Spatial and molecular mapping of *Pfkelch13* gene polymorphism in Africa in the era of emerging *Plasmodium falciparum* resistance to artemisinin: a systematic review

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The spread of *Plasmodium falciparum* isolates carrying mutations in the *kelch13* (*Pfkelch13*) gene associated with artemisinin resistance (*Pf*ART-R) in southeast Asia threatens malaria control and elimination efforts. Emergence of *Pf*ART-R in Africa would result in a major public health problem. In this systematic review, we investigate the frequency and spatial distribution of *Pfkelch13* mutants in Africa, including mutants linked to *Pf*ART-R in southeast Asia. Seven databases were searched (PubMed, Embase, Scopus, African Journal Online, African Index Medicus, Bioline, and Web of Science) for relevant articles about polymorphisms of the *Pfkelch13* gene in Africa before January, 2019. Following PRISMA guidelines, 53 studies that sequenced the *Pfkelch13* gene of 23 100 sample isolates in 41 sub-Saharan African countries were included. The *Pfkelch13* sequence was highly polymorphic (292 alleles, including 255 in the *Pfkelch13*-propeller domain) but with mutations occurring at very low relative frequencies. Non-synonymous mutations were found in only 626 isolates (2·7%) from west, central, and east Africa. According to WHO, nine different mutations linked to *Pf*ART-R in southeast Asia (Phe446Ile, Cys469Tyr, Met476Ile, Arg515Lys, Ser522Cys, Pro553Leu, Val568Gly, Pro574Leu, and Ala675Val) were detected, mainly in east Africa. Several other *Pfkelch13* mutations, such as those structurally similar to southeast Asia *Pf*ART-R mutations, were also identified, but their relevance for drug resistance is still unknown. This systematic review shows that Africa, thought to not have established PfART-R, reported resistance-related mutants in the past 5 years. Surveillance using *Pf*ART-R molecular markers can provide valuable decision-making information to sustain the effectiveness of artemisinin in Africa.

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See Online for appendix

Introduction

An estimated 219 million new malaria cases and 435 000 deaths occurred globally in 2017, with more than 75% of these cases coming from Africa.[1] In the absence of an effective vaccine, reducing the burden of *Plasmodium falciparum* malaria relies on the effectiveness of artemisinin-based combination therapies (ACTs).[1,2] ACTs combine the rapid antimalarial action (but short half-life) of artemisinin or its derivatives with the slower action (but longer half-life) of partner drugs.[3] However, one major challenge for malaria control and elimination efforts is the emergence and spread of *P falciparum* artemisinin resistance (*Pf*ART-R) from the Greater Mekong subregion in southeast Asia over the past decade.[4–6]

A reverse migration of *Pf*ART-R resistance towards Africa (by comparison with the evolutionary origin and spread of the parasite)[7] is a troubling scenario that could have severe consequences on the burden of malaria because alternative therapies are few.[8–10] As this resistance is not yet established in Africa, monitoring *Pf*ART-R on the continent is necessary from a global health perspective.[9] Therapeutic efficacy trials are the standard method for assessing *Pf*ART-R; however, insufficient funding restricts these studies in African countries.[3,11,12] The delayed parasite clearance obtained in clinical trials and the in-vitro ring-stage (trophozoite) survival assay are also useful for tracking the emergence of artemisinin resistance.[5,13–15]

The association of specific single nucleotide polymorphisms (SNPs) in the *P falciparum* kelch 13 gene (*Pfkelch13*) with delayed parasite clearance has raised the potential of molecular markers for the surveillance of *Pf*ART-R.[14,16,17] More than 100 *Pfkelch13* mutations have been reported in Africa, but there is still little evidence of *Pf*ART-R mutants circulating in the continent.[9,18] In this systematic review, we examine studies reporting *Pfkelch13* SNPs across different African countries to determine the relative frequencies and spatial distribution of parasites carrying mutations currently considered to be *Pf*ART-R markers.

Methods

Search strategy and selection criteria

Our systematic review follows the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines.[19,20] Seven electronic medical databases (PubMed, Embase, Scopus, African Journal Online, African Index Medicus, Bioline, and Web of Science) were searched for peer-reviewed articles published before January, 2019, that have the relevant population, intervention, comparator, outcomes, and study design (PICOS) framework (appendix p 2).

A predetermined search strategy used French and English versions of keyword terms of the Medical Subject Headings 2018 database and free terms, such as ("malaria" OR "falciparum" OR "paludisme") AND ("marqueur moléculaire" OR "molecular marker" OR

"*kelch13*" OR "*Pfkelch13*" OR "K13" OR "*Pf*K13") AND ("Africa" OR "Afrique" OR each name of the 54 African countries) as detailed in appendix p 3). No filters or limitations (ie, language) ensured a large inclusion of informative reports. Individual searches on the internet allowed for the inclusion of references identified in primary reports. Inaccessible full-text articles were requested from corresponding authors. Although previous review papers were not included in the final analysis, they were sources for additional reports. Studies with data from an unknown sampling site or country, systematic reviews, modelling reports, reports based only on genetically modified isolates, conference presentations, abstracts with insufficient information, and letters or correspondence to editors were excluded.

Selection of studies, quality assessment, and data management

A structured data collection process addressed any possibility of uncertainty or missing data. Two masked teams (NKK, DYM, and AR-A; ET-K, VPT, and YY) worked independently at each stage of the process (literature search, relevance assessment, classification report and validation, and data extraction) before cross-checking and merging related outcomes. Extracted study variables included general information (authors, title of study, year of publication, geographical location of the study), study characteristics (study design, sampling period, collection time during the survey, participant characteristics, setting, sample size, and analytical methods), and SNP information (loci sequenced, alleles found, number of isolates sequenced at specific loci, and number of isolates with individual alleles). The Newcastle-Ottawa scale assessed three quality criteria in selected studies: the representativeness of samples (maximum one star), the sample size (maximum one star), and the study outcome (maximum three stars).[21] Regarding the outcome, the accurate definition of the genotype (given the reference), and the correct nomenclature of mutations following standard recommendations in molecular diagnostics[22,23] awarded quality stars to articles. Only moderate (two to three stars) and high (four to five stars) quality studies were included in our systematic review.

For the **UNIPROT protein database** see https://www.uniprot.org/uniprot/Q8IDQ2

Definitions

*Pf*ART-R is defined by a delayed parasite clearance representing partial resistance that affects only ring-stage parasites.[17] *Pf*ART-R mutations are SNPs in the *Pfkelch13*-propeller domain associated with *Pf*ART-R in vitro or in vivo. Our systematic review uses the most updated list of 31 *Pf*ART-R molecular markers released by WHO in August, 2018,[17] to classify *Pf*ART-R mutations into three categories. First, validated molecular markers significantly associated with both slow parasite clearance and reduced drug in-vitro sensitivity: Phe446Ile, Asn458Tyr, Met476Ile, Tyr493His, Arg539Thr, Ile543Thr, Pro553Leu, Arg561His, and Cys580Tyr. Second, candidate molecular markers significantly associated with slow parasite clearance: Pro441Leu, Gly449Ala, Cys469Phe, Ala481Val, Pro527His, Asn537Ile, Gly538Val, Val568Gly, Pro574Leu, Phe673Ile, and Ala675Val. Finally, molecular markers suspected to be associated with slow parasite clearance without reaching statistical significance because of the low number of mutants: Asp452Glu, Cys469Tyr, Lys479Ile, Arg515Lys, Ser522Cys, Asn537Asp, Arg575Lys, Met579Ile, Asp584Val, Pro667Thr, and His719Asn.

Mutations similar to molecular markers of *Pf*ART-R are also highlighted in this Review because they are structurally close to the WHO *Pf*ART-R mutations, although their clinical relevance is not yet established.

Data summary measures and synthesis

Since studies reporting *Pfkelch13* SNPs had different designs and used mainly convenience sampling, their data were not suitable for combining in a meta-analytic approach. Sequencing processes provided variable lengths of genetic sequences resulting in diverse ranges of loci analysed for allele discovery in studies. A narrative synthesis was done to systematically organise the information. SNPs in the *Pfkelch13* sequence were defined on the basis of the reference wild-type *Pfkelch13* sequence PF3D7_1343700, available in the UNIPROT protein database. Relative frequencies of each allele were summarised using the median values and interquartile ranges. Proportions of mutants with at least one non-synonymous change in the *Pfkelch13* gene within each geographical site were calculated. Data from individual reports and locations were geo-referenced and locus-referenced before being uploaded on maps to display spatial and molecular patterns using the ggplot2,[24] ggmap,[25] and rgdal[26] packages in R (version 3.5.3).[27] The risk of bias was minimised by excluding studies on malaria cases exported outside Africa and repeated communications on the same isolates.

Results

A list of 3756 records reporting individual SNPs in the *Pfkelch13* gene were identified through database searching, including four records found manually. After removal of 2236 duplicated reports, we screened 1520 records of which 1467 were considered ineligible according to the PICOS approach (figure 1). 53 studies remained after exclusion, with *P falciparum* sample isolates successfully sequenced for *Pfkelch13* in 41 African countries;[9,18,28–77] baseline characteristics of these studies are detailed (appendix pp 4, 7). Analytical methods used in studies were nested PCR with subsequent Sanger sequencing (49 studies), next-generation sequencing of targeted amplicons (two studies), next-generation sequencing of whole *P falciparum* genomes with a genome-wide association study (one study), and mapping reads to targeted references (one study). Different protocols were used in the exploration of SNPs in the *Pfkelch13* gene, with sequence lengths ranging from 445 to 2438 base pairs.

44 studies identified both *Pfkelch13* wild-type and mutant isolates, and nine found only wild-type isolates.

A total of 24 652 *P falciparum* isolates were analysed in the selected studies, yielding 23 100 isolates successfully sequenced at loci in the *Pfkelch13* gene. The majority of isolates (22 474 isolates [97·3%]) carried a *Pfkelch13* gene with either a wild-type sequence or a sequence displaying only synonymous mutations. The remaining 626 isolates (2·7%) had non-synonymous mutations, 604 (2·6%) of which were classified as missense substitutions in the amino acid sequence. Other non-synonymous mutations included 15 insertions (0·06%), four deletions (0·02%), and three nonsense substitutions (0·01%) (appendix p 8).

All the 41 African countries with information about *Pfkelch13* polymorphisms were in sub-Saharan Africa (appendix p 9). *Pfkelch13* non-synonymous mutations were absent in 11 countries: Botswana, Burundi, Guinea-Bissau, Liberia, Mauritania, Sierra Leone, Somalia, South Africa, South Sudan, Sudan, and Zimbabwe. Sequence haplotypes were heterogeneous in the other 30 countries, with surveys detecting indistinct wild-type *Pfkelch13* and *Pfkelch13* non-synonymous mutations (at least a single isolate carrying a non-synonymous mutation). High (>50%) and intermediate (40–50%) relative proportions of isolates with non-synonymous *Pfkelch13* mutations were reported in west Africa (Senegal and Nigeria) and east Africa (Kenya and Uganda; figure 2).

The majority of *Pfkelch13* polymorphic loci (149 of 182 [81·9%]) were detected in the propeller domain sequence (ie, downstream of codon position 440), with 255 allelic variations (213 non-synonymous alleles) among a total of 292 alleles found in *Pfkelch13* sequencing studies (figure 3). Lys189Thr was the most frequently reported non-synonymous allele in *Pfkelch13* gene sequencing studies and Ala578Ser was the most frequently reported non-synonymous allele in partial sequencing studies of the *Pfkelch13*-propeller domain. Lys189Thr was observed in 145 mutants with relative frequencies in surveys ranging from 0·8% to 50% (median 32·8%), whereas Ala578Ser was reported in 98 mutants at relative frequencies in surveys from 0·2% to 7·1% (median 1·4%). Lys189Thr and Ala578Ser spanned over several countries at variable relative frequencies and there were no distinctive geographical patterns in their distribution (appendix p 10). Relative frequencies of non-synonymous mutations in the *Pfkelch13*-propeller domain in surveyed sites ranged from 0·08% to 10·3% (appendix p 8).

Only 35 (0·15%) of the total 23 100 sequenced isolates recorded in this systematic review had alleles classified as candidate *Pf*ART-R markers by WHO: Cys469Tyr (Uganda),[29,45] Arg515Lys (Zambia),[9] Ser522Cys (Togo, Central African Republic, Gabon, DR Congo, Uganda, and Kenya),[9,42,62,78] Val568Gly (Kenya),[36] Pro574Leu (Rwanda),[69] and Ala675Val (Uganda and Rwanda).[29,45,69] Moreover, WHO-validated *Pf*ART-R markers were found in at least one isolate in four countries: Phe446Ile (Mali),[65] Met476Ile (Tanzania),[47] and Pro553Leu (Kenya and Malawi; figure 4).[77]

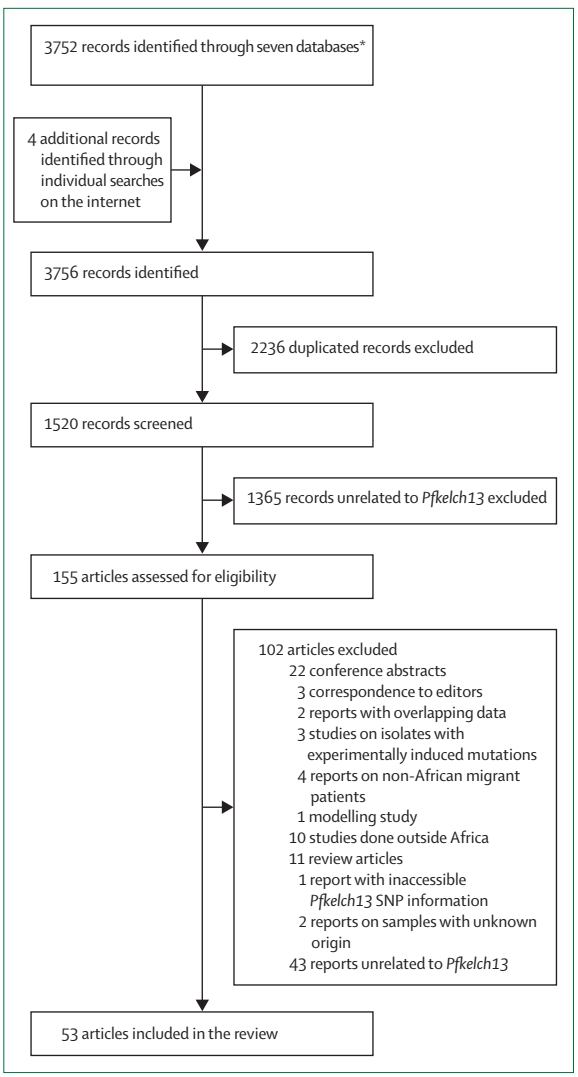

***Figure 1*: Flowchart of the article selection process**
Pfkelch13=*Plasmodium falciparum kelch13*. SNP=single-nucleotide polymorphisms. *PubMed (n=738), Scopus (n=101), Embase (n=754), African Index Medicus Database (n=1207), African Journals Online (n=10), Web of Science (n=926), Bioline (n=16).

16 alleles similar to WHO *Pf*ART-R markers were also found in 18 isolates (0·08% to 5·41% per sampling site) in seven countries: Gly449Asp and Gly449Ser (similar to Gly449Ala) in Mali,[65,77] Cys469Trp (similar to Cys469Tyr) in Kenya,[42] Met476Lys (similar to Met476Ile) in DR Congo,[62] Ser522Met and Ser522Arg (similar to Ser522Cys) in Togo, Uganda, and Kenya,[36,64,78] Arg539Ile and Arg539Lys (similar to Arg539Thr) in Senegal and Kenya,[36,70] Pro553Ile (similar to Pro553Leu) in Senegal,[70] Arg561Cys (similar to ArgR561His) in Mali and DR Congo,[9,77] Arg575Gly (similar to Arg575Lys) in Mali,[65] Asp584Glu, Asp584Asn, and Asp584Tyr (similar to Asp584Val) in Comoros, Mali, and Kenya;[36,44,65] and Pro667Arg and Pro667Ser (similar to Pro667Thr) in Kenya[36] (appendix p 11). There has been an increasing trend in reports of *Pf*ART-R molecular markers

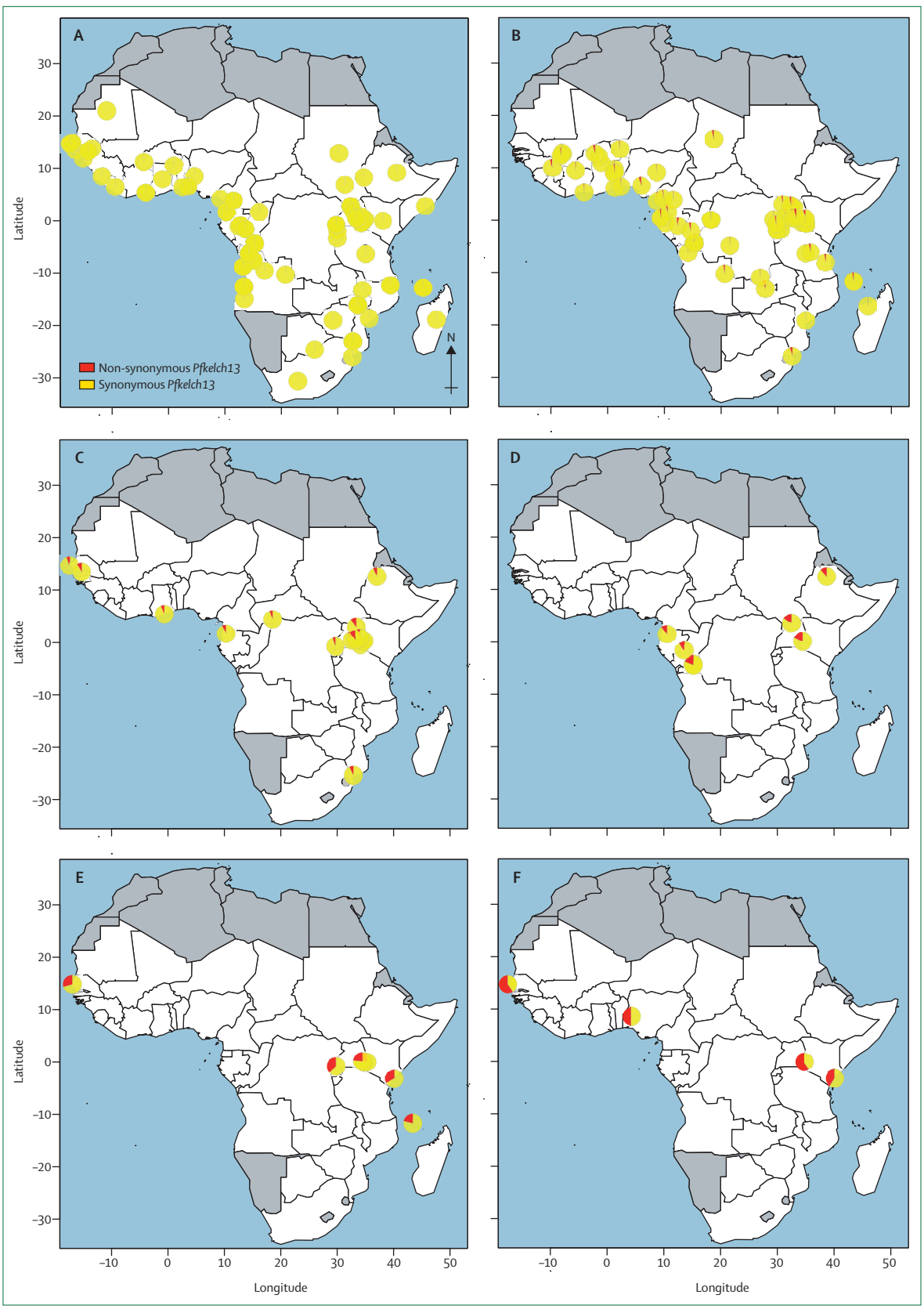

Figure 2: **Geographical maps displaying proportions of isolates carrying *Pfkelch13* with non-synonymous changes in Africa**
Pie charts displayed on these maps represent the proportions of isolates with only synonymous changes in the *Pfkelch13* gene, or with at least one non-synonymous change. Overall, isolates with non-synonymous changes were observed in sampling sites located within 30 of 41 surveyed countries in east, west, and central Africa. Moderate-to-high proportions of isolates with non-synonymous changes in the *Pfkelch13* sequence were found in east and west Africa. (A) Map showing surveys with only synonymous changes or wild-type isolates. (B) Map showing surveys with very low (>0% to <5%) proportions of isolates with non-synonymous *Pfkelch13* changes. (C) Low (5% to <10%) proportions of isolates with non-synonymous *Pfkelch13* changes. (D) Moderate (10% to 20%) proportions of isolates with non-synonymous *Pfkelch13* changes. (E) Low-intermediate (>20% to <40%) proportions of isolates with non-synonymous *Pfkelch13* changes. (F) Intermediate-high (40 to <80%) proportions of isolates with non-synonymous *Pfkelch13* changes.

and mutations similar to *Pf*ART-R markers in Africa since the first year of reporting in 2014 (figure 5).

Discussion

This paper is the first comprehensive and systematic review specifically focusing on polymorphisms of the *Pfkelch13* gene in Africa. *Pfkelch13* has exclusively been explored in sub-Saharan African countries, showing a highly polymorphic structure with most allelic variations located in the propeller domain. Despite low relative frequencies of non-synonymous mutations in this domain across the continent, we identified mutant alleles reported to be associated with diminished artemisinin responsiveness in southeast Asia and several alleles structurally similar to resistance mutations.

The large allelic variation in the *Pfkelch13*-propeller domain (despite being located in a conserved region of the *P falciparum* genome) is likely to reflect the adaptation of parasites to selective pressures in Africa (eg, the use of antimalarial drugs).[64,79] Therefore, this information should raise concerns among health policy makers to prevent the use of poor-quality (ie, counterfeit or substandard) artemisinin-based therapies and artemisinin monotherapies in Africa.[79,80] Sub-therapeutic antimalarial drug concentrations would provide ideal conditions for selecting *Pfkelch13*-propeller mutants, among which *Pf*ART-R mutants could emerge.[18,81] This hypothesis is supported by the generation of *Pfkelch13*-propeller mutations in isolates with an African genetic background that underwent in-vitro experimental artemisinin selective pressure, and the subsequent appearance of the *Pf*ART-R phenotype.[14,82]

Unlike the high frequency of non-synonymous mutations in southeast Asia (with proportions ranging from intermediate to fixation levels among sequenced parasites),[9] *Pfkelch13* non-synonymous mutations in Africa occur at very low relative frequencies, despite presenting high allelic variation. The delayed introduction

1	P441	F442L	M442V	P443	L444	V445	F446I	C447	I448L	I448M
	G449D	G449S	G450	F451	D452	G453	V454A	V454I	E455	Y456
	L457I	N458	S459S	M460	E461	L462	L463S	D464H	D464N	D464Y
	D464D	I465T	S466I	Q467H	Q467Q	Q468R	Q468Q	C469W	C469Y	C469C
	W470*	R471R	M472I	M472V	C473F	C473_	C473Y	T474T		
2	P475	M476I	M476K	S477Y	S477S	T478P	T478T	K479	K480R	A481
	Y482S	F483	G484R	S485	A486	V487I	L488S	N489S	S489K	N489N
	N490H	N490S	F491S	L492	Y493Y	V494A	V494I	V494L	F495L	G496G
	G496S	G497	N498I	N499D	N499O	Y500Y	D501G	Y502	K503K	A504G
	A504T	A504V	L505F	L505S	F506	E507E	T508	E509D	E509E	E509G
	V510M	V510_	V510V	Y511	D512N	R513R	R513S	L514	R515K	D516
	V517	W518C	Y519C	V520A	S521	S522C	S522M	S522R	S522S	N523T
	L524	N525	I526M							
3	P527	R528	R529K	N530I	N530K	N530Y	N531I	N531N	N531Y	C532S
	G533C	G533S	G533V	V534A	V534I	V534L	T535M	T535T	S536L	N537
	G538G	R539I	R539K	R539R	I540	Y541	C542G	C542Y	I543	G544R
	G545E	Y546	D547	G548D	G548G	G548S	S549P	S550	I551	I552M
	S552C	P553I	P553L	P553P	A554S	N554H	N554K	N554O	N554S	V555A
	V555L	E556K	E556V	A557S	Y558C	Y558H	D559N	H560L	R561C	M562T
	K563	A564	W565*	V566I	E567E	E567K	V568G	A569G	A569S	A569T
	P570L	L571	N572	T573A						
4	P574L	R575G	R575R	S576L	S577P	A578D	A578S	M579	C580	V581F
	V581V	A582	F583L	F583S	F583Y	D584E	D584N	D584Y	N585K	K586
	N587K	Y588C	L589I	V589I	V589V	I590F	G591D	G592E	G592G	G592R
	G592V	T593	N594	G595S	E596G	E596K	E596R	R597I	R597R	L598F
	L598L	N599	I601T	E602D	E602E	E602	V603	Y604	E605G	E606G
	K607	M608L	B609B	N609L	N609N	K610K	W611_	E612D	E612G	Q613E
	Q613H									
5	F614L	P615S	Y616N	A617T	A617V	L618	L619S	E620K	A621A	A621S
	R622G	R622I	S623	S624	G625R	A626P	A626S	A626T	A626V	A627
	F628L	N629T	Y630P	L631	N632	Q633R	Q633*	I634	Y635C	V636
	V637A	V637D	V637I	V637_	G638R	G639C	G639S	G639V	I640V	D641G
	N642	E643	H644	N645	I646	L647	D648H	S649	V650	E651
	Q652	Y653	Q654Q	P655	F656I	N657	K658	R659	W660C	Q661
	F662	L663	N664	G665C	G665S					
6	V666A	V666I	V666V	T667R	T667S	E668	K669K	K670	M671	N672
	F673	G674	A675V	A676S	A676V	T677A	T677K	L678V	S679	D680N
	S681	Y682	I683	I684	T685	G686	G687	E688	N689	G690C
	G690G	E691	V692	L693	N694	S695	C696	H697	F698F	F699
	S700	P701	D702	T703	N704	E705	W706	Q707	L708	G709
	P710	S711	L712	L713	V714	P715	R716	F717	G718	H719
	S720	V721	L722	I723	A724	N725	I726			

Wild-type residue or synonymous mutation
Non-synonymous mutation not classified by WHO as a *Pf*ART-R marker
Non-synonymous mutation classified by WHO as a *Pf*ART-R marker
Non-synonymous mutation mimicking a *Pf*ART-R marker

Figure 3: **Molecular map of alleles in the *Pfkelch13*-propeller domain of African isolates**

This figure shows allelic variations of amino acid residues within blades (1–6) of the *Pfkelch13*-propeller domain (after position 440) based on the *Plasmodium falciparum* 3D7 reference sequence. Of the 280 loci in this sequence, 150 were polymorphic with synonymous and non-synonymous alleles. Loci reported with either a wild-type residue or synonymous mutations are shaded in yellow. Colours in cells identify whether the non-synonymous mutation is classified as PfART-R marker (red), a mutation mimicking a known PfART-R (dark blue), and a non-PfART-R marker (light blue). Single-letter abbreviations for the amino acid residues are as follows: A=alanine, C=cysteine, D=aspartic acid, E=glutamic acid, F=phenylalanine, G=glycine, H=histidine, I=isoleucine, K=lysine, L=leucine, M=methionine, N=aspargine, P=proline, Q=glutamine, R=arginine, S=serine, T=threonine, V=valine, W=tryptophan, and Y=tyrosine. In mutants, reference amino acids at specified locations were substituted with other amino acids—eg, M476I indicates that methionine at codon position 476 was replaced by isoleucine. The asterisk (*) shows a substitution of a reference amino acid residue by a stop-codon within the mutant (nonsense mutation). The underscore (_) shows deletion of a reference amino acid residue at a given codon-position within the mutant (deletion). PfART-R=*Plasmodium falciparum* artemisinin resistance.

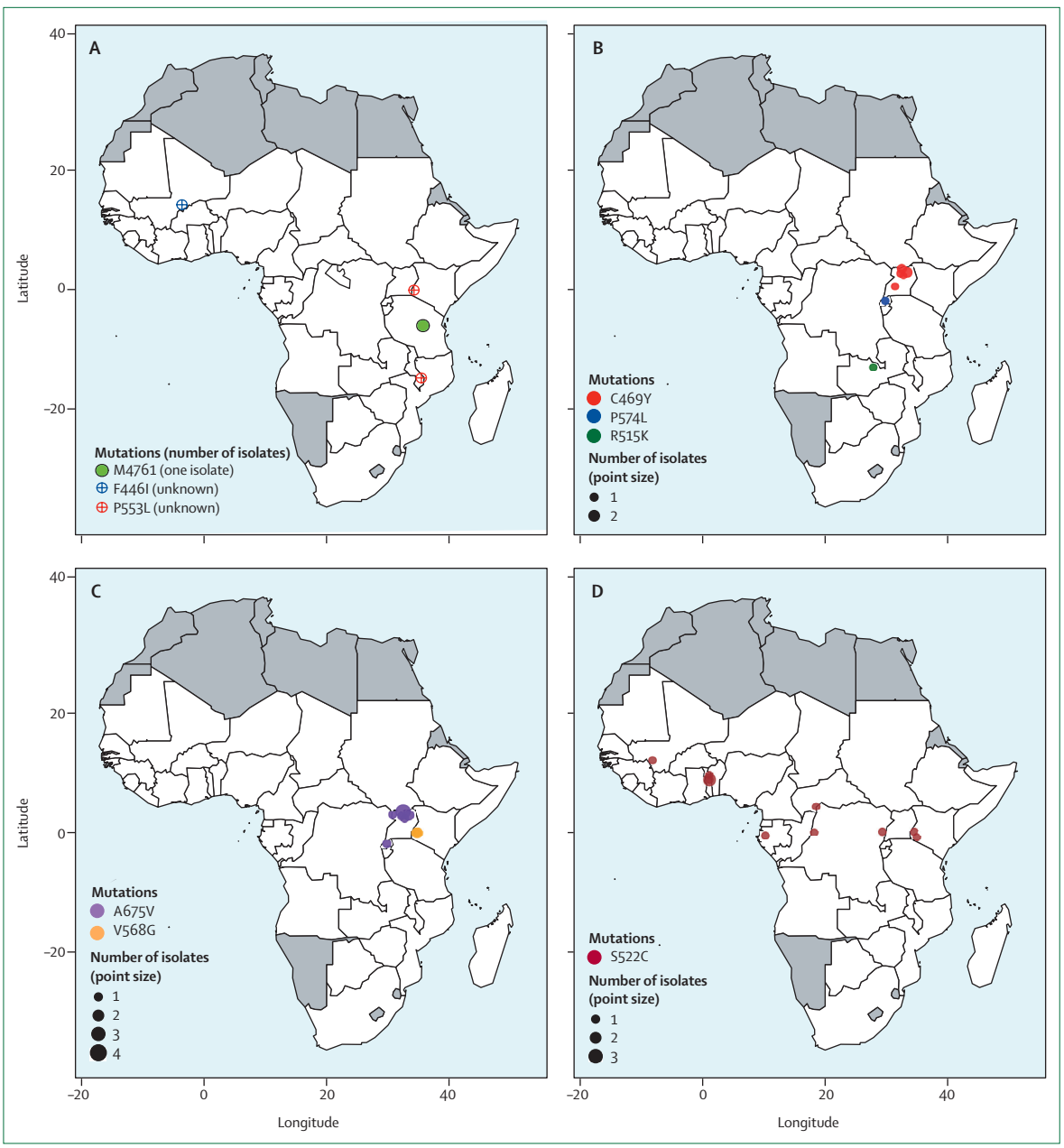

Figure 4: **Maps displaying sampling sites that recorded isolates carrying WHO *Pf*ART-R markers in Africa**
Points plotted on these maps represent isolates carrying mutations associated with *Pf*ART-R in southeast Asia according to the WHO 2018 list of *Pf*ART-R markers.[17] The point size is proportional to the number of isolates that were identified at each sampling location whenever it is known. Mutations with an unknown number of isolates are shown as crossed-out circles. (A) Map showing surveys that detected *Pf*ART-R markers validated by WHO in at least one isolate in four countries, including F446I in Mali, M476I in Tanzania, and P553L in Kenya and Malawi. (B–D) Maps showing surveys that recorded WHO candidate *Pf*ART-R markers including C469Y in Uganda, R515K in Zambia, S522C in Togo, Central African Republic, Gabon, DRC, Uganda, and Kenya, V568G in Kenya, P574L in Rwanda, and A675V in Uganda and Rwanda. Single-letter abbreviations for the amino acid residues are as follows: A=alanine, C=cysteine, F=phenylalanine, G=glycine, I=isoleucine, K=lysine, L=leucine, M=methionine, P=proline, S=serine, V=valine; and Y=tyrosine. Mutations show that reference amino acids at specified locations were substituted with other amino acids; for example, M476I indicates that a methionine at codon-position 476 was replaced by an isoleucine.
*Pf*ART-R=*Plasmodium falciparum* artemisinin resistance.

of artemisinin in Africa between 2000 and 2005, associated with a shorter time of drug pressure by comparison with the early use of artemisinin in southeast Asia since the 1970s, could explain why there is a scarcity of isolates with non-synonymous mutations in Africa.[83] Moreover, multi-country studies done in Africa, southeast Asia, and South America had suggested that non-synonymous *Pfkelch13* mutations reported in African countries could be due to local adaptation rather than to importation from Asia, because alleles were mostly Africa-specific

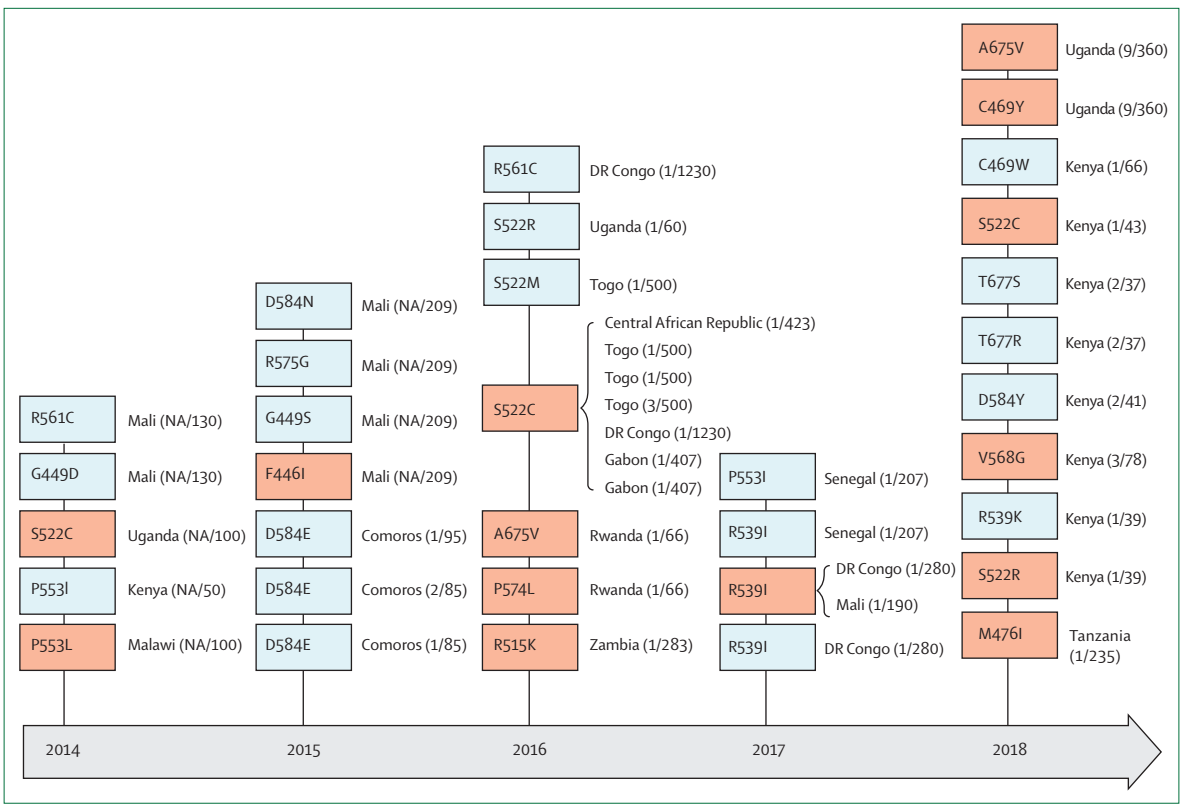

Figure 5: **Evolution in the reporting of *Pf*ART-R molecular markers during 2014–18**
Graph shows the discovery of different *Pf*ART-R markers (shaded in red) and mutations similar to *Pf*ART-R markers (shaded in dark blue), over the time in Africa. The years 2014–18 indicate the publication year of the articles reporting mutants. Single-letter abbreviations for the amino acid residues are as follows: A=alanine, C=cysteine, D=aspartic acid, E=glutamic acid, F=phenylalanine, G=glycine, I=isoleucine, K=lysine, L=leucine, M=methionine, N=aspargine, P=proline, R=arginine, S=serine, T=threonine, V=valine, W=tryptophan, and Y=tyrosine. In mutant alleles, reference amino acids at specified locations were substituted with other amino acids; for example, M476I shows that methionine at codon-position 476 was replaced by isoleucine. *Pf*ART-R=*Plasmodium falciparum* artemisinin resistance.

and structurally different from those observed in southeast Asia .[9,18,77]

The WHO list of African *Pfkelch13* alleles included nine non-synonymous mutations of the total 31 molecular markers of *Pf*ART-R based on studies done mainly in southeast Asia.[17] Six of these mutations, namely Phe446Ile, Met476Ile, Pro553Leu, Val568Gly, Pro574Leu, and Ala675Val, were reported to be strongly associated with delayed parasite clearance in vivo.[14,18,84,85] However, only Phe446Ile, Met476Ile, and Pro533Leu have shown a decreased response to artemisinin in vitro; they are being considered as validated artemisinin resistance markers by WHO.[14,16,17,86] Ala675Val, although not listed as a validated marker, has shown an altered response to artemisinin in vitro in one clinical isolate from Uganda.[45] Phe446Ile was only found in west Africa (Mali), whereas Ala675Val, Met476Ile, Pro553Leu, Pro574Leu, and Val568Gly were reported in the Great Lakes region in east Africa (Kenya, Uganda, Malawi, Zambia, Tanzania, and Rwanda). The other three mutations identified in African isolates (Ser522Cys, Cys469Tyr, and Arg515Lys) could also be related to causing slow parasite clearance by artemisinin; however, according to the WHO, there is no significant evidence for this association because of the low number of these mutants in surveys.[17] The Ser522Cys mutation that was found in several African countries (Mali, Gabon, Central African Republic, Togo, DR Congo, and Kenya),[9,42,69,78] had been correlated with delayed parasite clearance in one (from southeast Asia) of the three unique mutant isolates that have been assessed in vivo (one isolate being from DR Congo).[18,87] Similarly, two Cys469Tyr mutants have been linked to causing low parasite clearance and day 3 positive parasitaemia after treatment with artemisinin in southeast Asia.[87,88] However, in our systematic review, the unique African isolate with this mutation (from Uganda) assessed in vitro did not show a reduced response to artemisinin.[45] Unlike Ser522Cys and Cys469Tyr, the data accumulated for the Arg515Lys mutation deserves to be taken into account in future lists of *Pf*ART-R markers as more than ten mutants from southeast Asia have been consistently associated with delayed parasite clearance.[85,87,89]

Nine non-synonymous mutations that are classified by the WHO as either validated, candidate, or suspected artemisinin resistance markers, and 245 other *Pfkelch13*-propeller mutations were registered during the writing of this

systematic review. Most of them have never been experimentally explored because they did not fulfil the criteria for further functional exploration (eg, high frequency of new alleles with non-synonymous mutation, evidence of allele dissemination, or preliminary association with clinical resistance).[9] 16 amino acid substitutions among the unexplored mutations found in this review are structurally close to the WHO *Pf*ART-R mutations—ie, Gly449Asp, Gly449Ser, Cys469Trp, Met476Lys, Ser522Met, Ser522Arg, Arg539Ile, Arg539Lys, Prp553Ile, Arg561Cys, Arg575Gly, Asp584Glu, Asp584Asn, Asp584Tyr, Thr677Arg, and Thr677Ser. For instance, Gly449Asp and Gly449Ser observed in Mali[65] are able to resemble the *Pf*ART-R mutation Gly449Ala. They occur on a locus known to be accessible to glycine (Gly449) and establish a network of hydrogen bonds for the *Pf*kelch13 protein function.[90] These two African alleles could be structurally unfavourable to the structure of the *Pf*kelch13 propeller domain, resulting in potential markers for *Pf*ART-R.[90] Molecular modelling can thus provide more information about the phenotypic effect of unexplored mutations on the *Pf*kelch13 protein structure;[9,90] however, conventional research (in vitro or in vivo studies using either transgenic or clinical parasites) is always required to confirm their functional importance. Until the functional effect of these mutations that are similar to *Pf*ART-R markers is clarified, we suggest that they are included in any molecular surveillance of *Pf*ART-R in Africa when possible.

It is important to mention that African parasites might have their own genetic background preference to select *Pf*ART-R that would differ from parasites in southeast Asia.[8,91] The discovery of *Pfcoronin* in 2018 as a second *Pf*ART-R gene suggests that non-*Pfkelch13* types of resistance could independently emerge in natural settings in Africa.[92–94] *Pfcoronin* mutants associated with *Pf*ART-R were selected among parasites of Senegalese origin, using the same in-vitro selection experiment that had related *Pfkelch13* mutations to reduced artemisinin susceptibility in Tanzanian parasites.[14,90,92] Although the *Pfcoronin* mutations are not yet detected in clinical isolates, their structural similarity with *Pfkelch13* provides insights into the molecular mechanisms of artemisinin resistance.[93–95] Tracking the emergence of *Pfkelch13* mutant parasites in Africa is very important, but further research is needed to identify other possible *Pf*ART-R genes.[91]

Our systematic review has several limitations. First, no information is available from non-endemic malaria areas—eg, the Maghreb countries (eg, Algeria, Libya, Morocco, Egypt, Western Sahara, and Tunisia)—that have a lot of migration from sub-Saharan Africa, where the *Pf*ART-R threat might also exist. Future surveys should fill in the gaps of the existing map. Second, the heterogeneity in survey designs, sampling methods (eg, convenience samples), and analytical approaches in individual studies (eg, different lengths of analysed *Pfkelch13* sequences, different sampling time in surveys) did not allow pooling of reports for a standard meta-analysis. Third, primary information (eg, the precise collection time) was very variable or not available in some surveys, hindering the possibility of analysing temporal trends. Instead, we assessed the temporal trend of reporting *Pf*ART-R mutations. Fourth, the inaccuracy in the number of isolates carrying alleles in some reviewed studies and mutations reported with unknown origin that were excluded from the review could have led to an underestimation of allele frequencies (eg, Pro553Leu in Malawi[77] or Phe446Ile in Mali[65]). For these studies, the frequency of sites with these mutations was reported without assigning allele frequencies. Finally, our review did not include studies reporting migrating patients who returned to their home countries from Africa with isolates carrying *Pfkelch13* mutations. For instance, three *Pf*ART-R markers (Cys580Tyr, Met579Ile, and Arg539Thr) found in isolates of patients returning to Asia from Africa were excluded from this Review.[96,97] These mutations that favour *Pf*ART-R were probably acquired outside Africa as no autochthonous patients had these mutations, despite published reports extensively discussing that acquisition in Africa cannot be ruled out.

Despite the limitations above, we believe that our systematic review provides a valuable baseline reference for building and strengthening surveillance activities in African countries to prevent the emergence and later spread of *Pf*ART-R. The message in this paper is not a lack of confidence in ACTs, which are still very effective in Africa, but it is more a call for vigilance and increased surveillance efforts against possible *Pf*ART-R emergence locally. Therefore, proactive surveillance using *Pf*ART-R molecular markers and timely consolidation and interpretation of surveillance findings can contribute to decision making to further sustain the effectiveness of artemisinin in Africa. Any possible introduction by known *Pf*ART-R alleles, temporal increase in the frequency of autochthonous alleles, and expansion of non-synonymous mutations similar to *Pf*ART-R markers should be targeted by surveillance efforts. Enhanced research efforts (ie, more funding and field exploration) are required to uncover the functional importance of other *Pfkelch13*-propeller mutations and to identify other possible *Pf*ART-R genes like *Pfcoronin*.

Contributors

NS and NKK conceived the study. NKK, ET-K, AR-A, and BD wrote the protocol. NKK, DYM, AR-A, ET-K, VPT, and YY did the literature search, acquired the aggregate data, screened records, and extracted the data. NKK, ET-K and AR-A did the data analyses and made figures. NKK, ET-K, AR-A, VPT, BD, and NS wrote the first draft of the manuscript. DMM, GLM, DYM, PDM, M-PH, YY, and EOW revised the first draft of the manuscript. NS had full access to all the data in the study and had final responsibility for the decision to submit for publication. All authors provided conceptual input, and revised and approved of the final version of the manuscript.

Declaration of interests

We declare no competing interests.

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