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Tolerance of Gambian *Plasmodium falciparum* to Dihydroartemisinin and Lumefantrine detected by Ex vivo Parasite Survival Rate Assay (PSRA)

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Abstract

Monitoring of *Plasmodium falciparum* sensitivity to antimalarial drugs in Africa is vital for malaria elimination. However, the commonly used *ex-vivo/in-vitro* IC_{50} test is inconsistent for several antimalarials, while the alternative ring-stage survival assay (RSA) for artemisinin

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derivatives has not been widely adopted. Here we applied an alternative two-colour flow-cytometry based parasite survival rate assay (PSRA) to detect *ex-vivo* antimalarial tolerance in *P. falciparum* isolates from The Gambia.

PSRA infers parasite viability from quantifying re-invasion of uninfected cells following 3 consecutive days of drug exposure (10-fold the IC_{50} drug concentration of field isolates). The drug survival rate for each isolate is obtained from the slope of the growth/death curve. We obtained PSRA of 41 isolates for DHA and LUM, out of 51 infections tested by RSA against DHA. We also determined the genotypes for known drug resistance genetic loci in *Pfdhfr*, *Pfdhps*, *Pfmdr*, *Pfcrt* and *Pfk13* genes.

The PSRA for 41 Gambian isolates showed faster killing and lower variance by DHA compared to LUM, despite a strong correlation between both drugs. Four and three isolates were respectively tolerant to DHA and LUM, with continuous growth during drug exposure. Isolates with the PfMDR1-Y184F mutant variant had increased LUM survival though this was not statistically significant. Sulphodoxine/Pyrimethamine (SP) resistance markers were fixed, while all other antimalarial variants were prevalent in more than 50% of the population.

The PSRA detected *ex-vivo* antimalarial tolerance in Gambian *P. falciparum.* This calls for its wider application and increased vigilance against resistance to ACTs in this population.

Introduction

There has been a substantial decline in malaria morbidity and mortality in sub-Saharan Africa over the past decade [1]. This was mainly driven by the scale-up of control interventions such as long-lasting insecticidal nets and clinical case management with artemisinin-based combination therapy (ACT) [1]. Currently, ACTs are used in endemic countries for the

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45 treatment of clinical malaria, for individual chemoprevention in mass treatment campaigns [2].
46 These interventions increase pressure on the parasites which could result in the emergence of
47 resistance to both partner drugs and artemisinin-derivatives as confirmed in southeast Asia [3,4]
48 and delayed parasite clearance reports in Africa [5].

49 Currently, the WHO recommends regular efficacy testing of the locally used antimalarials in
50 humans, complemented by *in-vitro* (laboratory-based) assessment of parasite growth in
51 response to drug exposure [6]. Comparing the *in-vitro* efficacy of ACTs is complex as the
52 components have different mechanisms of action [5]. Moreover, most of the existing drug
53 susceptibility assays were developed when treatment was based on monotherapies [7,8]. The
54 most common assays are based on the IC_{50} ; drug concentrations required to inhibit parasite
55 growth by half, under a set of experimental conditions [9,10]. This approach is sensitive to
56 variations in drug concentrations used and inconsistency in data analysis [11]. IC_{50} assays also
57 do not assess the temporal course of parasite viability following exposure, and are not suited
58 for artemisinin derivatives with characteristically shorter half-lives [9].

59 New *in-vitro* methods assessing the efficacy of fast-acting drugs such as the Ring-stage
60 Survival Assay (RSA) [12], Piperaquine survival assay (PSA) [13] and Parasite Viability Fast
61 Assay (PVFA) [14] are now available. RSA and PSA determine parasite survival following
62 drug exposure and withdrawal, while the PVFA aims at discriminating fast-acting anti-
63 malarial drugs by assessing parasite killing kinetics over time. There are still critical gaps in
64 these assays; RSA was designed solely for fast-acting drugs and therefore cannot be used for
65 slow-acting antimalarials with longer half-lives [15]. The PVFA has only been used in
66 antimalarial development for screening candidate drugs.

67 Besides *in-vitro* assessment of drugs, molecular surveillance is recommended to monitor the
68 emergence and spread of resistance by determining the proportion of isolates in a given

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population with resistance associated alleles [16,17]. While the Kelch-13 molecular markers of artemisinin resistance have not been identified in sSA [18], resistant alleles in PfCRT and PfMDR1 for both current and previously used drugs, including partners in ACTs, are in circulation [19]. For instance, the use of lumefantrine in the ACT artemether-lumefantrine (AL) has been associated with an increase in copy numbers, the frequency of N86 allele, and the N86/184F/D1246 haplotype of PfMDR1 [20–25]. Additionally, Sulphodoxine/Pyrimethamine (SP) used in seasonal malaria chemoprevention (SMC) and intermittent preventive treatment in pregnancy (IPTp) select for mutant PfDHFR and PfDHPS alleles [26]. Combining molecular surveillance with *in-vitro* surveillance can therefore provide an early warning signal on the emergence of drug tolerant parasites. This is critical for a parasite population that is exposed to substantial pressure by drug and vector control interventions such as in The Gambia, where malaria transmission and prevalence are low to very low. The Gambia together with neighbouring Senegal is driving for malaria elimination by deploying SMC, while mass drug treatments with ACTs are being contemplated.

Therefore, the goal of this study was to evaluate a Parasite Survival-Rate Assay (PSRA) to estimate *ex-vivo* drug sensitivity of *P. falciparum* from The Gambia to the currently used ACT (AL). The PSRA mimics 3 days of exposure to an ACT, measuring parasite survival rates over this period. The assay assesses the survival and re-invasion potential of parasites following exposure to lumefantrine (LUM) and dihydroartemisinin (DHA); prototypes of slow- and fast-acting components of ACTs used in most endemic countries. The approach offers significant advantages over the standard IC_{50} determination assay due to its higher sensitivity in measuring parasite viability based on the production of invasive merozoites after drug exposure; an index of susceptibility or drug tolerability.

Materials and Methods

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Sample collection

Ethical clearance for this study was obtained from the Gambia Government/MRCG Joint Ethics committee and further approved by the Gambian Ministry of Health. The study was conducted as part of a therapeutic efficacy study (TES) of AL in collaboration with the National Malaria Control Programme (NMCP) at the Brikama Health Centre (Western Gambia) in 2017. Patients were included in the study following diagnosis of *P. falciparum* infection with a parasite density of at least 1000/µL. An informed consent or assent was obtained from eligible patients. Two millilitres of venous blood samples were collected at day 0 of the TES into EDTA tubes and blood spots made on Whatman filter papers (Scientific Laboratory Supplies). Filter papers were air dried and stored in sealed sample bags with silica gel desiccants. Samples were transported on ice to the MRCG at LSHTM culture facility and processed within 4 hours of collection.

Parasite processing for drug assays

Thin blood smears were made for all samples to identify parasite lifecycle stages. For each sample, 50 µL was used to estimate parasite density using a C6 flow cytometer (BD Accuri™, BD Biosciences) after DNA staining with SYBR Green I DNA intercalating dye (Applied Biosystems). To eliminate white blood cell populations from the analysis, gating was done on the red blood cell (RBC) population only using forward and side scatter parameters followed by gating of the SYBR green 1 positive population which effectively delineates parasitized RBCs. Plasma was separated from blood cells following centrifugation for 5 minutes at 1500 rpm. An equal volume of incomplete media (RPMI 1640 (Sigma-Aldrich, UK) supplemented with 35 mM HEPES (Sigma, St. Louis, MO), 24 mM $NaHCO_3$, 1 mg/l of hypoxanthine (Sigma), 5µg/ml of gentamicin (Gibco-BRL)) was added to the cell pellet and layered on 6 ml of lymphoprep (Axis-Shield, UK). The layered sample was

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centrifugated for 20 minutes at 2,500 rpm and leukocytes aspirated. The RBCs were washed thrice by re-suspending the pellet in incomplete media and centrifugated for 5 minutes at 1,500 rpm. The washed pellet was re-suspended in growth medium: incomplete medium with 0.5% Albumax (Gibco-BRL). The parasitemia was normalized to 0.5% (1000 parasites / µL) for all samples with parasitemia higher than 0.5% and 2% haematocrit using uninfected O^+ heterologous RBCs prior to PSRA and RSA. Four laboratory adapted strains were used as internal controls: 3D7, Dd2 and MRA-1239 which are sensitive to both LUM and DHA, and MRA-1241 which is sensitive to LUM but resistant to DHA. The isolates were routinely cultured with fresh O^+ RBCs and maintained at 2% haematocrit with growth media under standard incubation conditions of 37°C, 90% N_2, 5% O_2, and 5% CO_2. All laboratory adapted strains were synchronized twice with 5% D- sorbitol to obtain ≥ 80% ring stages prior to assay set-up. One hundred and seventy samples were obtained from Brikama Health Centre in 2017.

Parasite Survival Rate Assay (PSRA)

The parasite survival rate assay is based on re-invasion of surface labelled uninfected O^+ RBCs (uRBC) by merozoites emerging from ruptured schizonts that developed after drug exposure of infected samples. This was a modification of the protocol described [14]. Here, target uRBCs were pre-labelled with the amine-reactive fluorescent dye:7-hydroxy-9H-(1,3-dichloro-9, 9-dimethylacridin-2-one) succinimidyl ester (DDAO-SE; 10 µM; Invitrogen); a far-red cell dye as described [27]. A 2% haematocrit suspension of the uRBCs in incomplete media with 10 µM DDAO-SE was made and incubated at 37°C for 2 hours while shaking. The suspension was washed once, re-suspended with incomplete media and re-incubated for a further 30 minutes. DDAO-SE labelled uRBC suspension: $uRBC^{DDAO\text{-}SE}$ were washed and reconstituted with growth media for a final haematocrit of 2%.

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PSRAs were set up using laboratory isolates of ≥ 80% rings (*in vitro*) and field isolates within 4 hours of sample collection (*ex vivo*). The assay were done in triplicate for sensitivity to concentrations that were 10-fold higher than the median IC_{50} of the respective drugs determined in a previous study [19]. Briefly, 100 µL of parasite suspension at 0.5% parasitemia and 4% hematocrit was added to 48-well microtiter plates pre-coated with 100 µL of the respective drugs at twice the target concentration [(10-fold median IC_{50}) x 2]. This resulted in a final drug concentration of 10-fold median IC_{50} of DHA (8.1 nM) and LUM (398 nM) at 2% haematocrit. A no-drug control, substituted with 0.1% dimethyl sulfoxide: DMSO (Sigma-Aldrich, UK) was assayed for each sample. The samples in the microtiter plates were incubated for 24-, 48- and 72- hours respectively using standard incubation conditions. Drugs were refreshed every 24 hours after washing cells by incubating twice for 5 minutes with incomplete media. Fifty microliters of the drug-free suspension was transferred to a fresh 96-well microtiter plate containing 100 µL of $uRBC^{DDAO\text{-}SE}$ (1 in 3 dilution) which was further incubated for 48 hours (Figure 1).

Each sample was then washed and counterstained with 1:10,000 dilution of SYBR Green I in phosphate buffered saline (PBS). For this, 100 µL of diluted stain solution was added to each assay well of the microtiter plate and incubated in the dark at room temperature with shaking for 20 minutes. Stained cells were washed twice and re-suspended with 200 µL of PBS. A further 1 in 4 dilutions with PBS was done prior to flow cytometric counting using BD Accuri™ C6 flow cytometer. For acquisition, the fluorescence emission peak for SYBR Green I and DDAO-SE were set at 520nm and 657nm for the green and red channels respectively. For each assay well, 100,000 events were acquired, and data analysed using the BD Accuri™ C6 software.

Ring-stage survival assay (RSA)

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A modification of the RSA protocol [12,28]. was carried out to assess the re-invasion potential of parasites exposed to 700nM of DHA, replacing microscopy with two-colour flow cytometry similar to the PSRA protocol above. Leukocyte depleted infected RBCs (iRBCs) were set up in duplicates at 0.5% parasitemia and 2% hematocrit. Each isolate was exposed to 700 nM of DHA and 0.1% DMSO as control for exactly 6 hours under standard incubation conditions. DHA and DMSO were then washed off using incomplete media and parasites re-suspended with drug-free growth media. Fifty microliters of this suspension was added to 100 µL uRBC$^{DDAO-SE}$ in a separate 96-well microtiter plate and both plates incubated for a further 66 hours. Thin blood smears were then made and stained with Giemsa following the standard RSA protocol. Ring-stage survival rates were determined microscopically using the initial parasitemia before drug exposure (initial parasitemia: INI), DMSO control (non-exposed: NE) and DHA-exposed (DHA). Ring-stage survival was calculated for isolates with growth rate of ≥ 1% using the published formula:

Percentage survival (%) = (DHA/NE) x 100

The cells incubated with uRBC$^{DDAO-SE}$ were counterstained as above with SYBR Green I and acquired using BD Accuri^{TM} C6 flow cytometer to determine parasite re-invasion rates.

Genotyping of selected drug resistance loci

Genotyping was done by locus specific high-resolution melt (HRM) assays with parasite DNA extracted from filter paper dried blood spots (DBS). To recover parasite DNA, DBS were punched onto 96-deep well plates, using punchers and forceps that were rinsed in 1% bleach and alpha-Q water after each sample to limit cross-contamination. For each plate, 4 negative and 4 positive controls were included. Genomic DNA was manually extracted using the QIAamp® 96 DNA Blood Kit (Qiagen, Hilden, Germany) with manufacturer's instructions. The DNA concentration of the eluates were quantified using a Nanodrop 1000

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(Thermo Scientific) and stored at -20°C until use. One micromolar of gDNA of approximately 10pg-1ng/uL, was used for genotyping assays. HRM genotyping reactions were performed for the following alleles: *pfcrt* C72/M74/N75/K76, *pfmdr1* N86, *pfmdr1* Y184, *pfdhps* S436/A437, *pfdhfr* N51/C59 and *pfk13* C580 on the LightCycler® 96 Real-Time PCR System (Roche). The primers and probes used for PCR reactions with 2.5X LightScanner master-mix (Biofire) were as previously described [29]. Each reaction had a final forward and reverse primer concentration of 0.05 µM and 0.2 µM respectively (asymmetric PCR) and 0.2 µM for allele specific probes. The PCR conditions and analysis method used are as previously described [29].

Statistical analysis of drug survival rates

The analysis aimed mainly at evaluating the effect of drug exposure from the magnitude of decline in growth by re-invasion with time of exposure compared to drug free controls, i.e. LUM Vs no-drug and DHA Vs no-drug. We also aimed to explore patterns of variation in response to drug exposure between isolates. To first assess the effect of time of exposure on survival (growth), the re-invasion rates were log-transformed for normality and these values were used for descriptive statistics on responses at each time-point. Linear mixed effect models were then fitted to examine the heterogeneity in drug susceptibility allowing for interaction between time and drug treatment group (LUM or DHA) with random effects on subjects. Since there were three discrete time points of measurement, an indication of potential non-linear relationship between treatment response and time, we used time as categorical variable comparing differences in effect of treatment for 48- and 72- hours against 24 hours as reference. As such we could examine the effect of longer exposure. To explore the difference in susceptibility to each drug per isolate, we first obtained the drug effect from the difference between drug exposed and no-drug control at each time point. We then fitted a

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linear trend across timepoints for each isolate, deriving patterns of individual growth decay slopes. These estimated slopes represent individual parasite survival (or death) rates. Based on the derived decay patterns (individual trajectories), an isolate was assigned to one of the four classes: linear decrease (---), linear increase (+++), non-linear increase/decrease (+-+) and non-linear decrease/increase (-+-). In addition, we assessed the relationship between individual trajectories and their corresponding genotypes. All analyses were performed using the R package (RStudio version 1.2.5001) and Stata 14 (StataCorp, College Station, TX, USA). A *P* value of <0.05 was considered significant. Other plots were explored using Prism (GraphPad Prism version 7.0a).

Results

Plasmodium falciparum isolates collected from patients with uncomplicated malaria cases recruited across the malaria transmission season in 2017 from Western Gambia were analysed. A total of 79 out of 170 (46.5%) isolates had a parasitemia of ≥ 0.5% and these were set up for both PSRA and RSA assays. Analysis data was obtained for 41 (52%) and 51 (64.6%) samples which had a drug free *ex vivo* growth rate of ≥1% for PSRA and RSA respectively. Apart from the field isolates used in this study, the PSRA was tested against a panel of previously characterized isolates, including an artemisinin resistant parasite line: MR4-1241 with the K13 I543T mutation.

Ring stage survival rates of field isolates by Microscopy and Flow cytometry

Ring stage survival rates of 51 isolates were determined using conventional microscopy as per the initial RSA protocol [12] and modified using uRBC$^{DDAO\text{-}SE}$ and SYBR Green I for flow cytometric analysis. Following pulse exposure to DHA, 31 isolates (61%) had surviving parasites observed by microscopy, ranging from 0.05 to 1.2% (Figure 2a). Flow cytometric

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counting of re-invasion in pre-labelled uRBCs was more sensitive, showing all isolates to have post-drug exposure survival ranging from 0.14 to 1.53%. The mean survival rates determined by flow cytometric analysis was statistically higher than microscopy (P <0.0001). Despite this, there was a strong positive correlation between the two analysis methods, with R= 0.83 and $p = 2.7 \times 10^{-14}$ (Figure 2b). However, isolates with the highest ring survival rates by flow cytometry were not the same observed by microscopy. Based on flow cytometry only, % ring survival after 6 hours of exposure to 700nM of DHA significantly correlated with parasite survival rates following PSRA analysis (R = 0.53, p = 0.00038), (figure 2c). Overall, the median cumulative rates of survival over the 72 hours of exposure was not significantly different between DHA (-0.051 to 0.029) and LUM (-0.048 to 0.037), (p = 0.35) though the responses to LUM had a wider distribution (Figure 2d).

P. falciparum ex-vivo survival decreases with longer drug exposure

By comparing log of survival rates between isolates with different durations of drug exposure, the overall survival declined with increased exposure time for both drugs. Whereas, there was an increasing growth trend in the drug-free group over time (Figure 3). The mean differences between treatment and control groups was always significant and increased with time as treatment groups appeared to show a marked decline in predicted survival particularly after 72 hours (Supplementary figure 1). Pairwise comparison between the drug-treated groups against drug-free group showed significant differences at all three timepoints (Table 1). Using 24 hours as the reference, differences in predicted responses were seen for both DHA and LUM at 72 hours post drug exposure. At 48 hours, the differences in predicted responses were not statistically significant when compared to 24 hours. This could be due to the exponential increase in merozoite infected RBCs following a complete *P. falciparum*

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growth cycle [30], potentially resulting to the high responses seen at 48 hours in the control group (Figure 3c).

Distribution of PSRA sensitivities to AL

We derived individual responses to each drug from fitting a linear model on the differences in predicted responses between the drug treated and control with time. These *ex vivo* parasite survival rates ranged from -0.051 – 0.029 for DHA and from -0.048 – 0.037 for LUM. The majority of isolates had a negative slope with consistently reducing survival with time (Figure 3a and 3b). This was seen for 30 isolates for DHA and 35 for LUM representing 73% and 85% of isolates treated respectively. Conversely, 27% (11/41) and 15% (6/41) had a net increase in growth despite 72 hours of exposure to DHA and LUM, with similar or higher predicted responses under drug conditions compared to the controls with DMSO (Figure 3a and 3b, Supplementary figure 2a and 2b). The overall response and rates of growth decline was higher for DHA compared to LUM (Figure 3c). However, the survival rates between DHA and LUM showed a strong positive correlation (R = 0.77, p = 5.4e-09), (Figure 3d).

Consistent clusters of survival rate patterns to both DHA and LUM

We identified four patterns of responses based on the growth vs time curve for both drugs (Figure 4a and 4b). The most common pattern was a continuous decline in survival with increase in time of exposure. This first group of isolates defined as linear decrease (designated as "---" on figure 4) represented 46 % (19/41) and 51% (21/41) of isolates tested against DHA and LUM respectively. The second group of isolates had a peak in growth at 48 hours of drug exposure ("-+-") and these represented 19.5% (8/41) and 22% (9/41) of isolates tested. The third group were isolates with consistently linear increase ("+++") despite drug exposure, with 9.75% (4/41) and 7.3% (3/41) identified for DHA and LUM, and the fourth pattern were isolates with the lowest survival timepoint at 48 hours ("+-+") representing 24%

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(10/41) and 20% (8/41) of isolates. These patterns did not correlate with initial parasitemia (Supplementary figure 4) or other patient demographic information.

Frequencies of drug resistance alleles in western Gambia

We obtained genotypes for at least 39 isolates for *pfcrt* C72/M74/N75/K76, *pfmdr1* N86, *pfmdr1* Y184, *pfdhps* S436/A437, *pfdhfr* N51/C59 and *pfk13* C580 (Table 2, Figure 5). The *pfcrt* mutant haplotype was found in 79% of isolates with 2% of mixed infections. 93% of isolates were wildtype for *pfmdr1* N86 and 5% mixed, while 57% were mutant for *pfmdr1* Y184 and 12% mixed. For antifolate markers, 90% of isolates had mutant variants at *pfdhps* S436/A437 while all isolates were mutated for *pfdhfr* N51/C59. We excluded the analysis for the *pfdhfr* alleles: IT/NC as the scoring of the melting curves were ambiguous, showing up to 55% of mixed allele calls. *PfK13* C580 was wildtype for all isolates. Given the almost fixed frequencies of either wildtype or mutant at these loci tested, no association with the PSRA patterns could be determined. However, for *pfmdr1* codon 184, higher LUM responses were observed for isolates with the 184F mutant allele though the mean differences were not significant between these and isolates with Y184 wildtype variant (Figure 5c.ii).

Discussion

This study describes the *ex vivo* susceptibility rates of natural isolates from the Gambia, where transmission has declined, and we had seen increasing *ex-vivo* tolerance to Lumefantrine by IC_{50} as well as modest survival rates (26%) to DHA by ring-stage survival assay (RSA). These rates were obtained against DHA and LUM with a flow cytometry-based parasite survival rate assay (PSRA), with potential application to other drugs and antimalarial candidates. The potency of these drugs depend on the drug concentrations used and the length of exposure, with the assumption that cytotoxicity occurs when parasites are exposed to the

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active component of the drug for a prolonged time [31]. Here, we used drug concentrations that are 10- fold higher than the median IC_{50} of the respective drugs obtained from the assessment of field isolates from western Gambia in 2015 [19]. The use of 10-fold higher drug concentrations, though much lower than serum concentrations, proved to be the optimal concentration to determine the rate of kill of slow, medium and fast acting drugs. This concentration is sub-optimal, allowing for gradual effect of the drugs on the parasites [32].

The PSRA provided several advantages over the IC_{50} and RSA assays; it determines the effect of drugs over 72 hours of exposure and measures both parasite growth and viability by determining re-invasion even at low parasite densities. Unlike RSA, there is no requirement for assaying early rings which can be difficult to ascertain for natural isolates from malaria patients, thereby eliminating the need for further stressing isolates by synchronizing them with sorbitol. Similar to the *in vivo* parasite clearance rate for determining the efficacy of artemisinin derivatives [33], the PSRA determines clearance rates from the rate of *ex vivo* inhibition of growth over 72 hours of drug exposure. This duration of exposure allows rings that emerge from tolerant isolates over the first cycle (48 hours) to experience another round of drug exposure for 24 hours, followed by recovery in drug free medium. The overall outcome is the kinetics of parasite killing by the test drug over 72 hours. This assay is therefore a variant of the PVFA [14,32]. Like PVFA, the PSRA does not assess parasite metabolic activity or other parasite molecules to quantify survival or death indirectly [14]. It quantifies viability from a direct count of viable merozoites that emerge from drug-exposed schizonts and invade pre-stained uninfected RBCs: $uRBC^{DDAO\text{-}SE}$. Flow cytometry provided increased sensitivity by individually counting cells and distinguishing new autologous and heterologous infected cells. With a 2 to 1 ratio of $RBC^{DDAO\text{-}SE}$ to non-labelled RBCs, higher numbers of pre-labelled RBCs are present, skewing re-invasion to occur in these cells. As merozoites emerge after drug exposure, active re-invasion is proof of viability. This gives a

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331 good estimate of the number of parasites that survive following drug exposure. The rate of
332 death is therefore intrinsic to the level of drug tolerance by each isolate. Autologous re-
333 invasion of unlabelled RBCs are excluded from the analysis as they cannot be differentiated
334 from dead and arrested cells. Unlike RSA, the PSRA uses a much lower concentration of
335 drug but potent enough to kill isolates and to induce the delayed clearance phenotype in RSA
336 control isolate (MRA-1241). Hence, there was a high positive correlation between PSRA and
337 flow-cytometry modified RSA. With strong correlation with microscopy but improved
338 throughput, flow cytometry-based RSA and PSRA should allow for robust detection of
339 emerging drug tolerance in natural isolates. Future and wider application of this method is
340 warranted in Africa where drug pressure is substantial. This is the case for The Gambia
341 where the artemisinin-based combination therapy AL is used as first line treatment and other
342 ACTs are being considered for mass administration after several clinical trials.

343 Most of the isolates tested by PSRA in The Gambia had decreasing parasite survival with
344 increasing days of exposure to drugs. However, four isolates exposed to DHA and three to
345 LUM continued to grow and were considered potentially tolerant with one isolate surviving
346 in the presence of both drugs. More isolates would have been classified as tolerant if all those
347 that showed a rebound of growth at 72 hours were included. These suggest a state of reduced
348 drug sensitivity, allowing parasite growth and re-invasion to occur in the presence of sub-
349 lethal drug concentrations [34]. The six surviving isolates could be on a path towards a
350 persistent state of drug insensitivity that may result in resistance [35] and should be closely
351 monitored. Extending the assay time to 96 hours could also reveal clearer response profiles
352 for the isolates with non-linear responses over the 72-hour period. Importantly, the weak
353 correlation between initial patient parasitemia and parasite response suggests that the
354 responses seen are not driven by the rate at which parasites grew in the patient (*in vivo*). Most
355 isolates had similar response patterns for both drugs and their survival rates correlated

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positively. This could be an indication of common mechanisms that enable survival to several drugs, a factor that could lead to multidrug resistance. Multidrug resistance to artemisinin derivatives and partners has been confirmed in South East Asia [36]. We have already shown in The Gambia a consistent increase of LUM tolerance between 2012 and 2015 [19]. In the same study, 26% of the isolate in the 2015 population from western Gambia showed viable parasites by microscopy-based RSA for DHA. The presence of surviving parasites in this current study though at different proportions with both assays, suggests sustained low level of DHA tolerance and requires further investigation. These parasites survived and replicated in high concentration and prolonged length of DHA pressure with RSA and PSRA respectively. Malaria transmission in western Gambia has reduced drastically in the last decade, with prevalence of infection lower than 5% overall and 1% for children under 5 years of age. Despite this, various ACTs remain widely available and accessible through private and public vendors. While it is officially required that ACTs should be prescribed only upon a positive malaria diagnosis, this is hardly sustained given regular short supplies of Rapid Diagnostic Test kits. We can therefore speculate that the emergence of tolerant parasites is being driven by high drug pressure against low transmission which is hypothesized to be one of the main drivers in the emergence of antimalarial drug resistance in South East Asia. This calls for improved vigilance across Africa as elimination programs are implemented. ACT resistance has been shown to emerge on a backbone of known drug resistance including *Pfmdr1* and *Pfcrt* selected by LUM. The WHO recommends surveillance for known and emerging markers of resistance in natural populations.

We genotyped the isolates assayed for alleles at *Pfmdr1*, *Pfcrt*, *Pfdhfr*, *Pfdhps* and *Pfk13* loci that have been implicated in quinoline, antifolate or artemisinin resistance. We found high levels of resistance loci against the antifolates, an expected result given the use of SP by SMC and IPTp. We also found high levels of *Pfmdr1* N86, the wild type allele selected by

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LUM, a result aligning with what we had shown before for this population [19]. On the contrary, the *Pfcrt* 72-76 mutant haplotype was in over 80% of isolates, indicating continuous selection by chloroquine. Chloroquine had been withdrawn for treatment of malaria raising the question as to which drugs are driving selection at Pfcrt but not Pfmdr1 [37]. Selection of Pfcrt may be driven by amodiaquine which is available in combination with artesunate accessible from private vendors in The Gambia and is the ACT of choice in neighbouring Senegal with whom there is significant human migration. We expect to gain more insights on this considering current extensive temporal and spatial genome sequencing for these parasite population. With the high levels of mutant or wild alleles at drug resistant genes, an analysis of genetic association for the four different parasite PSRA profiles was not possible. However, higher survival rates against lumefantrine were seen for isolates with the mutant variant at *Pfmdr1* 184F though this was not significantly different from the distribution of rates in isolates with wild alleles. We assume that the responses observed for samples carrying multiple strains is a combined effect of the different strains present and the six isolates that survived following exposure to either of the two drugs have specific molecular signatures influencing their phenotypes which should be further investigated. Despite the number of isolates showing growth after 72 hours of exposure to DHA, no mutant alleles of *Pfk13* C580 were found in the population. Artemisinin associated Kelch13 variants are rare in African populations but high frequencies of other non-synonymous SNPs on *Pfk13* (kelch propeller domain) had been observed for isolates from The Gambia [38]. These further buttresses the need for routine and in-depth surveillance of this population.

This study highlights early signs of *ex-vivo* drug tolerance of parasites from western Gambia to the most common ACT components. These were derived by PSRA which provides a significant advancement in approaches for the determination of parasite susceptibility. A wider application of this approach across sSA to distinguish drug tolerance and resistance

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406 will support current and future chemoprevention and chemotherapeutic strategies against
407 malaria.

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Table 1. Effect of drug exposure on predicted responses of the treatment groups (DHA, LUM, DMSO-control) and exposure times (24-, 48- and 72- hours) for *P. falciparum* isolates analysed by PSRA.

Treatment groups	**Difference (95%CI)**	***P* value**
(DHA vs Control) 24h	-0.68 (-0.90, -0.47)	**<0.0001**
(DHA vs Control) 48h	-1.03(-1.25, -0.81)	**<0.0001**
(DHA vs Control) 72h	-1.20 (-1.42, -0.99)	**<0.0001**
(LUM vs Control) 24h	-0.31 (-0.52, -0.09)	**0.005**
(LUM vs Control) 48h	-0.62(-0.84, -0.41)	**<0.0001**

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Gene	Alleles	Codons	Frequency
(LUM vs Control) 72h		-0.99 (-1.21, -0.77)	**<0.0001**
(48h vs 24h) Control		0.25 (0.01, 0.48)	**0.04**
(48h vs 24h) DHA		-0.10 (-0.33, 0.13)	0.39
(48h vs 24h) LUM		0.07 (-0.30, 0.16)	0.57
(72h vs 24h) Control		0.14 (-0.13, 0.42)	0.31
(72h vs 24h) DHA		-0.38 (-0.65, -0.10)	**0.007**
(72h vs 24h) LUM		-0.54 (-0.81, -0.27)	**0.0001**

*24h = 24 hours; 48h = 48 hours; 72h = 72 hours; DHA = Dihydroartemisinin treatment; LUM = Lumefantrine treatment; Control: DMSO treatment.

Values in bold are significant P values determined by pairwise comparisons

547 **Table 2.** Allele frequencies of drug resistance genes for 41 parasite isolates with drug
548 phenotypic data (PSRA and RSA).

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pfcrt	C72, M74, N75, K76	CMNK (wildtype)	0.17
		CIET (mutant)	0.79
		CMNK/CIET (mixed)	0.02
pfmdr1	N86	N (wildtype)	0.93
		Y (mutant)	0
		N/Y (mixed)	0.05
	Y184	Y (wildtype)	0.29
		F (mutant)	0.57
		Y/F (mixed)	0.12
pfdhps	S436/A437	SA (wildtype)	0.02
		SG (mutant)	0.88
		FG (mutant)	0.02
		SA/SG (mixed)	0.05
		FG/SA/SG (mixed)	0.02
Pfdhfr	N51/C59	NC (wildtype)	0
		IR (mutant)	0.26
		IT/NC (mixed)	-
		IR/NR (mixed)	0.12
		NR/NC (mixed)	0.02

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pfk13	C580	C (wildtype)	1
		Y (mutant)	0

pfcrt = *P. falciparum* chloroquine resistance transporter; *pfmdr1* = *P. falciparum* multidrug resistance gene 1; *pfdhps* = *P. falciparum* dihydropteroate synthase; *pfdhfr* = *P. falciparum* dihydrofolate reductase; *pfk13*= *P. falciparum* kelch 13

549 **Figure 1.**

550 Schematic representation of *ex vivo* parasite survival rate assay. (1) iRBCs at 0.5%
551 parasitemia and 2% haematocrit are incubated with 10-fold median IC_{50}s of
552 Dihydroartemisinin and Lumefantrine for 24-, 48- and 72-hour time-points. (2) uRBCs are
553 labelled with the intracellular dye: DDAO-SE ($uRBC^{DDAO\text{-}SE}$). (3) Drugs are washed off from
554 step 1 every 24 hours, aliquots taken, and drugs replenished. (4) Post-exposure drug free
555 aliquots are incubated with 2 x $uRBC^{DDAO\text{-}SE}$ for a further 48 hours. (5,6) These are then
556 counterstained with SYBR Green I for flow cytometric analysis where 100,000 cells are
557 acquired (7) and double positive stained cells analysed.

558 **Figure 2**. (a) Percentage ring survival of 51 isolates using conventional microscopy to assess
559 viable parasites and flow cytometry to assess the number of re-invaded parasites following
560 pulse exposure and withdrawal of DHA with RSA. Each point on the plot represents an
561 isolate. The median survival rates of the isolates for each method are shown as the red broken
562 lines. T-test statistics gave a *P* value of <0.0001 using Wilcoxon rank sum test. (b)
563 Correlation analysis of percentage ring survival using flow cytometry and microscopy with a
564 Pearson correlation coefficient of $R = 0.83$ and $p = <0.0001$ and (c) correlation analysis of
565 percentage ring survival using RSA and parasite survival rates using PSRA analysis. Pearson

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correlation coefficient gave an *R* value of 0.53 and a *p* value of 0.00036. (d) Distribution of the parasite survival rates of 41 isolates treated with DHA and LUM at 3 timepoints over 72 hours with PSRA . Each point shows the rate at which each isolate survives following drug exposure with reference to DMSO-treated control. The red dotted lines are the median survival rates for both drug treatments with $p = 0.35$. *P* value of <0.05 represents statistical significance. All *ex vivo* assays were performed in triplicates.

Figure 3. Individual trajectories of 41 isolates following exposure to (a) DHA and (b) LUM relative to DMSO treated control at 24-, 48-, and 72- hour timepoints. Linear mixed effect model was used, and a linear trend fitted for each isolate across timepoints. The blue and red dotted lines show the isolates with decreasing and increasing responses over time respectively. The thick blue and red lines represent the mean log response of isolates with decreasing and increasing responses respectively. (c) Mean predicted parasite responses of all isolates following exposure to DHA (red broken line), LUM (blue broken line) and DMSO control (grey broken line) with the SEM shown as bars. (d) Correlation between parasite survival rates of isolates treated with DHA and LUM with R = 0.77 and $p < 0.001$. All *ex vivo* assays were performed in triplicates.

Figure 4. Grouped profiles of 41 isolates following exposure to DHA and LUM at 24, 48 and 72 hours with PSRA. Each point in the individual plots represent the difference between the predicted response of the (a) DHA treated and control and (b) LUM treated and control. The connecting lines give an indication of the response pattern of each isolate. The isolates are grouped based on their response profiles. (i) linear decrease (---), (ii) non-linear decrease/ increase (-+-), (iii) linear increase (+++), (iv) non-linear increase/decrease (+-+).

Figure 5. (a) Allele frequencies of 41 field isolates for the drug resistant genes: *pfcrt* C72/M74/N75/K76, *pfmdr1* N86, *pfmdr1* Y184, *pfdhps* S436/A437 and *pfk13* C580. Parasite

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survival rates of (b) DHA and (c) LUM for isolates with wildtype, mutant and mixed alleles for *pfcrt* C72/M74/N75/K76, *pfmdr1* Y184 and *pfdhps* S436/A437. Each point in the graphs represent the parasite survival rate of an isolate. The broken red lines indicate the median survival rates of the isolates with the same alleles.

List of abbreviations

ACT	artemisinin combination therapy
AL	artemether lumefantrine
DBS	dried blood spots
DHA	dihydroartemisinin
DMSO	dimethyl sulfoxide
HRM	high resolution melting
IC_{50}	50% inhibitory concentration
IPTp	intermittent preventive treatment in pregnancy
LUM	lumefantrine
PBS	phosphate buffered saline
PSA	piperaquine survival assay
PSRA	parasite survival rate assay
PVFA	parasite viability fast assay
RBC	red blood cells
iRBC	infected red blood cells
uRBC	uninfected red blood cells
RSA	ring-stage survival assay
SMC	seasonal malaria chemoprevention
SP	sulphodoxine/pyrimethamine

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614 TES therapeutic efficacy study

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624 **Author Contributions**

625 HM and AAN conceived and designed the experiment. HM carried out the experiments. FB
626 and FKJ assisted in validating the assay using laboratory adapted controls. ACJ contributed
627 in sample preparation. BN contributed in microscopic analysis as the second reader and NIM
628 supported all statistical analyses. JO was involved in the planning of the research. HM took
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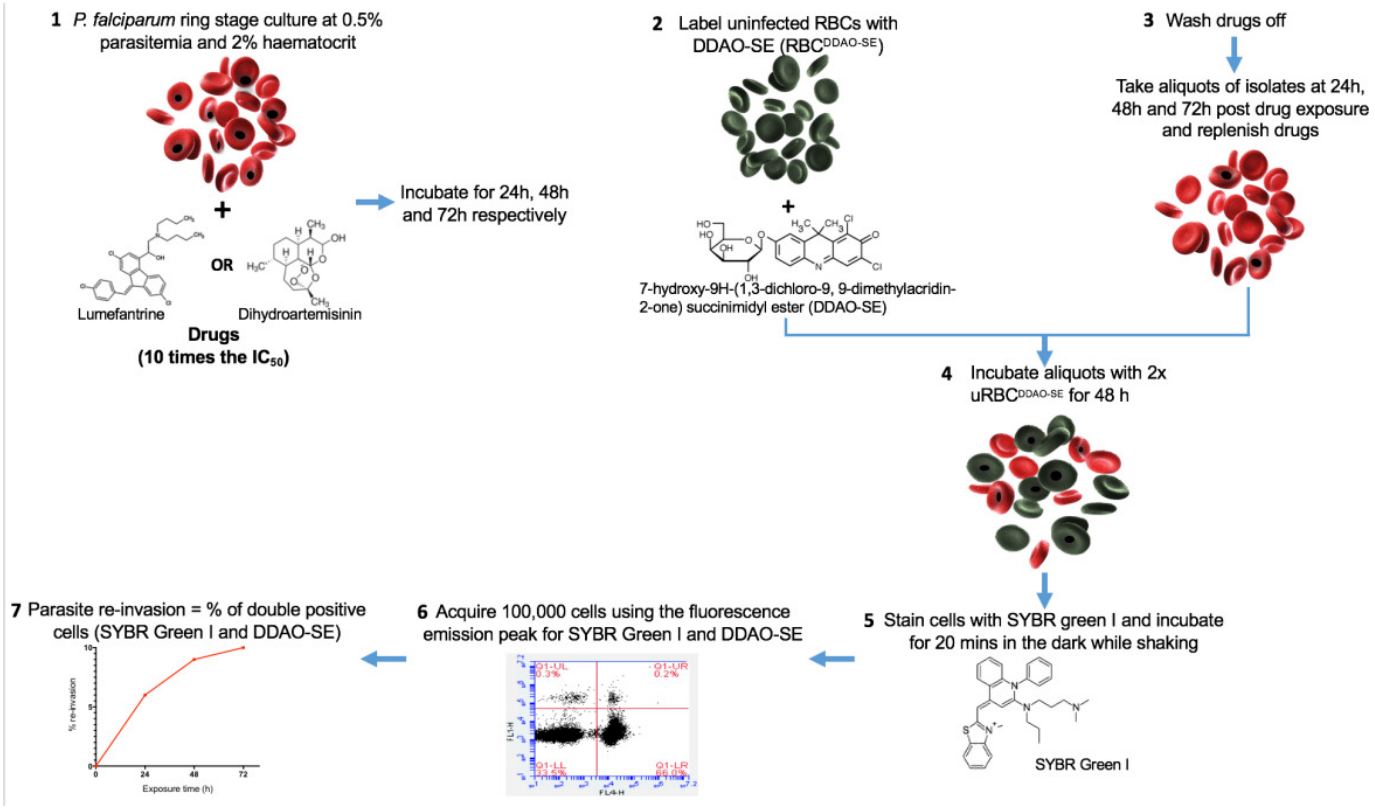

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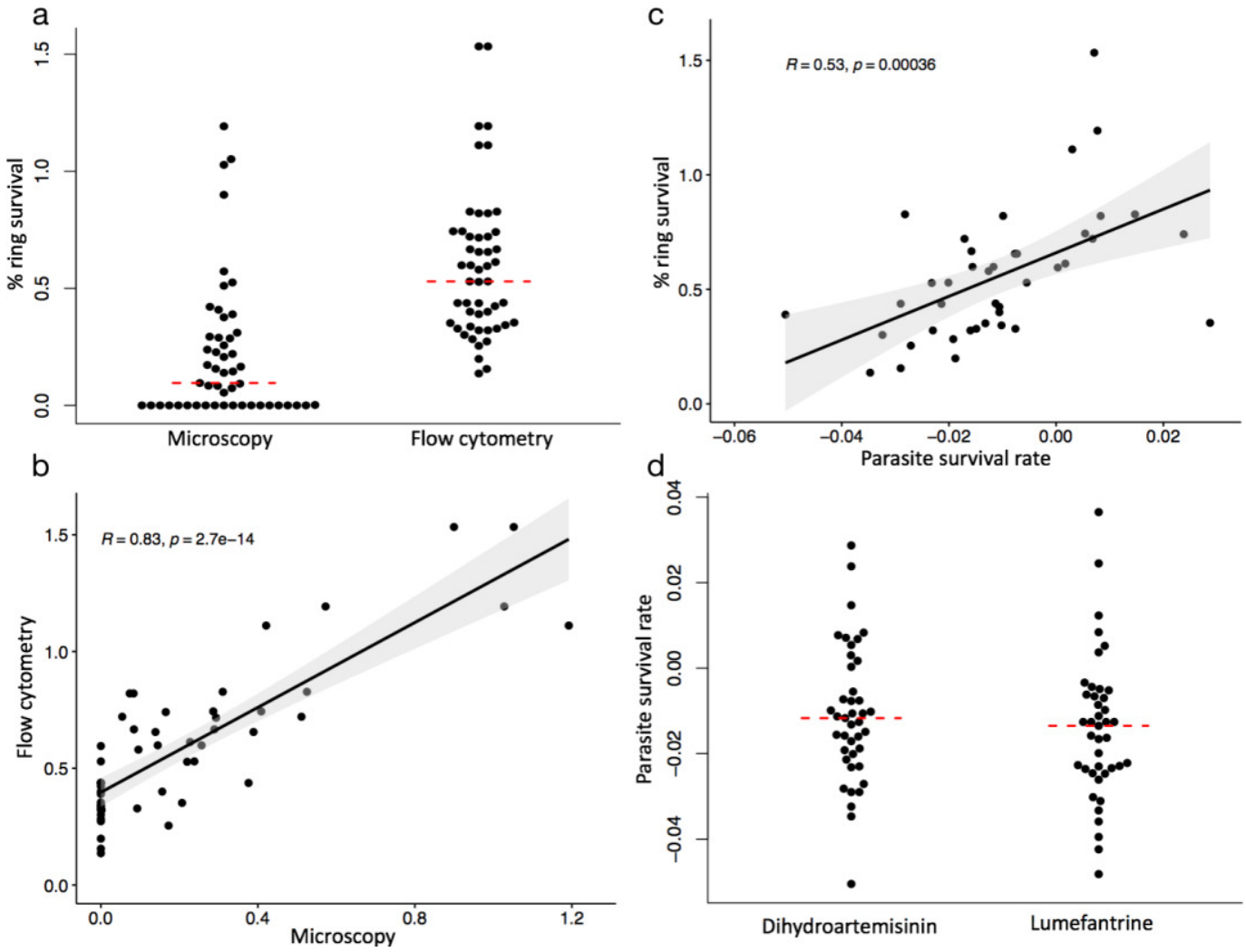

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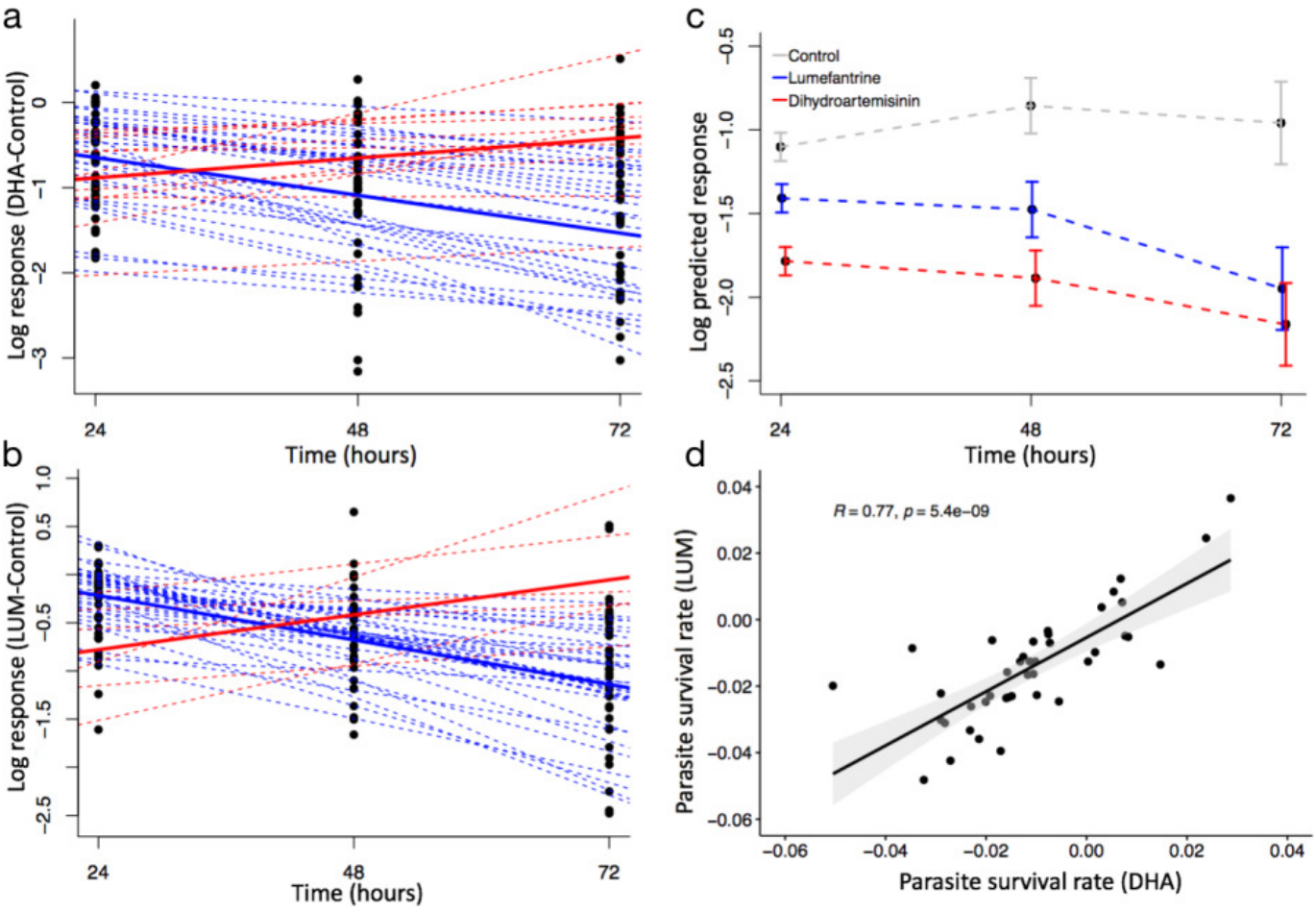

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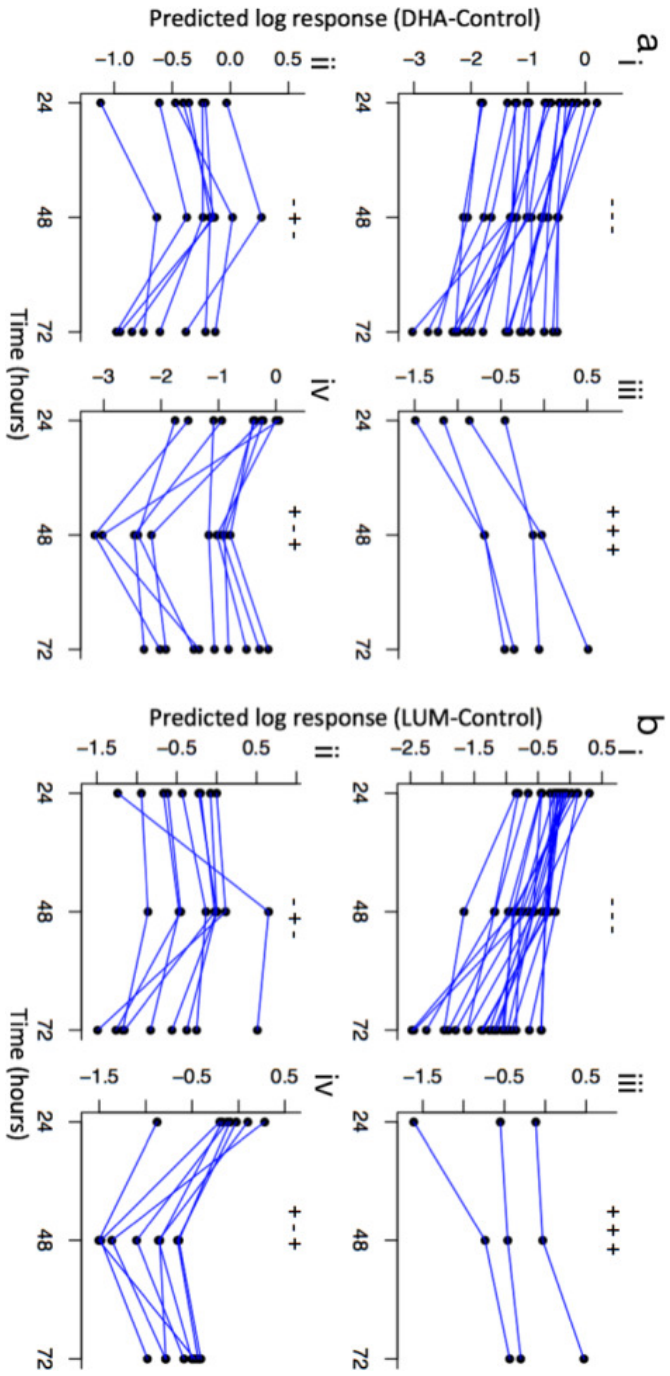

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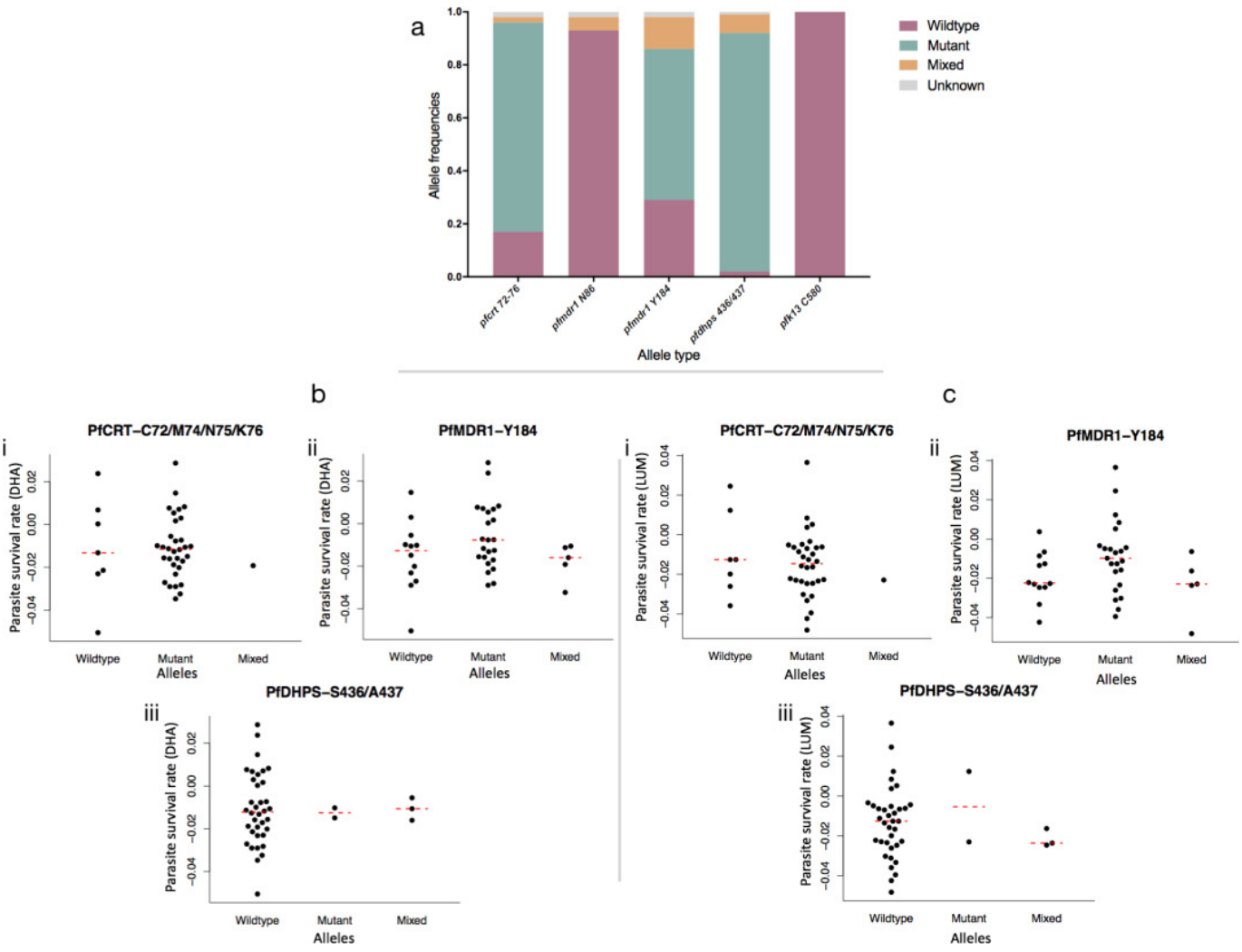

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