Special Issue Article

Advances in genetics and genomics: use and limitations in achieving malaria elimination goals

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Success of the global research agenda towards eradication of malaria will depend on the development of new tools, including drugs, vaccines, insecticides and diagnostics. Genetic and genomic information now available for the malaria parasites, their mosquito vectors and human host, can be harnessed to both develop these tools and monitor their effectiveness. Here we review and provide specific examples of current technological advances and how these genetic and genomic tools have increased our knowledge of host, parasite and vector biology in relation to malaria elimination and in turn enhanced the potential to reach that goal. We then discuss limitations of these tools and future prospects for the successful achievement of global malaria elimination goals.

Keywords: Parasitic disease, Eradication, Infectious disease, Genetic diversity, Plasmodium, Vaccines, Malaria control

Background

Elimination of malaria entails reducing to zero the incidence of locally acquired malaria infection in a specified geographic area as a result of deliberate efforts, with continued measures in place to prevent re-establishment of transmission.[1] The past decade witnessed unprecedented efforts to control malaria, including renewed political and financial commitment and increased availability of both old and new strategies and tools.[2] World Health Organization recommends a multi-pronged strategy to control and eliminate malaria, which includes vector control interventions, preventive therapies, diagnostic testing, treatment with quality-assured artemisinin-based combination therapies (ACT) and strong malaria surveillance.[3] Significant progress has been achieved in malaria control worldwide over the past decade, with impressive reductions in transmission occurring in many endemic regions,[1] including Sri Lanka.[4] These successes have stimulated renewed hopes for achieving the ambitious goal of global eradication of malaria.[5]

Currently, tools such as ACT, insecticide-treated bed nets (ITNs) and rapid diagnostic tests (RDT) have become widely available.[3] Many countries have adopted the use of ACT as first-line treatment as they are safe and highly effective in curing infection. Primaquine is currently the only drug available to treat the liver stage (hypnozoite) of *Plasmodium vivax* infection (i.e. radical treatment). There have been significant increases in the availability and use of parasitological testing (both RDTs and microscopy) in recent years. However, further funding and technical support are required to help countries to achieve universal diagnostic testing of suspected malaria.

Efforts to control and eliminate malaria in the present context relate to the combined use of antimalarial drugs, ITNs and indoor residual spraying of insecticides (IRS), with vaccine development remaining a long-term goal.[3] Genetic variation in the parasite population threatens to undermine these efforts, as the parasite evolves rapidly to evade host immune systems, drugs and vaccines.[6,7] Recently reported emergence of *Plasmodium falciparum* resistance to the front-line drug artemisinin is of great concern. It has been detected in five countries in the Greater Mekong Subregion: Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand and Vietnam,[3] and is likely to spread further despite efforts to contain it.[8,9] The Greater Mekong Subregion is the cradle of now widespread resistance to previous front-line antimalarial drugs,[10] which urgently calls for preemptive surveillance of the African parasite population for genetic markers of emerging drug resistance.[11] Losing the artemisinins to resistance would be a disaster for the control and treatment of malaria and would bring elimination efforts to a standstill.[12] The effectiveness of both IRS and ITNs is threatened by the development of insecticide resistance.[3,13]

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DOI 10.1179/2047773215Y.0000000015

Resistance to pyrethroid insecticides is of greatest concern as these are the major class of insecticides used in public health and the only insecticide class permitted for impregnation of mosquito nets. Since 2010, insecticide resistance has been reported in 49 countries, with pyrethroid resistance being the most commonly reported.[3]

Global eradication of malaria, therefore, would be more realistic with the development of new tools, including drugs, vaccines, insecticides and diagnostics. Recent years have seen tremendous advances in genetic and genomic technologies, which are accessible for less cost than ever before. Genomic information, which is now available for the malaria parasites, their mosquito vectors, and human host, can be leveraged to both develop these tools as well as monitor their effectiveness.[14] With resistance threatening to render ineffective the mainstay of current strategies for malaria elimination, taking advantage of these technologies is vital for realising the goal of malaria elimination. Therefore, this article attempts to review the current technological advances and how these genetic and genomic tools have increased our knowledge of host, parasite and vector biology in relation to malaria elimination. The limitations of these tools and future prospects for malaria elimination goals are also discussed.

Technological Advances that aid Elimination

Nucleic acid amplification techniques (NAT)

The invention of the polymerase chain reaction (PCR) by Kary Mullis in 1983 transformed many aspects of malaria research. Nucleic acid amplification techniques (NAT), which are several orders of magnitude more sensitive than microscopy or RDTs, are being used increasingly for epidemiological studies, investigating the origin of infection, analysis of pre-patent parasitaemia, in drug efficacy trials, drug resistance research and for the evaluation of new strategies/interventions aimed at transmission reduction.[15] A number of different PCR diagnostic techniques exist: single step, nested, multiplex and quantitative. Small subunit 18S ribosomal RNA (18SrRNA) molecular amplification, first exploited by Snounou *et al.*[16] using a nested PCR technique, is the most widely used NAT in malaria diagnostic research.[15] Alternative NAT have been developed which do not need thermocyclers, the most common being loop-mediated isothermal amplification (LAMP)[17–20] and nucleic acid sequence-based amplification (NASBA).[21,22] In countries where malaria transmission has decreased substantially and malaria elimination is feasible, more sensitive methods are required for active detection of infected individuals, especially those with low-density, asymptomatic infections. While molecular methods such as PCR are possible, the field deployment of these tests is problematic. Isothermal amplification of nucleic acids offers the opportunity of addressing some of the challenges related to the field deployment of molecular diagnostic methods.[23] Loop-mediated isothermal amplification is now being successfully deployed as point-of-care testing devices to detect and differentiate *Plasmodium* species.[24] Developments in the field therefore are encouraging, but simple, low cost and sensitive tools that could be used for mass screening of susceptible populations to detect sub-patent infections of Plasmodia species, including *P. vivax* remain as the need of the hour in malaria elimination settings.

Genotyping

Genetic variation in the parasite population threatens to undermine malaria control efforts as the parasite evolves rapidly to evade host immune systems, drugs and vaccines. Genotyping of parasite populations can provide insights into the basic parasite biology, its ability to adapt, and allows tracking of parasites as they respond to intervention efforts.[7] Genotyping methods of studying natural variation and population structure have evolved from the traditional microsatellite-length polymorphisms to shotgun sequencing, single nucleotide polymorphism (SNP) discovery, and genotyping using arrays or a variety of other high-throughput, low-cost approaches.[25] Since the first malaria genome was sequenced in 2002,[26] around 700 000 unique SNPs (with numbers continuing to increase) have been identified by concerted sequencing efforts,[7,27,28,29–32] genomic tiling arrays[25,33] as well as low-density SNP arrays.[34,35] Genome-wide maps of diversity in geographically diverse strains of *P. falciparum* have been produced[27,28] demonstrating the potential utility of SNP genotyping in identifying genes subject to recent natural selection and for understanding population genetic parameters of this parasite.

The use of PCR genotyping around a limited number of highly variable genes in *P. falciparum* parasites has become well established, both to describe diversity as well as to distinguish recrudescence from new infection.[36–38] The antigen genes *msp1*, *msp2* and *glurp* are the most commonly used molecular markers for *P. falciparum* and have given useful results;[37] while *Pvcsp*, *Pvmsp1* and *Pvmsp3* have been commonly used for *P. vivax* genotyping, particularly in the past.[37] Other vaccine candidate genes such as *Pvama1* and the Duffy binding protein (DBP) that display SNPs[37] have also been used for genotyping purposes. However, highly polymorphic but relatively neutral microsatellites (2–6 bp tandem repeats) that are more informative have been popularly utilised in the recent past for population genetic

studies of *P. vivax*.[39,40] These markers also have shown its potential utility in parasite tracking,[41] a critically important need in elimination settings, though more sensitive and robust tools would certainly be of better practical value.

Molecular barcodes

The first *P. falciparum* molecular barcode composed of 24 SNPs, which in combination created a unique fingerprint or signature for a parasite genome.[42] Such an assay is useful in distinguishing recrudescence from re-infection in drug trials, to monitor the frequency and distribution of specific parasites in a patient population undergoing drug treatment or vaccine challenge, or for tracking samples and determining purity of isolates.[42] However, these nuclear SNPs are constrained by a lack of geographic specificity and frequent recombination.[43] The synthesis of a 23-SNP barcode using polymorphisms from organellar (mitochondrion and apicoplast) genomes provide evidence that they are non-recombining and co-inherited, and enable identifying the geographic origin of *P. falciparum* strains to regional level.[43] However, resolution at this scale will only be of limited practical benefit for malaria elimination campaigns, as they will be unable to recognise parasites from neighbouring countries. Efforts along similar lines have also yielded successful results in *P. vivax,*[44] which is encouraging news for countries that labour efforts on wiping out *P. vivax* during the period of run up to elimination.

Single nucleotide polymorphism genotyping has thus far been useful in discovery of biomarkers of important traits in the parasite, vector and the host, which includes drug resistance, virulence factors, insecticide resistance, immune evasion and modulation mechanisms and infection resistance. Therefore, surveillance and monitoring of these biomarkers have enabled policy makers to make informed decisions regarding the choice of treatment and vector control measures. However, for genotyping to be of ideal value in malaria elimination, the biomarkers should be of low cost and easily and reliably identified in real-time in the field setting, so that informed point-of-care diagnosis and treatment options will be made possible. Use of such principles to make available high-tech but affordable, efficient and effective tools that are of practical value in the field might indeed be a reality, as evident by latest discoveries in the field.[45]

Microarray technology

Microarrays, along with other genetic analysis tools, have enabled the malaria research community to study the *P. falciparum* parasite (and *P. vivax* on a more limited scale) on a genome-wide level. While the range of applications for microarray technology is enormous, it is in widespread use for gene expression profiling and for comparative genomics to analyse genomic alterations such as SNPs, microsatellite variants (MSVs), insertions or deletions (indels) and copy number variants (CNVs).[14] Though limited to sophisticated laboratories, this technology has indeed been useful to answer specific questions in basic discovery research of malaria pathogenesis, molecular diagnosis of drug resistance and genetic epidemiology and surveillance studies. Availability of devices with probes to over several thousands of parasite and mosquito transcripts have enabled a wide variety of experiments including gene expression monitoring and SNP genotyping (www.affymetrix.com). A single microarray platform (though no longer available) that can assay CNV and genotype SNPs simultaneously has also been described.[46] A latest development, an integrated DNA lab-on-chip using microfluidics technology coupled with reverse transcription, PCR amplification, and microarray hybridisation has taken high-tech diagnostic tools to another level. This super-sophisticated device claims its use for simultaneous identification and differentiation of 26 tropical pathogens that cause 14 globally important tropical diseases including malaria,[47] though its practical diagnostic value in a tropical country setting remains debatable.

Microarrays are able to tolerate DNA of lower quantity and quality typical of patient-derived field samples, which contain far more human than parasite DNA. Thus, field samples unsuitable for full-genome sequencing may be amenable to microarray analysis,[48] which is a distinct advantage. However, with recent advancements and dramatic decline in sequencing costs, the popularity of next-generation sequencing (NGS) has increased rapidly, becoming a mainstream option for many laboratories. As costs become less prohibitive and methods become simpler, NGS is increasingly employed relative to microarrays as it allows for more complete and accurate data. The choice would ultimately depend on the research goals (e.g. for discovery or profiling), access to technology, maturity of applications, cost per sample and desired throughput that is required.

Whole genome sequencing (WGS)

DNA sequencing technology has evolved greatly since its inception in the mid-1970s.[49,50] The development of shotgun sequencing enabled the completion of revolutionary schemes such as the Human Genome Project (HGP). The core philosophy of massive parallel sequencing used in NGS is adapted from shotgun sequencing,[51–53] with NGS being capable of sequencing millions of fragments of DNA from a single sample at once.[54] During the past decade, the creation of several NGS platforms saw tremendous progress in terms of speed, read length and

throughput being provided at a low cost, and with widespread use paving the way for development of a large number of novel applications.[55] Today, some of the commercially available NGS platforms for DNA sequencing include: Thermo Fisher Life Technologies Ion Torrent PGM; Illumina sequencers, MiSeq, NextSeq, HiSeq and HiSeq X; Pacific Biosciences PacBio RS II; and the Oxford Nanopore sequencers MinION, PromethION and GridION. Currently, Illumina HiSeq X appears to offer the highest throughput per run and lowest per-base cost, while the Ion Torrent PGM provides fast run times, and the recently marketed MinION considered as a relatively inexpensive hand-held device.[55]

Whole transcriptome sequencing (RNA-seq)

Advances in NGS and assembly algorithm rapidly promote the development of transcriptome sequencing (RNA-seq), which can reconstruct the entire transcriptome in a selected species of interest and generate quantitative expression scores for each transcript.[56] This method of transcriptome analysis is now achievable at a lower cost than before, with a greater sequence yield, and high sensitivity for detection of less abundant and novel transcripts. It has thus enabled quantitative survey of RNA expression patterns in comparative genomic-levels and the discovery of more molecular markers.[57] However, the need for sophisticated laboratories and technical expertise together with the prohibitive costs limits its use in disease endemic countries.

Gene drive

Gene drive involves stimulating biased inheritance of particular genes to alter entire populations of organisms. It was first proposed more than a decade ago,[58] and since developed into a powerful and efficient tool for genome engineering using the CRISPR nuclease Cas9 (Clustered regularly interspaced short palindromic repeats – CRISPR: http://www.genome-engineering.org/crispr)[59] to cut sequences specified by guide RNA molecules.[60,61] This technique has now been adapted for use in *P. falciparum*.[62,63] Gene drives are potentially capable of altering entire populations of wild organisms,[64] and useful as a method to enable the spread of altered traits through wild populations over many generations. Gene drives would thus have the potential for use in containment of malaria transmission and reversal of the spread of insecticide resistance in vector mosquitoes.[65]

Genetic and genomic databases/repositories/ resources

The free online access of vast amounts of genetic information has virtually revolutionised the field leading to exponential growth in the use of bioinformatics in attempts to answer biological and operationally relevant questions in the field of infectious diseases, including malaria. One such example is PlasmoDB (http://PlasmoDB.org),[66] which is a functional genomic database for *Plasmodium* spp. that provides a resource for data analysis and visualisation in a gene-by-gene or genome-wide scale.[67] The current release of PlasmoDBv13.0 houses information from 10 parasite species and provides tools for intra- and inter-species comparisons. Sequence information is integrated with other genomic-scale data emerging from the *Plasmodium* research community, including gene expression analysis, microarray projects and proteomics studies.

The GeneDB project is a core part of the Sanger Institute's Pathogen Genomics activities. It provides reliable access to the latest sequence data and annotation/curation for the whole range of organisms sequenced by the group (GeneDB: http://www.genedb.org).[68] GeneDB currently provides access to more than 40 genomes, at various stages of completion.

Malaria Genomic Epidemiology Network (MalariaGEN) PanOptes offers exploration and visualisation of data from several different projects. One such platform is the pf3k (http://www.malariagen.net/apps/pf3k/release_2/index.html#start),[69] which provides an interactive view of pilot data from the Pf3k project, an outcome of a global collaboration that enables comprehensive analyses of genome variations in several thousands of highly diverse *P. falciparum* parasites. Malaria Genomic Epidemiology Network Ag1000G (http://www.malariagen.net/apps/ag1000g/phase1-AR2/index.html)[70] is yet another public resource that provides an interactive view of data released by the *Anopheles gambiae* 1000 genomes project, another international collaboration that uses whole-genome deep sequencing data to enable genetic variation studies in these mosquitoes. The latest version contains data on 765 mosquito specimens collected from eight countries and sequenced by the Wellcome Trust Sanger Institute. The data from the MalariaGEN *P. falciparum* Community Project (http://www.malariagen.net/apps/pf/3.1/#start)[71] is also a rich data resource that is open to the public and enable analysis of data from over 3000 isolates from 40 separate locations in 20 countries across the globe.

The National Center for Biotechnology Information (NCBI), a division of the National Library of Medicine (NLM) at the National Institutes of Health (NIH), USA, also continues to contribute immensely by way of online support for the research community at large. It has created automated systems that enable storage and analysis of knowledge related to molecular biology, biochemistry and genetics, which are widely utilised by scientists across the world (NCBI: http://www.ncbi.nlm.nih.

gov).[72] GenBank (http://www.ncbi.nlm.nih.gov),[73] a comprehensive database that contains publicly available nucleotide sequences of over 250 000 species[74] and clinical trials registry (https://clinicaltrials.gov),[75] which is a repository of clinical studies involving human participants conducted around the world are yet more examples of such free online resources available to the scientific community.

VectorBase (http://www.vectorbase.org)[76] is a bioinformatics resource focused exclusively on invertebrate vectors of human disease and contains data on 20 *Anopheles* genomes at present. VectorBase also provides a forum for discussion and distribution of news and information relevant to invertebrate vectors, as well as access to tools to facilitate the querying and analysis of the data sets accessible through that site.

The *Plasmodium* Genetic Modification (PlasmoGEM) database (http://plasmogem.sanger.ac.uk)[77] provides access to a resource of modular, versatile and adaptable vectors for genome modification of *Plasmodium* spp parasites. *Plasmodium* Genetic Modification currently consists of >2000 plasmids designed to modify the genome of *Plasmodium berghei*, with plans to expand further.[78]

The service provided to the scientific community by the Malaria Research and Reference Reagent Resource Centre (MR4), a repository of quality controlled malaria reagents and protocols, is undeniable. Supported by the NIAID, NIH, MR4 acquires, authenticates, preserves and distributes parasites, mosquito vectors and associated biological and molecular reagents at no cost to registered researchers from disease endemic countries (MR4: http://www.mr4.org).[79]

The Worldwide Antimalarial Resistance Network (WWARN) is a global network of academic experts that was established with the ultimate aim of identifying and tracking the global spread of malaria drug resistance.[80] As a multidisciplinary network, WWARN focuses on five aspects of drug resistance: viz. clinical, molecular, *in vitro*, pharmacology and antimalarial quality. Worldwide Antimalarial Resistance Network provides high-quality data resources, customised research tools and services and a global platform for exchanging scientific and public health information on malaria drug resistance (WWARN: http://www.wwarn.org).[81]

Role of Genetics and Genomic Tools in Achieving and Sustaining Elimination

The overall goal of malaria research discovery efforts with elimination targets in sight is to generate tools that can be used in the field for accurate diagnosis, effective prevention and treatment of malaria, to arrest transmission and prevent re-introduction of parasites to areas with zero prevalence. A significant milestone in scientific advancements of the past decade was the elucidation of the genomes, transcriptomes and proteomes of many pathogens, including malaria parasites. This information offers a clearer and more detailed picture that provides the foundation for new approaches to refine existing targets as well as to identify new target antigens for the development of more efficient and effective vaccines, drugs and diagnostic tests.[82] Advances in the field of bioinformatics with the availability and accessibility of new and better tools for data analysis also have helped the field in no small measure. Genetic mapping by linkage or genome-wide association studies (GWAS), selection of genomic regions under strong recent selection (candidate resistance loci), genetic manipulations that can demonstrate causality[83] and genome-wide expression analysis with genetic variation mapping studies that can determine differential expression patterns throughout the *Plasmodium* life cycle enabling identification of new drug and vaccine targets[84] are few notable examples.

Host genetics and genomics

Sanger sequencing was used to obtain the first consensus sequence of the human genome in 2001[85,86] and the first individual human diploid sequence in 2007.[87] Shortly thereafter, the second complete individual genome was sequenced using NGS technology.[88] The International HapMap Project launched in 2003[89] and the 1000 Genomes Project in 2008,[90] provide a comprehensive map of human genetic variation. With the availability of human genome sequence information, GWAS were widely applied to genomics to identify polymorphisms associated with human disease.[91,92] Since 2009, exome sequencing has enabled a more targeted and less costly capture of genetic associations in human disease.[93] Malaria, with its high mortality and widespread impact, represents one of the strongest evolutionary selective forces on the human genome in recent history[94,95] and uncovering all of the human genetic factors that confer resistance to malaria would provide clues to the molecular basis of protective immunity that would be invaluable for the development of vaccines.[96]

The concept of a heterozygote advantage against malaria was first proposed in 1949[97] and subsequently tested true with the observed association between the sickle gene (HbAS) and protection against malaria in Uganda.[98] It is now well established that heterozygotes for HbAS are relatively more protected against death from malaria.[98–100] Apart from HbS, a wide range of other variants of the erythrocyte system have also resulted from evolutionary selection by malaria. They include HbC[101,102] and possibly HbE;[103,104]

alpha and beta thalassaemia;[105–107] ovalocytosis;[108,109] the Duffy-negative blood group;[110] ABO blood groups;[111,112] glycophorins;[113] polymorphisms of the red cell enzyme gene G6PD, which causes glucose-6-phosphate dehydrogenase enzyme deficiency;[114,115] haptoglobins;[116] nitric oxide synthase 2[117,118] and *ATP2B4* gene.[119] In addition, genetic polymorphisms of the immune response genes also have been demonstrated to have an influence on susceptibility for or protection against disease severity in malaria, which include *HLA* polymorphisms (*HLA-B53, HLA-DRB1*); cytokine and related gene polymorphisms (TNF-alpha, *INFG, IRF1, CD40LG, IL1A/B, IL4,-FCGR2A, FCGR2B, TLR4, MBL2*); complement regulatory protein polymorphisms (*CR1*) and endothelial receptor polymorphisms (*ICAM-1*, *CD36*, *PECAM1*).[94,95,120]

Genome-wide association studies represent a powerful and systematic approach for exploring the whole genome for the genetic basis of human resistance to malaria.[119,121,122] With the availability of dense genotyping chips containing sets of hundreds of thousands of SNPs, together with appropriately large and well-characterised clinical samples, GWAS that are powered to detect plausible functional associations are now possible for malaria.[121,122] The Malaria Genomic Epidemiology Network is a multi-country collaborative effort, which is led by researchers at the Wellcome Trust Sanger Institute and the University of Oxford, UK. It provides the necessary infrastructure to conduct GWAS, in order to gain fundamental new insights into the effects of genetic variation on malaria susceptibility, and thereby on molecular mechanisms of protective immune responses and pathogenesis in endemic populations.[96,120] An example of fruition of this collaborative network is the reappraisal of the known malaria resistance loci in a large multicenter study using cases of severe malaria due to *P. falciparum*,[122] reconfirming evidence of association with the HBB, ABO, ATP2B4, G6PD and CD40LG loci. However, previously reported associations at 22 other loci did not replicate in this multi-centre analysis. The large sample size enabled identification of authentic genetic effects that are heterogeneous across populations or phenotypes, such as the main African form of G6PD deficiency, which reduces the risk of cerebral malaria but increases the risk of severe malarial anaemia.[122] These revelations have led to arguments in favour of the power of large-scale genomic epidemiology studies that appear to provide answers to basic questions that decades of immunological research have failed to resolve. Genome-wide association studies are also strengthened by its potential to enable the discovery of novel molecular pathways for protective immunity, providing critical insights in to the development of an effective malaria vaccine(s) with promising outcomes for the future.

Parasite genetics and genomics

The first published *P. falciparum* clone 3D7 genome sequence in 2002[26] materialised due to the establishment of the Malaria Genome Project in 1995,[123] and was determined by using whole chromosome shotgun sequencing.[26] The 24-Mb genome of the parasite is distributed among 14 linear chromosomes, and also contains two extra-chromosomal apicoplast and mitochondrial genomes. Since its first publication, efforts have continued to improve both the sequence and annotation with the latest version being continually updated to GeneDB and periodically to PlasmoDB. The current version (v3.0) has 5369 genes, some re-assembled regions of chromosomes 7, 8 and 13, comprehensive reannotation and no gaps.[124] To better understand *P. falciparum*-specific biology through genetics and genomics, there are many ongoing efforts at various stages of completion such as: the sequencing or genotyping of many hundreds of isolates;[125] sequencing of *P. falciparum* laboratory model, clone IT, using whole genome shotgun sequencing,[29] and more recently, using Illumina Sequence-by-Synthesis;[124] sequencing of two more clones HB3 (Honduras) and Dd2 (Indochina) with better coverage;[126] and resequencing of large numbers of *P. falciparum* isolates.[124] Sequence comparisons between clones with different phenotypes and characteristics will provide critical information about the underlying mechanisms of drug resistance as well as the transmissibility, immunogenicity and virulence of malaria.

The *P. falciparum* genome evolves in response to natural selection pressures of the human host immune system, the mosquito vector and various environmental factors, including drug treatment and changes in transmission intensity owing to specific interventions.[6] Such changes to *P. falciparum* population structure can potentially be used to identify and overcome survival strategies used by the parasite for achieving malaria control and elimination goals.[127] The first *Plasmodium* GWAS involving 189 drug-phenotyped *P. falciparum* parasites from Asia, Africa and the Americas were genotyped using a custom-built Affymetrix low-density array ($\sim$1 SNP per 7 kb). These efforts enabled valuable insights into the *P. falciparum* genome[35] that included information on population structure, variation in recombination rate and loci under positive selection as well as several novel drug resistance candidates. Van Tyne *et al.*[7] confirmed thereafter that natural selection and array-based GWAS methods are useful to identify clinically relevant genes as well as demonstrate, through functional validation, candidate genes that are involved in antimalarial drug resistance, while Park *et al.*[128] demonstrated the feasibility and power improvements gained by using whole genome

sequencing (WGS) for association studies. The value of deep population-specific genomic analyses for identification of selection signals of parasites which may correspond to local selection pressures was highlighted recently,[129] while Manske *et al.*[28] used deep sequencing and analysis of 86 158 SNPs to provide a comprehensive genome-wide estimate of allele frequency distribution, population structure and linkage disequilibrium. Recently, Van Tyne *et al.*[130] utilised the nCounter® platform[131] and imputation of global gene expression[132] to uncover the expression profiles of *in vivo* sequestered *P. falciparum* malaria parasites, further revealing the potential of such novel applications.

The blood stages of *Plasmodium* evade antibody-mediated host immunity by altering parasite-encoded antigens exposed on the surface of infected red blood cells through antigenic variation.[133] Antigenic variation results from amplification of hypervariable gene families coupled with the ability to switch expression of individual genes.[134] Structural and functional characterisation of these gene families will facilitate understanding of their role in immune evasion and malaria pathogenesis, which in turn will help to further vaccine development. The *P. falciparum* genome is equipped with several subtelomeric gene families implicated in parasite virulence and immune evasion,[26] such as *var*, *rif* (repetitive interspersed family), *stevor* (sub-telomeric variable open reading frame), *phist* (Plasmodium helical interspersed subtelomeric), *pfmc-2tm* (*P. falciparum* Maurer's clefts two transmembrane) and the *hyp* families 1–17.[134] The most polymorphic gene family in *P. falciparum* is the ~60 *var* genes that encode for the hypervariable surface proteins PfEMP1 (*P. falciparum* erythrocyte membrane protein 1) that are critical for pathogenesis (cytoadherence) and immune evasion in *P. falciparum*.[135] The high recombination rate of *var* exon 1 indicates that millions of new antigenic structures could potentially be generated each day in a single infected individual,[136] thus, effectively evading the host immune system and maintaining long-term, persistent infections. Genome-wide comparative transcriptional profiling revealed recently that *var* promoters play an essential part in singular gene choice, while transcription of non-*var* gene families is not subject to the same mode of transcriptional regulation.[134] Despite the remarkable diversity and rapid evolution of the *var* genes found within and among *P. falciparum* populations, the basic structure of these domains and the gene family show an old and stable evolutionary history.[137]

Genome variations, such as SNPs and CNVs, underpine *P. falciparum* drug resistance. Identification of causal mutations allows to delineate mechanisms of drug action, and can suggest ways in which drugs may be modified to restore efficacy.[83] Key SNPs that affect drug susceptibility in *P. falciparum* have been identified and include SNPs causing non-synonymous amino acid substitutions in the chloroquine (CQ) resistance transporter gene (*Pfcrt*)[138] and CNV in the multidrug resistance gene 1 (*Pfmdr1*),[139] which influence parasite susceptibility to CQ, quinine, lumefantrine and mefloquine. Similarly, mutations in the mitochondrial cytochrome b gene can mediate atovaquone resistance, and mutations in dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) underlie resistance to the antifolate drugs pyrimethamine and sulfadoxine, respectively.[83]

Plasmodium falciparum resistance to artemisinin derivatives in Southeast Asia threatens malaria control and elimination activities worldwide. An analysis of genome variation in 825 *P. falciparum* samples from Southeast Asia and West Africa identified multiple subpopulations of resistant parasites having widely differing genetic characteristics at the epicentre of artemisinin resistance in western Cambodia.[31] Defining genetic markers for these subpopulations will assist efforts in eliminating major foci of transmission through mapping of their geographic distribution and monitoring the spread of artemisinin resistance. Ariey *et al.*[140] used WGS of an artemisinin-resistant parasite line from Africa and clinical parasite isolates from Cambodia to reveal mutations in the PF3D7_1343700 kelch propeller domain ('K13-propeller') associated with artemisinin resistance *in vitro* and *in vivo*. Strong evidence of genetic linkage to a region of *P. falciparum* chromosome 13[141,142] has now been translated into the discovery of a marker useful for large-scale surveillance to contain artemisinin resistance in the Greater Mekong Subregion and prevent its global spread.[140]

The strategy adopted by Ariey and co-workers[140] was a combined approach to analyse mutations in laboratory-adapted parasite clones selected by continuous exposure to high doses of artemisinin *in vitro* for 5 years, and then using this information to guide analysis of polymorphisms in drug-resistant clinical parasite isolates. These studies have enabled the discovery of mutations that had eluded previous genetic studies. Since parasite clearance rates can be affected by non-genetic factors such as the stage of parasite development at the time of treatment and host related factors such as pharmacokinetics and immunity,[143] it appears that genomic approaches alone maybe insufficient in reliably discerning such markers and therefore, such considerations should also be made in the design of future drug resistance studies.

Plasmodium vivax causes extensive human malaria globally, but genomic data for this species are limited as it cannot be easily propagated *in vitro*. The ~26.8-Mb nuclear genome of *P. vivax* (Salvador I strain)

was sequenced by whole genome shotgun methods in 2008.[144] Publication of this genome sequence allowed the use of homology to describe biological pathways and infer possible functions for malaria genes.[144] In 2010, Dharia *et al.*[33] reported the WGS results obtained by NGS technology of a *P. vivax* Peruvian isolate (IQ07) obtained directly *ex vivo* without further propagation *in vitro* or in monkeys, revealing selective pressures on putative drug resistance genes including the *P. vivax* multidrug resistance-associated protein (PvMRP1, an ABC transporter associated with quinoline and antifolate tolerance in *P. falciparum*). There are now four more published reference genomes of *P. vivax*.[145] These strains from Asia (North Korean and India VII), West Africa (Mauritania I) and South America (Brazil I) provide the first genome-wide perspective of global variability within this species and its significantly greater genetic diversity, and greater ability to evolve, as compared to *P. falciparum* strains.[145] Chan *et al.*[146] used WGS of five *P. vivax* isolates from Madagascar and Cambodia, and generated a catalogue of approximately 80 000 SNPs distributed throughout the genome that will enable large-scale genotyping studies and contribute to a better understanding of *P. vivax* traits, such as drug resistance. Bright *et al.*[147] sequenced the entire genome of a *P. vivax* isolate derived from a traveller returning from Sudan, and reported the identification of several SNPs as candidate molecular markers of drug resistance, which also included mutations in the *pvmrp1* gene. Furthermore, the analysis of the population structure by Orjuela-Sanchez *et al.*[148] demonstrated the feasibility of GWAS in carefully selected populations of *P. vivax*.

The first cases of *P. vivax* CQ resistance were reported in 1989 in Papua New Guinea,[149] and are now present across most of the *vivax*-endemic regions.[150] The orthologue genes in *P. vivax, pvmdr1* and *pvcrt-o* genes have not revealed any point mutations correlating with CQ resistance[151–153] and the molecular basis of *P. vivax* CQ resistance still remains largely unknown.[154] The main difficulty in studying the mechanisms of *P. vivax* CQ resistance is the absence of a continuous *in vitro* culture system. Resistance to sulfadoxine-pyrimethamine (SP) is widespread in *P. vivax*. Twenty SNPs have been described in *P. vivax* dihydrofolate reductase (*pvdhfr*), with double mutants in *pvdhfr* (S58R and S117N) resulting in decreased binding of pyrimethamine and quadruple (F57L, S58R, S117N and I173L) resistance SNPs associated with SP treatment failure.[155]

Subtelomeric multigene families of malaria parasites encode virulent determinants. The *P. vivax* genome sequence revealed the largest subtelomeric multigene family of human malaria parasites, the *vir* superfamily, consisting of a total of 346 genes.[144] Lopez *et al.*[156] redefined the *vir* superfamily using a new computational approach which contributes to a new structural framing of this and other multigene families. The *vir* multigene family shares sequence homology with other *Plasmodium* species and is included within the variant gene superfamily (*Plasmodium* interspersed repeats, *pir*) together with *kir* in *Plasmodium knowlesi*, and the *cir/yir/bir* family in *Plasmodium chabaudi*, *Plasmodium yoelii* and *P. berghei*.[157] However, the function of the *vir* multigene family remains largely unknown. The presence of *pir* in rodent models allows the investigation of this gene family *in vivo* and thus their potential as vaccines or in other interventions in human *P. vivax* infections.

The 23.5-Mb nuclear genome of the simian and human malaria parasite *P. knowlesi* was produced by whole-genome shotgun sequencing.[158] *Plasmodium knowlesi* is now increasingly recognised as a significant cause of human malaria, particularly in Southeast Asia[159–161] and has a close phylogenetic relationship to *P. vivax*.[144,162] Further sequencing of the *P. knowlesi* H strain is underway using Illumina Sequence-by-Synthesis.[163] The genome sequence of another primate malaria parasite *P. cynomolgi*, which provided insight into *P. vivax* and the monkey malaria clade,[164] would no doubt increase in significance with the reporting of the first case of naturally acquired human infection from Malaysia.[165] Generating genetic maps of these sister species to *P. vivax* no doubt will help create robust model systems that can be utilised in further understanding of the important biological, pathogenic and immunological aspects of this neglected species.

The genomes of several non-human malaria parasites have also been published: *Plasmodium reichenowi*;[166] *P. yoelii*,[167,168] *P. berghei*,[168,169] *P. chabaudi*[168,169] and *Plasmodium gallinaceum*.[66] Species of malaria parasite that infect rodents have long been used as models for malaria disease research. Therefore, the complete rodent malaria parasite genomes, RNA-sequences and genotypic diversity data act as excellent and important resources to determine gene-function and post-genomic analyses and to better interrogate the *Plasmodium* biology.[168] Furthermore, *P. chabaudi* isolates make excellent tools for study of genotype–phenotype relationships and the improved classification of multigene families, enhanced studies on the role of exported proteins in virulence and immune evasion or modulation.[168]

Vector genetics and genomics

The genome of *A. gambiae*, the principal vector of *P. falciparum* malaria in Africa, first became publicly available in 2002.[170] The availability of this reference genome enabled the design of PCR-based tests and

provided a better understanding of genomic regions of importance for vector control such as parasite resistance or targets for insecticides. Jiang *et al.*[171] reported the draft genome sequence of the Indian strain of *A. stephensi*, which is a key vector of urban malaria throughout the Indian Subcontinent and Middle East and an emerging model for molecular and genetic studies of mosquito–parasite interactions. More recently, the genomes and transcriptomes of 16 anophelines from Africa, Asia, Europe and Latin America were sequenced and assembled by Neafsey and co-workers.[172] The analyses spanned ~100 million years of evolution and represented a range of evolutionary distances from *A. gambiae*.[172] The Ag1000G project that is currently underway is expected to provide a high-resolution view of genetic variation in natural populations of *A. gambiae* in Africa.[173] These resources have the potential to make future vector control campaigns 'custom-designed' with resultant improved efficiency and effectiveness by using ways such as selection of an insecticide(s) with no risk of resistance development, mapping target areas for insecticide spraying and using precise monitoring of vector populations in response to control campaigns.

Mosquito vectors experience a very intense selective pressure from insecticides used in malaria control programmes, through the use of ITNs and IRS, in addition to the heavy use of insecticides for agricultural purposes.[174] In several mosquito species and populations, the unique G119S mutation in the *ace-1* gene is responsible for organophosphates and carbamates resistance[175] and the L1014F mutation in the *para*-type sodium channel gene (*kdr-west* mutation) is responsible for pyrethroid resistance in mosquitoes from West Africa.[176] So far, *kdr* mutations have also been detected in at least 13 anopheline species in natural populations from three continents (African, Asian and, more recently, American), with seven mutational variants (L1014F, L1014S, L1014C, L1014W, N1013S, N1575Y and V1010L) being described so far.[177] The increase of frequency and distribution of *kdr* mutations clearly shows its importance in conferring pyrethroid resistance in mosquitoes. Development of more tools for large-scale genetic screening of natural populations would further enable reliable predictions to be made regarding the predisposition of mosquito populations to develop insecticide resistance.

Gene expression microarray-based approaches in *A. gambiae* have evolved from small custom-made arrays containing probes for gene families associated with insecticide detoxification[178] to whole genome arrays.[179] Recent application of NGS technologies to mosquito genomics offers exciting opportunities to expand the understanding of mosquito biology in many important vector species and harness the power of comparative genomics.[171] Genome-block expression-assisted association studies recently identified three malaria resistance genes (*A. gambiae adenosine deaminase, fibrinogen-related protein 30 and fibrinogen-related protein 1*) in *Anopheles gambiae*.[180] A comprehensive transcriptome sequencing and analysis of *A. sinensis*, a major malaria vector in China and Southeast Asia, are now available.[57] Transcriptome sequencing has also been successfully applied to mosquito species, *A. gambiae*,[181,182] *A. funestus*[183] and *A. albimanus*,[184] clearly demonstrating its utility for functional and evolutionary studies.[185] The improved knowledge is expected to reveal molecular targets that would aid the development of novel vector control and pathogen transmission-blocking strategies. Genetics and genomics have already made huge advances in the understanding of vector control, and also more widely in the fight against malaria, and therefore, will continue to be critically important in the path towards elimination of malaria as a public health problem and thereafter.

Vaccine development

In the backdrop of limited malaria treatment options and development of drug and insecticide resistance, appropriate means of disease control are currently threatened and the development of a vaccine appears as the only tool that might ensure long-term sustainability of malaria elimination and enable disease eradication.[186] The Rainbow Table of malaria vaccine projects summarised by the WHO,[187] and also reviewed by several authors,[188–190] provide a comprehensive summary of malaria vaccine studies at advanced pre-clinical and clinical stages globally, extending from studies related to preclinical antigens to the Phase III RTS,S/AS01 trials underway in Africa.

Three types of vaccine candidates targeting different stages in the life cycle of the malaria parasite have been intensively investigated: (i) transmission-blocking vaccines (TBVs); (ii) pre-erythrocytic vaccines; and (iii) blood-stage vaccines.[190] Transmission-blocking vaccines target antigens on gametes, zygotes and ookinetes to prevent parasite development in the mosquito midgut: the leading vaccine candidates in this group include the *P. falciparum* ookinete surface antigens Pfs25 and Pfs28 and their *P. vivax* homologues Pvs25 and Pvs28.[190] To improve immunogenicity, Pfs25 has been expressed as a recombinant protein chemically cross-linked to Exo-Protein A (Pfs25-EPA) and delivered as a nanoparticle and is currently undergoing Phase I trials in humans.[191]

Pre-erythrocytic vaccines target sporozoite and/or hepatic stage parasites to prevent asexual and

sexual blood-stage infection, thereby preventing both the occurrence of clinical malaria and block transmission of malaria to others. To date, the most advanced pre-erythrocytic vaccine candidate is RTS,S which consists of fusion between a portion of the *P. falciparum*-derived circumsporozoite protein (CSP) and the hepatitis B surface antigen. The lack of enduring protection remains a major disadvantage of this vaccine approach. Although it has shown 30–50% protection in human field trials in Africa, efficacy has declined over time to 16.8% at the end of 4 years.[192] As elaborated by Lorenz and others,[186] with a genome that comprises more than 5000 protein-coding genes, with only some expressed at specific stages, disappointing results with a vaccine candidate that triggers antibodies only against a single surface protein, is therefore not surprising. An efficacious malaria vaccine needs to induce an immune response against multiple malaria antigens,[193] a belief that has propelled the development of whole cell pre-erythrocytic malaria vaccines such as the *P. falciparum* sporozoite vaccine (PfSPZ).[194] The PfSPZ Vaccine, that is currently undergoing Phase IIa trials, is composed of radiation-attenuated, aseptic, purified, cryopreserved, and metabolically active but non-replicating *P. falciparum* sporozoites that are administered intravenously.[194]

Research on vaccines has progressed steadily during this last decade, and a number of new approaches are in the pipeline that could further improve the second generation of malaria vaccines. The clinically relevant vector-modified vaccinia virus Ankara (MVA) and the chimpanzee adenovirus (ChAd63) are promising delivery systems for malaria vaccines due to their safety profiles and proven ability to induce protective immune responses.[195] Promising second-generation vaccine candidates that include the cell-traversal protein for ookinetes and sporozoites (CelTOS); vaccines of ChAD63-MVA expressing CS (CSVAC), multiple epitope string with thrombospondin-related adhesion protein (ME-TRAP), apical membrane antigen (AMA) 1, and merozoite surface protein (MSP) 1; NMRC-M3V-Ad-PfCA, human adenovirus 5-vectored *P. falciparum* vaccine encoding CSP and AMA1; and GMZ2, fusion protein of *P. falciparum* MSP3 and glutamate-rich protein (GLURP), are in various stages of clinical trials.[187]

Blood-stage vaccines are designed to elicit anti-invasion and anti-disease responses. At present, several blood-stage antigens that are highly expressed on the surface of merozoites are in clinical trials: e.g. AMA1, erythrocyte-binding antigen-175 (EBA-175), GLURP, MSP1, MSP2 and MSP3 and serine repeat antigen 5 (SERA5).[190] New antigens that have been more recently discovered, such as *P. falciparum* reticulocyte-binding protein homologue 5 (PfRH5)[196] and rhoptry-associated leucine zipper-like protein 1 (RALP1)[197] show great potential as blood-stage vaccine candidates. The most advanced pregnancy-associated malaria vaccine approaches, although still at the preclinical stage, target var2CSA, which is preferentially expressed by placental parasites and is the target of acquired immunity over successive pregnancies.[198]

For *P. vivax*, a chimaeric CSP (VMP001) representing repeat regions of the two major alleles, VK210 and VK247, was adjuvanted with the AS01 adjuvant system and evaluated in a completed Phase II challenge study.[199] A *P. vivax* vaccine based on ChAd63-MVA that express PvTRAP was shown to be immunogenic in transgenic *P. berghei* parasites.[195] Another promising molecule is DBP of *P. vivax* that is vital for host erythrocyte invasion and a Phase Ia clinical trial is currently underway to assess the safety and immunogenicity of the *P. vivax* vaccine candidates ChAd63 PvDBP on its own and together with MVA PvDBP.[200] Pvs25 and Pvs28 are two TBV candidates that have been widely studied and shown to have limited sequence polymorphisms and believed to offer great promise as TBVs against *P. vivax* infection.[201,202]

Genetic surveillance

Molecular markers of drug-resistant malaria represent public health tools of great potential value. These markers can allow tracking of the spread of resistance alleles, map the distribution and rate of spread and inform local treatment policies.[203] They also provide better understanding of the disease evolution and strategies to curtail resistance.[83] Molecular markers of resistance to CQ,[138] sulfadoxine and pyrimethamine[83] were of little practical value because they were described only after resistance was already widespread. Molecular surveillance has been advocated in WHO policy guidelines on two past occasions, both relating to the use of sulfadoxine and pyrimethamine as prophylactic intermittent preventive treatment.[204,205] The identification of the K13-propeller locus before artemisinin resistance appeared in Africa means molecular surveillance could be used to provide policy makers with a forecast of impending problems, rather than confirmation of an already existing one.[206] This is especially valuable when drugs are used in combination, as molecular surveillance can suggest that one drug is failing even when it is still apparently clinically effective when used in combination. Furthermore, parasite populations can be monitored for the four well-defined mutations in the *fd*, *arps10*, *mdr2* and *crt* genes, allowing for risk assessment for the likelihood of emergence of drug resistance in different locations, thus helping to target high-risk regions even before resistant parasites take hold.[32]

The main vector control methods, IRS and ITNs, rely on insecticides but resistance to the limited number of available insecticides is already widespread, with some populations now showing resistance to all four classes.[207] The molecular targets of the major classes of insecticides are known, and mutation of target sites are well understood with the mechanisms of resistance.[177,208] The SNPs in these genes will facilitate monitoring of the frequency and spread of resistance alleles.[180] Thus, identification of genetic markers associated with resistance was included in the priorities of the WHO Global Plan for Insecticide Resistance Management (GPIRM).[209] The *kdr* mutations play an important role in the process of pyrethroid resistance, and several species-specific and highly sensitive methods have been designed in order to genotype individual mosquitoes for *kdr* in large scale, which may serve as important tools for monitoring the dynamics of pyrethroid resistance in natural populations.[177]

The nature and strength of population genomic signals expected at various stages of elimination have been studied with the use of SNPs and microsatellites[210–212] with promising results. Population genomics could potentially be used to track parasite genotypes through time and geographically across routes of human or vector migration.[43,211] Thus, knowledge about parasite genetic diversity can be made use of to monitor parasite populations as transmission levels change.

Surveillance of malaria is an essential strategy to achieve and maintain the status of disease elimination. The level of effectiveness of surveillance, however, depends on the performance of surveillance tools. Recent history of malaria in Sri Lanka, the first country in South Asia to reach near elimination targets, amply demonstrates the relative resistance of *P. vivax* to elimination strategies, when compared to *P. falciparum*.[4] Therefore, development of effective and more efficient tools to combat *P. vivax* remains a priority for malaria elimination settings. Hidden parasite reservoirs in *P. vivax*, continues to be a huge challenge for effective elimination, especially considering its ability to remain dormant in the liver for many months or years. Polymerase chain reaction-based diagnostic methods are accepted as being superior to light microscopy for identification of low parasitaemias.[213] Therefore, in order to accelerate malaria elimination and sustain the status thus achieved, some form of mass screening using PCR-based or comparably sensitive methods combined with radical cure approach has been recommended to identify and treat all sub-microscopic *P. vivax*-infected subjects.[213]

Limitations of Existing Genomic Tools

One of the major limitations of malaria parasite genomic studies to date is the paucity of appropriate samples and their links to clinically relevant phenotypes.[14] The ability to continuously culture *P. falciparum*[214] has allowed greater study of this species, although culture adapted parasites are both technically challenging and require significant resources. Despite its importance as a major human pathogen, *P. vivax* is little studied because it cannot be easily propagated in the laboratory except in non-human primates[144] leading to vast disparity in the state of knowledge regarding almost all aspects of the biology of this species.[145] Since efforts on genetic research on malaria have historically been spear-headed by studies on *P. falciparum*, the knowledge thus derived is generally used as an appropriate inference for *P. vivax*, a classic example being the search for CQ resistance markers. However, due to intrinsic biological dissimilarities, extrapolation from *P. falciparum* does not necessarily reflect the true mechanisms of *P. vivax*, thus limiting the number of genetic markers being studied.[154]

Comparative analysis of the parasite genome sequences to discover homologous genes plays an important role in elucidating the functions of many predicted proteins. However, the use of homology to discern gene function is limited in *P. falciparum* as the genome has a great number of hypothetical proteins ($\sim$60%),[215] posing a challenge for the use of genomics.[14] An alternative approach would be the use of genetic variation to infer the biological importance of loci.[27–32] Linkage analysis of laboratory crosses and GWAS are other approaches to map genetic loci contributing to drug resistance and virulence in malaria parasites.[216] However, the cost and intensive lab work of genetic crosses have limited its application for larger-scale functional analysis in human malaria parasites.[215] This situation is made even worse in the case of *P. vivax* due to the lack of practical methods for its long-term propagation and cloning *in vitro*, which are required to characterise phenotypes in the progeny of experimental crosses.[216]

The high human DNA contamination and low amounts of parasite DNA, present serious challenges to genotyping the large number of samples necessary for GWAS. The genotyping tools and statistical methods available for GWAS also struggle to deal with the high levels of genome diversity and population structure in Africa.[217] Authentic loci with strong genetic effects may fail to reach genome-wide significance because of weak LD between causal variants and the SNPs that are genotyped.[218] The limited amount of LD in *P. falciparum* that hinders the power of traditional array-based GWAS has been improved by the use of WGS for association studies.[128] Similarly, low levels of LD and high levels of population substructuring[219] have limited the utility of GWAS in *A. gambiae* and efforts

are now being directed to using NGS technology to study insecticide resistance traits.[13]

Both NGS and microarrays are used for a wide range of genomic applications such as: genotyping, gene expression profiling, chromatin immunoprecipitation (ChIP) and DNA methylation studies. Genotyping with microarrays are substantially less expensive than NGS and much more conducive to processing thousands of samples required for typical GWAS.[48] But microarrays are limited by the number of SNPs they contain and tend to focus more on relatively common variants, while sequencing allows for capture of both common as well as rare variants. Exome sequencing offers a more cost-effective approach in this regard.[93,220] Similarly, RNAseq methods provide a detailed view of the transcriptome, while arrays are limited to the regions of probe design. However, expression arrays are still cheaper and easier when processing large numbers of samples. Chromatin immunoprecipitation experiments have progressed rapidly from arrays to sequencing (ChIP-seq) as NGS provides much better peak resolutions, less noise and greater coverage.[128] Next-generation sequencing also provides a more complete picture of the methylome and better in aiding discovery, while microarrays are less costly and provides rapid profiling. However, both technologies are not feasible for use under field conditions, or cost effective as tools for screening and characterisation of large numbers of samples.[130]

The capacity of NGS technologies to sequence DNA at unprecedented speed has led to massive production of data, which presents a significant challenge for data storage, analyses, and management solutions with advanced bioinformatic tools being essential for the successful application of NGS technology.[221] While NGS technologies provide unprecedented power to explore the transcriptome of a species in great detail, the assembling of short reads into full-length gene and transcript models presents significant computational challenges.[222] New and more accurate transcript assembly tools are needed to further refine the genes and transcript models in view of the expanding biological knowledge about gene structure and variation and advances in sequencing technologies.[222]

Future

Success of the global research agenda towards eradication of malaria will essentially depend on the development of new tools, including drugs, vaccines, insecticides and diagnostics. The rapid advancements in genomic tools have revolutionised malaria research, providing access to annotated, genome sequences of malaria parasites, their vectors and the human host. Genetic diversity of the *Plasmodium* spp. is now being used to aid discovery of vulnerable targets for intervention strategies, and to identify biomarkers for diagnosis, surveillance and monitoring of malaria infections in humans or mosquitoes.[14] Furthermore, the study of epigenetics in *Plasmodium* has flourished due to improvement in genomic technologies.[223–225]

A number of promising antimalarial compounds are now undergoing Phase II clinical development. Of particular note are KAE609, KAF156 and DSM265 are chemical scaffolds, which are new to malaria chemotherapy and would truly diversify antimalarial options.[226] KAE609 (a synthetic spiroindolone) and KAF156 (imidazopyrazine) are effective against both, *P. falciparum* and *P. vivax*, while KAE609 offers hope as a single-dose combination therapy, and KAF156 is expected to kill parasites at its early, asymptomatic liver stage for *P. vivax*.[227] Imidazopyrazines target phosphatidylinositol-4-OH kinase (PI(4)K) and inhibits the intracellular development of multiple *Plasmodium* species at each stage of infection in the vertebrate host.[228] DSM265 is a triazolopyrimidine, a potent *P. falciparum* dihydroorotate dehydrogenase inhibitor, able to kill both sensitive and drug resistant strains of the parasite.[229] Tafenoquine (alternative for primaquine but with the added advantage of being effective when administered as a single dose) co-administered with CQ for the radical cure of *P. vivax* malaria and relapse prevention has moved onto clinical assessment in Phase III.[230]

The new found molecular markers useful for genetic surveillance appear promising for both drug- and insecticide-resistance monitoring. The K13-propeller mutations,[140] and the four mutations in the *fd*, *arps10*, *mdr2*, and *crt* genes,[32] have the potential to provide policy makers with a forecast of impending emergence and/or spread of resistance, thus enabling localisation of high-risk regions, and allowing appropriate containment strategies to be implemented. Similarly, the molecular targets of the major classes of insecticides and their mutations will facilitate monitoring of the frequency and spread of resistance alleles.[180] The species-specific and highly sensitive methods available for genotyping individual mosquitoes for *kdr* in large scale will serve as important tools for monitoring the dynamics of pyrethroid resistance in natural populations.[177] The recent finding that insecticide resistance impacts vector competence of *A. gambiae* towards *P. falciparum,* and probably parasite transmission, through increased sporozoite prevalence in *kdr*-resistant mosquitoes[174] is of great concern for the epidemiology of malaria, considering the widespread pyrethroid resistance currently observed in Sub-Saharan Africa and the efforts deployed to control the disease. The capsule suspension formulations of the organophosphate pirimiphos methyl, evaluated for IRS treatments in experimental

huts in Benin (where *A. gambiae* is resistant to pyrethroids but susceptible to organophosphates) have shown great promise for providing prolonged control of pyrethroid-resistant *A. gambiae* and for delaying pyrethroid resistance.[231] An alternative to DDT, giving year-round transmission control in Sub-Saharan Africa may now be a realistic prospect.[231]

It is clear that further advances are required for malaria vaccine development, based on empirical approaches and basic research, to identify new target antigens and provide improved understanding of how different adjuvants will affect the balance and durability of effector, memory and regulatory responses.[190] Emphasis should be on mixtures of antigens combined with powerful adjuvants, not only to induce the necessary effector responses but to increase the possibility of inducing at least partial cross-strain immunity by including a range of *Plasmodium* epitopes.[232] Taking advantage of the new tools and strategies available will speed up the development of a new generation of malaria vaccines that are highly efficacious. Innovative genome-based vaccine strategies have shown potential for a number of challenging pathogens, including malaria. A rational genome-based vaccine design, enabling the selection of the best possible targets by prioritising antigens according to clinically relevant criteria (frequency and magnitude of clinically relevant immune response and/or biological function), will overcome the problem of poorly immunogenic, poorly protective vaccines that has plagued malaria vaccine development for the past 25 years.[82]

Vaccine delivery systems are becoming increasingly more important for the effective use of new generation of vaccines that has led to efforts such as co-administration of immunostimulants and use of more than one antigen in the same system.[190] Recent attention has been directed towards the utility of nanoparticles as delivery vehicles for vaccines,[233] while the wheat germ cell-free protein synthesis system has shown to be an optimal tool for synthesis of quality malaria proteins and hence the discovery of novel malaria vaccine candidates.[234] Photosynthetic organisms, including terrestrial plants and algae, offer several advantages over conventional heterologous protein production systems and exist as a potential solution.[235] Vaccines are the most cost effective means for disease prevention and will be the key to eradicate malaria. However, such a vaccine(s) would need to be low cost, easily administered (ideally oral) and also should work in concert with current control methods.

The development of new tools and adaptation of existing tools for use by malaria researchers and clinicians to meet elimination and eradication goals is a priority. Ideal tools should give maximal information from minimal amounts of biological sample, and be both affordable and easy to use.[130] The rapidly developing high-throughput sequencing platforms promise even larger and faster data generation, while becoming more compact, easier to use and economical.[55,236,237] The use of gene drive technology could revolutionise ecosystem management. These emerging technologies with potential global effects are now put forward for public discussions with regard to environmental and security concerns and formulation and testing of safety features.[65] This powerful tool has the potential to re-programme mosquito genomes, leading to modification or eradication of vectors of disease including malaria, with possibilities for malaria eradication. To conclude, the overall prospects for expanding the 'map of malaria elimination' are clearly encouraging and potential opportunities are seemingly endless with technological advances moving at an unbelievably rapid pace. Therefore, in this context, even the far-off dream of achieving the status of eradication of malaria might not be too optimistic a view to hold.

Acknowledgements

Financial support by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number R01AI099602 to Nadira D Karunaweera and National Research Council of Sri Lanka (NRC grant ref: 14–69) to Sharmini Gunawardena are acknowledged.

Disclaimer Statements

Contributors Both authors (SG and NDK) contributed equally to writing of this manuscript.

Funding NIAID, NIH, USA. NRC, Sri Lanka.

Conflicts of interest The authors have declared no conflict of interest.

Ethics approval There is no ethical approval for this article.

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