The newly discovered role of endocytosis in artemisinin resistance

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Abstract
Artemisinin and its derivatives (ART) are the cornerstone of malaria treatment as part of artemisinin combination therapy (ACT). However, reduced susceptibility to artemisinin as well as its partner drugs threatens the usefulness of ACTs. Single point mutations in the parasite protein Kelch13 (K13) are necessary and sufficient for the reduced sensitivity of malaria parasites to ART but several alternative mechanisms for this resistance have been proposed. Recent work found that K13 is involved in the endocytosis of host cell cytosol and indicated that this is the process responsible for resistance in parasites with mutated K13. These studies also identified a series of further proteins that act together with K13 in the same pathway, including previously suspected resistance proteins such as UBP1 and AP-2μ. Here, we give a brief overview of artemisinin resistance, present the recent evidence of the role of endocytosis in ART resistance and discuss previous hypotheses in light of this new evidence. We also give an outlook on how the new insights might affect future research.

KEYWORDS
artemisinin, endocytosis, hemoglobin uptake, Kelch13, malaria, Plasmodium falciparum, resistance, vesicle trafficking
**INTRODUCTION**

Malaria is one of the deadliest infectious diseases, killing over half a million people every year.\(^1\) Malaria deaths have decreased by more than a third since 2004 despite global population growth.\(^1,2\) One of the factors making this reduction possible is efficient treatment which relies on artemisinin and its derivatives (ART), which are applied in combination with partner drugs, so-called ART combination therapies (ACTs).\(^3\)

Artemisinin was purified in the early 1970s\(^4\) and like its derivatives contains an unusual peroxide bridge in an endoperoxide 1,2,4-trioxane ring. In the parasite, this endoperoxide is activated by heme-derived iron.\(^5\) The resulting free radicals react with nucleophiles in proteins, lipids, and heme. The reaction with proteins causes non-specific protein unfolding and damage.\(^6,7\) This also includes damage to the proteasome, which disposes of unfolded and damaged proteins in healthy cells. As a result, the parasites are exposed to stress from damaged proteins which cannot be resolved.\(^8\)

### 1.1 Occurrence of ART resistance

In 2006, the WHO mandated ART to be used only in combination with other drugs to avoid the development of resistance. At that point, decreased sensitivity to the drug had emerged in Southeast Asia.\(^9\) This region had previously been the birthplace of resistance to two other antimalarials, chloroquine, and sulfadoxine–pyrimethamine.\(^10,11\)

In the peer-reviewed literature, ART resistance was first reported in Cambodia in 2008.\(^12\) By 2014, resistance had emerged in Thailand, Vietnam, Myanmar, and Laos.\(^13–16\) Around this time, single point mutations in the gene coding for K13 were determined as a molecular marker for ART resistance\(^17\) and shown to explain resistance in field isolates.\(^18\) As a result, endemic artemisinin resistance is currently defined as \(\geq 5\%\) of patients carrying K13 resistance-confirmed mutations, all of whom have been found to have either persistent parasitemia by microscopy on Day 3 or a half-life of the parasite clearance slope \(\geq 5\) hours after treatment.\(^19\) A K13 mutation is resistance-confirmed when it correlates with slow clearance in clinical studies and with increased survival in ring-stage survival assays (RSA) in vitro.\(^20\) The RSA is an in vitro assay to measure ART resistance by exposing early ring-stage parasites to a 6-h drug pulse (Figure 1). It mimics the pharmacokinetics of ART that result in only short effective serum concentrations.\(^23\) A list of confirmed K13 mutations is available.\(^20\) The resistance conferred by K13 mutations is a partial resistance as only ring-stage parasites survive ART treatment. Due to the short in vivo half-life of ART this can lead to delayed parasite clearance and treatment failure.\(^15,20,24–27\)

In 2016, nearly 70% of *Plasmodium falciparum* isolates from Cambodia harbored resistance mutations in K13.\(^28\) In Southeast Asia, K13 mutations spread by both clonal expansion and through multiple de novo occurrences.\(^29\) Outside of Asia (Oceania, Africa, South America) resistance mutations occurred de novo as early as 2010\(^20,31\) but are so far less common.\(^28,32–34\) In Guyana (South America), Papua New Guinea (Oceania), and Rwanda (Africa) resistant isolates are endemic at over 5%.\(^30,35–37\) Additionally, K13 resistance mutations, including C580Y, which is the most prominent mutation in Southeast Asia, were detected in Cameroon\(^38\) and Ghana.\(^39\) Despite these reports of potentially resistant parasites in Africa, significant clinical consequences such as the clearance delay on par with those observed in Southeast Asia have not been reported in African settings.

### 1.2 Understanding the mechanism of resistance

In an effort to understand which genetic factors determine resistance, several research groups created resistant parasites cell lines under laboratory settings by long-term selection with ART pulses. Ariey et al.\(^17\) first
demonstrated the presence of mutant \textit{kelch13} in such resistant lines and provided the genotype–phenotype link that led to establishing this gene as a marker for resistance in Southeast Asia. Recent detailed studies on the localization and functional characterization of K13 have since provided evidence that this protein is involved in the endocytosis of host cell hemoglobin. These findings and their implications for resistance will be discussed in this review. Other in vitro ART resistance selection regimes resulted in the detection of resistance conferring mutations in other genes.\textsuperscript{40–44} While none of these mutations were found to be predictive of resistance in patients, some of these were now found to act in the same pathway as K13 and will also be discussed here.

Other approaches to understand ART resistance were the study of resistant field isolates\textsuperscript{14,45,46} and—once K13 mutations were identified as causative in 2014—the study of K13 mutations in isogenic backgrounds.\textsuperscript{8,18,47–49} These studies led to hypotheses involving ubiquitination, stress response systems, and dormancy to explain how K13 mutation causes ART resistance. These hypotheses will be discussed in light of the recent discoveries on the role of K13 in endocytosis.

\textsc{FIGURE 1} Imaged ring-stage survival assay using dihydroartemisinin (DHA) with resistant (\textit{Kelch}\textsuperscript{C580Y})\textsuperscript{21} parasites, shows the maturation of a surviving parasite completing the cycle and giving rise to second-generation parasites (cycle 2) whereas the other parasites die as a result of the DHA pulse. Parasites were imaged using differential interference contrast long-term confocal time-lapse imaging\textsuperscript{22} starting immediately following DHA exposure. Single z-section of selected time points are shown. The age of parasites in each shown time point is given as time post-invasion (hpi). DHA affected (orange) and surviving (green) parasites, and second-generation parasites (blue) resulting from the surviving parasites are indicated by arrows. Released hemozoin from the ruptured schizont is also indicated by an arrow (white). Note that the life cycle time of in vitro cultured 3D7-derived parasites is typically several hours less than 48 h.
REDUCED ENDOCYTOSIS CAUSES ART RESISTANCE

2.1 Resistance mutations reduce K13 abundance

What effect do the mutations in the propeller domain have on K13? Ariey et al. predicted mutations would destabilize the K13 protein and alter its function. Indeed, resistant field isolates have lower levels of K13 protein and gene-edited K13 alleles harboring resistance-causing R539T or C580Y polymorphisms result in lower K13 protein abundance than their WT equivalents in isogenic backgrounds. In support of a causal connection between resistance and K13 abundance, reducing the amount of wild-type (WT) K13 at its active site by mis-localization results in ART resistance and overexpression of WT K13 or K13C580Y in resistant parasites, rendered the parasites sensitive. In conclusion, resistance mutations in the K13 propeller domain lead to reduced K13 levels, which is sufficient to render the parasite resistant. Hence, a change in function is not required to explain the resistance phenotype. While K13 protein levels are reduced in ART-resistant parasites, kelch13 messenger RNA levels are not. Therefore, the stability of K13 is likely reduced, as previously predicted.

2.2 K13 physiological function in endocytosis

A variety of cellular locations have been reported for K13. A GFP–K13 fusion expressed from the genomic k13 locus was found in foci close to the parasite’s food vacuole but did not colocalize with the endoplasmic reticulum (ER), Golgi, apicoplast, or the food vacuole itself. GFP-K13C580Y fully colocalized with K13WT. Antibody studies showed partial colocalization of K13 with various compartment markers of vesicular trafficking. Immune-electron microscopy using anti-K13 antibodies identified K13 in membrane-bound structures near ER, food vacuole, and parasite plasma membrane, some of which likely are cytostomes. Super-resolution microscopy found GFP-K13 in ring-shaped structures at the parasite surface, which could be part of the cytostomal neck based on their similar locations and diameter. While these results paint a heterogenous picture of K13 locations, it is likely that physiologically active protein is located at the cytostome (Figure 2A).

Further hints for K13 function were obtained using a novel inducible biotin-labeling approach (termed DiQ-BioID) to determine proteins in close proximity to K13. The resulting list of associated proteins contained 10 uncharacterized proteins (KIC1–10) as well as MCA2, Eps15, UBP1, and PFK9. DiQ-BioID of K13 C580Y yielded the same hits as K13 WT, supporting the conclusion that K13 resistance mutations only alter abundance, not function. Application of DiQ-BioID to Eps15, one of the candidates resulting from the K13 screen, resulted in a similar list of proteins, validating the approach. Subsequent fusion of the endogenous genes of the KICs as well as Eps15 and UBP1 with the sequence encoding GFP revealed colocalization of all candidates with K13, confirming that all these proteins were part of the compartment K13 locates to K13 compartment. Exceptions were PFK9 and KIC10, which did not colocalize with K13. PFK9 was also found in an unrelated DiQBioID and was concluded to be a nonspecific contaminant.

Because Eps15, the top hit among the annotated proteins, is involved in endocytosis in other organisms, a role for K13 in endocytosis was suspected. By using correlative light and electron microscopy, Eps15 was found in the proximity of host cell cytosol-filled (consisting mainly of hemoglobin) membranous structures, possibly cytostomes. Previously, electron microscopy studies found that in asexual parasites, hemoglobin is taken up into vesicles, originating from endocytic events, which are transported to and fuse with the parasite food vacuole (Figure 2A). Some of these vesicles and compartments contain phosphoinositide 3-phosphate (PI(3)P), a typical feature of endosomal vesicles in model organisms. The molecular mechanism of P. falciparum host cell cytosol uptake (HCCU), however, was—and remains—poorly understood.

Using a food vacuole bloating assay, AP-2μ (discussed in detail below), UBP1, KIC7, and Eps15, but not K13, were found to contribute to endocytic transport of hemoglobin to the food vacuole in trophozoites. While
inactivation of AP-2μ, UBP1, KIC7, and Eps15 negatively affected all life cycle stages, K13 was only important in ring stages. Reduction of K13 levels was found to reduce endocytosis in rings, as seen by fluorescent dextran uptake,51 and delayed or arrested growth in rings depending on the remaining K13 levels.21,51 A delay in ring stage development was also observed in resistant parasites,46,51,65 linking the phenotype observed by reducing active protein with the finding that mutant parasites contain less K13 protein.50 These findings indicated that K13-compartment proteins influence endocytosis in all asexual life cycles stages (involvement in rings and trophozoites is already known),51 with exception of K13 which is only required for endocytosis in rings (Figure 2B).
Two independent studies aimed to identify interactors of K13 by co-immunoprecipitation (IP) using anti-K13 antibodies. The resulting data sets of interactors included proteins from many different cellular compartments, including also several proteins involved in endocytosis in eukaryotes. Four proteins were shared between the co-IPs of the two studies, two ribosome proteins, one translation initiation factor and SAMS, a protein involved in methionine metabolism. Only PFK9 was shared with the proteins identified in the proximity of K13 by BioID where it had been considered a contaminant. In contrast to the interactors found by BioID, which affect the same endocytic pathway when inactivated, the function of the co-IP hits in ART resistance remains to be investigated. One of the studies using IPs suggested interactions between K13 and mitochondrial proteins, congruent with alterations seen in mitochondrial function in ART-resistant parasites. Future work will need to address how a protein such as K13 could get into contact with mitochondrial proteins. As opposed to BioID, which investigates proximity in intact cells, co-IP informs on physical interactions under the conditions of cell lysis. Co-IP is better suited to detect stable rather than dynamic interactions. The differing results between BioID and co-IPs might therefore indicate that the BioID identified K13 compartment proteins represent dynamic interactors. In this respect, it is noteworthy that the K13-BioID hit Eps15 was co-immunoprecipitated with K13 after crosslinking of parasites which would retain dynamic interactions.

2.3 AP-2 functions with K13, not with clathrin

In model organisms, a prominent form of endocytosis is mediated by clathrin-coated vesicles, which depends on clathrin and the AP-2 adaptor complex. Surprisingly, GFP-K13 colocalized with AP-2μ in live cells but not with the clathrin heavy chain (CHC). This finding was confirmed by immunofluorescence assays which found that AP-2μ colocalizes with K13 but not with CHC. AP-2μ also immunoprecipitates with all four AP-2 complex subunits but not with clathrin light or heavy chain. CHC, on the contrary, immunoprecipitated with all subunits of the AP-1 complex and to a lesser extent also with the AP-3 complex subunits but not with AP-2 complex-specific subunits. In a CHC DiQ-BioID, CHC was found to be in close proximity to subunits of the AP-1 complex, the AP-4 complex, and to a nonstatistically significant extent AP-3 subunits. AP-2 subunits were not significantly enriched. It, therefore, seems likely that AP-2 function is independent of clathrin in P. falciparum parasites.

Clathrin-independent action of the AP-2 complex had previously only been observed in Aspergillus nidulans, where AP-2 is also involved in endocytosis. While clathrin-independent endocytosis mechanisms are common, these do not depend on AP-2, which typically has the function to connect cargo receptors with the forming clathrin vesicle coat. The initiation of endocytic events to internalize hemoglobin may therefore be unusual in malaria parasites.

Interestingly, there is no evidence for a direct interaction between K13 and AP-2 complex subunits, despite the colocalization that has been observed through fluorescence microscopy and despite Eps15 (which associates with K13) harboring potential AP-2 binding sites. The AP-2 subunit was not significantly enriched in the K13 or Eps15 BioID, nor was K13 found in AP-2μ immunoprecipitation. It is therefore possible that there are substructures in the K13 compartment as previously proposed.

Other interactors of AP-2μ identified in high abundance in immunoprecipitation were Kelch10 (K10), EH domain-containing protein (EHd, PF3D7_0304200), and a GTPase activating protein. The IP interaction with K10 was confirmed by reciprocal IP. K10, like K13, contains Kelch motifs, yet there is only low sequence similarity (e-value 1e−3) between the two Kelch proteins, no similarity outside the Kelch domain and K10, as an N-propeller protein containing four predicted Kelch blades, belongs to a different type of Kelch protein than K13, which is an N-dimer-C-propeller protein containing six Kelch blades. A K10 codon 623 polymorphism has previously been identified as co-selected with K13 variants. As Kelch propeller domains occur in a large number of proteins with diverse functions, it cannot be concluded that K13 and K10 have equivalent roles. More work will be needed to elucidate whether K10 has a function in endocytosis and in ART resistance. EHd was previously connected with...
endocytosis-dependent processes and with lipid storage. Whether it does indeed colocalize with AP-2μ and other K13-compartment proteins and is involved in endocytosis or ART resistance remains to be determined.

Taken together, these studies showed that K13 and associated proteins are involved in endocytosis and are part of a compartment that colocalizes with AP-2μ, but is distinct from clathrin (Figure 2). This suggests an unusual clathrin-independent endocytosis pathway in malaria parasites.

2.4 | Inactivation of K13 and K13 compartment proteins causes ART resistance

The connection between endocytosis and K13-mediated ART resistance became obvious when K13 was conditionally inactivated by mislocalization to the nucleus (knock sideways) which resulted in reduced ART susceptibility in RSA and in increased abundance of hemoglobin-like peptides, suggesting reduced hemoglobin catabolism. Reduced hemoglobin digestion was also previously detected in culture-adapted resistant field isolates. Parasites with a resistance-conferring mutation in K13 endocytosed less host cell cytosol in young rings, further supporting the connection between resistance and endocytosis.

Previous work had shown that reduction of hemoglobin digestion, either by using inhibitors or by conditional inactivation of falcipains, which are food vacuole proteases, resulted in reduced susceptibility to ART. Hemoglobin digestion leads to the release of heme, which can activate ART. The role of K13 in hemoglobin endocytosis and resistance indicates that K13-mediated resistance is achieved by downregulation of the HCCU, which leads to reduced ART activation, resulting in a reduced effective drug concentration.

Further support comes from experiments in which lipocalin-like protein PV5 (PF3D7_0925900, PfLCN) was knocked down. These PV5-deficient parasites are less efficient in polymerizing toxic heme to hemozoin and were hypersensitive to artesunate, chloroquine, and atovaquone, suggesting that increased levels of heme result in increased susceptibility to artemisinin and other drugs related to hemoglobin digestion. However, it is worth noting that decreased hemozoin formation also leads to an increased level of free radicals, which might lead to additional cellular stress. It had also been observed previously that reduced temperature leads to higher survival rates of ART pulses in otherwise sensitive parasites. This is consistent with the role of endocytosis in resistance because vesicular trafficking often is a temperature-dependent process. Finally, the levels of PI(3)P, through regulation by kinase PI3K, have been reported to modulate ART resistance. This could also modulate host cell cytosol uptake—a hypothesis that remains to be tested.

Disruption of nonessential K13-compartment proteins KIC4, KIC5, and MCA2, as well as inactivation of essential K13-compartment proteins AP-2, UBP1, KIC7, and Eps15 also resulted in ART resistance in RSAs. Hence, all K13-compartment proteins that were involved in endocytosis, also resulted in resistance upon their inactivation. Further, the stage-specific transcription of the corresponding genes was found to be similar, suggesting they were part of a coregulated pathway. The relevance of this pathway for resistance was further illustrated by the finding that the R3138H mutation in UBP1, which had been identified as a possible factor of ART resistance in a genomic survey of parasite field samples, resulted in resistance in RSA.

In conclusion, there is an entire endocytic pathway centered around the K13-compartment, and reduced endocytic activity from this compartment, rather than a K13-specific qualitative change, causes resistance. This explains why reduction of the activity of any of the involved proteins leads to resistance. Identification of this pathway also validated UBP1 and AP-2μ to be involved in ART resistance, as had been suspected previously, and connected them with the K13-mediated resistance mechanism. Nevertheless, K13 mutations are the most prominent resistance mutations observed in the field. ART resistance is associated with a fitness cost and reduced endocytosis might be the reason for this. It was proposed that the role of K13 only in ring stages compared with the additional role of other K13 compartment proteins in trophozoites, causes K13 mutations to incur lower fitness costs. This could be the reason why K13 mutations are most commonly found in the field.
3 | NEW PERSPECTIVES ON PREVIOUS EVIDENCE

3.1 | Potential involvement of K13 in ubiquitin ligation

Before the studies on K13’s role in endocytosis, it was unclear how the K13 mutations would lead to ART resistance because data on the physiological function of K13 were lacking. Yet even today, it is unknown what mechanistic role K13 plays in endocytosis. It is also unclear how the role in endocytosis relates to previous and recent observations indicating wide-ranging alterations in ART-resistant parasites which either could result from K13-unrelated changes needed for resistance (see Section 3.5.1 below), downstream effects of reduced endocytosis or from additional roles of K13.46,49,52,53,57,84–86

*Pfkelch13* is an intron-free gene located on chromosome 13. The encoded 84 kDa protein K13 consists of a *Plasmodium*-specific region at the N-terminus, followed by a BTB/POZ domain and a C-terminal propeller domain consisting of six kelch motif repeats (residues 444–721) (Figure 3A).54 All resistance mutations20,82 lie in the propeller domain and most occur in the first three repeats (Figure 3A).54 Interestingly, none of the resistance mutations fall onto the fifth and large parts of the fourth Kelch repeat which are located opposite of the region of the propeller connecting to the BTB/POZ domain (Figure 3). Whether Kelch repeats four and five are less suitable for resistance mutations because they are functionally too constrained to be mutated or so dispensable that mutations confer no resistance, is not clear. The substitutions that confer resistance do not follow a recognizable pattern; neither charge, hydrophobicity, size nor position in the Kelch repeat predict their effect on resistance and fitness. The latter is relevant because a high fitness cost of substitutions could curb the benefit of resistance in some strains.78 A possible determinant of ART resistance could be tertiary structure stability, even though it is difficult to predict given the unknown native oligomeric state of K13 and the incomplete structural information. Models based on the dimeric partial crystal structures 4YY8 and 4ZGC, nonetheless, seem to have some predictive power regarding resistance and fitness effects.55 The first indication of the function of K13 came from the similarity with other proteins of known function that contain a BTB/POZ dimerization domain and kelch propeller domains. This combination of domains is also found in the human Kelch-like (KLHL) proteins, which act as substrate adaptors to bring a Cullin 3 RING E3 ligase close to its substrates.87 Ubiquitin is then attached to the substrates in a process...
involving E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and the E3 ubiquitin ligase. KLHL proteins bind Cullin 3 via the BTB domain and their substrates via the shallow groove of their Kelch domain formed collectively by the Kelch repeat motives. In K13, no resistance mutations affect the surface of this shallow pocket, suggesting it might be required for the correct function of K13 (Figure 3B).

The most similar human protein, KLHL-8, also has a BTB/POZ dimerization domain and kelch propeller (30% protein sequence identity, 40% coverage, e-value 4e−36). KLHL-8 is required for the ubiquitination and regulated degradation of rapsyn in neuromuscular junctions. Motivated by the hypothesis that an enhanced cellular stress response might be the mechanism of ART resistance (see below), similarity to human protein KEAP1 (KLHL19) was previously highlighted (28% protein sequence identity, 44% coverage, e-value 2e−26; <1 Å RMSD, PDB 2DYH, PDB 4YY8). In mammals, KEAP1 regulates oxidative stress. Seven KLHLs, including KLHL8, were found in a genome-wide screen to be needed for endocytosis. However, given the wide variety of substrates regulated by KLHL proteins, the level of similarity to a particular human KLHL likely is not predictive of its substrate specificity. Hence, neither the similarity to KLHLs involved in endocytosis nor to KEAP1 is likely predictive of the process Kelch13 is involved in but illustrates the diversity of processes that can be regulated by different KLHLs.

It is conceivable that K13 modulates endocytosis through ubiquitination. A similar role has been observed for several KLHLs and for human cullin 3 in CD22 endocytosis. Involvement of K13 in endocytosis and subsequent vesicular trafficking would therefore not be unexpected with the predicted function of this protein in the cullin pathway. If K13 has this regulatory role as a ubiquitination adapter, it might affect several pathways. Such a heterogeneous function might explain the various effects that have been observed in K13 mutants besides endocytosis, on the life cycle, the mitochondria, on DNA damage repair, and on stress response proteins. Alternatively, some or all of these might be downstream effects of a single direct effect of K13. Future work will need to address which of these effects are a direct result of Kelch13 function. In addition, it will be important to determine which effects cause resistance and which are a result rather than a cause of resistance, including compensatory effects.

Despite the similarity to human KLHLs, the lack of E1, E2, and E3 proteins in K13 interactomes is puzzling. Does it indicate that K13 plays a role independent of the canonical cullin pathway? The presence of the putative ubiquitin hydrolase UBP1 could indicate a regulation through de-ubiquitination, particularly as for example the function of Eps15 in endocytosis is known to be regulated by ubiquitination. The endocytic process involved in K13-mediated ART resistance is significantly divergent from endocytosis in mammals, as indicated by AP-2 acting without clathrin and by the many Plasmodium-specific proteins involved (KICs). It, therefore, remains a possibility that the function of K13, too, may have diverged from that of KLHL proteins. It was also noted that the BTB/POZ domain of K13 is not typical for KLHLs. It, therefore, remains to be shown whether the similarity to KLHL is indicative of the mechanism by which K13 influences endocytosis or other processes associated with ART resistance.

### 3.2 K13-independent resistance

Nonsynonymous mutations in four proteins, K13, AP-2µ, UBP1, and coronin, as well as deletions or knockdown of several others have been shown to cause ART resistance in vitro. K13 mutations are responsible for ART resistance in most patients. As no common mechanism for K13 and the other proteins was known, it was thought that several mechanisms of resistance may exist. The new understanding of the role of K13 in endocytosis revealed that at least some of the other proteins in that pathway confer resistance by the same mechanism: reducing ART activation by reducing HCCU. Here, we discuss, which proteins can confer K13-independent ART resistance by modulating hemoglobin uptake and degradation and how the resulting resistances might differ from K13-mediated ART resistance. Possible HCCU-independent ART resistance mechanisms, based on dormancy and stress response pathways, are discussed in the subsequent paragraphs.
AP-2μ, now known to be a K13-compartment protein,51,68 was first associated with ART resistance when the AP-2μ I568T mutation occurred at the same time as resistance in a Plasmodium chabaudi strain selected under drug pressure in laboratory conditions.40 The authors suggested two possible mechanisms. The one confirmed by recent studies was that reduced endocytosis leads to reduced hemoglobin digestion. As an alternative mechanism, endocytosis was suggested to be a route for uptake of ART and potentially other drugs and reduced endocytosis might foster resistance through reducing drug uptake.40 This hypothesis has not received much attention and could be further investigated. However, endocytic uptake of ART likely would not be affected by genetic or chemical inhibition of hemoglobin digestion,76,77 favoring the first hypothesis that AP-2μ influences resistance through reduced hemoglobin uptake rather than uptake of the drug.

When the AP-2μ mutation was engineered into P. falciparum parasites, they became less susceptible to DHA than WT parasites of isogenic background.96 However, the corresponding mutation was not found in clinical P. falciparum isolates,40,100 yet parasites from Kenya, which showed submicroscopic persistence 3 days after treatment, showed variants of the pfcrt, pfmdr1, pfap2-μ, and pfubp1 genes.100 The AP-2μ polymorphisms S160N/T, found in this study, were significantly enriched after treatment with dihydroartemisinin–piperaquine.100 Overexpression of the AP-2μ S160N in a WT background resulted in lower sensitivity to ART than the overexpression of AP-2μ WT in the same background.98 Interestingly, this indicates that AP-2μ S160N is a dominant mutation that exerts its effect in the presence of AP-2μ WT as opposed to K13 mutations which are not dominant.51

The reduced susceptibility of parasites expressing AP-2μ S160N was less pronounced than that of K13 resistance mutations, but interestingly resulted in additional reduced susceptibility to quinine.98 The study also provides some evidence for decreased susceptibility to chloroquine and lumefantrine.98 This raises the concerning possibility that K13-compartment proteins which are required throughout the asexual cycle, like AP-2μ and UBP1, might contribute to reduced sensitivity to antimalarials besides ART (Figure 2B).

Another study also found co-resistance to quinine after selecting parasites exclusively with ART.101 Interestingly, the resistant parasites displayed increased IC50 values, suggesting that the resistance mechanism was not limited to ring stage parasites. Unfortunately, a causative mutation was not identified, but K13 and UBP1 mutations were excluded.

UBP1 polymorphisms associated with posttreatment parasite persistence have been observed in P. falciparum isolates from Kenya100,102 and from Thailand,73 of which the latter was validated to confer ART resistance in RSA (R3138H51). In in vitro-selected P. chabaudi parasites, UBP1 V2697F occurred in artesunate-resistant parasites and UBP1 V2728F in ART-, chloroquine-, and mefloquine-resistant parasites.42,43 To confirm whether these mutations were causative of reduced ART susceptibility, equivalent mutations were introduced in P. falciparum and Plasmodium berghei and compared with isogenic parasites.96,102 Studies in all three organisms agree that the PcUBP1 V2697F mutation (and equivalent mutations) results only in ART resistance, but not in chloroquine resistance. PcUBP1 V2728F and the equivalent mutation in P. berghei were shown to cause ART and chloroquine resistance, while in a different assay, an equivalent mutation in P. falciparum resulted in no detectable resistance to either drug.42,43,96,103 Whether this is because of species-specific differences or different in vitro and in vivo assays used is not clear.

The observation that AP-2μ and UBP1 mutations confer low-level resistance to other drugs, while K13 mutations confer only ART resistance, might be due to the different roles these proteins play in endocytosis (Figure 2). K13 is
required in the ring stage and mutations reduce endocytosis in rings, leading to their survival of short drug pulses. This is relevant because of the short (<1 h) half-life of ART in vivo.\textsuperscript{104} Other K13 compartment proteins are involved in endocytosis in rings and trophozoites.\textsuperscript{51} This includes AP-2μ and UBP1 and might explain why some mutations in these proteins, but not K13 mutations, also conferred resistance to quinine and chloroquine, which have a longer serum half-life.

Common to these drugs and ART is that they depend on hemoglobin digestion, which is, in turn, dependent on endocytosis. Reduced endocytosis, therefore, reduces the drug action either by reducing the amount of active drug (ART) or by reducing the amount of available target of the drug (lumefantrin, chloroquine, quinine, mefloquine) (Table 1).

| Mode of action | (Potential) mode of action | Resistance mechanism |
|----------------|---------------------------|---------------------|
| Sulfadoxine-pyrimethamine\textsuperscript{105} | Yes | - Inhibits two enzymes in the folate biosynthesis pathway: DHP S and DHFR | - Quintuple SNPs: pfdhfr (51I, 59R, 108N) and pfdhps (437G, 540E) |
| Lumefantrine\textsuperscript{105} | No | - Interferes with heme detoxification | - pfcrt K76 SNP - pfmdr1 N86 SNP - Protein transporters may be involved |
| Amodiaquine\textsuperscript{105} | Yes | - Accumulates in DV, binds to toxic heme, prevents the formation of inert/nontoxic hemozoine - Similar to chloroquine | - SNPs in pfcrt and pfmdr1 in vivo association - pfct 76T and pfmdr 86T in vitro |
| Piperaquine\textsuperscript{105} | No | - Inhibition of steps in hemoglobin degradation - Interferes with heme detoxification | - CNV in plasmpesin II and III (debated)\textsuperscript{106,107} - pfct 76T SNP |
| Mefloquine | No | - May inhibit heme detoxification - May inhibit PfMDR1 - May target cytoplasmic ribosome | - CNV in pfmdr1 - SNPs in pfmrp1 and pfmrp2 |
| Pyronaridine\textsuperscript{105} | No | - Interferes with hemozoin formation | - No resistance reported - In vitro decreased susceptibility associated with pfct 76T Note that the use is not yet recommended by WHO |
| ART | Yes | - Activated by heme\textsuperscript{5} - Free radicals cause protein damage and block protein stress response\textsuperscript{6} | - SNPs in K13\textsuperscript{17} - Less frequent, SNPs in UBP1 and AP-2μ\textsuperscript{96,103} |

Abbreviations: ACT, artemisinin combination therapy; ART, artemisinin and its derivatives; CNV, copy number variant; SNP, single-nucleotide polymorphism.
The requirement for AP-2μ and UBP1 in rings and trophozoites, may result in a higher fitness cost compared with mutations in K13 which is only required in rings. This would also explain why K13 mutations result in higher RSA survival than mutations in AP-2μ and UBP1 and consequently why K13 mutations are more common in field isolates. However, mutations in AP-2μ, UBP1 and possibly other K13-compartment proteins pose the risk of one-step resistance to the two drugs of a combination therapy. Yet, at least in Southeast Asia, K13 mutations are most prevalent, suggesting that the clinical relevance of this risk might be slim.

3.2.4 | Coronin and ART resistance

Mutations in the coronin gene have also been shown to cause ART resistance in vitro compared to parasites with isogenic background. As observed for K13 mutations, only susceptibility measured by RSA, not EC50, was reduced in coronin mutants, indicating that parasites remain sensitive to long ART exposure, similar to parasites with K13 mutations. Resistance to other drugs was not investigated. G50E, R100K, and E107V were observed by in vitro selection but have not been observed in patient isolates to date. Coronin organizes actin filaments and is required for Plasmodium parasite motility and has been speculated to contribute to the same endocytic process as K13, by influencing actin. While coronin has so far not been observed to interact with K13-compartment proteins, a recent study found that the K13 C580Y mutation masked the effect of coronin mutations in two different isogenic backgrounds, and suggested that both K13 and coronin act in the same pathway. Further investigation of the roles of coronin and actin during intracellular blood stage development will be needed to elucidate whether and how coronin-mediated resistance modulates hemoglobin uptake.

3.2.5 | Other proteins

Deletion or knockdown of several K13 compartment proteins, as well as falcipain 2a and 3, causes ART resistance. It, therefore, seems possible that polymorphisms in the corresponding genes could cause ART resistance and several, such as in KIC6, EPS15, and MyoC have already been suspected. However, because they were unknown until recently, they have not been systematically screened for association with ART resistance in patients yet.

Many other proteins have been associated with ART resistance (discussed in Section 3.5). Yet, no proteins that were mutated, knocked down or deleted and compared with isogenic strains were so far found to cause resistance independent of hemoglobin uptake and degradation, with the possible exception of coronin, whose involvement in endocytosis remains to be investigated.

3.3 | Dormancy

Even after multiple doses of ART, recrudescence was reported in patients. This has been attributed to dormant parasites that remain in an inert state and later on reinitiate development. Several studies describe such recrudescence from in vitro culture, mice, monkeys, and humans. However, at the estimated 100 dormant parasites per milliliter of blood they are difficult to detect individually. Dormant parasites occur in both, ART-sensitive and -resistant parasites, and based on their low frequency, they are unlikely to contribute to the K13-mediated ART resistance but were proposed to be a separate mechanism that might be more important in sensitive parasites. As dormant parasites are believed to be arrested ring stages, they likely digest reduced amounts of hemoglobin which would—similar to K13-mediated ART resistance—reduce their susceptibility to ART. Another possibility is that parasites might survive treatment in privileged niches where ART might be less effective, as has been shown for P. berghei that can survive at sites of hematopoiesis and might act as a reservoir for the parasite.
It will be critical to directly track the development of individual dormant parasites that reinitiate growth again to understand the mechanism of dormancy and be able to exclude with certainty that the phenomenon of long-term recrudescence is not derived from a small population of surviving non-dormant parasites.

3.4 | Stress response

Before the role of K13 in endocytosis was established, a prominent hypothesis was that an enhanced stress response in K13 mutants is responsible for their resistance.49,57,85,91 This was a tempting hypothesis because ART kills by causing oxidative stress and unfolded protein stress.6,8,123,124 But this mode of action also made it difficult to discern ART-induced effects from resistance-causing effects and from resistance-associated side effects of K13 mutations.

Evidence of cellular stress was observed by monitoring phosphorylation of eIF2α, levels of ubiquitinated proteins or by transcriptomics.8,41,64,49,85 Susceptible parasites, when exposed to lethal doses of ART, have higher stress response levels than resistant parasites.85 This suggests that elevated stress responses alone are not sufficient to protect against ART. Resistant parasites already show some increased phosphorylation of eIF2α compared to wild type in the absence of ART,50,85 suggesting the K13 mutation itself causes some stress but basal protein turnover was found to be similar in resistant and sensitive parasites, as was the response to ER stress.50 The elevated eIF2α phosphorylation may be related to the fitness cost of the K13 mutations that was previously observed.83,108 It was hypothesized that the reduced hemoglobin uptake and digestion in resistant parasites may restrict the level of resistance due to a reduced availability of amino acids in ring stages.51 The lower amino acid levels due to the K13-based resistance mechanism (apart from isoleucine, which is not present in hemoglobin) may be a reason for the increased eIF2α phosphorylation in resistant parasites.

A K13-independent resistance mechanism related to cellular stress was proposed based on two parasite lines obtained by in vitro selection, which showed upregulated stress response proteins in a transcriptional analysis.41 These parasites had, among other mutations, regions of increased copy number. Overexpression of three genes related to stress response that were found in these regions, pf6pgd, pftrx1, and pfspp, increased the ART dose tolerated for 4 h in ring stage by up to threefold. This contrasts with a 10- and 100-fold increase in ART dose tolerated in the selected resistant cell lines. These genes are therefore not the only contributors to resistance. The selected parasites also had single-nucleotide polymorphisms (SNPs), including a non-sense mutation in falcipain 2a and a non-synonymous mutation in AP-2α, respectively. Given the new understanding of the involvement of hemoglobin uptake and degradation in ART resistance, it would be interesting to test whether these changes contributed to the resistance phenotype.

Even though elevated stress responses only seem to make a small contribution to resistance, an intact stress response is required to survive even sublethal effective doses of ART, independent of the K13 genotype.59 It has further been suggested that an elevated stress response is required as a second component in addition to reduced endocytosis to survive DHA exposure.71 Inhibitors affecting the stress response, like deubiquitinase inhibitors and proteasome inhibitors, or increasing stress therefore synergize with ART49,97,125 and make Plasmodium-specific proteasome inhibitors, which are also potent as monotherapy, promising candidates for new partner drugs.126-130

3.5 | Genetic background and geographic distribution

3.5.1 | Genetic background

There are two general groups of genes besides K13 that influence ART resistance. With our improved understanding of the K13-resistance mechanism and the pathway in which it is involved, we can begin to classify these
proteins. The first group comprises genes that are sufficient to influence ART resistance on their own. This includes endocytosis proteins such as UBP1 and AP-2μ for which also resistance mutations were observed in field samples but also proteins known or suspected to be involved in endocytosis such as KIC4, 5, 7, MCA-2, coronin, and PI3K for all of which so far only laboratory data exists. This group also includes proteins that confer resistance independent of endocytosis, like Falcipain 2a and Falcipain 3. It is likely that there are additional proteins in this category that can confer resistance by influencing endocytosis or through novel mechanisms, which are not yet known. However, given the prominence of K13 mutations, at least in Southeast Asia, the clinical relevance of such genes likely is not high.

The second group of mutations comprises changes that do not by themselves confer resistance but act in combination with resistance-causing mutations. These could either be changes that provide a background on which K13 mutations can arise more easily or that contribute to optimal growth after resistance has occurred. This could, for instance, be compensatory mutations to reduce the fitness cost of resistance. Evidence for a role of the genetic background in K13-mediated resistance comes from studies showing that the same K13 mutation results in very different levels of resistance in RSA when introduced into different genetic backgrounds. Candidates for this group of genes were identified in studies that observed mutations overrepresented in parasites with K13 resistance mutations in patient samples and in in vitro selected parasite strains. Over 30 genes have been associated with K13 resistance mutations and were recently reviewed. However, assigning genes to this category is the most difficult and no confirmed examples can be given. In the future, introducing candidate mutations and comparing to parasites of isogenic background might enable the elucidation of the potential roles of genetic background mutations.

A third group, that we call hitchhiker mutations, may also be found to be associated with K13 mutations in patients as the result of confounding influences, but does not influence ART resistance. This includes polymorphisms that are overrepresented because they happened to be present in a K13 mutant parasite that clonally expanded. Additionally, mutations causing resistance to partner drugs might frequently occur together with K13 resistance mutations because reduced selection pressure from one drug eases resistance development to other drugs.

### 3.5.2 Geographic distribution

K13 mutations and ART resistance occur at much higher frequencies in Southeast Asia than in the rest of the world. Especially striking is the near-absence of K13 polymorphisms in Africa, where 93% of all malaria cases occur. In fact, K13 resistance mutations were initially found to be absent in Africa, but have now been detected in several African countries. One hypothesis to explain the occurrence of ART resistance mainly in Southeast Asia proposed a specific genetic background is required for the K13 mutation to arise. However, in vitro experiments showed that K13 mutations can be introduced in parasites of African origin and confer in vitro resistance. In fact, the first study that ever found the K13 mutation and linked it to resistance in Southeast Asia, used African parasites. Together with the detection of K13 resistance mutations in African parasites, this suggests that the genetic background of African parasites is, in principle, permissive for K13 mutations that result in ART resistance. Further, Rwandan parasites harboring K13 resistance mutations had no altered mutation frequency in 10 candidate “genetic background” genes. This could suggest that additional mutations making up the permissive background are more important in vivo or that the genetic background is not the main reason for the geographic distribution of K13 mutations.

Alternatively, the geographic distribution of resistant parasites could be independent of parasite genetic backgrounds. A defining feature of many African settings compared with other malaria endemic regions, including Southeast Asia, are their high endemicity levels. The ensuing higher level of immunity results in lower treatment frequencies while at the same time multiple infections in a single host are common, exacerbating the fitness cost of
resistance. Hence, while ART pressure is infrequent in high transmission settings, parasite fitness is critical, both of which could slow the spread of resistance. In contrast, the major selection pressure in Southeast Asia is treatment with ACTs. If lower treatment rates are the cause for the lower occurrence of resistance in Africa, resistance is expected to only slowly spread to the African continent, as has been the case for other resistances. Chloroquine and sulfadoxine–pyrimethamine resistance, for example, also evolved in Southeast Asia and spread from there, suggesting that the geographic difference may not be an ART-specific effect but possibly that low transmission settings are generally more permissive to the development of resistance.

The high levels of host immunity in many African regions might itself contribute. Antibodies have been reported to be more effective against K13-mutant parasites than against wild type. The higher immunity in high transmission areas has been predicted to slow the emergence of resistance by 10 years compared with low transmission areas.

Finally, there are different partner drug combinations that are used in different regions. For instance, artemether–lumefantrine (AL) is more prominently used in Africa whereas several countries in Southeast Asia use dihydroartemisinin–piperaquine, although these combinations are not exclusive to each of these regions. Different ACTs differ in the mechanism of action of the partner drug (Table 1) and in the frequency with which the ACT is applied, and both could potentially influence the spread of ART resistance. Notably, AL is applied at 12-h intervals, compared with 24-h intervals for other ACTs, a factor that has been hypothesized to counter the spread of resistance. Depending on their mechanism of action, different partner drugs might also influence resistance, by acting synergistically or antagonistically with the ART action and resistance mechanism. The mode of action of the frequently used partner drugs lumefantrine, piperaquine, and amodiaquine is related to hemoglobin catabolism and could influence the K13-mediated ART resistance pathway, for example, by modifying the amount of ART-activating hemoglobin degradation products. However, as the different partner drug combinations are not exclusive to Africa and Southeast Asia, they are unlikely to explain the geographic difference in the development of resistance on their own but might be a contributing factor.

4 | OUTLOOK

4.1 | Implications for treatment

The development of new antimalarial drugs has the potential to decrease our dependence on ART for malaria treatments. New or improved ART, such as second-generation ozonide antimalarials, have longer in vivo half-lives and in this way may partially overcome K13-mediated ART resistance. Further, new or improved partner drugs can help to manage partner-drug resistance, which is widespread especially in Southeast Asia. Knowledge of the ART resistance mechanism can now be used to select partner drugs that have unrelated or synergistic targets.

What can be done until new drugs are available? The fact that K13 mutants only survive short pulses of ART, because the effect of K13 mutations to reduce endocytosis is restricted to ring stages, may prevent higher resistance levels. Furthermore, the tradeoff between resistance and hemoglobin uptake which provides amino acid to the young ring likely further restricts the levels of resistance achievable. K13 C580Y seems to be the SNP that best strikes the balance of maximized resistance and minimized fitness cost, and is, therefore, most commonly found. K13 mutations R539T and I543T confer higher levels of resistance but also have higher fitness costs and are less commonly found. Overall, it therefore seems that optimal resistance levels are already reached and it is possible that higher resistance levels from K13 mutants will not emerge. In accordance with this, there is some evidence that the majority of strains with reduced ART susceptibility may still be treatable with modified timings and frequencies of ART doses which might prolong the usefulness of ACTs.

While 3-day regimens of artesunate monotherapy are not sufficient to clear parasites from the bloodstream on Day 3, a 4-day regimen monotherapy has been predicted to be effective against K13-mutant parasites. This is
supported by the observation that a 6-day regimen of ACT was effective in patients with resistant parasites.\textsuperscript{14} Split-dose regimens, where half the dose is taken every 12 h instead of the full dose every 24 h, have also been predicted to improve clearance times of K13 mutant-resistant parasites based on computational models of the disease.\textsuperscript{49,148,149} However, clinical data of parasites (K13 status was not assessed) showed no enhanced cure rates with split dosing.\textsuperscript{155}

Apart from K13, other proteins involved in the same pathway are also needed for endocytosis in trophozoites. Mutations in these proteins, for example, AP-2μ and UBP1, could therefore result in reduced susceptibility to ART throughout the life cycle.\textsuperscript{42,43,97,98} Resistance caused by these mutations might not be overcome by prolonged administration of ART and might additionally reduce the susceptibility to other drugs. However, mutations causing such multidrug resistance have so far conferred lower levels of resistance to ART than K13 mutations,\textsuperscript{42,43,51,97,98} probably because of the negative effect mutations in these genes have on later stages, restricting the possible reduction of endocytosis. Additionally, it was suggested that even strong effects on the IC\textsubscript{50} would not have a large effect on survival because the short half-life of ART in the blood reduces the benefit of full resistance (which increases IC\textsubscript{50}) compared with the partial resistance caused by K13 mutations.\textsuperscript{148}

To reduce the chance of treatment failure, it is important to reduce the selection pressure on partner drugs now. Extended ACT regimens, sequential treatment with different ACTs, shorter intervals between ACT doses, and combinations of multiple ACTs (Refs. 24,156 and in clinical trials NCT03923725, NCT02297477, PACTR202010540737215) might be ways to achieve that.

4.2 | Diagnostics and monitoring

K13 resistance mutations have proven to be a good proxy for the occurrence of resistant parasites in Southeast Asia. The recent detection of such mutations in Africa, Oceania, and South America emphasizes the importance to further monitor K13 resistance mutations globally.

The seven additional proteins that act in the same pathway as K13 and cause resistance when impaired in function\textsuperscript{51} or unrelated proteins influencing the same pathway such as falcipains\textsuperscript{77} are candidates for new resistance-conferring mutations. Several reports of rare delayed parasites clearance in Africa, where the parasites do not carry K13 mutations, might be due to mutations in other K13-pathway genes.\textsuperscript{33,34,100,141,157} Specifically, it would be interesting to monitor the occurrence of SNPs and CNVs in UBP1, AP-2μ, coronin, EPS15, KIC4, KIC5, KIC7, MCA2, and possibly PI3K in isolates from patients that present with delayed clearance. Vice versa, SNPs associated with resistance could indicate further genes potentially involved in endocytosis or otherwise influencing hemoglobin catabolism.

4.3 | Further research on the K13 mechanism

The role of K13 in endocytosis highlights the clinical relevance of endocytosis in \textit{Plasmodium} blood stages (for a review of apicomplexan endocytosis see Spielmann et al.\textsuperscript{64}). Apart from ART, many other antimalarials, including all but one ACT partner drug capitalize on the parasite-specific process of host cell cytosol uptake (Table 1). Nonetheless, this process remains poorly understood. The recent studies reviewed here have brought some important findings but also opened up many more fascinating questions about endocytosis.

Malaria parasites employ AP-2 in a clathrin-independent manner. Is this a unique adaptation or a wider-spread clathrin-independent endocytosis mechanism previously overlooked in model organisms? How do the newly identified K13-compartment proteins orchestrate endocytosis? Is actin (and coronin) involved? When and where do vesicles bud? How is curvature generated? How is the rate of endocytosis regulated? What is the function of non-
essential KICs at the K13 compartment? Are they redundant? Are any K13 compartment proteins with their low or absent homology to human proteins suitable targets for new drugs? A better understanding of endocytosis might also improve our understanding of ACT partner drugs, many of which still have only partially characterized mechanisms of action and resistance.

Accelerated genetic manipulation through selection-linked integration and conditional inactivation systems have enabled the recent elucidation of K13-associated HCCU.\textsuperscript{21,50,51,68} Genome editing using zinc-finger nucleases and CRISPR/CAS9 were instrumental in confirming K13 mutations in resistance.\textsuperscript{18,95} The improved genetic tractability and improved tools for gene function studies for \textit{P. falciparum} parasites\textsuperscript{21,95,158–162} are expected to allow to determine the roles and contributions of the many proteins previously associated with the emergence of K13 mutations and with resistance.\textsuperscript{136} Which mutations confer resistance? Which alleviates fitness costs? Which are hitchhiker mutations? Which are unrelated? Which of the effects observed in K13 mutants cause resistance and which are compensatory or downstream effects? Proteins like coronin\textsuperscript{64} and PI3K\textsuperscript{163} have been implicated in resistance and a role in endocytosis seems possible but has not yet been confirmed. It will be useful to know whether HCCU-independent resistance mechanisms exist to inform the selection of partner drugs to avoid synergistic resistance mechanisms. Furthermore, compensatory changes highlight vulnerabilities of the parasite that could be exploited to maintain the usefulness of ART in the future.

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