide association studies identified a novel mutation C350R in South American isolates that conferred PPQ resistance and restored CQ sensitivity in gene-edited 7G8+C350R parasites. Other PfCRT mutations (T93S, I218F, F145I) that evolved on the CQ-resistant Dd2 isoform increased rapidly in frequency in Cambodia between 2010 and 2016 and have now taken over the *P. falciparum* populations across the region. T93S and I218F conferred moderate resistance whereas the F145I mutation was the most resistant *in vitro*. All three mutations were associated with an increased risk of treatment failure. Relative to the highly unfit F145I mutation, T93S and I218F showed a reduced fitness cost, explaining their rapid ascendance in recent years. Prof Fidock's team also engineered F145I into the GB4 haplotype (the most common PfCRT variant in Africa), with gene-edited parasites showing a gain of PPQ resistance *in vitro* that came at a substantial fitness cost. Almost all PPQ-resistant PfCRT isoforms lost their resistance to CQ. Prof Fidock and his colleagues also used cryo-EM to solve a 3.2Å structure of the PfCRT isoform from the South American CQ-resistant, PPQ-sensitive 7G8 isoform (Kim *et al.*, 2019). The mutations associated with CQ and PPQ resistance localized primarily to moderately conserved sites on distinct helices that line a central negatively-charged binding cavity. Biochemical studies with purified protein revealed that these mutations conferred a gain of drug transport without altering drug binding. Overall, these novel data provide atomic-level insights into the molecular and genetic basis by which PfCRT confers PPQ resistance and contributes to PPQ treatment failures.

Polymorphisms in the *P. falciparum* multidrug resistant protein 1 (MDR1) and PfCRT, both located on the digestive vacuole membrane, alter the parasite's susceptibility to many current and developmental drugs that act, accumulate, and/or are activated in the vacuole. Whilst both the drug transport and natural functions of PfCRT are becoming increasingly well understood, the function of PfMDR1 - and the mechanism(s) by which it modulates drug sensitivity - remains unclear. PfMDR1 belongs to the ABC transporter superfamily, members of which expend ATP to pump solutes against their electrochemical gradients and is orientated in the digestive vacuole membrane such that it will pump solutes from the parasite cytosol into the vacuole. In order to elucidate PfMDR1's function, and to undertake direct measurements of drug transport, **Dr Rowena Martin** (Australian National

University, Australia) developed a strategy for expressing PfMDR1 in *Xenopus* oocytes. *P. falciparum* transporters are very difficult to express, whereas the expression of human transporters is less challenging. The human homologue of PfMDR1 (P-gp) was therefore expressed in the oocyte system and its function confirmed using a known P-gp substrate (vinblastine) and inhibitors (e.g. vanadate and nifedipine). Application of the P-gp expression conditions and optimised transport assay to PfMDR1 resulted in the successful expression of this parasite pump in oocytes. The interactions of various antimalarials (lumefantrine, CQ, amodiaquine, quinine, and PPQ) with different field isoforms of PfMDR1 were characterised, with transport shown to be time-dependent, ATP-dependent, and saturable. PfMDR1-mediated transport was also potently inhibited by two developmental antimalarial drugs that have been postulated to target the pump. This system provided novel data on the role of PfMDR1 in multidrug resistance and of its simultaneous hypersensitization of *P. falciparum* to certain antimalarials. Importantly, the PfMDR1 oocyte system can be used to test novel antimalarial candidates for the ability to interact with different isoforms of the pump.

The artemisinin resistance phenotype is characterised by prolonged parasite clearance times following treatment with artemisinin derivatives, which is strongly associated with nonsynonymous mutations in the *P. falciparum* *kelch13* (K13) gene. **Prof Zybdek Bozdech** from Nanyang Technological University, Singapore, led MAM2020 attendees through the population transcriptomic investigations in the tracking resistance to artemisinin collaboration (TRACI, 2011-2013 and TRACII 2016-2018) (Zhu *et al.*, 2018). Prof Bozdech undertook transcriptomic and genomic (eQTL) analysis in TRACI and TRACII samples and demonstrated that during the intervening ~5 years, the main global transcriptomic features, including upregulation of Unfolded Protein Response (UPR) /oxidative damage, protein synthesis and turnover, exported proteins (PHIST/FIKK) and at the same time deceleration of the transcriptional cascade of the Intraerythrocytic developmental Cycle (IDC), were generally similar in resistant parasites. *In vivo* transcriptional analysis of samples collected 6 h after artemisinin treatment showed that a number of genes get upregulated in sensitive parasites and these are already upregulated in resistant parasites (including K13). Prof Bozdech's work highlighted that an artemisinin resistance associated transcriptional profile exists which may have arisen from an initial transcriptional response (on drug exposure) which then cemented in and stayed on. Artemisinin resistance continues to evolve in the Greater Mekong Subregion *P. falciparum* population with new markers/effectors emerging. These markers may emerge and come together to produce a strong resistance phenotype and identification of these markers will help global surveillance efforts for artemisinin resistance.

Genetic crosses are used to link phenotypes with genotypes, but ethics prevent the use of chimpanzees and humans to complete the parasite life cycle. Therefore, to study linkage analysis in *P. falciparum*, **Asst Prof Ashley Vaughan** from Seattle Children's Research Institute, USA, presented an elegant novel genetic crossing platform using mice carrying human hepatocytes and infused with human red blood cells (the human liver chimeric FRG huHep/huRBC mouse) as the vertebrate host (Vendrely, Kumar, Li & Vaughan, 2020). This approach allows complete *P. falciparum* liver stage development and transition to blood stage parasitemia, allowing for cloning and characterization of recombinant progeny. A sympatric cross was completed using two SE Asian patient-derived strains

- an artemisinin sensitive strain, and a resistant strain with the common C580Y *kelch13* mutant (Li *et al.*, 2019). In addition, an allopatric cross was set up between an artemisinin resistant parasite from a SE Asian patient with an artemisinin sensitive laboratory parasite strain, NF54, of African origin. Initial cloning from the sympatric cross isolated sixty unique recombinant progeny from 63 overall recombinants and numerous artemisinin resistant parent progeny resulting from selfing. Genome sequencing of progeny revealed high recombination rates and a balanced inheritance of the genome, likely due to the geographic proximity of the two parents. Bulk segregant analysis was used to assess parasite fitness throughout the lifecycle and interestingly, artemisinin resistant parent parasites were out-competed upon transition to *in vitro* asexual culture by the recombinant parasites. Conversely, in the allopatric African/Asian cross, 84 unique recombinant progeny have been isolated, with minimal inbreeding and genome sequencing of the recombinants revealed extreme skews in marker segregation. This indicates that allopatric strains preferentially outcross but there are barriers to successful recombination. Although extreme segregation distortion can lower the probability of detecting linkage in skewed regions of the genome, the skews themselves may reveal genes underlying traits of interest. This novel platform, with major advances in the number of unique progeny generated (double the number generated in the previous 30 years), provides a powerful opportunity to rapidly map biomedically important parasite traits.

Prof John Adams from the University of South Florida, USA, developed a large-scale phenotypic screening pipeline of piggyBac mutants to identify *P. falciparum* genetic factors critical for parasite survival of lethal febrile temperatures which would enable sustained infection and onwards transmission of the parasite. A phenotypic screen for Pf piggybac heat shock mutants involved heat shocking parasites at 41°C for 8 h for 3 cycles using parasites from the Pilot library (128 single insertion clones from MR4) and 1K library (922 uncloned mutants) and identifying heat shock sensitive and resistant ones. Various processes were identified that drove heat shock survival. Highly essential, evolutionarily conserved pathways for chaperone binding, transport, regulating protein folding, proteasome-mediated degradation, ribosomes, heat shock proteins and apicoplast isoprenoid synthesis were all upregulated in response to heat shock. Given the similarity of these innate febrile-response mechanisms to reported transcriptome changes associated with artemisinin resistance, Prof Adams interrogated the *P. falciparum* mutant library and discovered that heat shock sensitive mutants were more sensitive to artemisinin-derivatives and the proteasome inhibitor Bortezomib than heat-shock tolerant parasites. The heat shock response was also correlated with artemisinin-resistance gene expression in field isolates and Prof Adams hypothesised that the parasite exploits its innate febrile-response mechanisms for resistance to artemisinin.

Accurately measuring parasite clearance after antimalarial treatment is critical to inform antimalarial drug development and to detect emerging drug resistance. However, measuring parasite clearance curves using microscopy or qPCR is complicated by parasite viability, because viable and non-viable parasites cannot be distinguished. **Dr Maria Rebelo** from QIMR Berghofer Medical Research Institute, Australia, investigated the viability of artemisinin-resistant (K13) and artemisinin-sensitive (3D7) *P. falciparum* parasites after artesunate treatment in human volunteer infection studies (Watts *et al.*, 2020). Parasite growth was assessed in pre- and post-treatment samples using flow cytometry on ex vivo cultures then the percentage of viable parasites estimated using exponential growth

mathematical models. Sensitive parasites cleared quickly after treatment whereas in resistant infections there was ~2-fold difference in parasite clearance rate. The *ex vivo* parasite growth data revealed that ~0.25% of sensitive parasites are viable after artesunate treatment compared to >50% of artemisinin resistant parasites. Dr Rebelo subsequently showed that measuring circulating parasites underestimates antimalarial drug activity (artesunate and other drugs) when non-viable parasites remain in circulation after treatment. Measuring parasite viability provides critical insights into antimalarial killing activity and may prove a useful standard parameter to assess drug efficacy in volunteer infection studies.

Novel technologies and approaches to advance malaria research

This was the final session of the conference and was chaired by Dr Kelly Rogers (The Walter and Eliza Hall Institute, Australia) and Dr Paul Gilson (the Burnet Institute, Australia). Invited speaker **Prof Andrea Cristanti** who was scheduled to kick off this session had to give his talk earlier in the conference after being called back to Europe because of the escalating Covid-19 crisis. The focus of his talk was on the recent developments and challenges in using CRISPR/CAS9-based gene drive technology to selectively edit a mosquito population, to either impair female fertility or interfere with the mosquito transmitting *Plasmodium*. Such edited populations have been spread from few laboratory individuals to large caged mosquito populations. These laboratory experiments have also supported mathematical modelling predicting how gene technology has the potential to eradicate malaria transmission in a span of few years from vast regions of Africa. Technical challenges in the development of a gene drive technology suitable for release include the development nuclease-resistant functional gene variant that would block the spreading of the drive as well as off target activity of the CAS9 nuclease that may generate undesirable mutations at other loci. Prof Cristanti discussed a number of gene editing solutions they have developed that are extremely efficient in achieving complete population suppression without inducing resistance (Kyrou *et al.*, 2018; Simoni *et al.*, 2020).

Asst Prof Ellen Yeh from Stanford University, USA, described an elegant chemical genetics screen for understanding the development of the relict plastid, or apicoplast (Tang *et al.*, 2019). By chemically rescuing parasites that lacked an apicoplast, a fluorescent reporter-based conditional system was developed that allowed for the identification of *Plasmodium* genes required for apicoplast development. This approach identified many genes with previously undefined functions and others with interesting evolutionary histories. This work has paved the way for further functional characterization of the gene hits which is currently underway and could uncover new drug targets.

Prof Malcolm McConville from University of Melbourne, Australia, described the development of a new multiplex, stable isotope resolved metabolomics approach to identify both the 'expected' and unexpected, or 'dark' metabolome, of erythrocytes following infection with malaria parasites. Many new metabolites were identified that were not predicted from genome annotations, including some that may result from enzyme promiscuity, adding to the complexity of metabolic reconstructions. The team also used conditional gene expression systems to characterize the function of possible enzymes

with no known function, including a novel mitochondrial protein involved in the provision of one-carbon units for sustaining the parasite mitochondria and growth.

The final act was given by invited speaker **Dr Kelly Rogers** from the Walter and Eliza Hall Institute, who showcased 4-dimensional microscopy of *P. falciparum* merozoite invasion of the erythrocyte using a lattice light-sheet microscope built onsite at the institute by her team. The study provided unparalleled live-imaging resolution of invasion and highlighted morphological and biophysical changes in the erythrocyte membrane during host cell entry by *P. falciparum* merozoites, providing unprecedented imaging of the nascent parasitophorous vacuole membrane surrounding the newly invaded parasite. An image of a trophozoite captured using the lattice light-sheet microscope is presented in Figure 4. The presentation demonstrated the power of lattice light-sheet microscopy for characterizing the fascinating cell biology of malaria parasites and provided the ideal ending to the conference.

Closing remarks

This MAM conference highlighted yet again the incredible research that is being conducted in different laboratories and field sites across the world and the molecular, technical, translational and fundamental knowledge advances that have been made since the last MAM conference four years ago. Presently, Covid-19 is posing significant challenges, both to scientists and malaria affected communities. Therefore, it is imperative that we do not slow our collective efforts and considerable momentum in advancing malaria research, particularly in malaria endemic countries, in order to rise to the challenge of malaria eradication.

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References

- Abraham, M., Gagaring, K., Martino, M.L., Vanaerschot, M., Plouffe, D.M., Calla, J., . . . Winzeler, E.A. (2020). Probing the Open Global Health Chemical Diversity Library for Multistage-Active Starting Points for Next-Generation Antimalarials. *ACS Infect Dis*, 6, 613-628. <https://doi.org/10.1021/acsinfecdis.9b00482>.
- Alam, M.M., Sanchez-Azqueta, A., Janha, O., Flannery, E.L., Mahindra, A., Mapesa, K., . . . Tobin, A.B. (2019). Validation of the protein kinase PfCLK3 as a multistage cross-species malarial drug target. *Science*, 365. <https://doi.org/10.1126/science.aau1682>.
- Amato, R., Lim, P., Miotto, O., Amaratunga, C., Dek, D., Pearson, R.D., . . . Fairhurst, R.M. (2017). Genetic markers associated with dihydroartemisinin-piperaquine failure in *Plasmodium falciparum* malaria in Cambodia: a genotype-phenotype association study. *Lancet Infect Dis*, 17, 164-173. [https://doi.org/10.1016/s1473-3099\(16\)30409-1](https://doi.org/10.1016/s1473-3099(16)30409-1).
- Barber, B.E., Russell, B., Grigg, M.J., Zhang, R., William, T., Amir, A., . . . Yeo, T.W. (2018). Reduced red blood cell deformability in *Plasmodium knowlesi* malaria. *Blood Adv*, 2, 433-443. <https://doi.org/10.1182/bloodadvances.2017013730>.
- Bernabeu, M., Gunnarsson, C., Vishnyakova, M., Howard, C.C., Nagao, R.J., Avril, M., . . . Smith, J.D. (2019). Binding Heterogeneity of *Plasmodium falciparum* to Engineered 3D Brain Microvessels Is Mediated by EPCR and ICAM-1. *mBio*, 10. <https://doi.org/10.1128/mBio.00420-19>.
- Birnbaum, J., Scharf, S., Schmidt, S., Jonscher, E., Hoeijmakers, W.A.M., Flemming, S., . . . Spielmann, T. (2020). A Kelch13-defined endocytosis pathway mediates artemisinin resistance in malaria parasites. *Science*, 367, 51-59. <https://doi.org/10.1126/science.aax4735>.
- Chenet, S.M., Akinyi Okoth, S., Huber, C.S., Chandrabose, J., Lucchi, N.W., Talundzic, E., . . . Udhayakumar, V. (2016). Independent Emergence of the *Plasmodium falciparum* Kelch Propeller Domain Mutant Allele C580Y in Guyana. *J Infect Dis*, 213, 1472-1475. <https://doi.org/10.1093/infdis/jiv752>.
- Cooper, D.J., Plewes, K., Grigg, M.J., Rajahram, G.S., Piera, K.A., William, T., . . . Barber, B.E. (2018). The effect of regularly dosed paracetamol versus no paracetamol on renal function in *Plasmodium knowlesi* malaria (PACKNOW): study protocol for a randomised controlled trial. *Trials*, 19, 250. <https://doi.org/10.1186/s13063-018-2600-0>.
- Davies, H., Belda, H., Broncel, M., Ye, X., Bisson, C., Introini, V., . . . Treeck, M. (2020). An exported kinase family mediates species-specific erythrocyte remodelling and virulence in human malaria. *Nat Microbiol*, 5, 848-863. <https://doi.org/10.1038/s41564-020-0702-4>.
- Ecker, A., Lehane, A.M., Clain, J. & Fidock, D.A. (2012). PfCRT and its role in antimalarial drug resistance. *Trends Parasitol*, 28, 504-514. <https://doi.org/10.1016/j.pt.2012.08.002>.
- Favuzza, P., de Lera Ruiz, M., Thompson, J.K., Triglia, T., Ngo, A., Steel, R.W.J., . . . Cowman, A.F. (2020). Dual Plasmepsin-Targeting Antimalarial Agents Disrupt Multiple Stages of the Malaria Parasite Life Cycle. *Cell Host Microbe*, 27, 642-658.e612. <https://doi.org/10.1016/j.chom.2020.02.005>.
- Fernandez-Ruiz, D., Ng, W.Y., Holz, L.E., Ma, J.Z., Zaid, A., Wong, Y.C., . . . Heath, W.R. (2019). Liver-Resident Memory CD8(+) T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection. *Immunity*, 51, 780. <https://doi.org/10.1016/j.immuni.2019.09.019>.
- Fierro, M.A., Asady, B., Brooks, C.F., Cobb, D.W., Villegas, A., Moreno, S.N.J. & Muralidharan, V. (2020). An Endoplasmic Reticulum CREC Family Protein Regulates the Egress Proteolytic Cascade in Malaria Parasites. *mBio*, 11. <https://doi.org/10.1128/mBio.03078-19>.

- Galaway, F., Yu, R., Constantinou, A., Prugnolle, F. & Wright, G.J. (2019). Resurrection of the ancestral RH5 invasion ligand provides a molecular explanation for the origin of *P. falciparum* malaria in humans. *PLoS Biol*, 17, e3000490. <https://doi.org/10.1371/journal.pbio.3000490>.
- Gruszczyk, J., Huang, R.K., Chan, L.J., Menant, S., Hong, C., Murphy, J.M., . . . Tham, W.H. (2018a). Cryo-EM structure of an essential *Plasmodium vivax* invasion complex. *Nature*, 559, 135-139. <https://doi.org/10.1038/s41586-018-0249-1>.
- Gruszczyk, J., Kanjee, U., Chan, L.J., Menant, S., Malleret, B., Lim, N.T.Y., . . . Tham, W.H. (2018b). Transferrin receptor 1 is a reticulocyte-specific receptor for *Plasmodium vivax*. *Science*, 359, 48-55. <https://doi.org/10.1126/science.aan1078>.
- Hale, V.L., Watermeyer, J.M., Hackett, F., Vizcay-Barrena, G., van Ooij, C., Thomas, J.A., . . . Saibil, H.R. (2017). Parasitophorous vacuole poration precedes its rupture and rapid host erythrocyte cytoskeleton collapse in *Plasmodium falciparum* egress. *Proc Natl Acad Sci U S A*, 114, 3439-3444. <https://doi.org/10.1073/pnas.1619441114>.
- Henry, B., Roussel, C., Carucci, M., Brousse, V., Ndour, P.A. & Buffet, P. (2020). The Human Spleen in Malaria: Filter or Shelter? *Trends Parasitol*, 36, 435-446. <https://doi.org/10.1016/j.pt.2020.03.001>.
- Hoeijmakers, W.A.M., Miao, J., Schmidt, S., Toenhake, C.G., Shrestha, S., Venhuizen, J., . . . Bártfai, R. (2019). Epigenetic reader complexes of the human malaria parasite, *Plasmodium falciparum*. *Nucleic Acids Res*, 47, 11574-11588. <https://doi.org/10.1093/nar/gkz1044>.
- Howick, V.M., Russell, A.J.C., Andrews, T., Heaton, H., Reid, A.J., Natarajan, K., . . . Lawniczak, M.K.N. (2019). The Malaria Cell Atlas: Single parasite transcriptomes across the complete *Plasmodium* life cycle. *Science*, 365. <https://doi.org/10.1126/science.aaw2619>.
- Kanjee, U., Grüning, C., Chaand, M., Lin, K.M., Egan, E., Manzo, J., . . . Duraisingh, M.T. (2017). CRISPR/Cas9 knockouts reveal genetic interaction between strain-transcendent erythrocyte determinants of *Plasmodium falciparum* invasion. *Proc Natl Acad Sci U S A*, 114, E9356-e9365. <https://doi.org/10.1073/pnas.1711310114>.
- Kariuki, S.N., Marin-Menendez, A., Introini, V., Ravenhill, B.J., Lin, Y.C., Macharia, A., . . . Rayner, J.C. (2020). Red blood cell tension protects against severe malaria in the Dantu blood group. *Nature*. <https://doi.org/10.1038/s41586-020-2726-6>.
- Kim, J., Tan, Y.Z., Wicht, K.J., Erramilli, S.K., Dhingra, S.K., Okombo, J., . . . Mancina, F. (2019). Structure and drug resistance of the *Plasmodium falciparum* transporter PfCRT. *Nature*, 576, 315-320. <https://doi.org/10.1038/s41586-019-1795-x>.
- Kone, A., Sissoko, S., Fofana, B., Sangare, C.O., Dembele, D., Haidara, A.S., . . . Djimde, A.A. (2020). Different *Plasmodium falciparum* clearance times in two Malian villages following artesunate monotherapy. *Int J Infect Dis*, 95, 399-405. <https://doi.org/10.1016/j.ijid.2020.03.082>.
- Kyrou, K., Hammond, A.M., Galizi, R., Kranjc, N., Burt, A., Beaghton, A.K., . . . Crisanti, A. (2018). A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nat Biotechnol*, 36, 1062-1066. <https://doi.org/10.1038/nbt.4245>.
- Li, X., Kumar, S., McDew-White, M., Haile, M., Cheeseman, I.H., Emrich, S., . . . Vaughan, A.M. (2019). Genetic mapping of fitness determinants across the malaria parasite *Plasmodium falciparum* life cycle. *PLoS Genet*, 15, e1008453. <https://doi.org/10.1371/journal.pgen.1008453>.
- Medicines for Malaria Venture. (2020). [WWW document]. URL <https://www.mmv.org/research-development>

- Mukherjee, A., Gagnon, D., Wirth, D.F.& Richard, D. (2018). Inactivation of Plasmepsins 2 and 3 Sensitizes Plasmodium falciparum to the Antimalarial Drug Piperaquine. *Antimicrob Agents Chemother*, 62. <https://doi.org/10.1128/aac.02309-17>.
- Odorizzi, P.M., Jagannathan, P., McIntyre, T.I., Budker, R., Prah, M., Auma, A., . . . Feeney, M.E. (2018). In utero priming of highly functional effector T cell responses to human malaria. *Sci Transl Med*, 10. <https://doi.org/10.1126/scitranslmed.aat6176>.
- Pilosof, S., He, Q., Tiedje, K.E., Ruybal-Pesántez, S., Day, K.P.& Pascual, M. (2019). Competition for hosts modulates vast antigenic diversity to generate persistent strain structure in Plasmodium falciparum. *PLoS Biol*, 17, e3000336. <https://doi.org/10.1371/journal.pbio.3000336>.
- Ross, L.S., Dhir, S.K., Mok, S., Yeo, T., Wicht, K.J., Kümpornsin, K., . . . Fidock, D.A. (2018). Emerging Southeast Asian PfCRT mutations confer Plasmodium falciparum resistance to the first-line antimalarial piperaquine. *Nat Commun*, 9, 3314. <https://doi.org/10.1038/s41467-018-05652-0>.
- Rudlaff, R.M., Kraemer, S., Marshman, J.& Dvorin, J.D. (2020). Three-dimensional ultrastructure of Plasmodium falciparum throughout cytokinesis. *PLoS Pathog*, 16, e1008587. <https://doi.org/10.1371/journal.ppat.1008587>.
- Schaber, C.L., Katta, N., Bollinger, L.B., Mwale, M., Mlotha-Mitole, R., Trehan, I., . . . Odom John, A.R. (2018). Breathprinting Reveals Malaria-Associated Biomarkers and Mosquito Attractants. *J Infect Dis*, 217, 1553-1560. <https://doi.org/10.1093/infdis/jiy072>.
- Simoni, A., Hammond, A.M., Beaghton, A.K., Galizi, R., Taxiarchi, C., Kyrou, K., . . . Crisanti, A. (2020). A male-biased sex-distorter gene drive for the human malaria vector Anopheles gambiae. *Nat Biotechnol*, 38, 1054-1060. <https://doi.org/10.1038/s41587-020-0508-1>.
- Skinner-Adams, T.S., Fisher, G.M., Riches, A.G., Hutt, O.E., Jarvis, K.E., Wilson, T., . . . Andrews, K.T. (2019). Cyclization-blocked proguanil as a strategy to improve the antimalarial activity of atovaquone. *Commun Biol*, 2, 166. <https://doi.org/10.1038/s42003-019-0397-3>.
- Tang, Y., Meister, T.R., Walczak, M., Pulkoski-Gross, M.J., Hari, S.B., Sauer, R.T., . . . Yeh, E. (2019). A mutagenesis screen for essential plastid biogenesis genes in human malaria parasites. *PLoS Biol*, 17, e3000136. <https://doi.org/10.1371/journal.pbio.3000136>.
- Thomas, J.A., Tan, M.S.Y., Bisson, C., Borg, A., Umrekar, T.R., Hackett, F., . . . Blackman, M.J. (2018). A protease cascade regulates release of the human malaria parasite Plasmodium falciparum from host red blood cells. *Nat Microbiol*, 3, 447-455. <https://doi.org/10.1038/s41564-018-0111-0>.
- Toro-Moreno, M., Sylvester, K., Srivastava, T., Posfai, D.& Derbyshire, E.R. (2020). RNA-Seq Analysis Illuminates the Early Stages of Plasmodium Liver Infection. *mBio*, 11. <https://doi.org/10.1128/mBio.03234-19>.
- Uwimana, A., Legrand, E., Stokes, B.H., Ndikumana, J.M., Warsame, M., Umulisa, N., . . . Menard, D. (2020). Emergence and clonal expansion of in vitro artemisinin-resistant Plasmodium falciparum kelch13 R561H mutant parasites in Rwanda. *Nat Med*, 26, 1602-1608. <https://doi.org/10.1038/s41591-020-1005-2>.
- Valencia-Hernandez, A.M., Ng, W.Y., Ghazanfari, N., Ghilas, S., de Menezes, M.N., Holz, L.E., . . . Fernandez-Ruiz, D. (2020). A Natural Peptide Antigen within the Plasmodium Ribosomal Protein RPL6 Confers Liver T(RM) Cell-Mediated Immunity against Malaria in Mice. *Cell Host Microbe*, 27, 950-962.e957. <https://doi.org/10.1016/j.chom.2020.04.010>.
- Vendrey, K.M., Kumar, S., Li, X.& Vaughan, A.M. (2020). Humanized Mice and the Rebirth of Malaria Genetic Crosses. *Trends Parasitol*. <https://doi.org/10.1016/j.pt.2020.07.009>.

- Watts, R.E., Odedra, A., Marquart, L., Webb, L., Abd-Rahman, A.N., Cascales, L., . . . McCarthy, J.S. (2020). Safety and parasite clearance of artemisinin-resistant *Plasmodium falciparum* infection: A pilot and a randomised volunteer infection study in Australia. *PLoS Med*, 17, e1003203. <https://doi.org/10.1371/journal.pmed.1003203>.
- World Health Organisation (2019). WHO: Status of antimalarial drug efficacy report December 2019.
- Zhu, L., Tripathi, J., Rocamora, F.M., Miotto, O., van der Pluijm, R., Voss, T.S., . . . Bozdech, Z. (2018). The origins of malaria artemisinin resistance defined by a genetic and transcriptomic background. *Nat Commun*, 9, 5158. <https://doi.org/10.1038/s41467-018-07588-x>.

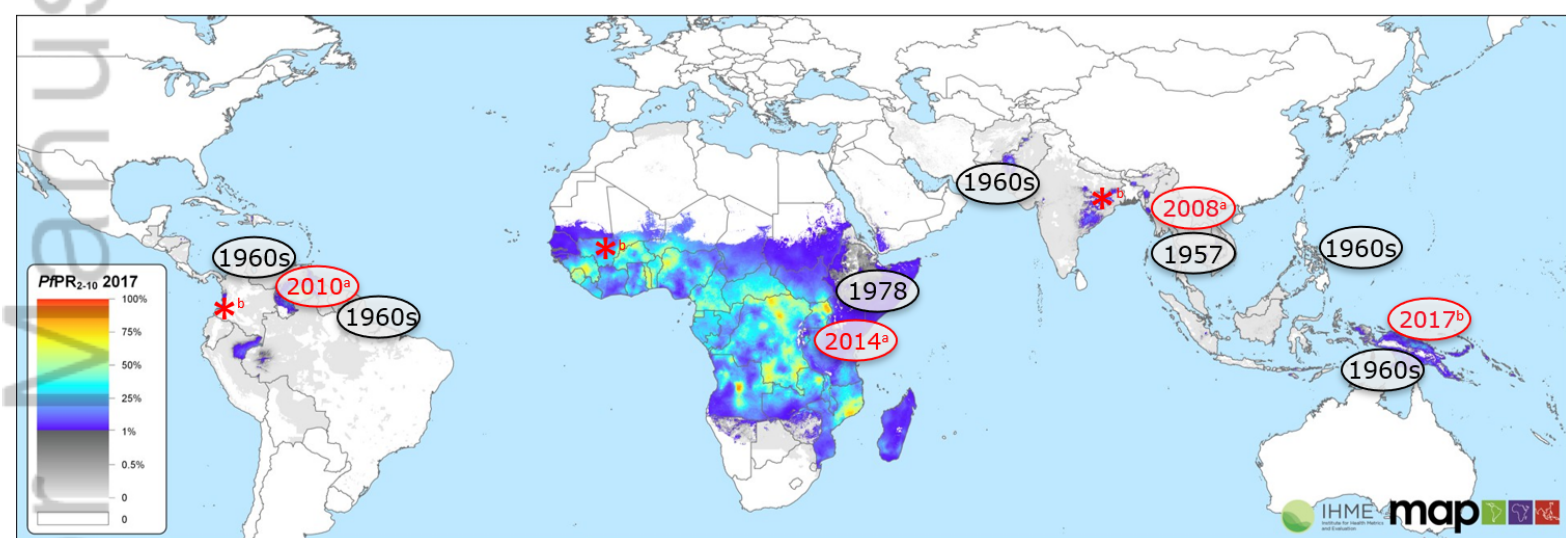
Figure 1: A word cloud depicting the major themes and topics discussed at MAM2020. Image credit: Win Han Oo.

Figure 2: Emergence of *P. falciparum* strains resistant to chloroquine and artemisinin derivatives. Emergence to artemisinin derivatives were first reported in Western Cambodia and promptly followed by other sites within the Greater Mekong Sub-region of South-East Asia (in red). Unlike chloroquine resistance (in black, adapted from (Ecker, Lehane, Clain & Fidock, 2012)), which also first emerged in the Greater Mekong sub-Region and went onto to spread into South Asia and sub-Saharan Africa, artemisinin resistant *P. falciparum* emerged independently outside the Greater Mekong and have now been reported in Guyana, Rwanda and Papua New Guinea (Chenet *et al.*, 2016; World Health Organisation, 2019; Uwimana *et al.*, 2020). Data presented at the conference (denoted by *) indicated that artemisinin resistance associated SNPs in kelch13 are yet to be detected in Colombia, India and Mali, however increases in parasite clearance time were observed in Mali and ongoing surveillance is necessary for early detection of emergence of resistant strains ^a reported by WHO (World Health Organisation, 2019), ^b reported at the conference. Image credit: Katherine O’Flaherty.

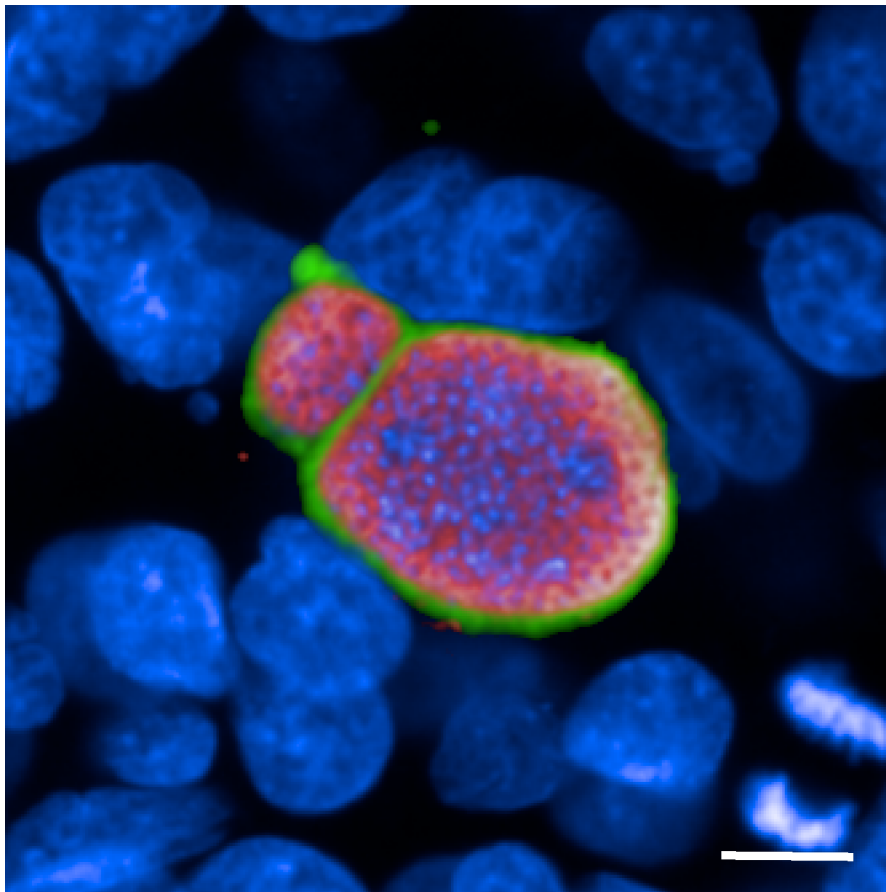
Figure 3: The pre-erythrocytic liver stage. Shown is a *Plasmodium berghei* exoerythrocytic form expressing mCherry (red) in the cytoplasm and UIS4 (green) at the parasitophorous vacuole membrane within a HepG2 hepatocyte. Scale bar, 10 µm. Image credit: Ryan Steel and Justin Boddey.

Figure 4: A 3D rendered trophozoite with membranes and DNA labelled captured by lattice light sheet microscopy. A slice montage of the same image is presented below. Image credit: Michal Pasternak and Niall Geoghegan.

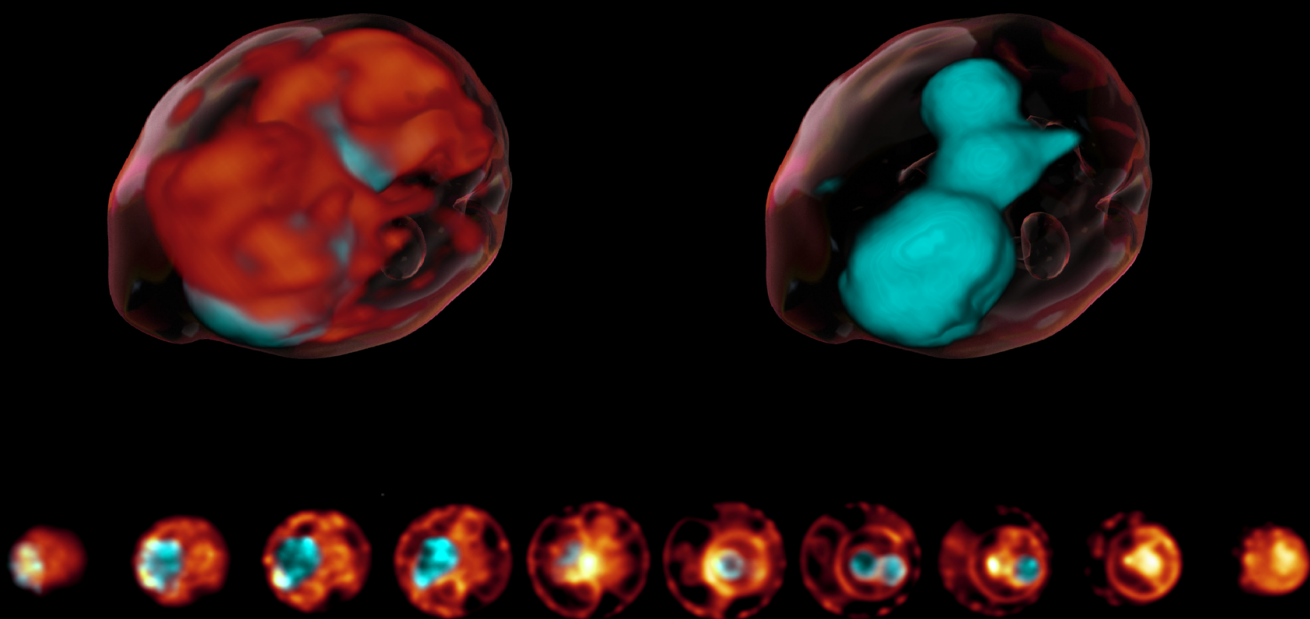
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CMI_13289_Figure 2.png



CMI_13289_Figure 3_.tif



CMI_13289_Figure 4.png