Integration of population and functional genomics to understand mechanisms of artemisinin resistance in \textit{Plasmodium falciparum}

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\section*{A B S T R A C T}

Resistance to antimalarial drugs, and in particular to the artemisinin derivatives and their partner drugs, threatens recent progress toward regional malaria elimination and eventual global malaria eradication. Population-level studies utilizing whole-genome sequencing approaches have facilitated the identification of regions of the parasite genome associated with both clinical and \textit{in vitro} drug-resistance phenotypes. However, the biological relevance of genes identified in these analyses and the establishment of a causal relationship between genotype and phenotype requires functional characterization. Here we examined data from population genomic and transcriptomic studies in the context of data generated from recent functional studies, using a new population genetic approach designed to identify potential favored mutations within the region of a selective sweep (iSAFE). We identified several genes functioning in pathways now known to be associated with artemisinin resistance that were supported in early population genomic studies, as well as potential new drug targets/ pathways for further validation and consideration for treatment of artemisinin-resistant \textit{Plasmodium falciparum}. In addition, we establish the utility of iSAFE in identifying positively-selected mutations in population genomic studies, potentially accelerating the time to functional validation of candidate genes.

\section*{1. Introduction}

Although malaria incidence has declined over the last decade, progress toward malaria elimination has stalled over the last 5 years as incidence has plateaued (\textit{World Health Organization, 2019}). In addition, these gains are threatened by the continued evolution of resistance in both the parasite and mosquito vector in response to interventions. For example, the parasite continues to evolve resistance to antimalarial drugs, with \textit{Plasmodium falciparum} having developed resistance to most antimalarials used to treat clinical infection, including former first-line antimalarials such as chloroquine, the antifolates, and most recently the artemisinin derivatives and their partner drugs used in artemisinin-based combination therapies (ACTs) [Reviewed in (Menard and Dondorp, 2017)]. Indeed, in some malaria endemic areas, such as the eastern Greater Mekong Subregion, \textit{P. falciparum} is resistant to multiple approved ACTs, severely limiting treatment options (\textit{World Health Organization, 2019}).

The advent of technologies allowing whole-genome sequencing of \textit{P. falciparum} at epidemiological scales has facilitated the use of population-level approaches to identify regions of the parasite genome associated with drug-resistance phenotypes. These studies have been successful in identifying molecular markers of resistance and the genetic architecture of resistant parasites and have provided insights into the underlying mechanisms of resistance (Agrawal et al., 2017; Amato et al., 2017; Arisy et al., 2014; Borrman et al., 2013; Cerqueira et al., 2017; Cheeseman et al., 2012, 2015; Demas et al., 2018; Miotto et al., 2013, 2015; Mu et al., 2010; Mukherjee et al., 2017; Park et al., 2012; Takala-Harrison et al., 2013, 2015; Van Tyne et al., 2011; Wang et al., 2016; Widdler et al., 2014; Witkowski et al., 2010; Yuan et al., 2011; Zhu et al., 2018). However, these studies are limited by the fact that

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observed associations are not necessarily causal and that, in the region of a selective sweep, several polymorphisms in multiple genes may be associated with the phenotype of interest, making it difficult to pinpoint the gene driving the observed associations. For example, our initial genome-wide association study of delayed parasite clearance following artemisinin treatment identified a region of chromosome 13 (containing 12 genes) that was associated with parasite clearance half-life (Takala-Harrison et al., 2013). In a similar timeframe, other population-genomic studies also identified a region of chromosome 13 that appeared to be under recent positive selection and highly differentiated in artemisinin resistant parasite populations (Cheeseman et al., 2012; Miotto et al., 2013). In these studies, it was not clear which gene(s) contained the causal mutation until the following year, when results from in vitro selection experiments indicated that mutations within the kelch13 gene were responsible for artemisinin resistance (Ariey et al., 2014). Since these initial studies, several subsequent population-level genomic and transcriptomic studies have identified multiple genomic regions and differentially expressed genes associated with resistance to the artemisinin derivatives or their partner drugs (Agrawal et al., 2017; Amato et al., 2017; Borrmann et al., 2013; Cerqueira et al., 2017; Cheeseman et al., 2015; Demas et al., 2018; Miotto et al., 2013, 2015; Mok et al., 2015; Mukherjee et al., 2017; Takala-Harrison et al., 2015; Wang et al., 2016; Wendler et al., 2014; Zhu et al., 2018); however, as in the case of kelch13, the function and biological relevance of these genes has not always been clear.

The development of higher-throughput experimental genetic approaches has increased the capacity for functional characterization of gene products as they relate to various phenotypes of interest (Birnbaum et al., 2020; Cowell et al., 2018; Demas et al., 2018; Gnädig et al., 2020; Li et al., 2019; Oberstaller et al., 2021; Zhang et al., 2020), including drug resistance. Results from such genome-scale screens provide an opportunity to re-interpret previous findings of population-level genomic and transcriptomic studies for which the functional relevance of identified genes is not understood. In this study, we aimed to integrate findings from previous population-level studies with new functional data to better understand drug resistance and compensatory mechanisms utilized by multidrug-resistant malaria parasites. In addition, we utilized a new population-genetic approach (i.e., Integrated Selection of Allele Favored by Evolution (iSAFE)), designed to identify favored mutation(s) within the genomic region of a positive selective sweep (Akbari et al., 2018), to identify potentially novel functional targets that may inform rational strategies to identify new drugs to counter resistance.

2. Methods

2.1. Literature review

We mined the literature for genes/genomic regions identified in multiple population-genomic or transcriptomic studies of resistance to artemisinin derivatives or ACTs. We curated gene IDs from 22 published population-genomic or transcriptomic studies and 8 published functional screens encompassing 28 total publications (Table 1). Studies were published between the years 2010 and 2020 and were conducted in malaria-endemic areas of Asia, Africa, South America, and Oceania. Genes in genomic regions either significantly associated with a resistance phenotype or identified based on estimated signals of positive selection were compiled, and any outdated gene IDs were corrected using PlasmoDB release 48 (https://plasmodb.org) (Aurrecoechea et al., 2009). Each gene was given an evidence score equal to the number of independent genomic, transcriptomic or functional studies in which it was identified (Supplementary Table 1). Genes containing more than one SNP associated with a phenotype (e.g., kelch13) were only counted once per publication.

| Table 1 | Publications providing source-data for this meta-analysis by evidence type. |
|---|---|
| **Data-sources by evidence-type** | Genomic | Transcriptomic | Functional |
| Agrawal et al. (2017) | Mok et al. (2015) | Birnbaum et al. (2020) |
| Amato et al. (2017) | Witkowski et al. (2010) | Cowell et al. (2018) |
| Ariey et al. (2014) | Zhu et al. (2018) | Demas et al. (2018) |
| Borrmann et al. (2013) | Cerqueira et al. (2017) | Li et al. (2019) |
| Cheeseman et al. (2012) | Cheeseman et al. (2015) | Mbengue et al. (2015) |
| Demas et al. (2018) | Miotto et al. (2013) | Pradhan et al. (2015) |
| Miotto et al. (2013) | Mu et al. (2010) | Yuan et al. (2011) |
| Miotto et al. (2015) | Mukherjee et al. (2017) |  |
| Park et al. (2012) | Takala-Harrison et al. (2013) |  |
| Takala-Harrison et al. (2015) | Van Tyne et al. (2011) |  |
| Wang et al. (2016) | Wendler et al. (2014) |  |
| Wendler et al. (2014) | Witkowski et al. (2017) |  |
| Zhu et al. (2011) | Zhou et al. (2018) |  |

2.2. Down-selection of genes for iSAFE analysis

We aimed to 1) estimate the degree to which population-level associations or selection signals can be explained by new functional evidence and 2) identify potentially novel functions contributing to resistance. To accomplish the latter goal, we first sought to identify genes with population-genomic or transcriptomic evidence, but not functional evidence, for further analysis to identify favored mutations driving associations with resistance. The down-selection strategy is illustrated in Fig. 1. From among genes identified in the literature search, we first excluded genes that were not identified in at least one population-genomics study. We then excluded genes that were identified in one or more functional screens (to identify potential new functional targets). Finally, based on the inflection point of a plot of the number of citations per gene (Fig. 2), we excluded all genes observed in fewer than three independent studies.

2.3. Integrated Selection of Allele Favored by evolution (iSAFE) analysis

The remaining genes were analyzed using Integrated Selection of Allele Favored by Evolution (iSAFE), a new approach that uses population genetic signals to identify the favored mutation within the broad genomic region contained within a recent positive selective sweep (Akbari et al., 2018). The iSAFE score is based on the distribution of each derived mutation in sampled haplotypes and the relative frequency of those haplotypes, where the favored mutation is expected to be associated with a small number of distinct haplotypes that are at high frequency in the population. First, a SAFE score is calculated for each derived mutation, which is a function of the fraction of haplotypes containing the mutation, the total number of derived mutations in those haplotypes, and the relative frequency of each haplotype (Romer et al., 2015). The iSAFE score for each mutation then combines the SAFE score across several consecutive, small (low-recombination) genomic windows. iSAFE exploits slight differences in signal expected between the selected mutation and those closely linked to it, which lie on the shoulders of the sweep. iSAFE operates more accurately when favored mutations are at or near fixation (derived allele frequency > 0.9). To account for these types of mutations, samples chosen randomly from a non-target population can be used as a control, to modulate the derived allele frequency of the favored mutation.

Using iSAFE, we analyzed publicly available whole-genome sequencing data from 61 P. falciparum isolates (Supplementary
Table 2), including 34 isolates that harbor the Kelch13 C580Y mutation and belong to the same genetic subpopulation (i.e., “cases”) and 27 isolates that lack Kelch13 mutations and belong to the pre-artemisinin resistance Southeast Asian genetic subpopulation (i.e., “controls”) (Dwivedi et al., 2017; Miotto et al., 2013; Takala-Harrison et al., 2015).

Variant-calling was performed using Haplotype Caller to generate genomic variant call format (GVCF) files for each sample and joint single nucleotide polymorphism (SNP) calling was performed using GATK (v4.1) (McKenna et al., 2010). Variant calls were filtered for quality based on the following criteria: (QUAL < 50 || DP < 12 || FS > 14.5 || MQ < 20.0).

We examined 100 kb regions centered around the 612 genes of interest to identify genes containing putative favored mutations within that genomic region. The 612 windows included an average of 27 genes/window, and combined, covered 2727 unique genes across the genome (Fig. 1, Supplementary Figure 1). Several iSAFE parameters were modified from defaults (designed for human genomes) to be appropriate for the much-smaller \textit{P. falciparum} genome: minimum region-size was decreased to 20 bp or 20 polymorphic sites, with a sliding window of 10 polymorphic sites and a step-parameter of 5 polymorphic sites. SNPs with an iSAFE score >0.5 and iSAFE rank above 5 were considered possible favored mutations (Supplementary Table 3; Supplementary Figure 2). Variant annotation information, including predicted phenotypic consequences of each candidate mutation was assessed using SNPeff (v.4.3) (Supplementary Table 4). Genes of interest were grouped into three categories based on whether they contained possible favored mutations: 1) genes linked to known artemisinin resistance determinants or genetic background genes as identified by Miotto and colleagues (Miotto et al., 2015) (e.g., Kelch13, PfCRT, etc.); 2) genes predicted to contain a putative favored mutation, and 3) genes not containing a putative favored mutation and not “hitchhiking” with known artemisinin resistance determinants. Genes linked to known artemisinin resistance determinants were excluded from further analysis. Genes predicted to contain a putative favored mutation underwent Gene Ontology (GO) enrichment analysis to identify significantly enriched functions representing potential new functional targets. Genomic regions centered on genes that were neither hitchhiking with known resistance determinants nor harboring possible favored mutations themselves were examined for linked genes predicted to contain a favored mutation. These linked genes with favored mutations also underwent a GO analysis to identify enriched functions and were cross-referenced with genes having functional evidence to determine the extent to which population-level signals are corroborated by data from recent functional screens.
2.4. Gene ontology (GO) enrichment analysis

All GOenrichment analyses were performed testing GOTerms mapped to genes in the category of interest against a background of GOTerms mapped to all other genes in the analysis. The GOTerm database was created from the latest curated \textit{P. falciparum} ontology available at the time of analysis, downloaded from GeneDB (accessed May 2, 2019) (Logan-Klumpler et al., 2012). GO enrichment analyses were performed in R using the topGO package from bioconductor (v. 2.40.0) (Alexa and Rahnenfurh, 2020). Genes selected for iSAFE analysis containing favored mutations (n = 145; Fig. 3A, Supplementary Table 6) were compared to a background of all genes with population genomic evidence but not functional evidence (n = 3310 genes; Fig. 3A, Supplementary Table 1). Genes driving the signal of nearby iSAFE input genes of interest (n = 653; Fig. 3A, Supplementary Table 6) were compared to a background of all genes with population genomic evidence identified in the literature search, including those with functional evidence (n = 3984; Fig. 3A, Supplementary Table 1). GO terms with a weighted Fisher/elim hybrid p-value $\leq 0.05$ were further examined as significantly enriched biological processes, molecular functions, or cellular compartments.

3. Results

3.1. Genomic regions identified in multiple independent studies of artemisinin resistance or ACTs

A total of 4460 genes were identified within the 28 publications reviewed for this analysis (Table 1, Fig. 1). Of these 4460 genes, 78.6% were only observed in one or two independent studies, while 0.5% were observed in $\geq 5$ independent studies (Fig. 1), including genes encoding Kelch13 (PF3D7_1343700), PICRT (PF3D7_0709000), prodrug activation and resistance esterase (PF3D7_0709700), autophagy-related protein 7 (PF3D7_1126100), heat shock protein 90 (PF3D7_0708400), and K13-interacting candidates 1 and 5 (KIC1, KIC5), among others.

3.2. iSAFE analysis

To verify that iSAFE was working as expected with our parameters and genomic data set (representative of a pre- and post-Kelch13 C580Y selective sweep), we performed iSAFE on the 100 kb region centered around the gene encoding Kelch13. The top favored mutation identified by iSAFE encodes C580Y, indicating that iSAFE is detecting known mutations favored by selection (Supplementary Table 5). We next applied iSAFE to examine 100 kb genomic regions centered on the 612 genes of interest (n = 2727 unique genes) and identified 34 genes that were linked to known artemisinin-resistance determinants or genetic background genes (Supplementary Table 6). 145 of the 612 genes of interest were predicted to contain favored mutations that were likely driving the selection signal (Supplementary Tables 3-4) and represent potential new functional targets that require validation. Genomic windows comprising the 433 genes that were neither “hitchhiking” with known resistance determinants nor harboring favored mutations themselves contained 653 genes that were previously identified in at least one population genomics study and were predicted to contain a putative favored mutation. Of these 653 genes, 137 have been identified in recent genome-scale functional screens (Supplementary Table 7), representing a significant enrichment of genes with functional evidence among genes with putative favored mutations in genomic regions identified in population-level studies (Fisher’s exact test, p-value < 0.001).

3.3. Genes identified in functional screens that are supported by population-level studies

A GO enrichment analysis was performed on the 653 genes containing favored mutations that were linked to genes identified in...
population-level studies. Significantly enriched GO terms (hybrid Fisher/elim p-value<0.05; Supplementary Table 8) are shown in Fig. 3B (purple), with genes corresponding to those terms shown in Supplementary Table 9. Significantly enriched molecular functions or biological processes include microfilament motor activity (GO:0000146) and actin filament-based movement (GO:0051258, DNA helicase activity (GO:0017116, GO:0032508, GO:0043138), and alpha-amino acid metabolic process (GO:1901605). Significantly enriched cellular compartments include the cell periphery, food vacuole, nucleus, and nuclear periphery.

Of these 653 genes, 137 have been identified in recent functional screens of antimalarial drug resistance (Supplementary Table 7), including 40 conserved proteins of unknown function. Among these 137 genes, several have been shown to encode products involved in processes implicated in artemisinin resistance, such as proteasomal degradation, endocytosis, vesicular trafficking, or stress response (Fig. 3B, yellow; Table 2; Supplementary Tables 10-11). Notable examples include amino acid transporter AAT1 (PF3D7_0629500), clathrin heavy chain (PF3D7_1219100), DER-1 like protein (PF3D7_1032500), Epis15-like protein (PF3D7_1025000), actin II (PF3D7_1412500), myosin F and J (PF3D7_1329100, PF3D7_1229800), ubiquitin conjugating enzyme E2 (PF3D7_0812600), KIAI interacting protein KIC6 (PF3D7_0609700), and other proteins shown to interact with K13 (Gnädig et al., 2020). In addition, proteins involved in erythrocyte invasion, such as apical membrane antigen 1 (AMA1), merozoite surface protein 1 (MSP1), and rhoptry neck protein 2 (RON2) were also among those proteins having both functional and population-level evidence. Interestingly, a number of genes encoding exported proteins were among the 137 genes with functional evidence, including genes encoding members of the PfEMP1, PHIST, RIFIN, and CLAG protein families (Table 2).

3.4. Potential new targets from population-genomic studies

145 genes evaluated in the iSAFE analysis contained favored mutations (Supplementary Table 6). A GO enrichment analysis indicated significant enrichment (hybrid Fisher/elim p-value<0.05; Supplementary Table 11) in gene products involved in intramolecular transport activity (GO:0016866), small GTPase binding (GO:0031267), and other proteins shown to interact with K13 (Gnädig et al., 2020). In addition, proteins involved in erythrocyte invasion, such as apical membrane antigen 1 (AMA1), merozoite surface protein 1 (MSP1), and rhoptry neck protein 2 (RON2) were also among those proteins having both functional and population-level evidence. Interestingly, a number of genes encoding exported proteins were among the 137 genes with functional evidence, including genes encoding members of the PfEMP1, PHIST, RIFIN, and CLAG protein families (Table 2).

### Table 2

| Gene_ID | Total score | Annotation Functional Categories |
|---------|-------------|---------------------------------|
| PF3D7_1118200 | 5 | Heat shock protein 90, putative | Stress response |
| PF3D7_1329100 | 5 | Mpsin F, putative | Vesicular trafficking |
| PF3D7_1412500 | 4 | Actin II | Vesicular trafficking |
| PF3D7_0629500 | 4 | Amino acid transporter AAT1 | Hemoglobin digestion/digestive vacuole |
| PF3D7_1133400 | 4 | Apical membrane antigen 1 | Exported protein, invasion |
| PF3D7_0202800 | 4 | Cytoadherence linked asxual protein 2 | Exported protein, host-cell remodelling |
| PF3D7_0629200 | 4 | DnaJ protein, putative | Exported protein, host-cell remodelling |
| PF3D7_0823800 | 4 | DnaJ protein, putative | Exported protein, host-cell remodelling |
| PF3D7_0930000 | 4 | Merozoite surface protein 1 | Exported protein, invasion |
| PF3D7_0902700 | 4 | Plasmodium exported protein (PHIST)b | Exported protein, host-cell remodelling |
| PF3D7_1016800 | 4 | Plasmodium exported protein (PHIST)c | Exported protein, host-cell remodelling |
| PF3D7_0609700 | 4 | Protein KIC6 | K13-mediated endocytosis |
| PF3D7_0822600 | 4 | Protein transport protein SEC23 | Vesicular transport; protein export |
| PF3D7_1452000 | 4 | Rhoptry neck protein 2 | Exported protein, invasion |
| PF3D7_0702400 | 4 | Small, exported membrane protein 1 | Exported protein |
| PF3D7_0319700 | 3 | ABC transporter I family member 1, putative | Stress response |
| PF3D7_0935800 | 3 | Cytoadherence linked asxual protein 9 | Exported protein, host-cell remodelling |
| PF3D7_0711700 | 3 | Erythrocyte membrane protein 1, PfEMP1 | Exported protein, host-cell remodelling |
| PF3D7_1229800 | 3 | Myosin J, putative | Vesicular trafficking |
| PF3D7_0608800 | 3 | Ornithine aminotransferase | Stress response |
| PF3D7_1119000 | 3 | Protein transport protein SEC16, putative | Vesicular trafficking |
| PF3D7_0395900 | 3 | Ring-exported protein 1 | Exported protein, host-cell remodelling |
| PF3D7_1145600 | 3 | TME65 domain-containing protein, putative | Stress response |
| PF3D7_1417400 | 3 | Ubiquitin carboxy-terminal hydrolase, putative | Protein degradation |
| PF3D7_1025000 | 2 | Epis15-like protein | K13 interactor/ protein degradation |
| PF3D7_0531000 | 2 | Erythrocyte membrane protein 1 (PfEMP1) | Exported protein, host-cell remodelling |
| PF3D7_0712900 | 2 | Erythrocyte membrane protein 1, PfEMP1 | Exported protein, host-cell remodelling |
| PF3D7_1476600 | 2 | Plasmodium exported protein, unknown function | Exported protein |
| PF3D7_1472600 | 2 | Protein disulfide-isomerase | Oxidative stress (redox)/protein degradation |
| PF3D7_1340700 | 2 | ras-related protein Rab-11B | Vesicular trafficking |
| PF3D7_0712500 | 2 | RIFIN, pseudogene | Exported protein, host-cell remodelling |
| PF3D7_1352500 | 2 | Thioredoxin-related protein, putative | Oxidative stress |
| PF3D7_0812600 | 2 | Ubiquitin-conjugating enzyme E2, putative | Protein degradation |

4. Discussion

Population-genomic and transcriptomic studies have identified genomic regions and differentially expressed genes associated with resistance phenotypes; however, the function and biological relevance of these genes has not always been clear. Recent advances in both targeted and forward-genetic approaches are providing insight into the function of many *Plasmodium* genes as it relates to phenotypes of public health interest, including drug resistance. In this study, we conducted a meta-analysis of population-level studies of resistance to artemisinin derivatives or ACTs, identified genes or genomic regions identified in multiple studies, and used a new population-genomic tool, iSAFE, to identify genes likely to contain favored mutations driving a selective sweep or that are linked to genes with possible favored mutations. We found that multiple prongs of the increasingly complex network of interactions implicated in artemisinin resistance, including those related to proteasomal degradation, endocytosis, vesicular trafficking and stress...
response are well-supported in the data obtained from previous population-level studies (Fig. 4). Our results suggested a role for exported proteins in artemisinin resistance and identified potential new drug targets/pathways to consider for treatment of artemisinin-resistant \textit{P. falciparum}. The large number of uncharacterized gene products with both population-level and functional evidence emphasizes the need for further genetic screens to understand the role of these gene products in established or yet-undiscovered mechanisms contributing to parasite artemisinin resistance.

Artemisinin resistance was first reported in western Cambodia in 2006–2007 (Dondorp et al., 2009; Noedl et al., 2008), and primarily manifested as delayed clearance of parasitemia following treatment with artemisinin derivatives or ACTs. Although Kelch13 was identified as a major determinant of this phenotype in 2014 (Ariey et al., 2014), the molecular mechanisms underlying this clinical phenotype have been more difficult to resolve. Artemisinin derivatives are thought to be activated by reduced iron that results from parasite hemoglobin digestion (Klonis et al., 2011), resulting in reactive carbon-centered radicals and oxygen species that cause extensive cellular damage and lethal proteotoxic stress. Parasite artemisinin resistance/tolerance has been hypothesized to result from decreased endocytosis and hemoglobin digestion, as well as increased cellular repair in surviving parasites (reviewed in (Sutherland et al., 2020; Xie et al., 2020). The complete picture of all Kelch13 functions has not yet been elucidated; however, Kelch13 and its interacting partners have been localized to parasite cytostomes where they are hypothesized to play a role in hemoglobin uptake from the host cytosol (Birnbaum et al., 2020; Yang et al., 2019). Kelch13 abundance is reduced in mutant parasites (Gnádig et al., 2020), leading to decreased hemoglobin uptake, disrupted hemoglobin catabolism, and reduced artemisinin activation. Several proteins involved in endocytosis and vesicular trafficking, including proteins shown to interact with Kelch13 (Birnbaum et al., 2020; Gnádig et al., 2020), have been identified in this meta-analysis, offering additional support for this resistance mechanism based on earlier population-level studies. Likewise, gene products involved in oxidative stress and protein damage responses (e.g., thioredoxin-related protein, PF3D7_1352500; Der1-like protein, PF3D7_1032500) are well-supported in previous population genomic studies, based on our analysis. Using isSAFE, we have also identified genes with favored mutations that are functionally-related to these established processes, that are well-supported by population-level studies, but have not been characterized in functional studies for their specific contributions to artemisinin resistance, including genes encoding phosphatidylinositol-4-phosphate 5-kinase, PIP5K (PF3D7_0110600), the histone acetyltransferase, PfMYST (PF3D7_1118600), and calcium-dependent protein kinase 5, CDPK5 (PF3D7_1357800) (Table 3, Fig. 4).

Phosphatidylinositol-4-phosphate 5-kinase, PIP5K (PF3D7_0110600) is thought to be a bifunctional molecule with a PIP5K domain at its C-terminus and a neuronal calcium sensor (NCS)-like domain (i.e., calcium trigger that causes protein conformational changes in response to calcium) at its N-terminus (Leber et al., 2009). In eukaryotes, PIP5K plays a role in membrane trafficking, cytoskeletal reorganization and cell signaling by regulating the levels of PtdIns(4,5)P2 in conjunction with phosphatidylinositol 4-kinases (PI4Ks) (Martin, 2012). Based on experimental data from other eukaryotes (Kakuchi et al., 2007; Liu et al., 2018; Voisinne et al., 2016), PI4Ks on both chromosomes 4 (PF3D7_0419900) and 5 is thought to be a bifunctional molecule with a PIP5K domain at its C-terminus and a neuronal calcium sensor (NCS)-like domain (i.e., calcium trigger that causes protein conformational changes in response to calcium) at its N-terminus (Leber et al., 2009). In eukaryotes, PIP5K plays a role in membrane trafficking, cytoskeletal reorganization and cell signaling by regulating the levels of PtdIns(4,5)P2 in conjunction with phosphatidylinositol 4-kinases (PI4Ks) (Martin, 2012). Based on experimental data from other eukaryotes (Kakuchi et al., 2007; Liu et al., 2018; Voisinne et al., 2016), PI4Ks on both chromosomes 4.
enriched among gene products with functional and/or population encoded by the could be explored for activity on multidrug-resistant parasites. As observed in some prior studies, exported proteins, such as those encoded by the var, pfist, rfn, and cag multigene families, were enriched among gene products with functional and/or population genomic evidence (Mok et al., 2015; Rocamora et al., 2018; Siddiqui et al., 2020; Zhu et al., 2018). This observation suggests a potential role for host-cell remodeling in artemisinin resistance.

PMYST (PF3D7_1118600) is a histone acetyltransferase (HAT) that has been demonstrated to play a role in regulation of gene expression, cell cycle control, and DNA repair (Miao et al., 2010). PMYST is the only HAT of its kind in P. falciparum. It is expressed in both long and short forms and localizes to both the nucleus and the cytoplasm (Miao et al., 2010). Efforts to knock out pfmyst have not been successful, suggesting that it is an essential gene; however, overexpression of PMYST led to changes in cell cycle progression. Specifically, parasites with overexpression of full-length PMYST displayed a shorter intraerythrocytic developmental cycle (IDC), while parasites expressing truncated PMYST had a more prolonged IDC compared to control parasites, with IDC changes occurring primarily in the schizont stage (Miao et al., 2010). Changes in the duration of the parasite IDC are also a hallmark of artemisinin-resistant parasites, although it is the ring stage that has been shown to be prolonged, likely as a result of reduced hemoglobin catabolism. PMYST was also demonstrated to participate in var gene activation, as well as DNA repair, and to modulate sensitivity to genotoxic agents. HAT inhibitors have been shown in previous studies to have parasiticidal effects (Andrews et al., 2000; Cui et al., 2007; Darkin-Rattray et al., 1996; Sen et al., 2020), lending credence to the possibility of histone acetylation enzymes as antimarial drug targets.

Multiple studies have implicated DNA damage-repair mechanisms in K13-associated drug-resistance, supporting that DNA repair functions are utilized by the parasite to cope with cellular damage induced by activated artemisinins (for example Gibbons et al., 2018; Sheriff et al., 2021; Suthram et al., 2020; Xiong et al., 2020). An enhanced capacity for DNA-repair may be an underlying advantage common to resistance-phenotypes more generally that may also hasten their spread (Xiong et al., 2020). Several DNA damage-response genes met our criteria for iSAFE analysis, with two recently characterized double-strand break repair proteins identified as harboring possible favored mutations (DNA replication-factor C subunit 1, RFC1; the RecQ helicase BLM) (Sheriff et al., 2021; Suthram et al., 2020). A small molecule-inhibitor of PBLM was also found to act synergistically with both artemisinin and chloroquine against multi-drug-resistant parasites, suggesting the utility of targeting these pathways for promising co-therapies (Suthram et al., 2020).

Calcium-dependent protein kinase 5, CDPK5 (PF3D7_1337800) is an enzyme that has been shown to regulate parasite egress from the host cell (Dvorin et al., 2010). The CDPKs are unique to plants and some protists, including apicomplexans. Blomqvist et al. recently identified 50 proteins with significantly reduced phosphorylation using a conditional CDPK5 knockdown (Blomqvist et al., 2020). An enrichment analysis of these 50 proteins based on gene ontology showed significant enrichment for proteins with transport and transmembrane transport activity, as well as proteins located in vesicles and the cell periphery, consistent with artemisinin resistance mechanisms involving endocytosis. Existing compounds, including kinase inhibitors within the Tres Cantos anti-malaria compound set (TCAMS) (Gamo et al., 2010), have been predicted to target CDPK5, among other kinases, and these compounds could be explored for activity on multidrug-resistant parasites.

In addition to providing further evidence in support of recently identified mechanisms of artemisinin resistance, our analysis also identified the gene encoding FIKK4.2 as potentially containing favored mutations in artemisinin resistant parasites. FIKK4.2 (PF3D7_0424700) is an exported kinase belonging to the FIKK multigene family, which is comprised of 20 genes on 11 chromosomes (Davies et al., 2020; Nunes et al., 2007). The FIKK kinases are specific to apicomplexans and are thought to play a role in host cell remodeling in response to intracellular, and possibly extracellular, changes in infected erythrocytes (Nunes et al., 2007). FIKK4.2 is unique in that it contains a large central repetitive region and, of the human malaria parasite species, is specific to P. falciparum (Kats et al., 2014). FIKK4.2 localizes to a compartment of the infected erythrocyte distinct from the Maurer’s clefts or J-dots, and disruption of the gene encoding FIKK4.2 leads to dramatically altered mechanical properties and cytoadherence of infected erythrocytes (Kats et al., 2014). Specifically, erythrocytes infected with FIKK4.2 knockout parasites were less rigid and less adhesive than erythrocytes harboring parasites with functional FIKK4.2 (Kats et al., 2014), a phenotype also observed in cells infected with artemisinin-resistant parasites (Ndour et al., 2015; Wojnarski et al., 2019). Further studies would be required to determine whether mutations in FIKK4.2 contribute to changes in deformability observed in artemisinin resistant parasites.

In addition to providing further evidence in support of recently identified mechanisms of artemisinin resistance, our meta-analysis of population genomic and transcriptomic studies identified potential new or related targets that may be leveraged to counter artemisinin-resistant parasites. These new targets will require functional characterization to validate their contribution to reduced artemisinin susceptibility, as it is possible that the selection signals identified could be driven by other selective pressures, including partner drugs or other pressures not related to drug treatment. In addition, because our iSAFE analysis compared parasites from the predominant mutant Kelch13 lineage circulating in the eastern Greater Mekong (Agrawal et al., 2017; Amato et al., 2017) to parasites from the pre-artemisinin resistant genetic subpopulation (Mietto et al., 2013), it is possible that our analysis may not have captured the contribution of gene products involved in non-kelch-mediated mechanisms of resistance. Likewise, our analysis focused on biallelic SNPs, and therefore would not have detected signals from gene copy number variants, which are also commonly observed in drug-resistant parasites (Cowell et al., 2018) and which are more difficult to detect because they can arise on different genetic backgrounds (i.e., a soft sweep).
5. Conclusions

In conclusion, we have implemented a novel approach integrating findings from previous population genomic and transcriptomic studies with new data acquired in genome-wide functional screens. By looking at the union of results from these different types of studies, we can more easily differentiate determinants of drug resistance from determinants of successful in vitro culture, as most population-level studies utilize sequencing data generated directly from clinical isolates without culture adaptation. Our analysis has corroborated the contribution of altered processes related to proteasomal degradation, endocytosis, vesicular trafficking and stress responses in artemisinin resistance, and has shown the utility of iSAFE to narrow the list of possible favored mutations within the region of a selective sweep, potentially accelerating time to functional validation. In addition, our analysis emphasizes the importance of future studies of the role of exported proteins and host-cell remodeling in artemisinin resistance.

Declaration of competing interest

The authors have nothing to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.05.006.

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