PONTIFICIA UNIVERSIDAD CATÓLICA DEL ECUADOR

FACULTAD DE CIENCIAS EXACTAS Y NATURALES

ESCUELA DE CIENCIAS BIOLÓGICAS

Effect of selective drug pressure and host human migration in antimalarial resistance in circulating *Plasmodium falciparum* in Ecuador

Disertación previa a la obtención del título de Licenciado en Ciencias Biológicas

KEVIN ISAAC ÑACATA IZA

Quito, 2022

Certifico que la Disertación de Licenciatura en Ciencias Biológicas del Sr. Kevin Isaac Ñacata Iza ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

Firmado electrónicamente por:
FABIAN ERNESTO SAENZ CALDERON

Dr. Fabián Ernesto Sáenz Calderón

Director de Disertación

Quito, 03 de agosto de 2022

Mediante la ciencia y la tecnología hemos penetrado en este mundo, pero apenas hemos empezado a explorarlo.

Edward O. Wilson

AGRADECIMIENTOS

Agradezco a la Pontificia Universidad Católica del Ecuador, en especial al Centro de Investigación para la Salud en América Latina (CISeAL) por el financiamiento de esta investigación.

A los docentes e investigadores de la Facultad de Ciencias Exactas y Naturales, en especial de la Escuela de Ciencias Biológicas por inspirarme durante toda mi carrera.

A mi director de disertación, Dr. Fabián Sáenz por acogerme en su laboratorio y darme la oportunidad de hacer investigación.

A mis lectoras, Dra. Iliana Alcocer y Dra. Anita Villacís por su retroalimentación en la realización de este trabajo de disertación.

A mi familia por apoyarme incondicionalmente en todo el proceso de mi carrera universitaria.

A mis amigos del CISeAL, en especial a Diego Mena por su apoyo y compartir su conocimiento conmigo; a Eduarda y Daniela, pasantes del laboratorio de malaria por su ayuda.

A todos mis amigos y compañeros de biología y microbiología por su apoyo incondicional y únicos momentos que pasamos juntos durante la carrera.

TABLA DE CONTENIDOS

ABBREVIATIONS LIST

Abbreviation	Meaning
ACT	Artemisinin-based combination therapy
AL	Artemether-Lumefantrine
AQ	Amodiaquine
AR	Artemether
ART	Artemisinin
AS	Artesunate
CD	Clindamycin
CISeAL	Centro de Investigación para la Salud en América Latina
CQ	Chloroquine
CQR	Chloroquine resistant
DV	Digestive vacuole
LF	Lumefantrine
MPH	Ministry of Public Health
MQ	Mefloquine
PCR	Polymerase Chain Reaction
Pfaat1	Amino acid transporter 1 of *P. falciparum*
Pfcrt	Chloroquine resistance transporter of *P. falciparum*
Pfdhfr	Dihydrofolate reductase of *P. falciparum*
Pfdhps	Dihydropteroate synthetase of *P. falciparum*

ABBREVIATIONS LIST

Abbreviation	Meaning
Pfk13	Kelch 13 of *P. falciparum*
Pfmdr-1	Multidrug resistance transporter 1 of *P. falciparum*
PQ	Primaquine
QN	Quinine
RDT	Rapid Diagnostic Test
SNP	Single Nucleotide Polymorphism
SP	Sulfadoxine-Pyrimethamine
µl	Microliter
WGS	Whole-genome sequencing
WHO	World Health Organization

FIGURE LIST

TABLE LIST

1 RESUMEN

Introducción: La resistencia antimalárica en *P. falciparum* es un problema de salud pública para combatir la malaria en Ecuador. Caracterizar la epidemiología molecular de genes de resistencia a fármacos ayuda a entender el surgimiento y dispersión de parásitos resistentes en un área en eliminación de la malaria. Este estudio determina el efecto de la presión selectiva de drogas y la migración humana en la resistencia antimalárica en *P. falciparum* en la costa norte de Ecuador.

Métodos: Se analizaron 63 muestras de *P. falciparum* del noroeste de Ecuador entre 2019 y 2021. Se identificaron mutaciones puntuales en *Pfcrt*, *Pfdhps*, *Pfdhfr*, *Pfmdr-1*, *Pfk13 and Pfaat1* por secuenciación Sanger y secuenciación de genoma completo para determinar genotipos de resistencia a fármacos. Además, se realizó una comparación de las frecuencias de los haplotipos entre los periodos de 2013-2015 y 2019-2021. También, se calculó la diversidad nucleotídica (π), diversidad haplotípica (H) y se realizó una prueba de neutralidad (Tajima's D) en *Pfcrt*, *Pfdhps*, *Pfdhfr* y *Pfmdr-1*.

Resultados: Las frecuencias de los haplotipos mutantes CVM**ET** en *Pfcrt* y C**I**C**N**I en *Pfdhfr* incrementaron de 2013-2015 a 2019-2021. NED**F**S**D**F**Y** en *Pfmdr-1* fue detectado por primera vez en Ecuador, mientras que, los haplotipos salvajes SAKAA en *Pfdhps* y MYRIC en *Pfk13* se mantuvieron dominantes. En general, las muestras obtenidas de Colombia y San Lorenzo presentaron mayor diversidad genética que Esmeraldas y Tobar Donoso. Además, la prueba de Tajima's D mostró selección positiva en *Pfcrt*, *Pfdhfr* y *Pfmdr-1*.

Conclusión: Este estudio muestra que la resistencia a cloroquina y pirimetamina en *P. falciparum* ecuatorianos ha aumentado en los últimos años, pero se mantiene la sensibilidad a sulfadoxina y artemisininas. Por lo tanto, el actual tratamiento es todavía útil contra *P. falciparum*. Los resultados muestran que *P. falciparum* ha pasado por un proceso de selección debido a la presión de fármacos y la migración frecuente de hospederos humanos entre Ecuador y Colombia ha contribuido a la dispersión de parásitos resistentes.

Palabras claves: *Plasmodium falciparum*, resistencia, antimaláricos, presión selectiva, migración humana

2 ABSTRACT

Background: Antimalarial resistance in *P. falciparum* is a public health problem in the fight against malaria in Ecuador. Characterizing the molecular epidemiology of drug resistance genes helps to understand the emergence and spread of resistant parasites in a malaria eliminating area. In this study, the effect of selective drug pressure and human migration in antimalarial resistance in *P. falciparum* is determined.

Methods: Sixty-three *P. falciparum* samples from northwestern Ecuador between 2019 and 2021 were analyzed. Point mutations in *Pfcrt*, *Pfdhps*, *Pfdhfr*, *Pfmdr-1*, *Pfk13* and *Pfaat1* were identified by Sanger sequencing and whole-genome sequencing to determine drug resistance genotypes. In addition, a comparison of the frequencies of the haplotypes was made between the periods of 2013-2015 and 2019-2021. Also, nucleotide diversity (π), haplotype diversity (H) and a neutrality test (Tajima's D) were calculated for *Pfcrt*, *Pfdhps*, *Pfdhfr* and *Pfmdr-1.*

Results: The frequencies of the mutant haplotypes CVM**ET** in *Pfcrt*, C**I**C**N**I in *Pfdhfr* increased from 2013-2015 to 2019-2021. NED**F**S**D**F**Y** in *Pfmdr-1* was detected for the first time in Ecuador, while the wild-type haplotypes SAKAA in *Pfdhps* and MYRIC in *Pfk13* remained dominant. In general, samples obtained from Colombia and San Lorenzo presented more genetic diversity than Esmeraldas and Tobar Donoso. Furthermore, Tajima's D test showed positive selection in *Pfcrt*, *Pfdhfr* and *Pfmdr-1.*

Conclusions: This study shows that resistance to chloroquine and pyrimethamine in Ecuadorian *P. falciparum* has increased in recent years, but sensitivity to sulfadoxine and artemisinins is maintained. Therefore, the current treatment is still useful against *P. falciparum*. The results show that *P. falciparum* has undergone a selection process due to drug pressure and the frequent migration of human hosts between Ecuador and Colombia has contributed to the spread of resistant parasites.

Keys words: *Plasmodium falciparum*, resistance, antimalarials, selective pressure, human migration.

REVISTA

Malaria Journal

TÍTULO

Effect of selective drug pressure and host human migration in antimalarial resistance in circulating *Plasmodium falciparum* in Ecuador.

AUTORES

Isaac Ñacata[1]

Email: kinacata@puce.edu.ec

Angela M Early[2 3]

Email: early@broadinstitute.org

Daniel E Neafsey[2 3]

Email: neafsey@hsph.harvard.edu

Fabián E. Sáenz[1*]

Email: fsaenz213@puce.edu.ec

*Corresponding author email: fsaenz213@puce.edu.ec

[1] Centro de Investigación para la Salud en América Latina, Facultad de Ciencias Exactas y Naturales, Pontificia Universidad Católica del Ecuador, Quito, Ecuador

[2] Infectious Disease and Microbiome Program, Broad Institute of MIT and Harvard, Cambridge, MA, USA

[3] Department of Immunology and Infectious Disease, Harvard T.H. Chan School of Public Health, Boston, MA, USA

Effect of selective drug pressure and host human migration in antimalarial resistance in circulating *Plasmodium falciparum* in Ecuador.

Isaac Ñacata[1], Angela M. Early[2 3], Daniel E. Neafsey[2 3], Fabián E. Sáenz[1*]

[1] Centro de Investigación para la Salud en América Latina, Facultad de Ciencias Exactas y Naturales, Pontificia Universidad Católica del Ecuador, Quito, Ecuador

[2] Infectious Disease and Microbiome Program, Broad Institute of MIT and Harvard, Cambridge, MA, USA

[3] Department of Immunology and Infectious Disease, Harvard T.H. Chan School of Public Health, Boston, MA, USA

*Corresponding author

Tel: 593-22991-700 ext. 2923, fax: 593-22991-689, email: fsaenz213@puce.edu.ec

3 BACKGROUND

Malaria is a vector-borne infectious disease that is endemic in tropical and subtropical regions of the world (1). *Plasmodium falciparum* causes the most severe malaria in humans and the highest morbidity and mortality worldwide (2). According to the World Health Organization (WHO), in 2020, 241 million cases and 627 000 deaths by malaria occurred in the world (3), and 650 000 cases and an incidence of 4.6 cases per 1000 inhabitants occurred in the Americas (4). In this region, the highest malaria incidence is attributed to *P. vivax* followed by *P. falciparum* (1,5).

Since the approval of artemisinin-based combination therapy (ACT) against *P. falciparum*, several South American countries have implemented it in their treatment schemes (6,7). Artemether (AR) and artesunate (AS) are the most common artemisinins (ARTs) used in the ACT (6). Currently, seven of eleven countries in this region, including Ecuador, use artemether-lumefantrine (AL) + primaquine (PQ) as first-line treatment and quinine (QN) + clindamycin (CD) as second-line treatment (6,8).

Antimalarial resistance in *P. falciparum* is a public health problem that complicates malaria control in endemic regions (9,10). Resistance is an evolutionary response to pressure exerted by drugs over parasites that allows them to survive and reproduce (fitness) during exposure to antimalarials (7). This advantage is due to mutations that modify protein function and reduce antimalarial efficacy (11,12).

Chloroquine (CQ) resistance in *P. falciparum* originated in Southeast Asia in the late 1950s (13). Worldwide, *P. falciparum* exhibits CQ resistance mediated by mutations in *Pfcrt* that change the configuration of the chloroquine resistance transporter (PfCRT), located in the digestive vacuole (DV), allowing drug expulsion (14,15). High concentrations of CQ in the DV prevent the release of toxic endogenous products, which cause the death of the parasite (16). Mutations at codons 72-76 are associated with CQ resistance (17,18). In the early 1960s, CQ resistance emerged independently in South America (13), and CVMN**T**, CVM**ET** and CV**EIT** haplotypes have been found to predominate on the Pacific coast and **S**VMN**T** in the Amazon region (19–21). Specifically, in Ecuador, CVMN**T**, CVM**ET** and **S**VMN**T** have been previously reported (19,22). In addition, other mutations such as H97**Q**, A220**S**, Q271**E**, N326**D**, C350**S**, I356T/**L** and R371**I** in *Pfcrt* contribute to different degrees of CQ resistance (14,23,24). H97**Q**, A220**S**, N326**D**, S334**N**, I356**L** and R371**I**/**T** have been reported in South America (16,23,25,26). While in Ecuador, A220**S**, N326**D** and I356**L** were identified in Ecu1110 (isolate from 1990) (15,19).

Resistance to combined drugs sulfadoxine-pyrimethamine (SP) in *P. falciparum* originated in Southeast Asia in the 1960s (13). SP resistance is associated with mutations in *Pfdhps* and *Pfdhfr*, which encode synergistic enzymes: dihydropteroate synthetase and dihydrofolate reductase, respectively (27,28). Both enzymes participate in the folate synthetic pathway and the presence of SP cause competitive inhibition of the active site, causing parasite death (29,30). Mutations at codons 436, 437, 540, 581 and 613 in *Pfdhps* confer sulfadoxine resistance. In South America, SAKAA (wild type), **F**AKAA, **FG**KAA, S**G**KAA, SA**E**AA, S**G**K**G**A and S**GEG**A have been reported (20,31–34), while only SAKAA and SA**E**AA have been found in

Ecuador (19). On the other hand, mutations at codons 50, 51, 59, 108 and 164 in *Pfdhfr* confer pyrimethamine resistance (35). In South America, CNCSI (wild-type), CNC**N**I, C**I**C**N**I, C**I**C**NL**, CNC**T**I and **RI**C**N**I haplotypes have been reported (20,32,33), while CNCSI, CNC**N**I and C**I**C**N**I have been found in Ecuador (19). Additionally, SP treatment failure has been registered in South America since the 1980s (20).

Mutations in *Pfmdr-1* as well as its copy number are associated with resistance to several drugs such as CQ, QN, amodiaquine (AQ), mefloquine (MQ) and lumefantrine (LF) (14,36). Mutations at codons 86, 130, 144, 184, 1034, 1042, 1226 and 1246 change the charge and structure of the multidrug resistance transporter (PfMDR1), located in the DV, causing drugs expulsion (37,38). N86**Y**, Y184**F**, S1034**C**, N1042**D** and D1246**Y** mutations have been reported in South America (24,36), while Y184**F** and N1042**D** have been found in Ecuador (19).

ART resistance in *P. falciparum* was first reported in Southeast Asia in 2006 (39). Initially, ARTs are activated by heme and iron to then cause protein and proteasome damage, leading to prolonged cellular stress and parasite death (40). Mutations at codons 476, 493, 539, 543 and 580 in *Pfk13* are associated with ART resistance (36,40,41). In South America, C580**Y** has only been found in Guyana since 2010 (41,42), while Ecuador, Colombia, Peru, Venezuela, Brazil, Suriname and French Guiana have not reported any mutation associated with ART resistance (19,42,43).

In recent years, putative amino acid transporter 1 (*Pfaat1*) has been associated with resistance to quinolines such as QN, AQ MQ and especially CQ (44–46). A study suggests that PfAAT1 protein, located in the DV, transports amino acids out of the DV while allowing entry of quinolines (46,47). However, the presence of mutations such as T162**E** changes the charge of PfAAT1, blocking drug entry into the DV (46). On the Pacific coast of Ecuador and Colombia, S258**L** mutation in *Pfaat1* has been related to CQ resistance (48).

In South America, *P. falciparum* populations are mostly clonal as a result of bottlenecks caused by efforts to eliminate malaria in the region (12,21,49). In Esmeraldas, Ecuador, a malaria outbreak was caused by a clonal expansion in 2013 (21). In addition, malaria outbreaks are influenced by the spread of parasites through human hosts between endemic regions (21,22,50). In malaria-endemic countries of the Americas, 7775 imported cases were reported in 2016 and 8411 in 2017, while in Ecuador, the latest records of imported infections reported 395 cases, 41.5% of them were due to *P. falciparum* between 2015 and 2017 (6,51).

In 2012, Ecuador was considered one of 21 countries in the world with the potential to eliminate malaria by 2020 due to a reduction in cases since 2002 of more than 99% (2). The decrease in malaria infections was largely due to the success of the malaria elimination programs; however, infections have increased since 2015 (5). This increase may be associated with factors such as the restructuring of the health system, environmental changes and, indiscriminate drug use and the spread of parasites (52,53). Genetic surveillance helps to understand if the emergence of new

resistant genotypes in *P. falciparum* as a result of de-novo mutations or the spread of parasites by human host migration.

This study focuses on the molecular epidemiology of drug resistance genes to determine the effect of selective drug pressure and host human migration in antimalarial resistance in *P. falciparum.* This work aims to characterize the current epidemiological situation of resistance, contribute to the strengthening of health programs and notify the efficiency of the treatment schemes against *P. falciparum* in Northwest Ecuador.

4 MATERIALS AND METHODS

Ethics statement

The study was approved by the Research Ethics Committee on Human Beings of Pontificia Universidad Católica del Ecuador registered in the document CEISH-571-2018 and by the Ministry of Public Health (MPH) of Ecuador registered in the document MSP-DIS-2019-044-O.

Study area and parasite population

The study was carried out in Ecuador (South America), with samples collected in Esmeraldas and Carchi provinces located in the Northwest on the border with Colombia (Fig. 1). Sixty-three blood samples were collected on Whatman filter paper from patients diagnosed with *P. falciparum* by microscopy or RDTs (Rapid Diagnostic Tests). Samples were collected by personnel of the MPH between 2019

and 2021 and delivered to CISeAL (Centro de Investigación para la Salud en América Latina) for molecular analysis. During this period, 587 infections by *P. falciparum* were registered in Northwest Ecuador (5). Samples of local Ecuadorian cases corresponded to the counties of Esmeraldas (42.86%), San Lorenzo (7.94%) and Tobar Donoso (28.57%). The remaining 20.63% corresponded to migrants from Nariño, Colombia diagnosed in San Lorenzo. Additionally, data of *P. falciparum* samples (2013-2015 period) from a previous study in Ecuador were included for spatial and temporal comparisons (19).

DNA extraction

DNA extraction of *P. falciparum* was performed from blood samples on Whatman filter paper stored at 4 °C, using the commercial Purelink™ Genomic DNA Mini Kit, INVITROGEN following the protocol recommended by the manufacturer (Invitrogen, Life Technology, USA), and stored at -20 °C for its molecular analysis.

Amplification of drug resistance genes

DNA was amplified for molecular markers of drug resistance genes: *Pfcrt* (codons 72-76), *Pfdhps* (codons 436, 437, 540, 581 and 613), *Pfdhfr* (codons 50, 51, 59, 108 and 164), *Pfmdr-1* (codons 86, 130, 144, 184, 1034, 1042, 1226 and 1246) and *Pfk13* (codons 476, 493, 539, 543 and 580).

Polymerase Chain Reaction (PCR) solution contained 12.5 µl of Colorless GoTaq Master Mix (Promega, Madison, WI, USA), 2.5 µl of $MgCl_2$ (25 mM) (Promega,

Madison, WI, USA), 3 µl of ultrapure H_2O, 1 µl of primers forward and reverse (10 µM) and 5 µl of DNA. Nested PCR protocols (Table. 1) established in previous studies were used (19). Amplifications were carried out in SureCycler 8800 (Agilent Technologies, USA).

Amplicon sizes were verified in 2% agarose gel and observed in UVP BioDoc-It digital transilluminator (UVP, CA, UK). After confirmation, amplified DNA was sent for purification and Sanger sequencing to Macrogen, South Korea.

Determination of genotypes in drug resistance genes

NF54 strain of *P. falciparum* was used as a control to identify SNPs (Single Nucleotide Polymorphisms) in *Pfcrt* (codons 72-76), *Pfdhps* (codons 436, 437, 540, 581 and 613), *Pfdhfr* (codons 50, 51, 59, 108 and 164), *Pfmdr-1* (codons 86, 130, 144, 184, 1034, 1042, 1226 and 1246) and *Pfk13* (codons 476, 493, 539, 543 and 580). All sequences were analyzed and aligned in Geneious software version 2020.0.1 (Biomatters, Inc, Newark, NJ, USA).

Whole Genome Sequencing

Fifty-four DNA samples of *P. falciparum* were sent to the Harvard School of Public Health/Broad Institute for whole-genome sequencing (WGS) as part of a collaboration between Dr. Fabián Sáenz and Dr. Daniel Neafsey. Data for drug resistance markers was obtained for this study. WGS results allowed to complete and confirm the genotypes obtained by Sanger sequencing.

Data Analysis

GraphPad Prism version 7.02 was used to calculate frequencies and make comparative graphs of *Pfcrt*, *Pfdhps*, *Pfdhfr*, *Pfmdr-1* and *Pfk13* haplotypes in each locality from the 2013-2015 and 2019-2021 periods. The number of polymorphic sites (S), the average number of pairwise nucleotide differences (k), nucleotide diversity (π), number of haplotypes (h), haplotype diversity (Hd) and Tajima's D (neutrality test) were calculated in DnaSP software version 6.12.03.

5 RESULTS

Genotypes of resistance

Pfcrt (codons 72-76), *Pfdhps* (codons 436, 437, 540, 581 and 613), *Pfdhfr* (codons 50, 51, 59, 108 and 164), *Pfmdr-1* (codons 86, 130, 144, 184, 1034, 1042, 1226 and 1246) and *Pfk13* (codons 476, 493, 539, 543 and 580) were analyzed to identify drug resistance genotypes.

The *Pfcrt* haplotypes found in 61 samples were: CVM**ET** (63.93%) and CVMN**T** (36.07%). CVM**ET** was dominant in Esmeraldas (100%). In San Lorenzo, the CVM**ET** proportion (60%) was higher than CVMN**T** (40%). In Tobar Donoso, CVMN**T** had the higher proportion (88.89%), followed by CVM**ET** (11.11%). In samples obtained from Colombia, CVM**ET** (69.23%) was the most prevalent haplotype followed by CVMN**T** (30.77%) (Fig. 2a). The CVM**ET** and CVMN**T** haplotypes show N75**E** and K76**T** mutations associated with CQ resistance (23).

The *Pfdhps* haplotypes found in 49 samples were: the wild-type SAKAA (98%) and S**G**KAA (2%). SAKAA was dominant in Esmeraldas, San Lorenzo and Tobar Donoso (100%). While in samples obtained from Colombia, SAKAA was most prevalent (83.33%) followed by S**G**KAA (16.67%) (Fig. 2b). In addition, a synonymous mutation (AA**G**) at codon 540 was found in a sample from Esmeraldas and Tobar Donoso and two samples from Colombia. The S**G**KAA haplotype shows A437**G** mutation associated with sulfadoxine resistance (34).

The *Pfdhfr* haplotypes found in 63 samples were: C**I**C**N**I (63.49%), the wild-type CNCSI (34.92%), and CNC**N**I (1.59%). C**I**C**N**I was dominant in Esmeraldas (100%). In San Lorenzo, the C**I**C**N**I proportion (60%) was higher than CNCSI (40%). All Tobar Donoso samples presented the wild-type, CNCSI (100%). In samples obtained from Colombia, C**I**C**N**I (76.92%) was the most prevalent followed by CNCSI (15.38%) and CNC**N**I (7.69%) (Fig. 2c). The C**I**C**N**I and CNC**N**I haplotypes show N51**I** and S108**N** mutations associated with pyrimethamine resistance (30).

The *Pfmdr-1* haplotypes found in 34 samples were: NED**F**S**D**FD (55.88%) and NED**F**S**D**F**Y** (44.12%). NED**F**S**D**FD proportion was higher in Tobar Donoso (100%) than Esmeraldas (14.29%), while NED**F**S**D**F**Y** was predominant in Esmeraldas (85.71%). In San Lorenzo and samples obtained from Colombia, both haplotypes had the same proportion (50% each one) (Fig. 2d). Haplotypes could not be detailed for the rest of the 28 samples where amplifications were unsuccessful for codons 1034, 1042 and 1226. However, at the other codons (86, 130, 144, 184 and 1246),

NED**F**D was found in two samples from Esmeraldas (22.22%), one sample from San Lorenzo (100%), three samples from Tobar Donoso (100%) and six samples obtained from Colombia (75%). While NED**FY** was found in seven samples from Esmeraldas (77.78%) and two samples obtained from Colombia (25%).

The *Pfk13* haplotype found in 52 samples was the wild-type MYRIC (100%). MYRIC was present in all samples from Esmeraldas, San Lorenzo, Tobar Donoso and samples obtained from Colombia (Fig. 2e).

Whole-genome sequencing

Forty nine samples showed results in *Pfcrt* (codons 72-76, 97, 118, 220, 285, 326, 334, 356 and 371), *Pfdhps* (codons 44, 50, 51, 59, 108, 114, 142, 164, 223, 521 and 629), *Pfdhfr* (codons 16, 436, 437, 540, 581 and 613), *Pfmdr-1* (codons 35, 36, 86, 130, 144, 184, 661, 662, 1034, 1042, 1226 and 1246), *Pfk13* (codons 189, 341 and 344) and *Pfaat1* (27, 52, 68, 76, 80, 85, 119, 121, 126, 134, 179, 195, 231, 239, 258 and 317).

In *Pfcrt*, WGS confirmed the mutations found at codons 72-76 by Sanger sequencing in 36 samples. Also, it was found that 53% (19/36) had H97**Q**, 100% (36/36) had A220**S**, 41% (15/37) had N326**D**, 41% (15/37) had S334**N**, 42% (16/38) had I356**L** and 55% (21/38) had R371**T.** Insertions were found in 100% of samples at codon 118 (T or A) and 285 (T) (Fig. 3).

In *Pfdhps*, WGS confirmed the mutations found at codons 436, 437, 540, 581 and 613 by Sanger sequencing in 37 samples. Also, it was found that 44% (16/36) had a synonymous mutation (AA**A**) at codon 142. Insertions were found in 100% of samples at codon 44 (G or GA), 114 (A or AT), 223 (G), 521 (T), and 629 (C) (Fig. 3).

In *Pfdhfr*, WGS confirmed the mutations found at codons 50, 51, 59, 108 and 164 by Sanger sequencing in 35 samples. Also, it was found that 7% (2/30) had the A16**V** mutation (Fig. 3).

In *Pfmdr-1*, WGS confirmed the mutations found at codons 86, 130, 144, 184, 1034, 1042, 1226 and 1246 by Sanger sequencing in 34 samples. Also, it was found that 9% (2/23) had I35**K**. An insertion was found in 100% (25/25) at codon 36 (T or TA). Deletions were found in 100% of samples at codon 661 (G or GATA) and 662 (A or ACAAAAT) (Fig. 3).

In *Pfk13,* it was found that 100% of samples had K189**T** and 11% (2/19) had A344**V**. An insertion was found in 100% of samples at codon 341 (A or AT) (Fig. 3).

In *Pfaat1,* it was found that 100% (7/7) had D80**E**, 58% (19/33) had V231**D** and 49% (17/35) had S258**L**. Insertions were found in 100% samples at codon 27 (G or GT), 52 (A), 68 (A), 76 (A or AT), 85 (G), 119 (C), 121 (C or CTTT), 126 (A or AT), 179

(T or TA), 195 (G or GA), 239 (C or CT) and 317 (G). Also, a deletion was found in 100% (32/32) at codon 134 (CATA) (Fig. 3).

Comparative frequencies of drug resistance haplotypes over time

The spatial and temporal comparison allowed to characterize the fluctuation of haplotype frequencies between the 2013-2015 and 2019-2021 periods in northwest Ecuador. In *Pfcrt*, CVM**ET** frequency increased in San Lorenzo (from 0.22 to 0.60), Esmeraldas (from 0.03 to 1) and appeared in Tobar Donoso (from 0 to 0.11). On the other hand, CVMN**T** frequency decreased in San Lorenzo (from 0.78 to 0.40), Tobar Donoso (from 1 to 0.89) and disappeared in Esmeraldas (from 0.97 to 0) (Fig. 4a). In *Pfdhps*, SAKAA frequency remained high in San Lorenzo, Esmeraldas and Tobar Donoso (Fig. 4a). In *Pfdhfr*, the wild-type CNCSI frequency decreased in San Lorenzo (from 0.83 to 0.4) and disappeared in Esmeraldas (from 0.97 to 0), but it remained high in Tobar Donoso. CNC**N**I disappeared in San Lorenzo (from 0.13 to 0), while C**I**C**N**I frequency increased in Esmeraldas (from 0.03 to 1) and San Lorenzo (from 0.04 to 0.60) (Fig. 4a). In *Pfmdr-1*, NED**F**S**D**FD frequency decreased in Esmeraldas (from 0.97 to 0.14) and San Lorenzo (from 0.78 to 0.50), but it remained high in Tobar Donoso. While NED**F**S**D**F**Y** appeared in Esmeraldas (from 0 to 0.86) and San Lorenzo (from 0 to 0.5). Surprisingly, haplotypes with single mutations NED**F**SNFD (Y184**F**) and NEDYS**D**FD (N1042**D**) found in the 2013-2015 period disappeared in San Lorenzo (from 0.22 to 0) and Esmeraldas (from 0.03 to 0), respectively (Fig. 4a). Finally, in *Pfk13*, the wild-type MYRIC frequency has remained high in San Lorenzo, Esmeraldas and Tobar Donoso (Fig. 4a).

306 **Genetic diversity and neutrality test of drug resistance genes**

307 The nucleotide diversity (π) and haplotype diversity (Hd) were calculated to
308 determine the genetic diversity of *Pfcrt*, *Pfdhps*, *Pfdhfr* and *Pfmdr-1* in *P. falciparum*
309 from northwest Ecuador and samples obtained from Colombia. The nucleotide
310 diversity (π) measured the degree of DNA polymorphism, while haplotype diversity
311 (Hd) measured the singularity of a haplotype in relation to sample size (54). In *Pfcrt*,
312 samples obtained from Colombia (π=0.062, Hd=0.460) and San Lorenzo (π=0.080,
313 Hd=0.600) showed higher diversity than Tobar Donoso (π=0.028, Hd=0.209) and
314 no diversity was found in Esmeraldas. In *Pfdhps*, samples obtained from Colombia
315 (π=0.080, Hd=0.600) had higher diversity than Tobar Donoso (π=0.008, Hd=0.118)
316 and Esmeraldas (π=0.006, Hd=0.091) and no diversity was found in San Lorenzo.
317 In *Pfdhfr*, San Lorenzo (π=0.080, Hd=0.600) had higher diversity than Colombia
318 (π=0.040, Hd=0.410) and no diversity was found in Esmeraldas and Tobar Donoso.
319 In *Pfmdr-1,* samples obtained from Colombia (π=0.040, Hd=1.000) and San
320 Lorenzo (π=0.030, Hd=0.667) had higher diversity than Esmeraldas (π=0.010,
321 Hd=0.264) and no diversity was found in Tobar Donoso (Table 2). *Pfk13* was
322 excluded from the analysis because it did not exhibit polymorphisms in the molecular
323 markers analyzed.

324

325 The Tajima's D neutrality test was performed to evaluate if *Pfcrt*, *Pfdhps*, *Pfdhfr* and
326 *Pfmdr-1* have gone through a selection process in northwest Ecuador and samples
327 obtained from Colombia. In *Pfcrt*, Tajima's D values were positive for Colombia
328 (Tajima's D=1.214) and San Lorenzo (Tajima's D=1.459) and negative for Tobar
329 Donoso (-0.685). In *Pfdhps*, Tajima's D values were negatives for Colombia
330 (Tajima's D=-0.447), Esmeraldas (Tajima's D=-1.162) and Tobar Donoso (Tajima's

D=-1.164). While in *Pfdhfr*, Tajima's D values were positive for Colombia (Tajima's D=0.097) and San Lorenzo (Tajima's D=1.459). Finally, in *Pfmdr-1,* Tajima's D values were positive *for* San Lorenzo (Tajima's D=1.633) and negative for Esmeraldas (Tajima's D=-0.341) (Table 2). The Tajima's D value could not be calculated for the localities of Esmeraldas in *Pfcrt*, San Lorenzo in *Pfdhps*, Esmeraldas and Tobar Donoso in *Pfdhfr* and Tobar Donoso and Colombia in *Pfmdr-1*, because they did not show polymorphisms in drug resistance genes or sample size was less than four.

6 DISCUSSION

The efficacy of antimalarials has contributed to the reduction of morbidity and mortality rates of malaria (55). However, the spread and increase of antimalarial resistance contribute to the emergence of malaria outbreaks and hinder malaria elimination (24,42). This study focused on the molecular epidemiology of drug resistance genes (*Pfcrt*, *Pfdhps*, *Pfdhfr*, *Pfmdr-1*, *Pfk13* and *Pfaat1*) in *P. falciparum* in northwestern Ecuador between 2019 and 2021.

CVMN**T** and CVM**ET** haplotypes were found in *Pfcrt* (Fig. 2a), showing CQ resistance in 100% of Ecuadorian *P. falciparum* samples. CVMN**T** has been previously reported in Ecuador, Colombia and Peru, while CVM**ET** in Ecuador, Colombia and Venezuela (19–21,31). *In vitro* studies show that all chloroquine-resistant (CQR) parasites have the K76**T** mutation, which is essential to confer CQ resistance (14,18). In Ecuador, CVMN**T** was found in Ecu1110 (isolate from 1990) (19,20). In 2002, CQR parasites with K76**T** were found in Esmeraldas province (22).

Then in northwest Ecuador, CVMN**T** and CVM**ET** were found circulating between 2013 and 2015 (19). The fixation of K76**T** mutation is clear evidence of pressure exerted by CQ over parasites since 1950 when CQ was implemented as an official treatment in Ecuador (48,56). In 1976, CQ resistance was evidenced in Ecuador (57), but it was officially recognized in 1980 by the MPH (58). In 2005, CQ was used in combination with SP and in, 2006, CQ was finally withdrawn from the treatment scheme (Fig. 5) (56). So, CQ caused strong pressure in Ecuadorian *P. falciparum* for more than five decades.

It is evident that in Ecuador, as in the rest of South America, CQ resistance has become fixed by the presence of CVMN**T**, CVM**ET**, CV**EIT** and **S**VMN**T** haplotypes in *Pfcrt* (19–21). However, reversal of CQ resistance has not been seen in this region even though CQ is no longer used against *P. falciparum* (20,59). On the other hand, in French Guiana, reversal of CQ resistance is acquired by the C350**R** mutation in *Pfcrt* (60). The massive administration of CQ to combat malaria in South America since 1950, even as prophylaxis in cooking salt (20,61), caused a strong pressure that resulted in the emergence of CQR parasites (14,57). Likewise, the indiscriminate use of CQ reported in several areas of the Pacific coast during the 1990s contributed to the selection of CQR parasites (62). Besides, clonal expansions, very common in South America, may have caused the fast spread and increase of CQR parasites (21), as well as the loss of CQ-sensitive parasites that can compete in absence of CQ pressure.

Other mutations such as H97**Q**, A220**S**, Q271**E,** N326**D**, C350**S**, I356**T/L** and R371**I** in *Pfcrt* have been associated with CQR parasites (16,25). H97**Q**, A220**S**, N326**D**, S334**N**, I356**L** and R371**T** were found in this study (Fig. 3). Previously in Ecuador, A220**S**, N326**D** and I356**L** with CVMN**T** were found in Ecu1110, an isolate CQ resistant from 1990 (15,59). While in Colombia, H97**Q**, A220**S** and R371**T** with CVM**ET** were found in JAV strain that confer a high level of CQ resistance (59). Meanwhile, A220**S**, N326**D** and I356**L** with **S**VMN**T** present in 7G8 strain from Brazil have also been associated with CQ and AQ resistance (23,26,63). It has been suggested that at least three complementary mutations to K76**T** are necessary to confer high degrees of CQ resistance (17). Consequently, the presence of these mutant alleles may have contributed to CQ resistance in Ecuadorian *P. falciparum*.

SAKAA (wild-type) haplotype was found in *Pfdhps* of Ecuadorian *P. falciparum* (Fig. 2b), showing sulfadoxine sensitivity in 100% of samples from Ecuador. SAKAA has previously been reported in Ecuador, Colombia, Peru, Brazil and Venezuela (19,20,31,32). In Ecuador, SAKAA was found in Ecu1110 (isolate from 1990) (19,20). In 2002, a study found that all parasites from Esmeraldas province had this haplotype (22). Then in northwestern Ecuador, SAKAA was found circulating between 2013 and 2015 (19). So, sulfadoxine sensitivity has remained high over time in the coast of Ecuador. Besides, the dominance of wild-type haplotype (SAKAA) suggests that selection in *Pfdhps* has been weak in northwest Ecuador. In addition, a synonymous mutation at codon 540 (AA**G**) was found in *Pfdhps*, which it was already circulating in Esmeraldas since 2013 and also in Peru (19,31). On the other hand, a sample obtained from Colombia presented the S**G**KAA haplotype (Fig.

2b), which has been previously reported in Colombia, Peru, Venezuela and Brazil (20,32–34).

CNCSI (wild-type) haplotype was found in *Pfdhfr* (Fig. 2c), showing pyrimethamine sensitivity in only 34.92% of Ecuadorian *P. falciparum* samples. CNCSI has previously been reported in Ecuador, Colombia and Brazil (20,33). Otherwise, the mutant haplotypes found in *Pfdhfr* were: CNC**N**I (1.59%) and C**I**C**N**I (63.49%) (Fig. 2c). CNC**N**I has previously been reported in Ecuador, Colombia, Peru, Brazil and Venezuela, while C**I**C**N**I in Ecuador, Colombia and Venezuela (20,33,64). *In vitro* studies show that S108**N** is the first mutation to emerge in *Pfdhfr*, which is essential to cause pyrimethamine resistance (35,65). In Ecuador, CNCSI (wild-type) was found in Ecu1110 (isolate from 1990) (19,20). Then in 2002, N51**I,** C59**R**, S108**N**/**T** and I164**L** mutations were found circulating in Esmeraldas province (19,22). In 2008, an unpublished study identified NCSI and NC**N**I in this same region (65). Later, in northwest Ecuador, CNCSI, CNC**N**I and C**I**C**N**I were found between 2013 and 2015 (19).

In South America, pyrimethamine was also used massively as malaria prophylactic in the 1950s (13,20,57,66). Later, in 1970, pyrimethamine was co-administered with sulfadoxine to combat CQR parasites (20,57). Consequently, SP resistance has been reported in South America since 1980 (20). Specifically, in Ecuador, SP was implemented as a second-line treatment in 1950 (48,56). Then, in 2005, SP was used with CQ as first-line treatment and, in 2006, it was changed by SP+AS (Fig. 5) (48,56). Therefore, the emergence of resistant parasites was caused by the

pressure exerted by SP for more than five decades. However, the intermittent use of SP reduced temporarily SP pressure, which may have caused SP-sensitive parasites continue to circulate in the region. So, the wild-type haplotypes in *Pfdhps* and *Pfdhfr* are still observed in Ecuador as in the rest of South America. Favorably, this intermittent use has prevented SP resistance from becoming fixed in the region.

The A16**V** mutation (Fig. 3) with CNCSI haplotype in *Pfdhfr* was identified in two samples from Tobar Donoso. In South America, A16**V** was detected for the first time in combination with CNCSI and C**I**C**N**I in Nariño, Colombia between 2012 and 2013 (33). A16**V** is often found together with S108**N**/**T** mutations and it causes high resistance to cycloguanil, the active metabolite of proguanil (13,29,67). In South America, Suriname has been the only country that used proguanil for several decades; however, A16**V** has not yet been found (68). On the other hand, atovaquone-proguanil has been commercialized in southwest Colombia, so it has been suggested that the emergence of A16**V** has been caused by the use of atovaquone-proguanil as prophylaxis against malaria (33). Therefore, the presence of A16**V** in Tobar Donoso could be explained by the recent spread of parasites from Nariño. So, it is crucial to identify regions that use proguanil or other factors that can cause the emergence and spread of this mutation.

NED**F**S**D**FD (55.88%) and NED**F**S**D**F**Y** (44.12%) haplotypes found in *Pfmdr-1* of Ecuadorian *P. falciparum* (Fig. 2d) had Y184**F**, N1042**D** and D1246**Y** mutations associated with multidrug resistance. NED**F**S**D**F**Y** has been found in Colombia (69), NED**F**S**D**FD in Ecuador and Colombia (19,69), Y184**F** and N1042**D** mutations in

Brazil (70), and D1246**Y** in Venezuela, Peru, Brazil and Bolivia (20). In Ecuador, NED**F**S**D**FD has been reported in Ecu1110 (isolate from 1990) (19). Then in northwest Ecuador, NED**F**SNFD, NEDYS**D**FD and NED**F**S**D**FD were found circulating between 2013 and 2015 (19). Mutations in *Pfmdr-1* have an important role in response to different antimalarials such as CQ, QN, AQ, MQ and LF (7,13,14). However, several *in vitro* studies have not shown reliability in molecular markers in *Pfmdr-1* because the response to antimalarials differ in several strains and isolates (67–70). Therefore, it has been suggested that the interaction with mutations in other genes located in the DV such as *Pfcrt* and *Pfaat1* may be influencing in the response to resistance to CQ, QN, AQ, MQ and LF (12,69). However, South American *P. falciparum* have not shown phenotypic changes in drug response even though Y184**F**, N1042**D** and D1246**Y** are commonly present (19,75). An *in vitro* assay tested parasites from Nariño with NED**F**S**D**FD and NED**F**S**D**F**Y** haplotypes, which were shown to be sensitive to AQ, MQ and LF (75). In Esmeraldas, ESM-2013 (isolate from 2013) with Y184**F** and N1042**D** mutation showed sensitivity to QN, LF and AR (19).

The prevalence of MYRIC haplotype (wild-type) in *Pfk13* (Fig. 2e) shows sensitivity to ARTs in 100% of tested Ecuadorian *P. falciparum* samples. Previous studies in Ecuador, Colombia, Peru, Venezuela, Brazil, Suriname and French Guiana have not found any mutation in *Pfk13* associated with ART resistance (19,42,43). However, in Guyana, C580**Y** mutation was already detected since 2010 (41,42). Therefore, there is a risk that C580**Y** could spread to other regions of South America. So, molecular studies would help to understand how this mutation might spread in the future. In addition, K189**T** mutation in *Pfk13* was identified in this study and

recently reported in Ecuador, Colombia, Venezuela, Brazil, Guyana and French Guiana (42,48). It has been suggested that K189**T** could be used to identify parasites from this region (34,72). In Ecuador, AS was used with SP as a first-line treatment in 2006; however, AL was already used as a second-line treatment in 2005 (56). Then in 2012, AL treatment was officially implemented as first-line to date (Fig. 5) (48,56). So, ART-based treatments have been used for a too short time to cause the emergence of ART-resistant parasites.

In the last decade, resistance to quinolines has been associated with mutations in *Pfaat1* (44–46). An in vitro assay showed that the insertion of T162**E** mutation in the 3D7 strain (CQ sensitivity) caused resistance to CQ, QN, AQ and MQ (46). Similarly, a genome study showed high polymorphism in *Pfaat1* associated with CQ resistance (44). Likewise, an *in vitro* study showed resistance to three compounds not yet cataloged (MMV668399, MMV011895 and MMV007224) when P380**S**, K238**N**, V185**L**, F230**L** mutations and insertion at codon 35 were present in *Pfaat1* (47). The S258**L** mutation found in this study in Ecuadorian *P. falciparum* (Fig. 3) has recently been reported on the Pacific coast of Ecuador and Colombia and associated with CQ resistance (48). *Pfaat1* is a highly conserved gene (46), so the emergence of mutations in *Pfaat1* may be related to pressure from quinolines. This pressure could have been caused mostly by CQ and QN, used historically in South America (20,76), and AQ which was introduced in several areas of South America, including the Pacific coast, since 1949 (24,59,77).

The presence of resistant haplotypes with multiple mutations in Colombian parasites is related to historical changes in the treatment scheme against *P. falciparum* (48). So, Colombia could be considered a hot spot of the emergence of antimalarial resistance compared to Ecuador (69). On the other hand, in Ecuador, treatments against *P. falciparum* have been maintained for many years (Fig. 5), which could explain the dominance of wild-type haplotypes in *Pfdhps* (SAKAA) and *Pfdhfr* (CNCSI) and single mutants in *Pfcrt* (CVMN**T**) and *Pfmdr-1* (NED**F**SNFD, NEDYS**D**FD) in years previous to 2015 (19). However, the recent increase in proportions of mutant haplotypes in Ecuador such as CVM**ET** (double mutant) in *Pfcrt*, C**I**C**N**I (double mutant) in *Pfdhfr* and the appearance of NED**F**S**D**F**Y** (triple mutant) in *Pfmdr-1* (Fig. 4) could be explained by clonal expansions caused by parasites from migrant humans and not by the pressure by CQ and SP treatments, because they have not been used against *P. falciparum* in the last two decades. A previous analysis of neutral microsatellites found that outbreaks that occurred in Tobar Donoso and Esmeraldas in 2019 and 2020, respectively, were mostly caused by a single parasite clone (78). Furthermore, CVM**ET** in *Pfcrt*, C**I**C**N**I in *Pfdhfr* and NED**F**S**D**F**Y** in *Pfmdr-1* were dominant in samples from migrant human hosts in this study (Fig. 2), and they have been found circulating in Nariño since 1999 (20,24,33). While in northwest Ecuador, CVM**ET** and C**I**C**N**I were reported in low proportions in 2013-2015 (19), and NED**F**S**D**F**Y** was absent, but they are now common in recent samples in this study. This suggests that recent outbreaks that occurred in Ecuador were the result of the spread of parasites by migrant humans from Nariño.

Previously, an important human migratory flow that contributes to the spread of parasites on the Pacific coast of Colombia has already been reported (50). In the

present study, the main routes of cross-border and intra-border human migration contributing to the spread of malaria parasites in the North coast of Ecuador and the South coast of Colombia are proposed (Fig. 6). Specifically, the spread of resistant parasites to Ecuador could have occurred in two ways: 1) by Ecuadorians who traveled to Colombia and became infected with parasites from there, and 2) directly by infected Colombians who arrived to Ecuador. The main migration route between Nariño and San Lorenzo for several years has been by their seaports. Therefore, Colombian parasites could have reached the coast of Ecuador by the constant migratory flow along the Pacific coast due to the shellfish trade, legal and illegal mining, agriculture and illegal activities such as drug trafficking through the seaports of Nariño, San Lorenzo and Esmeraldas (79–81). In addition, it has recently been found that *P. falciparum* populations are shared between Nariño and San Lorenzo (48,49). Currently, a new road has been opened in this area between Colombia and Ecuador, the bridge over Mataje River that connects Nariño and San Lorenzo (82). This road will definitely represent an important passage of malaria infected people between Ecuador and Colombia. Finally, the E15 road that connects San Lorenzo and Esmeraldas is an important corridor in the spread of malaria parasites. In addition, the presence of A16**V** in *Pfdhfr* (Fig. 3) in Tobar Donoso also suggests a process of human migration because this mutation has only recently been found in Nariño (33). Possibly, the migration of human hosts occurred along of the Mira River, which comes from Nariño, and it is frequently used by Colombians to trade and transport supplies to Tobar Donoso (83). Additionally, migration by the E10 road, which connects San Lorenzo and Carchi, could also be contributing to the spread of parasites to Tobar Donoso because it is frequently used to reach the south of Carchi province. From there second and third-order improvised roads and walking trails

connect with Tobar Donoso. It is important to note that a helicopter from Esmeraldas transports food, medicine and soldiers to monitor the border at least once a month (83), and could also be considered a route of entry of parasites from Esmeraldas to Tobar Donoso.

South America is considered a low malaria transmission region with low genetic diversity of *P. falciparum* (84,85). An analysis using neutral microsatellite markers found that genetic diversity is low in northwest Ecuador (78). The findings in this study show that the genetic diversity in drug resistance genes (*Pfcrt*, *Pfdhps*, *Pfdhfr*, and *Pfmdr-1*) is also low. In general, it was found that San Lorenzo and Colombia presented more genetic variability than Esmeraldas and Tobar Donoso. The low diversity in Esmeraldas and Tobar Donoso was mostly due to the fact that samples came from a clonal outbreak, for which a single haplotype was predominant. While in San Lorenzo and Colombia, the genetic diversity was caused by the variability of haplotypes present in these localities.

Drug resistance genes such as *Pfcrt*, *Pfdhps*, *Pfdhfr*, *Pfmdr-1*, *Pfk13* and *Pfaat1* are constantly subject to natural selection due to beneficial mutations increasing the fitness of *P. falciparum* (12). Recently, a hard selective sweep in *Pfcrt* and soft or incomplete selective sweep in *Pfdhps*, *Pfdhfr* and *Pfmdr-1* has been evidenced in parasites from the Pacific coast of Ecuador and Colombia (48). In the present study, positive selection was evident in *Pfcrt*, *Pfdhfr* and *Pfmdr-1*. However, the negative values of Tajima's D in *Pfcrt* in Tobar Donoso and *Pfmdr-1* in Esmeraldas are explained by the low frequency of CVM**ET** and NED**F**S**D**FD, respectively. On the

other hand, *Pfdhps* did not show a selection process in Esmeraldas, Tobar Donoso and Colombia because only SAKAA (wild-type) haplotype was present. Finally, these results show that factors such as drug pressure and host human migration influence the emergence and spread of resistant parasites which contribute to the appearance of malaria outbreaks and complicate malaria control and elimination in Ecuador.

7 CONCLUSIONS

The findings of this study indicate that resistance to chloroquine and pyrimethamine in Ecuadorian *P. falciparum* has increased in recent years, but sensitivity to sulfadoxine and artemisinin derivatives is maintained. Therefore, the current treatment (artemether + lumefantrine) against *P. falciparum* appears to be still successful in the country. In addition, the results show that *P. falciparum* have undergone a selection process due to drug pressure in previous decades and the frequent migration of human hosts between the Ecuador-Colombia border has contributed to the spread of resistant parasites. Finally, this study shows the importance of the epidemiological characterization of resistance in *P. falciparum* in a region in the process of eliminating malaria. Therefore, genetic surveillance of drug resistance genes is key to respond in a timely manner in case of therapeutic failure and prevent the spread of malaria.

8 REFERENCES

1. PAHO. Epidemiological Update: Malaria in the Americas in the context of the COVID-19 pandemic. Pan American Health Organization. 2020.

2. WHO. World malaria report. World Health Organization. 2019. https://www.who.int/malaria/media/world-malaria-report-2019/es/. Accessed 21 Nov 2021.

3. WHO. Malaria. World Health Organization. 2021. https://www.who.int/news-room/fact-sheets/detail/malaria. Accessed 3 Dec 2021.

4. WHO. World malaria report 2021. 2021;1–15. https://cdn.who.int/media/docs/default-source/malaria/world-malaria-reports/world-malaria-report-2021-global-briefing-kit-spa.pdf?sfvrsn=8e5e915_23&download=true. Accessed 10 Dec 2021.

5. MSP. Malaria: Gacetas vectoriales. Ministerio de Salud Pública del Ecuador. Quito; 2022. https://www.salud.gob.ec/gacetas-vectoriales/#search. Accessed 21 Dec 2021.

6. PAHO. Malaria Situation Report in the Americas - 2017. Pan Am Heal Organ. 2017.https://www.paho.org/hq/index.php?option=com_docman&view=download&category_slug=datos-estadisticos-mapas-8110&alias=48336-situation-of-malaria-in-the-region-of-the-americas-2017-1&Itemid=270&lang=es. Accessed 22 Dec 2021.

7. Venanzi E, López-Vélez R. Resistencia a los antimaláricos. Rev Española Quimioter - Publicación Of la Soc Española Quimioter. 2016;29(1):72–5.

8. WHO. World malaria report. World Health Organization. 2016. https://apps.who.int/iris/bitstream/handle/10665/252038/9789241511711-eng.pdf?sequence=1. Accessed 4 Jan 2022.

9. Ranjitkar S, Schousboe ML, Thomsen TT, Adhikari M, Kapel CM, Bygbjerg

IC, et al. Prevalence of molecular markers of anti-malarial drug resistance in *Plasmodium vivax* and *Plasmodium falciparum* in two districts of Nepal. Malar J. 2011;10(1):75. doi: https://doi.org/10.1186/1475-2875-10-75.

10. Jadan-Solis KP, Alban-Meneses C de J, Salazar-Carranza A, Cruz-Fonseca L de los Á, Torres-Céspedes I, Scrich-Vázquez AJ. Characterization of the malaria like endemic illness in Ecuador. Rev Arch Médico Camagüey. 2019;23(4):540–58.

11. Hastings I, Donnelly M. The impact of antimalarial drug resistance mutations on parasite fitness, and its implications for the evolution of resistance. Drug Resist Updat. 2005;8(1–2):43–50. doi: https://doi.org/10.1016/j.drup.2005.03.003.

12. Pedreros MF. Determination of circulating genotypes of loci associated with resistance to antimalarial drugs in *Plasmodium falciparum* in the population of Guapi, Department of Cauca, Colombia. National University of Colombia; 2017.

13. Hodoameda P. *P. falciparum* and Its Molecular Markers of Resistance to Antimalarial Drugs. In: *Plasmodium* Species and Drug Resistance. IntechOpen; 2021.

14. Ibraheem ZO, Abd Majid R, Noor SM, Sedik HM, Basir R. Role of Different Pfcrt and Pfmdr-1 Mutations in Conferring Resistance to Antimalaria Drugs in *Plasmodium falciparum*. Malar Res Treat. 2014;1–17. doi: https://doi.org/10.1155/2014/950424.

15. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, et al. Mutations in the *P. falciparum* Digestive Vacuole Transmembrane Protein

643 PfCRT and Evidence for Their Role in Chloroquine Resistance. Mol Cell.
644 2000;6(4):861–71. 10. doi: https://doi.org/10.1016/S1097-2765(05)00077-8.

645 16. Ecker A, Lehane AM, Clain J, Fidock DA. PfCRT and its role in antimalarial
646 drug resistance. Trends Parasitol. 2012;28(11):504–14. doi:
647 https://doi.org/10.1016/j.pt.2012.08.002.

648 17. Atroosh WM, Al-Mekhlafi HM, Mahdy MA, Surin J. The detection of pfcrt and
649 pfmdr1 point mutations as molecular markers of chloroquine drug resistance,
650 Pahang, Malaysia. Malar J. 2012;11(1):251. doi: https://doi.org/10.1186/1475-
651 2875-11-251.

652 18. Djimdé A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourté Y, et al.
653 A Molecular Marker for Chloroquine-Resistant Falciparum Malaria. N Engl J
654 Med. 2001;344(4):257–63. doi: 10.1056/NEJM200101253440403.

655 19. Valenzuela G, Castro LE, Valencia-Zamora J, Vera-Arias CA, Rohrbach P,
656 Sáenz FE. Genotypes and phenotypes of resistance in Ecuadorian
657 *Plasmodium falciparum*. Malar J. 2019;18(1):415. doi:
658 https://doi.org/10.1186/s12936-019-3044-z.

659 20. Cortese JF, Caraballo A, Contreras CE, Plowe C V. Origin and dissemination
660 of *Plasmodium falciparum* drug-resistance mutations in South America. J
661 Infect Dis. 2002;186(7):999–1006.

662 21. Sáenz FE, Morton LC, Okoth SA, Valenzuela G, Vera-Arias CA, Vélez-
663 Álvarez E, et al. Clonal population expansion in an outbreak of *Plasmodium*
664 *falciparum* on the northwest coast of Ecuador. Malar J. 2015;14(1):497. doi:
665 https://doi.org/10.1186/s12936-015-1019-2

22. Arróspide N, Hijar-Guerra G, de Mora D, Diaz-Cortéz CE, Veloz-Perez R, Gutierrez S, et al. Mutant alleles associated to chloroquine and sulfadoxine-pyrimethanime resistance in *Plasmodium falciparum* of the Ecuador-Peru and Ecuador-Colombia borders. Rev Peru Med Exp Salud Publica. 2014;31(2):282–7.

23. Valderramos SG, Valderramos J-C, Musset L, Purcell LA, Mercereau-Puijalon O, Legrand E, et al. Identification of a Mutant PfCRT-Mediated Chloroquine Tolerance Phenotype in *Plasmodium falciparum*. Kazura JW, editor. PLoS Pathog. 2010;6(5). doi: https://doi.org/10.1371/journal.ppat.1000887.

24. Echeverry DF, Holmgren G, Murillo C, Higuita JC, Björkman A, Gil JP, et al. Short report: Polymorphisms in the pfcrt and pfmdr1 genes of *Plasmodium falciparum* and *in vitro* susceptibility to amodiaquine and desethylamodiaquine. Am J Trop Med Hyg. 2007;77(6):1034–8.

25. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, et al. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell. 2000;6(4):861–71. doi: https://doi.org/10.1016/S1097-2765(05)00077-8.

26. Beshir K, Sutherland CJ, Merinopoulos I, Durrani N, Leslie T, Rowland M, et al. Amodiaquine Resistance in *Plasmodium falciparum* Malaria in Afghanistan Is Associated with the pfcrt SVMNT Allele at Codons 72 to 76. Antimicrob Agents Chemother. 2010;54(9):3714–6. doi: https://doi.org/10.1128/AAC.00358-10**.**

27. Jiang T, Chen J, Fu H, Wu K, Yao Y, Eyi JUM, et al. High prevalence of Pfdhfr–Pfdhps quadruple mutations associated with sulfadoxine–pyrimethamine

690 resistance in *Plasmodium falciparum* isolates from Bioko Island, Equatorial
691 Guinea. Malar J. 2019;18(1):101. doi: https://doi.org/10.1186/s12936-019-
692 2734-x.

693 28. Gatton ML, Martin LB, Cheng Q. Evolution of Resistance to Sulfadoxine-
694 Pyrimethamine in *Plasmodium falciparum*. Antimicrob Agents Chemother.
695 2004;48(6):2116–23. doi: https://doi.org/10.1128/AAC.48.6.2116-2123.2004**.**

696 29. Costanzo MS, Brown KM, Hartl DL. Fitness Trade-Offs in the Evolution of
697 Dihydrofolate Reductase and Drug Resistance in *Plasmodium falciparum*. von
698 Seidlein L, editor. PLoS One. 2011;6(5). doi:
699 https://doi.org/10.1371/journal.pone.0019636.

700 30. Sridaran S, McClintock SK, Syphard LM, Herman KM, Barnwell JW,
701 Udhayakumar V. Anti-folate drug resistance in Africa: meta-analysis of
702 reported dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps)
703 mutant genotype frequencies in African *Plasmodium falciparum* parasite
704 populations. Malar J. 2010;9(1):247. doi: https://doi.org/10.1186/1475-2875-
705 9-247.

706 31. Griffing SM, Mixson-Hayden T, Sridaran S, Alam MT, McCollum AM, Cabezas
707 C, et al. South American *Plasmodium falciparum* after the Malaria Eradication
708 Era: Clonal Population Expansion and Survival of the Fittest Hybrids. Idnurm
709 A, editor. PLoS One. 2011;6(9):e23486. doi:
710 https://doi.org/10.1371/journal.pone.0023486.

711 32. Corredor V, Murillo C, Echeverry DF, Benavides J, Pearce RJ, Roper C, et al.
712 Origin and Dissemination across the Colombian Andes Mountain Range of
713 Sulfadoxine-Pyrimethamine Resistance in *Plasmodium falciparum*.

714 Antimicrob Agents Chemother. 2010;54(8):3121–5. doi:
715 https://doi.org/10.1128/AAC.00036-10.

716 33. Fierro B. Genotipos de *Plasmodium falciparum* asociados a la resistencia a
717 sulfadoxina/pirimetamina en Colombia. Universidad Nacional de Colombia;
718 2017.

719 34. Mita T, Venkatesan M, Ohashi J, Culleton R, Takahashi N, Tsukahara T, et
720 al. Limited Geographical Origin and Global Spread of Sulfadoxine-Resistant
721 dhps Alleles in *Plasmodium falciparum* Populations. J Infect Dis.
722 2011;204(12):1980–8. doi: https://doi.org/10.1093/infdis/jir664.

723 35. Brown KM, Costanzo MS, Xu W, Roy S, Lozovsky ER, Hartl DL.
724 Compensatory Mutations Restore Fitness during the Evolution of
725 Dihydrofolate Reductase. Mol Biol Evol. 2010;27(12):2682–90. doi:
726 https://doi.org/10.1093/molbev/msq160.

727 36. Veiga MI, Dhingra SK, Henrich PP, Straimer J, Gnädig N, Uhlemann A-C, et
728 al. Globally prevalent PfMDR1 mutations modulate *Plasmodium falciparum*
729 susceptibility to artemisinin-based combination therapies. Nat Commun.
730 2016;7(1):11553. doi: https://doi.org/10.1038/ncomms11553.

731 37. Duraisingh MT, Cowman AF. Contribution of the pfmdr1 gene to antimalarial
732 drug-resistance. Acta Trop. 2005;94(3):181–90. doi:
733 https://doi.org/10.1016/j.actatropica.2005.04.008.

734 38. Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski
735 C, et al. Resistance to Antimalarials in Southeast Asia and Genetic
736 Polymorphisms in pfmdr1. Antimicrob Agents Chemother. 2003;47(8):2418–
737 23. doi: https://doi.org/10.1128/AAC.47.8.2418-2423.2003**.**

738 39. Oberstaller J, Zoungrana L, Bannerman CD, Jahangiri S, Dwivedi A, Silva JC,
739 et al. Integration of population and functional genomics to understand
740 mechanisms of artemisinin resistance in *Plasmodium falciparum*. Int J
741 Parasitol Drugs Drug Resist 2021;16:119–28. doi:
742 https://doi.org/10.1016/j.ijpddr.2021.05.006.

743 40. Xie SC, Ralph SA, Tilley L. K13, the Cytostome, and Artemisinin Resistance.
744 Trends Parasitol. 2020;36(6):533–44. doi:
745 https://doi.org/10.1016/j.pt.2020.03.006.

746 41. Mathieu LC, Cox H, Early AM, Mok S, Lazrek Y, Paquet J-C, et al. Local
747 emergence in Amazonia of *Plasmodium falciparum* k13 C580Y mutants
748 associated with *in vitro* artemisinin resistance. Elife. 2020;9. doi:
749 https://doi.org/10.7554/eLife.51015.

750 42. Mathieu LC, Singh P, Monteiro WM, Magris M, Cox H, Lazrek Y, et al. Kelch13
751 mutations in *Plasmodium falciparum* and risk of spreading in Amazon basin
752 countries. J Antimicrob Chemother. 2021;76(11):2854–62. doi:
753 https://doi.org/10.1093/jac/dkab264.

754 43. Ménard D, Khim N, Beghain J, Adegnika AA, Shafiul-Alam M, Amodu O, et al.
755 A Worldwide Map of *Plasmodium falciparum* K13-Propeller Polymorphisms.
756 N Engl J Med. 2016;374(25):2453–64. doi: 10.1056/NEJMoa1513137.

757 44. Wang Z, Cabrera M, Yang J, Yuan L, Gupta B, Liang X, et al. Genome-wide
758 association analysis identifies genetic loci associated with resistance to
759 multiple antimalarials in *Plasmodium falciparum* from China-Myanmar border.
760 Sci Rep. 2016;6(1):33891. doi: https://doi.org/10.1038/srep33891.

761 45. Amambua-Ngwa A, Amenga-Etego L, Kamau E, Amato R, Ghansah A,

762 Golassa L, et al. Major subpopulations of *Plasmodium falciparum* in sub-
763 Saharan Africa. Science. 2019;365(6455):813–6. doi:
764 10.1126/science.aav5427.

765 46. Tindall SM, Vallières C, Lakhani DH, Islahudin F, Ting K-N, Avery S V.
766 Heterologous Expression of a Novel Drug Transporter from the Malaria
767 Parasite Alters Resistance to Quinoline Antimalarials. Sci Rep.
768 2018;8(1):2464. doi: https://doi.org/10.1038/s41598-018-20816-0.

769 47. Cowell AN, Istvan ES, Lukens AK, Gomez-Lorenzo MG, Vanaerschot M,
770 Sakata-Kato T, et al. Mapping the malaria parasite druggable genome by
771 using *in vitro* evolution and chemogenomics. Science. 2018;359(6372):191–
772 9. doi: 10.1126/science.aan4472.

773 48. Carrasquilla M, Early A. Resolving drug selection and migration in an inbred
774 South American *Plasmodium falciparum* population with identity by descent
775 analysis. PLoS One. 2022. doi: https://doi.org/10.1101/2022.02.18.480973

776 49. Vera-Arias CA, Castro LE, Gómez-Obando J, Sáenz FE. Diverse origin of
777 *Plasmodium falciparum* in northwest Ecuador. Malar J. 2019;18(1):1–11. doi:
778 https://doi.org/10.1186/s12936-019-2891-y.

779 50. Taylor AR, Echeverry DF, Anderson TJC, Neafsey DE, Buckee CO. Identity-
780 by-descent with uncertainty characterises connectivity of *Plasmodium*
781 *falciparum* populations on the Colombian-Pacific coast. Sirugo G, editor.
782 PLOS Genet. 2020;16(11):e1009101. doi:
783 https://doi.org/10.1371/journal.pgen.1009101.

784 51. PAHO. Initiative 2020: countries towards the elimination of malaria in the
785 Americas, 2018. Pan American Health Organization. 2018.

786 52. Mendoza Meza DL. Malaria y la resistencia a antimalaricos. Duazary.
787 2004;1(2):114–23.

788 53. OPS. Situación de la Malaria en la Región de las Américas, 2000-2016.
789 Organización Panamericana de la Salud. 2016. p. 10.

790 54. Nei M, Li WH. Mathematical model for studying genetic variation in terms of
791 restriction endonucleases. Proc Natl Acad Sci. 1979;76(10):5269–73. doi :
792 https://doi.org/10.1073/pnas.76.10.5269.

793 55. Rathmes G, Rumisha SF, Lucas TCD, Twohig KA, Python A, Nguyen M, et
794 al. Global estimation of anti-malarial drug effectiveness for the treatment of
795 uncomplicated *Plasmodium falciparum* malaria 1991–2019. Malar J.
796 2020;19(1):374. doi: https://doi.org/10.1186/s12936-020-03446-8.

797 56. Aguilar H, Real C, Veloz R, Torres C. Enfermedades transmisibles en el siglo
798 XXI en Ecuador: análisis de enfermedades trazadoras. In: Editor B, editor.
799 Ciencias en salud colectiva y terapias integrativas. Quito, Ecuador; 2018.

800 57. Mita T, Tanabe K, Kita K. Spread and evolution of *Plasmodium falciparum*
801 drug resistance. Parasitol Int. 2009;58(3):201–9. doi:
802 https://doi.org/10.1016/j.parint.2009.04.004.

803 58. Aguilar H. Malaria y espacio en el Ecuador del verde de París a la eliminación
804 de la enfermedad. Universidad Andina Simón Bolívar; 2021.

805 59. Sá JM, Twu O, Hayton K, Reyes S, Fay MP, Ringwald P, et al. Geographic
806 patterns of *Plasmodium falciparum* drug resistance distinguished by
807 differential responses to amodiaquine and chloroquine. Proc Natl Acad Sci.
808 2009;106(45):18883–9. doi: https://doi.org/10.1073/pnas.0911317106.

809 60. Pelleau S, Moss EL, Dhingra SK, Volney B, Casteras J, Gabryszewski SJ, et
810 al. Adaptive evolution of malaria parasites in French Guiana: Reversal of
811 chloroquine resistance by acquisition of a mutation in pfcrt. Proc Natl Acad
812 Sci. 2015;112(37):11672–7. doi: https://doi.org/10.1073/pnas.1507142112.

813 61. Payne D. Did medicated salt hasten the spread of chloroquine resistance in
814 *Plasmodium falciparum*? Parasitol Today. 1988;4(4):112–5. doi:
815 https://doi.org/10.1016/0169-4758(88)90042-7.

816 62. Gómez GF, Vasquez MF. Factores no biologicos relacionados con el
817 desarrollo de resistencia de *Plasmodium falciparum* a la cloroquina. Rev CES
818 Med. 2011;5(1):1–12.

819 63. Kim J, Tan YZ, Wicht KJ, Erramilli SK, Dhingra SK, Okombo J, et al. Structure
820 and drug resistance of the *Plasmodium falciparum* transporter PfCRT. Nature.
821 2019;576(7786):315–20. doi: https://doi.org/10.1038/s41586-019-1795-x.

822 64. McCollum AM, Mueller K, Villegas L, Udhayakumar V, Escalante AA.
823 Common Origin and Fixation of *Plasmodium falciparum* dhfr and dhps
824 Mutations Associated with Sulfadoxine-Pyrimethamine Resistance in a Low-
825 Transmission Area in South America. Antimicrob Agents Chemother.
826 2007;51(6):2085–91. doi: https://doi.org/10.1128/AAC.01228-06**.**

827 65. Morales F. Estudios moleculares de *Plasmodium falciparum* y *P. vivax* en la
828 provincia de Sucumbíos, Ecuador. Universidad San Francisco de Quito; 2010.

829 66. Gregson A, Plowe C V. Mechanisms of Resistance of Malaria Parasites to
830 Antifolates. Pharmacol Rev. 2005;57(1):117–45. doi:
831 https://doi.org/10.1124/pr.57.1.4.

67. Sirawaraporn W, Sathitkul T, Sirawaraporn R, Yuthavong Y, Santi D V. Antifolate-resistant mutants of *Plasmodium falciparum* dihydrofolate reductase. Proc Natl Acad Sci. 1997;94(4):1124–9. doi: https://doi.org/10.1073/pnas.94.4.1124.

68. Van Gool T, Panchoe D, Peek R, Bus E, Resida L, Greve S. Drug resistance and genetic diversity of Plasmodium falciparum parasites from Suriname. Am J Trop Med Hyg. 2005;73(5):833–8. doi: https://doi.org/10.4269/ajtmh.2005.73.833.

69. Montenegro M, Neal AT, Posada M, De las Salas B, Lopera-Mesa TM, Fairhurst RM, et al. K13 Propeller Alleles, mdr1 Polymorphism, and Drug Effectiveness at Day 3 after Artemether-Lumefantrine Treatment for *Plasmodium falciparum* Malaria in Colombia, 2014-2015. Antimicrob Agents Chemother. 2017 Dec;61(12). doi: https://doi.org/10.1128/AAC.01036-17**.**

70. Aguiar ACC, Pereira DB, Amaral NS, De Marco L, Krettli AU. *Plasmodium vivax* and *Plasmodium falciparum* ex vivo susceptibility to anti-malarials and gene characterization in Rondônia, West Amazon, Brazil. Malar J. 2014;13(1):73. doi: https://doi.org/10.1186/1475-2875-13-73.

71. Lim P, Wongsrichanalai C, Chim P, Khim N, Kim S, Chy S, et al. Decreased *In Vitro* Susceptibility of *Plasmodium falciparum* Isolates to Artesunate, Mefloquine, Chloroquine, and Quinine in Cambodia from 2001 to 2007. Antimicrob Agents Chemother. 2010;54(5):2135–42. doi: https://doi.org/10.1128/AAC.01304-09**.**

72. Dahlström S, Aubouy A, Maïga-Ascofaré O, Faucher J-F, Wakpo A, Ezinmègnon S, et al. *Plasmodium falciparum* Polymorphisms Associated with

Ex Vivo Drug Susceptibility and Clinical Effectiveness of Artemisinin-Based Combination Therapies in Benin. Antimicrob Agents Chemother. 2014;58(1):1–10. doi: https://doi.org/10.1128/AAC.01790-12**.**

73. Wurtz N, Fall B, Pascual A, Fall M, Baret E, Camara C, et al. Role of Pfmdr1 in In Vitro *Plasmodium falciparum* Susceptibility to Chloroquine, Quinine, Monodesethylamodiaquine, Mefloquine, Lumefantrine, and Dihydroartemisinin. Antimicrob Agents Chemother. 2014;58(12):7032–40. doi: https://doi.org/10.1128/AAC.03494-14**.**

74. Cheng W, Song X, Tan H, Wu K, Li J. Molecular surveillance of anti-malarial resistance pfcrt, pfmdr1, and pfk13 polymorphisms in African *Plasmodium falciparum* imported parasites to Wuhan, China. Malar J. 2021 Dec 1;20(1):209. doi: https://doi.org/10.1186/s12936-021-03737-8.

75. Montenegro LM, de las Salas B, Neal AT, Tobon-Castaño A, Fairhurst RM, Lopera-Mesa TM. State of Artemisinin and Partner Drug Susceptibility in *Plasmodium falciparum* Clinical Isolates from Colombia. Am J Trop Med Hyg [Internet]. 2021;104(1):263–70. doi: https://doi.org/10.4269/ajtmh.20-0148.

76. Achan J, Talisuna AO, Erhart A, Yeka A, Tibenderana JK, Baliraine FN, et al. Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. Malar J. 2011;10(1):144. doi: https://doi.org/10.1186/1475-2875-10-144.

77. Carmona JF, Tobón A, Álvarez G, Blair S. Treatment with amodiaquine–sulfadoxine–pyrimethamine is 98% effective against uncomplicated falciparum malaria. Latreia. 2005;18.

78. Cartagenova J. Poblaciones de *Plasmodium falciparum*, en las provincias de

Esmeraldas y Carchi en 2019 y 2020. 2021. Pontificia Universidad Católica del Ecuador; 2021.

79. Mosquera-Romero M, Zuluaga-Idárraga L, Tobón-Castaño A. Challenges for the diagnosis and treatment of malaria in low transmission settings in San Lorenzo, Esmeraldas, Ecuador. Malar J. 2018;17(1):440. doi: https://doi.org/10.1186/s12936-018-2591-z.

80. Estupiñan L. Influencia de las redes migratorias en las decisiones tomadas por los colombianos en situación de refugio. Caso cantón San Lorenzo del Pailón-Esmeraldas, 2000-2012. 2013.

81. Salazar B, Vera C, Tamayo M, Yumiseva C, Cagua J, Gomez J, et al. Alta prevalencia de infecciones asintomáticas de malaria en la frontera Ecuador Colombia. Práctica Fam Rural. 2020;5(2). doi: https://doi.org/10.23936/pfr.v5i2.157.

82. Rivera F, Ortega H, Larreátegui P, Riaño-Alcalá P. Migración forzada de colombianos. In: Migración forzada de colombianos: Colombia, Ecuador, Canadá. Medellín, Colombia; 2007. p. 207.

83. Vasco C. Problemas Fronterizos En Ecuador Con Colombia II: Provincia Del Carchi. Rev Ciencias Segur y Def. 2016;1(4):27–42.

84. Anderson TJC, Haubold B, Williams JT, Estrada-Franco§ JG, Richardson L, Mollinedo R, et al. Microsatellite Markers Reveal a Spectrum of Population Structures in the Malaria Parasite *Plasmodium falciparum*. Mol Biol Evol. 2000 ;17(10):1467–82.doi: https://doi.org/10.1093/oxfordjournals.molbev.a026247.

85. Yalcindag E, Elguero E, Arnathau C, Durand P, Akiana J, Anderson TJ, et al.

903 Multiple independent introductions of *Plasmodium falciparum* in South
904 America. Proc Natl Acad Sci. 2012;109(2):511–6. doi:
905 https://doi.org/10.1073/pnas.1119058109.

906

9 FIGURES

Figure 1. Study sites. Samples collected in northwest Ecuador (Esmeraldas (n=27), San Lorenzo (n=5), Tobar Donoso (n=18) and patients from Colombia (n=13)).

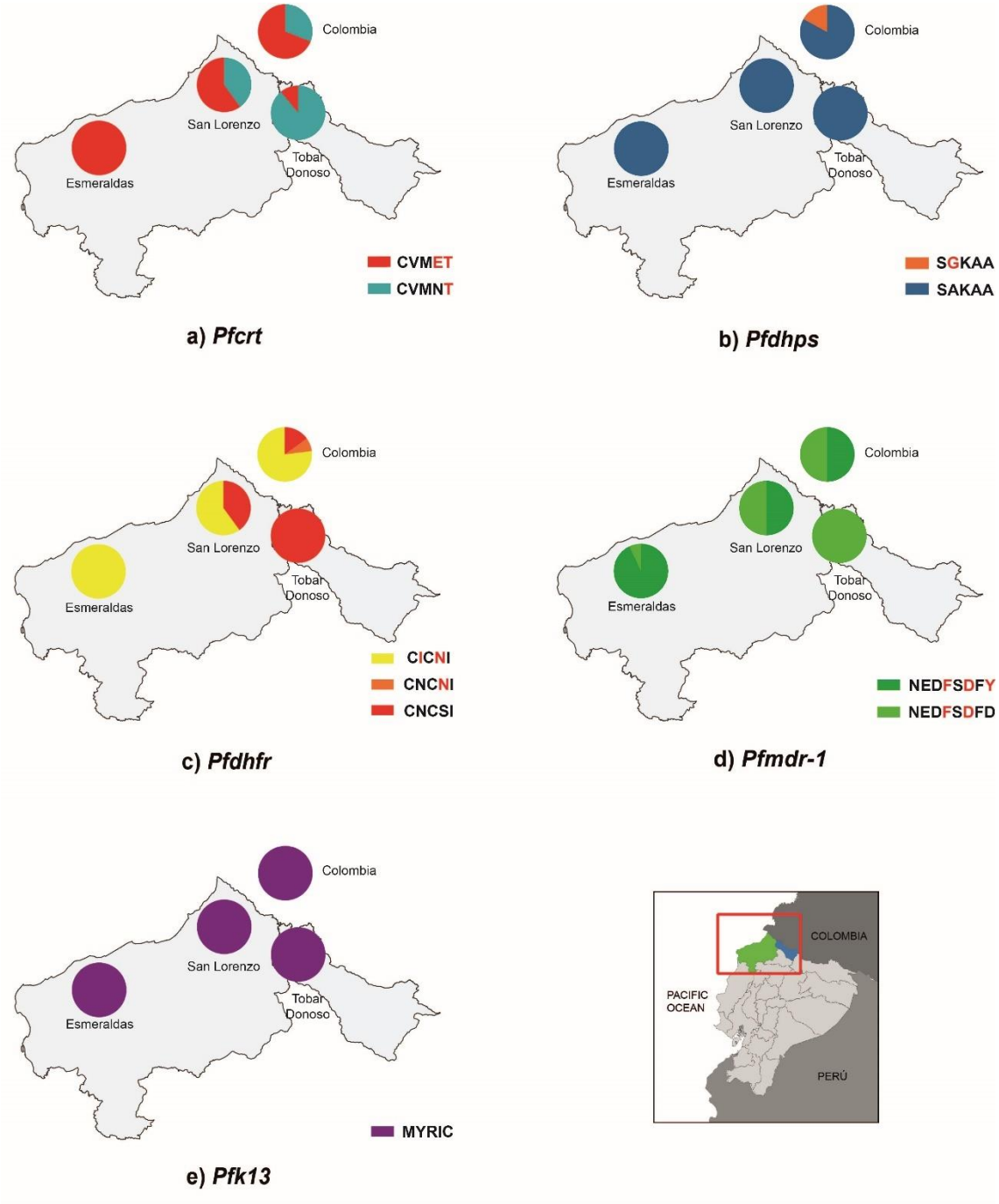

Figure 2. Geographic distribution of frequencies of drug resistance haplotypes in the 2019-2021 period. a. Haplotypes of *Pfcrt* (72-76), b. Haplotypes of *Pfdhps* (436, 437, 549, 581 and 613), c. Haplotypes of *Pfdhfr* (50, 51, 59, 108 and 164), d. Haplotypes of *Pfmdr-1* (codons 86, 130, 144, 184, 1034, 1042, 1226 and 1246), e. Haplotypes of *Pfk13* (codons 476, 493, 539, 543 and 580).

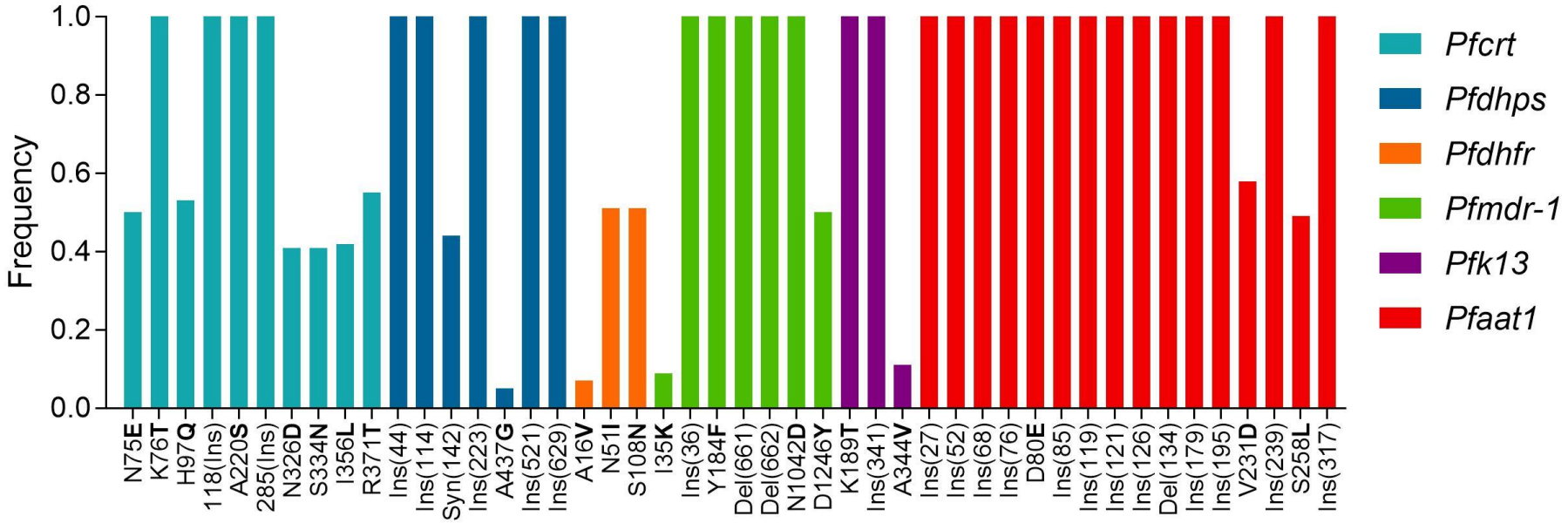

Figure 3. Frequencies of complementary mutations found in drug resistance genes obtained by WGS. Ins: Insertion, Syn: Synonymous mutation, Del: Deletion.

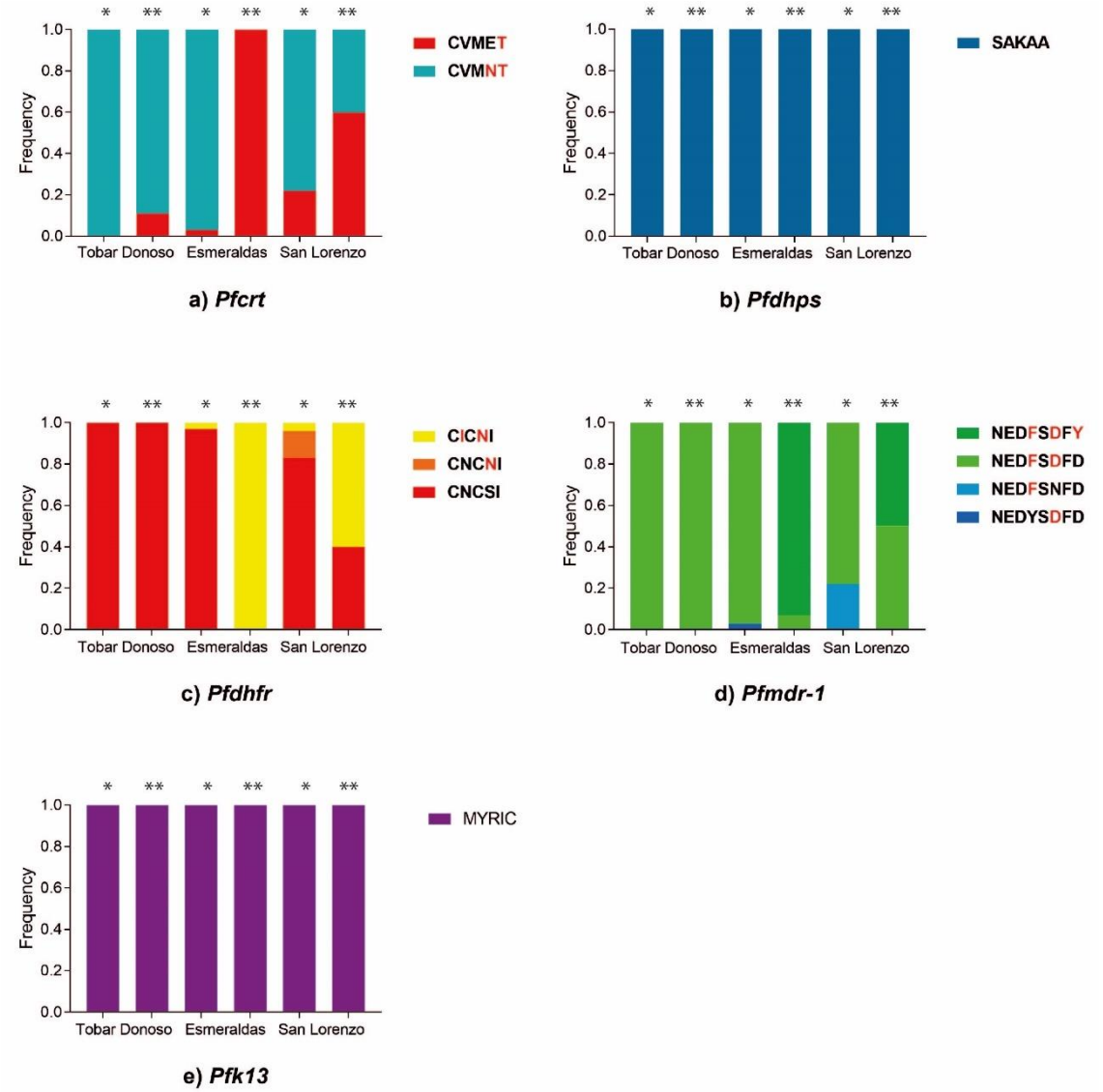

Figure 4. Comparative frequencies of drug resistance haplotypes in Northwest Ecuador between the 2013-2015 and 2019-2021 periods. a. Haplotypes of *Pfcrt* (72-76), b. Haplotypes of *Pfdhps* (436, 437, 549, 581 and 613), c. Haplotypes of *Pfdhfr* (50, 51, 59, 108 and 164), d. Haplotypes of *Pfmdr-1* (codons 86, 130, 144, 184, 1034, 1042, 1226 and 1246), e. Haplotypes of *Pfk13* (codons 476, 493, 539, 543 and 580). * 2013-2015 ** 2019-2021.

Figure 5. Antimalarial treatments against *P. falciparum* in Ecuador. Timeline of antimalarial treatments since 1950 according to the Ministry of Public Health of Ecuador. Chloroquine (CQ), primaquine (PQ), sulfadoxine-pyrimethamine (SP), artemether-lumefantrine (AL), artesunate (AS), quinine (QN) and clindamycin (CM). Source: Aguilar, 2018 (56).

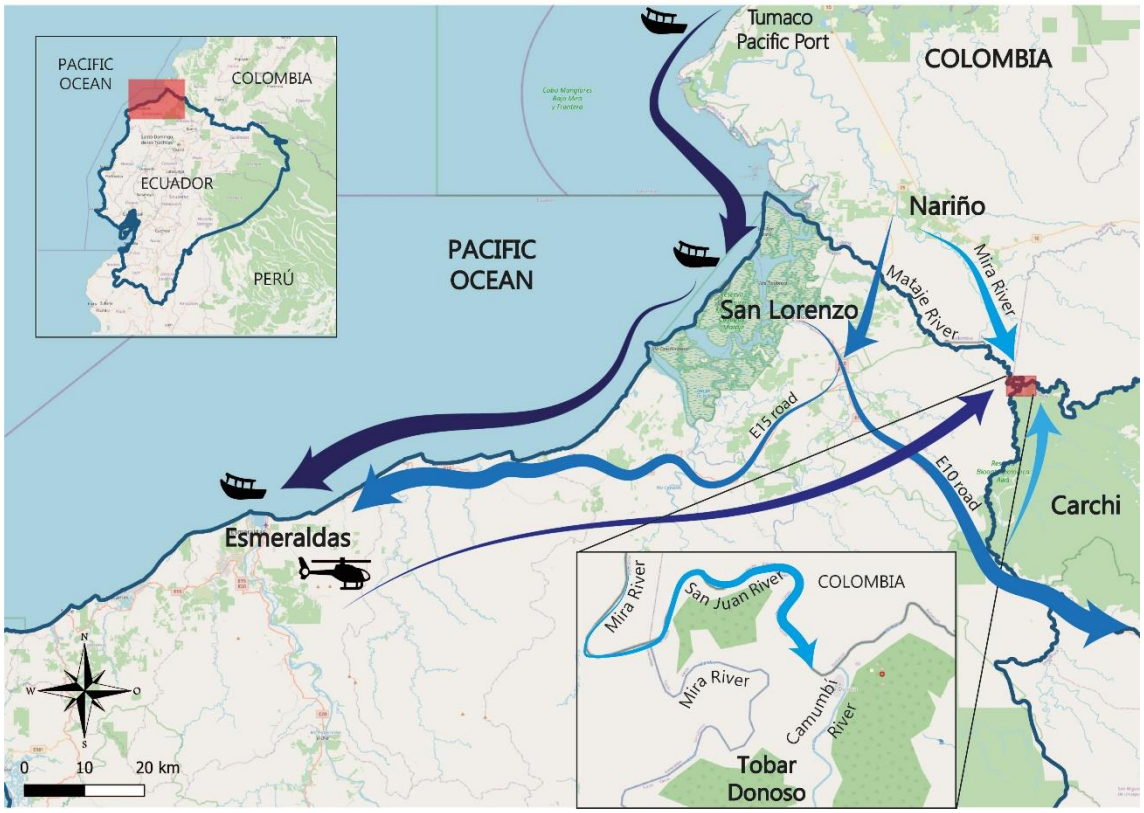

Figure 6. Cross-border and intra-border routes of entry of *P. falciparum* in northwestern Ecuador. Possible routes of the human migration used for the passage of *P. falciparum* in the south coast of Colombia and north coast of Ecuador. Elaborated according to the information collected from the bibliography.

10 TABLES

Table 1. Primers used for the nested PCR for molecular markers of drug resistance genes in *P. falciparum*.

Gen	Primers 5'-3'	Amplicon
Pfcrt	**F1:** AGC AAA AAT GAC GAG CGT TAT AG	559 pb
	R1: ATT GGT AGG TGG AAT AGA TTC TC	
	F2: TTT TTC CCT TGT CGA CCT TAA C	264 pb
	R2: AGG AAT AAA CAA TAA AGA ACA TAA TCA TAC	
Pfdhfr	**F1:** TCC TTT TTA TGA TGG AAC AAG	633 pb
	R1: AGT ATA TAC ATC GCT AAC AGA	
	F2: TTT ATG ATG GAA CAA GTC TGC	616 pb
	R2: ACT CAT TTT CAT TTA TTT CTG G	
Pfdhps	**F1:** AAC CTA AAC GTG CTG TTC AA	711 pb
	R1: AAT TGT GTG ATT TGT CCA CAA	
	F2: ATG ATA AAT GAA GGT GCT AG	528 pb
	R2: TCA TTT TGT TGT TCA TCA TGT	
Pfmdr-1	**F1:** CCG TTT AAA TGT TTA CCT GCAC	1014 pb
(86)	**R1:** TGG GGT ATT GAT TCG TTG CAC	
	F2: GTA TGT GCT GTA TTA TCA GGA G	746 pb
	R2: AGC CTC TTC TAT AAT GGA CAT G	
Pfmdr-1	**F1:** GCA TTT AGT TTC AGA TGA TGA AAT G	1016 pb
(1034)	**R1:** CCA TAT GGT CCA ACA TTT GTA TC	
	F2: TAT GCA TAC TGT TAT TAA TTA TGG	910 pb
	R2: TTC GAT AAA TTC ATC TAT AGC AG	
Pfk13	**F1:** GCA AAT AGT ATC TCG AAT	2126 pb
	R1: CTG GGA ACT AAT AAA GAT	
	F2: GAT AAA CAA GGA AGA ATA TTCT	748 pb
	R2: CGG AAT CTA ATA TGT TAT GTT CA	

Table 2. Genetic diversity and neutrality test of molecular markers of drug resistance genes in *P. falciparum*.

Gen	Locality	n	S	k	π	h	Hd	Tajima's D
Pfcrt	Tobar Donoso	18	2	0.418	0.028	2	0.209	-0.685
	Esmeraldas	25	0	0.000	0.000	1	0.000	-
	San Lorenzo	5	2	1.200	0.080	2	0.600	1.459
	Colombia	13	2	0.923	0.062	2	0.500	1.214
Pfdhps	Tobar Donoso	17	1	0.018	0.008	2	0.118	-1.164
	Esmeraldas	22	1	0.091	0.006	2	0.091	-1.162
	San Lorenzo	4	0	0.000	0.000	1	0.000	-
	Colombia	6	3	1.200	0.080	3	0.600	-0.447
Pfdhfr	Tobar Donoso	18	0	0.000	0.000	1	0.000	-
	Esmeraldas	27	0	0.000	0.000	1	0.000	-
	San Lorenzo	5	2	1.200	0.080	2	0.600	1.459
	Colombia	13	2	0.670	0.040	3	0.410	0.097
Pfmdr-1	Tobar Donoso	14	0	0.000	0.000	1	0.000	-
	Esmeraldas	14	1	0.260	0.010	2	0.264	-0.341
	San Lorenzo	4	1	0.670	0.030	2	0.667	1.633
	Colombia	2	1	1.000	0.040	2	1.000	-

n: Number of samples, S: Number of polymorphic sites, k: Average number of pairwise nucleotide differences, π: Nucleotide diversity, h: Number of haplotypes, Hd: Haplotype diversity.

Declarations

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee on Human Beings of Pontificia Universidad Católica del Ecuador (approvals #: CEISH-571-2018) and the Ministry of Public Health of Ecuador (approvals #: MSP-DIS-2019-044-O).

Consent for publication

Not applicable

Availability of data and materials

Data analyzed in this study is available in CISeAL in charge of Dr. Fabián Sáenz.

Competing interests

The authors declare that they have no competing interests.

Funding

Pontificia Universidad Católica del Ecuador.

Author's contribution

IÑ performed the experiments; IÑ and FES designed the study and wrote the manuscript; AME and DEN provided WGS data.

Acknowledgements

We thank to the personnel of the Ministry of Public Health of Ecuador for providing the samples for this study and patients who participated. CISeAL and PUCE for financing the development and publishing of this study.

11 SUBMISSION GUIDELINE

https://malariajournal.biomedcentral.com/submission-guidelines/preparing-your-manuscript/research

Research

Criteria

Malaria Journal publishes primary research from the full spectrum of malaria research. Subjects covered include any aspects of the biology of malaria parasites and their vectors; clinical features and disease pathophysiology; biochemistry and molecular biology; case management and treatment; drug development and drug resistance; immunology, immunogenetics and vaccine development; genomes and transcriptomes of malaria parasites and their vectors; epidemiology and transmission dynamics; vector control; health system, policy and planning issues relating to prevention and treatment; economic, sociological and anthropological issues; poverty and gender issues, land use and global environmental change; intersectoral issues; intervention studies and operational research; control policy and planning.

Malaria Journal requires that all datasets on which the conclusions of the paper rely should be available to readers. We encourage authors to ensure that their datasets are either deposited in publicly available repositories (where available and appropriate) or presented in the main manuscript or additional supporting files whenever possible. Please see Springer Nature's information on recommended repositories.

Preparing your manuscript

The information below details the section headings that you should include in your manuscript and what information should be within each section.

Please note that your manuscript must include a 'Declarations' section including all of the subheadings (please see below for more information).

Title page

The title page should:

- present a title that includes, if appropriate, the study design e.g.:
    - "A versus B in the treatment of C: a randomized controlled trial", "X is a risk factor for Y: a case control study", "What is the impact of factor X on subject Y: A systematic review"
    - or for non-clinical or non-research studies a description of what the article reports
- list the full names and institutional addresses for all authors
    - if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual PubMed records, please include this information in the “Acknowledgements” section in accordance with the instructions below
- indicate the corresponding author

Abstract

The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. Reports of randomized controlled trials should follow the CONSORT extension for abstracts. The abstract must include the following separate sections:

- **Background:** the context and purpose of the study
- **Methods:** how the study was performed and statistical tests used
- **Results:** the main findings
- **Conclusions:** brief summary and potential implications
- **Trial registration:** If your article reports the results of a health care intervention on human participants, it must be registered in an appropriate registry and the registration number and date of registration should be in stated in this section. If it was not registered prospectively (before enrollment of the first participant), you should include the words 'retrospectively registered'. See our editorial policies for more information on trial registration

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

Methods

The methods section should include:

- the aim, design and setting of the study
- the characteristics of participants or description of materials
- a clear description of all processes, interventions and comparisons. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses
- the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study reported.

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

- Ethics approval and consent to participate
- Consent for publication
- Availability of data and materials
- Competing interests
- Funding
- Authors' contributions
- Acknowledgements
- Authors' information (optional)

Please see below for details on the information to be included in these sections.

If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.

Ethics approval and consent to participate

Manuscripts reporting studies involving human participants, human data or human tissue must:

- include a statement on ethics approval and consent (even where the need for approval was waived)
- include the name of the ethics committee that approved the study and the committee’s reference number if appropriate

Studies involving animals must include a statement on ethics approval and for experimental studies involving client-owned animals, authors must also include a statement on informed consent from the client or owner.

See our editorial policies for more information.

If your manuscript does not report on or involve the use of any animal or human data or tissue, please state “Not applicable” in this section.

Consent for publication

If your manuscript contains any individual person’s data in any form (including any individual details, images or videos), consent for publication must be obtained from that person, or in the case of children, their parent or legal guardian. All presentations of case reports must have consent for publication.

You can use your institutional consent form or our consent form if you prefer. You should not send the form to us on submission, but we may request to see a copy at any stage (including after publication).

See our editorial policies for more information on consent for publication.

If your manuscript does not contain data from any individual person, please state “Not applicable” in this section.

Availability of data and materials

All manuscripts must include an ‘Availability of data and materials’ statement. Data availability statements should include information on where data supporting the results reported in the article can be found including, where applicable, hyperlinks to publicly archived datasets analysed or generated during the study. By data we mean the minimal dataset that would be necessary to interpret, replicate and build upon the findings reported in the article. We recognise it is not always possible to share research data publicly, for instance when individual privacy could be compromised, and in such instances data availability should still be stated in the manuscript along with any conditions for access.

Authors are also encouraged to preserve search strings on searchRxiv https://searchrxiv.org/, an archive to support researchers to report, store and share their searches consistently and to enable them to review and re-

use existing searches. searchRxiv enables researchers to obtain a digital object identifier (DOI) for their search, allowing it to be cited.

Data availability statements can take one of the following forms (or a combination of more than one if required for multiple datasets):

- The datasets generated and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS]
- The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
- All data generated or analysed during this study are included in this published article [and its supplementary information files].
- The datasets generated and/or analysed during the current study are not publicly available due [REASON WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.
- Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.
- The data that support the findings of this study are available from [third party name] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [third party name].
- Not applicable. If your manuscript does not contain any data, please state 'Not applicable' in this section.

More examples of template data availability statements, which include examples of openly available and restricted access datasets, are available here.

BioMed Central also requires that authors cite any publicly available data on which the conclusions of the paper rely in the manuscript. Data citations should include a persistent identifier (such as a DOI) and should ideally be included in the reference list. Citations of datasets, when they appear in the reference list,

should include the minimum information recommended by DataCite and follow journal style. Dataset identifiers including DOIs should be expressed as full URLs. For example:

Hao Z, AghaKouchak A, Nakhjiri N, Farahmand A. Global integrated drought monitoring and prediction system (GIDMaPS) data sets. figshare. 2014. http://dx.doi.org/10.6084/m9.figshare.853801

With the corresponding text in the Availability of data and materials statement:

The datasets generated during and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS].[Reference number]

If you wish to co-submit a data note describing your data to be published in *BMC Research Notes*, you can do so by visiting our submission portal. Data notes support open data and help authors to comply with funder policies on data sharing. Co-published data notes will be linked to the research article the data support (example).

Competing interests

All financial and non-financial competing interests must be declared in this section.

See our editorial policies for a full explanation of competing interests. If you are unsure whether you or any of your co-authors have a competing interest please contact the editorial office.

Please use the authors initials to refer to each authors' competing interests in this section.

If you do not have any competing interests, please state "The authors declare that they have no competing interests" in this section.

Funding

All sources of funding for the research reported should be declared. The role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript should be declared.

Authors' contributions

The individual contributions of authors to the manuscript should be specified in this section. Guidance and criteria for authorship can be found in our editorial policies.

Please use initials to refer to each author's contribution in this section, for example: "FC analyzed and interpreted the patient data regarding the hematological disease and the transplant. RH performed the histological examination of the kidney, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript."

Acknowledgements

Please acknowledge anyone who contributed towards the article who does not meet the criteria for authorship including anyone who provided professional writing services or materials.

Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgements section.

See our editorial policies for a full explanation of acknowledgements and authorship criteria.

If you do not have anyone to acknowledge, please write "Not applicable" in this section.

Group authorship (for manuscripts involving a collaboration group): if you would like the names of the individual members of a collaboration Group to be searchable through their individual PubMed records, please ensure that the title

of the collaboration Group is included on the title page and in the submission system and also include collaborating author names as the last paragraph of the “Acknowledgements” section. Please add authors in the format First Name, Middle initial(s) (optional), Last Name. You can add institution or country information for each author if you wish, but this should be consistent across all authors.

Please note that individual names may not be present in the PubMed record at the time a published article is initially included in PubMed as it takes PubMed additional time to code this information.

Authors' information

This section is optional.

You may choose to use this section to include any relevant information about the author(s) that may aid the reader's interpretation of the article, and understand the standpoint of the author(s). This may include details about the authors' qualifications, current positions they hold at institutions or societies, or any other relevant background information. Please refer to authors using their initials. Note this section should not be used to describe any competing interests.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

References

Examples of the Vancouver reference style are shown below.

See our editorial policies for author guidance on good citation practice

Web links and URLs: All web links and URLs, including links to the authors' own websites, should be given a reference number and included in the reference list rather than within the text of the manuscript. They should be provided in full, including both the title of the site and the URL, as well as the date the site was accessed, in the following format: The Mouse Tumor Biology Database. http://tumor.informatics.jax.org/mtbwi/index.do. Accessed 20 May 2013. If an author or group of authors can clearly be associated with a web link, such as for weblogs, then they should be included in the reference.

Example reference style:

Article within a journal

Smith JJ. The world of science. Am J Sci. 1999;36:234-5.

Article within a journal (no page numbers)

Rohrmann S, Overvad K, Bueno-de-Mesquita HB, Jakobsen MU, Egeberg R, Tjønneland A, et al. Meat consumption and mortality - results from the European Prospective Investigation into Cancer and Nutrition. BMC Medicine. 2013;11:63.

Article within a journal by DOI

Slifka MK, Whitton JL. Clinical implications of dysregulated cytokine production. Dig J Mol Med. 2000; doi:10.1007/s801090000086.

Article within a journal supplement

Frumin AM, Nussbaum J, Esposito M. Functional asplenia: demonstration of splenic activity by bone marrow scan. Blood 1979;59 Suppl 1:26-32.

Book chapter, or an article within a book

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology. London: Academic; 1980. p. 251-306.

OnlineFirst chapter in a series (without a volume designation but with a DOI)

Saito Y, Hyuga H. Rate equation approaches to amplification of enantiomeric excess and chiral symmetry breaking. Top Curr Chem. 2007. doi:10.1007/128_2006_108.

Complete book, authored

Blenkinsopp A, Paxton P. Symptoms in the pharmacy: a guide to the management of common illness. 3rd ed. Oxford: Blackwell Science; 1998.

Online document

Doe J. Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry. 1999. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999.

Online database

Healthwise Knowledgebase. US Pharmacopeia, Rockville. 1998. http://www.healthwise.org. Accessed 21 Sept 1998.

Supplementary material/private homepage

Doe J. Title of supplementary material. 2000. http://www.privatehomepage.com. Accessed 22 Feb 2000.

University site

Doe, J: Title of preprint. http://www.uni-heidelberg.de/mydata.html (1999). Accessed 25 Dec 1999.

FTP site

Doe, J: Trivial HTTP, RFC2169. ftp://ftp.isi.edu/in-notes/rfc2169.txt (1999). Accessed 12 Nov 1999.

Organization site

ISSN International Centre: The ISSN register. http://www.issn.org (2006). Accessed 20 Feb 2007.

Dataset with persistent identifier

Zheng L-Y, Guo X-S, He B, Sun L-J, Peng Y, Dong S-S, et al. Genome data from sweet and grain sorghum (Sorghum bicolor). GigaScience Database. 2011. http://dx.doi.org/10.5524/100012.

Figures, tables and additional files

See General formatting guidelines for information on how to format figures, tables and additional files.

Submit manuscript