Molecular surveillance uncovers early resistance markers in *Plasmodium falciparum* at the Brazilian Amazon border

Yanka E. A. R. Salazar[1,2,#], Antonio M. Rezende[3], Maria C. S. B. Puça[1,2], Sonja Lagström[4], Danielle Fletcher[5], Julian Muwanguzi-Karugaba[6], Colin J. Sutherland[6], Khalid B. Beshir[6], Maria E. P. Mascarenhas[1], Jaime Louzada[7], Joseli Oliveira-Ferreira[8], Abdoulaye Djimde[9], Dinora Lopes[10], José P. Gil[2,10], Tais N. de Sousa[1,2]

[1] Molecular Biology and Malaria Immunology Research Group, Instituto René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, Minas Gerais, Brazil; [2] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Solna, Sweden; [3] Biotechnology Applied to Pathogens Research Group, Instituto René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, Minas Gerais, Brazil; [4] Life Sciences and Diagnostics Markets Group, Agilent Technologies Sweden AB, Solna, Sweden; [5]Life Sciences and Diagnostics Markets Group, Agilent Technologies LDA UK Limited, Cheadle, UK; [6]Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom; [7]Universidade Federal de Roraima, Boa Vista, Roraima, Brazil; [8]Laboratório de Imunoparasitologia, IOC, Fiocruz, Rio de Janeiro, Brazil; [9]Malaria Research and Training Center (MRTC), Faculty of Pharmacy, University of Science, Techniques and Technologies of Bamako; Bamako, Mali; [10]Clinical Tropical Medicine (CTM), Global Health and Tropical Medicine, Institute of Hygiene and Tropical Medicine, Nova University of Lisbon, Lisbon, Portugal

Malaria elimination in the Brazilian Amazon is increasingly threatened by the emergence of antimalarial drug resistance in a region shaped by intense cross-border mobility, informal drug use, and social and territorial inequities. Surveillance strategies that rely predominantly on conventional Sanger sequencing have limited sensitivity to detect low-frequency resistance variants, delaying recognition of early warning signals. Using Illumina-based next-generation sequencing, we developed and validated a targeted ultra-deep sequencing panel covering the full coding regions of 48 resistance-associated genes in *Plasmodium falciparum*. Validation using 3D7:Dd2 mock mixtures and clinical samples demonstrated high depth and coverage, accurate allele-frequency estimation, and robust detection of minority variants, including dried blood spot samples. The approach was applied to 99 *P. falciparum* isolates collected in Roraima State, northern Brazil (2016–2020), a tri-border region, connecting Brazil, Venezuela, and Guyana. High-resolution analysis identified molecular resistance markers, including the *pfmdr1*-NFD haplotype (6%) and the *pfpm2/3* hybrid (4%), both reported for the first time in Brazil, as well as *pfcrt*-C350R (7%) and *pfpm2/3* amplifications (5% and 6%), all associated with reduced susceptibility to ACT partner drugs. No mutations linked to artemisinin partial resistance were detected, indicating a narrow window. The detection of these markers in border areas overlapping Indigenous territories, including Yanomami land, and other vulnerable populations shows that resistance emergence is established

and extends beyond national borders. These findings place clear responsibility on national and regional malaria programs to strengthen cross-border coordination, update surveillance strategies, and anticipate resistance-driven threats before they result in treatment failure and increased disease burden in the Amazon. Funding: WANECAM2 (EDCTP2); Swedish Research Council; FAPEMIG; CNPq; PrInt-Fiocruz-CAPES.