*Two alternative titles:*

An alternative pathway to reduced artemisinin susceptibility in *Plasmodium falciparum*

An actin-binding protein can modulate malaria parasite artemisinin susceptibility

Ryan Henrici & Colin J. Sutherland\*

Department of Immunology & Infection,

Faculty of Infectious & Tropical Diseases,

London School of Hygiene & Tropical Medicine.

\*Corresponding author

Malaria case management across endemic regions of the globe relies on artemisinin (ART) combination therapies for the rapid treatment of acute *Plasmodium* infection and prevention of severe disease. However, the efficacy of combination therapy is threatened by reduced sensitivity of *Plasmodium falciparum* to ART and partner drugs, which in turn may compromise the progress of control and elimination campaigns. Clinically, this phenomenon is most prevalent in Southeast Asia, where it manifests *in vivo* as measurably slower clearance of parasitaemia (1-3). There are also limited reports of patients with recrudescent infections of African origin (4-6), but slow clearance as measured by the microscopic criterion deployed in the Greater Mekong region is not present. Much effort has gone into understanding the genetic basis of ART susceptibility and, following the development of an *in vitro* correlate of clinical ART susceptibilityby Witkowski *et al.* (7), it was demonstrated that variants in the *pfk13* locus mediate the slow clearance phenotype in southeast Asia (8). The genetic diversity of this locus worldwide has since been studied extensively *in vivo* (9-12) and *in vitro* (7,11). Further, reverse genetic approaches with genome editing technologies including zinc finger nucleases and CRISPR-Cas9 have validated the involvement of these *pfkelch13* mutations in ring-stage ART resistance (12,13). Now, in PNAS, Demas *et al.* report and validate the role of the actin-binding protein Coronin in reducing *P. falciparum* ART susceptibility *in vitro* (14).

Conventional approaches to evolving and studying parasite resistance to ART have been challenging. Unlike other clinically-approved antimalarial drugs, artemisinin does not seem to inhibit a single target or pathway in the cell. Instead, the drug rapidly and promiscuously oxidises intracellular material, overwhelming the parasite cell’s damage response machinery (15). Despite its disseminated action, artemisinin derivates are fully metabolised and cleared from *in vivo* circulation within hours, meaning that most of parasite development occurs in the absence of drug. Consistent with this, early studies of recrudescent parasites revealed that ‘ART resistance’ results from a transient reduction in sensitivity during the first few hours of intra-erythrocytic development, but the same parasites are still fully susceptible to ART later in development (7, 15). Thus, in a treated febrile patient, a proportion of early stage parasites may survive the brief exposure to artemisinin that occurs after each dose, opening the door for recrudescent parasitaemia.

So far, there is no evidence that the K13 protein itself is the target of artemisinin. Instead, parasites expressing resistance-associated variants seem to be resilient to oxidative stress and have altered transcriptional activity in heat shock, redox, and ER stress response gene families (16). K13 has recently been implicated in PI3P-dependent vesicular traffic, and the function, regulation, and disruption of this pathway is of particular interest (17).

From a general perspective, these previous studies have provided valuable insight into the SE Asian ART resistance phenotype and the important role of *pfkelch13* mutations. But so far, there is no evidence of the same resistance-associated *pfkelch13* mutations in parasite isolates from Sub-Saharan Africa, nor the presence of other variants in this locus in documented cases of treatment failure (5,6,10). Additionally, recent studies have reported *pfkelch13-*independenttreatment failure in Southeast Asian patients, supporting the hypothesis that other loci are involved in modulation of ART susceptibility.

In their current report in the *Proceedings*, Demas *et al*. provide concrete evidence for this by experimentally evolving ART resistance in recently culture-adapted, ART-sensitive clinical isolates from Pikine and Thiès, Senegal (14). Importantly, the authors pulsed parasite cultures with dihydroartemisinin (the active metabolite of ART) repeatedly over four years. Clones of the resulting parasite lineages, Pikine-R and Thiès-R, displayed significantly reduced ring-stage susceptibility phenotypes (7.8 ± 1.0% and 7.6 ± 1.5% ring-stage survival, respectively; compared to <1% in parental), approaching the *in vitro* survival ability of the ART-R lineages in Southeast Asia. Parental lineages cultured continuously during the resistance evolution experiment remained fully sensitivity to ART. Both evolved lineages harboured unchanged, parental *pfkelch13* alleles. Neither lineage had mutations in ART-associated loci, including *pfk13, mdr1, mdr2, arps10,* and *crt.*

When compared to their progenitors, whole genome sequencing of Pikine-R and Thiès-R revealed ten unique SNPs outside of sub-telomeric regions. Interestingly, the authors found mutations in PF3D7\_1251200, which encodes PfCoronin, in both Pikine-R and Thiès-R. In Thiès-R, the authors found a mutation encoding PfCoronin(G50E), and in Pikine-R, two mutations encoding PfCoronin(R100K, E107V) (14).

Importantly, the authors then deployed Cas9 editing to validate the involvement of these mutations in modulating parasite susceptibility to ART. Introduction of the corresponding PfCoronin (G50E) mutation onto the Thiès background produced transgenic parasites with similar reduced ring-stage ART susceptibility. The same was found in clones of transgenic parasites expressing PfCoronin (R100K, E107V) on the Pikine background. Though the authors did not explore the evolved lineages further in this work, it would be interesting to understand the phenotypic contributions of each of the Pikine mutations. Individually, perhaps they have limited effects but synergise to cause a significant ART-R phenotype when introduced together. Alternatively, one of the two may be primarily responsible for the observed ART-R phenotype. Characterising the contributions of the other mutations identified by whole genome sequencing to artemisinin susceptibility and parasite fitness will also be informative. Notably, other developmental stages of both ART-R lineages were fully sensitive, strongly paralleling *pfk13-*mediated reduced susceptibility. Susceptibility to other antimalarials, including commonly deployed ART partner drugs, was not reported.

The strengths of this work lie in the authors’ use of two recent isolates as the backbone of their selection experiment and validation of *pfcoronin* mutations with Cas9 editing. Though it is unclear whether *in vitro* ring stage survival is predictive of clinical treatment outcome with these genotypes, Demas *et al* definitively show that *pfcoronin* is a contributor to artemisinin susceptibility in African parasites *in vitro*. Ariey *et al* identified *pfkelch13* mutants, which are now recognised as a major determinant of artemisinin treatment failure, in a similar experimental approach. Characterising the allelic diversity and evidence for directional selection of *coronin* variants in circulating patient isolates will be important and a focus of both retro- and prospective studies in the near future.

This new Coronin story deepens the relevance of a profound biological question: what cellular processes render early ring-stage *P. falciparum* parasites exquisitely susceptible to ART, and how are they exploited to evade or recover from drug action? From a cell biology perspective, it is tempting to speculate if and how Coronin, an actin-binding protein, might contribute to, interleave with, and diverge from the mechanisms underpinning the ART-resistance phenotype that have been defined in *pfk13*-mutant parasites. Recent evidence is supportive of a role for the inducible ER stress response in parasite susceptibility to artemisinin, and the authors note that actin dynamics and intracellular traffic are important extensions of this process (16, 18). K13 and Coronin both have beta-propeller domains but have different functions in higher eukaryotes. However, KEAP1, the orthologue of K13 in mammals is involved in regulation of a nuclear transcription factor, whereas K13 may be involved in intracellular traffic and protein turnover in *Plasmodium* (16, 17). Recent screens have suggested Coronin is not required for asexual survival in *P. falciparum*, which makes the biological role of the mutations reported by Demas *et al* all the more curious.

This work has important implications for surveillance of drug efficacy in endemic areas, particularly sub-Saharan Africa, but also including Asia and South America, where *pfk13*-independent treatment failure has been reported.Demas *et al.* demonstrate that variants of genes other than *pfk13* can reduce ART susceptibility *in vitro,* andsimply monitoring for resistance through genotyping at the *k13* locus will therefore miss novel genotypes. The authors also indicate other loci implicated in reduced susceptibility, but not as yet fully validated by reverse genetics, such as *pfubp1* and *pfap2mu* (19-20), which share with *pfcoronin* and *pfk13* putative roles in protein dynamics and intra-cellular trafficking. These data strongly suggest that phenotypicsurveillance of ART combination efficacy across malaria endemic areas of the world is essential to ensure emerging ART resistance in *P. falciparum*, and in other species in the genus, is identified in time to introduce corrective action.

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**Figure Legend Impact of transient ring-stage ART exposure on intra-erythrocyitc growth of *P. falciparum***

Parasites expressing certain genetic variants of Coronin (14) and Kelch 13 protein (8) have increased chance of survival following a brief ring-stage pulse of ART *in vitro*. Other genes such as *pfap2mu* and *pfubp1* may also encode variant proteins conferring a similar phenotype, but have not yet been fully validated. ART exposure at any other stage of intra-erythrocytic development, later than 6-8hr post-invasion, is lethal for all genotypes.

ART: artemisinin

mut: mutant form

WT: wild-type