**Shifts in *P. falciparum* genetic structure and gametocyte markers in the transition to elimination**

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### **Supplemental Methods**

***Quantitative polymerase chain reaction***

DNA was extracted from a half cut of the DBS using a QIAamp DNA Mini kit (Qiagen). Real-time quantitative PCR (qPCR) targeting *P. falciparum* (*Pf)* 18S rRNA on an ABI PRISM 7500 HT Real-Time System (Applied Biosystems) was used. Parasitemia was quantified by extrapolation of cycle thresholds (Ct) from a standard curve of *Pf* ring infected erythrocytes. Samples without amplification (no Ct detected) were considered negative. A negative control with no template DNA was run in all reactions 1,2 3.

***Library preparation for amplicon deep sequencing***

The *pfcsp* (PF3D7\_0304600; nucleotides 824-1142; ASM276v2) and *pfama1* (*PF3D7\_1133400:* nucleotides 423-779; ASM276v2) genes were amplified separately in 25μl reactions including 5μl of template DNA, 0.4μM of each forward and reverse gene specific plus overhanging primers and 1xHOT FirePol Master Mix (Solis BioDyne; Riia, Tartu, Estonia), reaction volume was raised by PCR-grade water. Primers contained a non-annealing overhang for the indexing primers during the second PCR (**table s1**). In a thermocycler, both the genes amplification followed the same PCR conditions except the annealing temperatures of 52°C of 1 min for *pfcsp* gene and 56°C of 1 min for *pfama1* gene. The template DNA was denatured at 95°C for 15min, followed by 35 cycles of amplification (95°C for 1min, 52°C or 56°C for 1min, and 72°C for 1min 30s) and a final extension at 72°C for 10min. A nested index-specific PCR amplified the primary PCR products using the PCR index primers. In brief, amplification was performed in 50μl reaction including 5μl of primary PCR product, 0.2μM of each forward and reverse index primers and 1x HOT FirePol Master Mix, reaction volume was raised by PCR-grade water. The indexing PCR program consisted of the following steps: Heat activation at 95°C for 15minutes, 20 cycles of denaturation at 95°C for 1minute, annealing at 60°C for 1 minute, and elongation at 72 °C for 1 minute, and one final elongation at 72 °C for 10 minutes. The expected size of PCR products for *pfcsp* and *pfama1* genes was 455bp and 493bp, respectively. PCR products were run on 2% agarose (Invitrogen, Carlsbad, CA, USA) gels in 1× TBE buffer (Invitrogen, Carlsbad, CA, USA) to determine the presence, size of the amplified DNA and the concentration (approximately) of each amplicon by comparing the amplicon against the known concentration of 100bp ladder (New England Biolabs, MA, USA) bands. PCR products were visualized using a UV trans-illuminator. QIAquick® Gel Extraction Kit (QIAGEN, Netherlands) was used to purify PCR products following the manufacturer’s instructions. Approximately 4nM of the pooled libraries were sequenced in an Illumina MiSeq instrument using paired end 2×300bp reads and a MiSeq v3 (600 cycles) flow cell. The sequencing quality and performance were assessed using gDNA extracted from *Pf* strains (3D7, 7G8, Dd2 and HB3) mixed at different concentrations(**table s2**)as well as 40 *Pf* isolates collected in Manhiça District (southern Mozambique) with the complexity of infection (COI) determined on the basis of the length of the amplicon size of *pfmsp1* and *pfmsp2* 4. Concentrations of mixture controls, isolates of 2006, pre-MDA and post-MDA isolates purified libraries were assessed by Qubit analysis followed by pooling of libraries at equimolar amount. A total of eight libraries were prepared for mixture controls, isolates of 2006, pre-MDA and post-MDA isolates.

***Performance of the amplicon deep sequencing***

The quality of the sequence run was assessed by investigating the sequencing error rate in sequence read of the *Pf* strains HB3. To censor regions of high mismatch rates, forward and reverse sequence reads for both genes were trimmed before any further analyses to a length of 275 and 253 nucleotides for *pfama1* and *pfcsp*, respectively. After trimming, the mean mismatch rate per nucleotide of HB3 control reads for the *pfcsp* gene was 0.43% and 0.82% for forward and reverse ends, respectively, and for the *pfama1* gene was 0.53% and 0.56% for forward and reverse ends, respectively. The LOD of minority clone, as assessed under controlled conditions using defined mixtures of *Pf* strains (HB3, DD2, 7G8 and 3D7). was 0.07% and 10% for *pfcsp* and *pfama1* genes, respectively (**table s2**). Mixtures with different COI (1-4) were identified successfully by the assay. However, the mixture with a COI of 3 was underestimated by the *pfama1* gene, and *pfcsp* gene detected the mixture with COI of 2 in only one of the replicates. Finally, COI in 40 field samples assessed by *pfmsp1*/*pfmsp2* genotyping 4 and *pfama1*/*pfcsp* sequencing were well correlated (*ρ* =0.67 and 0.75 for *pfama1* and *pfcsp*, respectively; p<0.001 in both cases; **figure s1**). Overall, *pfmsp1*/*pfmsp2* genotyping detected 1.3 more infections than *pfama1* (95% limit of agreement -1,58, 4.18) and *pfcsp* (95% limit of agreement: -0.37, 2.97) sequencing. No difference in mean COI was observed when comparing *pfama1* and *pfcsp* sequencing (95% limit of agreement: -2,31, 2,31). Across all successfully genotyped infections, read coverage achieved for both genes was similar in samples collected in 2015 and 2017 (**table s5**), with a mean of 5983 (SD 6427) in 2015 and 6449 (SD 4250) in 2017 for *pfama1* (p=0.589) and 8724 (SD 7137) and 7234 (SD 4339) for *pfcsp* (p=0.112). Parasite densities of samples that were successfully genotyped tended to have higher parasite densities, compared to those that were not (110, IQR: 17-825 and 41, IQR: 5-798, p=0.089; **figure s2**).

***Genetic similarity indices***

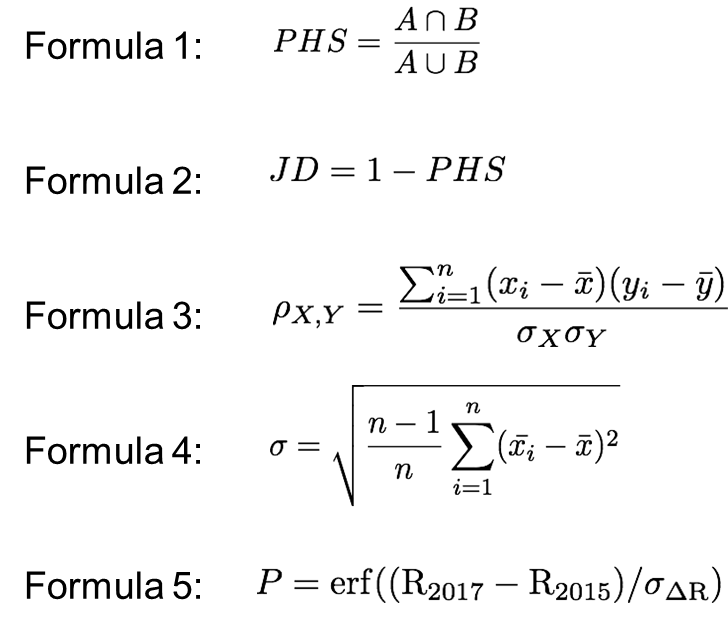
Genetic relatedness of pre and post-intervention populations was quantified through the following metrics:

a) Binary sharing (formula 1): It indicates whether it exists a common haplotype between two samples (1) or not (0).

b) Jaccard distance: the inverse of the Proportional Haplotype Sharing, which measures the fraction of haplotypes that two samples have in common with respect to all the haplotypes common in any of the samples (formula 2, where A and B represent the existence or not of all the haplotypes of the two samples). *JD* = 0 when the two samples have the same haplotype combination (even if they have different frequencies) and *JD* = 1 when they have no haplotypes in common.

c) Pearson correlation coefficient, which measures the linear correlation of the haplotype frequencies between two samples as follows (formula 3, where *x* and *y* are the haplotype frequencies of two samples, *i* represents the *i*th haplotype, and σX,σY are the standard deviations of the two samples). The Pearson CC gives values from -1 to 1, being -1 for completely anticorrelated samples, 0 for uncorrelated samples and 1 for completely correlated samples.

To obtain the relatedness on a population for any of the metric, the mean relatedness over all sample pairs is obtained. A Jack-Knife approach was used to calculate standard deviation of each similarity metric, using as many subgroups as samples in the data for the resampling, excluding a different sample each time. The error bars have been obtained through a Jack-Knife approach as described in the Definitions and data analysis section. For this, the population has been split in *n* groups, and the mean has been obtained *n* times, excluding one of the groups each time. The error obtained corresponds to the renormalised standard deviation of these measurements (formula 4). The change in relatedness from 2015 to 2017 has been obtained from the difference between the two measurements of relatedness. Since the two measurements are independent, the error of the differences has been obtained using error propagation of the errors of the measurements from 2015 and 2017. The power corresponds to the error function erf of the distance of the difference to 0 over the error (formula 5). The change in the fraction of sample pairs with similarity indices above different thresholds was calculated using the Jack-Knife approach with the same resampling scheme to obtain the error. The analyses were done using Python 3 programming language with modules numpy, scipy, pandas and matplotlib, and using Jupyter notebook software.



***RT-qPCR quantification of gametocyte-specific RNAs***

*Pf*-positive filter papers stored in RNAprotect were used for RNA extraction using Maxwell® RSC simplyRNA Blood Kit and Maxwell® RSC 48 Instrument (Promega). Extracted total RNA (400-800ng) was subjected to reverse transcription using PrimeScript™ RT Master Mix reagents (Takara) and resulting cDNA was eluted in 35 μl. Transcript levels of *pfs25*, *pfs230*, *pfap2G* and *gexp02* genes were assessed using a RT-qPCR described previously 5-8 and a 7500 HT Real-Time System (Applied Biosystem)[.](#bookmark1) *PF08\_0085* (ubiquitin-conjugating enzyme) gene was used as a housekeeping (HK) gene 5. Each reaction mixture had 10μl of 2X Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Warrington, WA, UK), 1μM of each forward and reverse primers and 5μl of template cDNA. Amplifications were performed with a holding for 3mins at 50°C, initial denaturation for 10mins at 95°C, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Non-template controls were tested in every plate. Samples with a Ct>40 for any gene target were considered as not amplified. The specificity of primer pairs against human gDNA was also determined. The 7500 System SDS software v1.4 was used to analyze the collected data. Transcript copy number of each gene was quantified by extrapolation against a standard curve of five serially diluted points was prepared from an *in vitro* culture of 3D7 strain (gDNA) containing known numbers of ring-infected erythrocytes. Zeros were eliminated from the copy number data by adding to all values half of the limit of detection (LOD) of the qPCR, as calculated by the lowest concentration of target gene that can be detected in triplicate serial dilutions of 3D7 gDNA obtained from in vitro cultures of known parasitemia assessed by microscopy (**table s3**). The relative copy number (RCN) was calculated as the ratio of transcript copy numbers between the target and the HK gene.

Samples with a Ct>40 were considered as not amplified. Transcript copy number of each gene was quantified by extrapolation against a standard curve of five serial dilutions of gDNA obtained from the 3D7 strain containing known numbers of ring-infected erythrocytes. Zeros were eliminated from the copy number data by adding to all values half of the limit of detection (LOD) of the qPCR, as calculated by the lowest concentration of target gene that can be detected in triplicate serial dilutions of 3D7 gDNA obtained from in vitro cultures of known parasitemia assessed by microscopy (**table s3**). The relative copy number (RCN) was calculated as the ratio of transcripts detected from target and HK genes.

**Figure s1.** Parasite densities of *P. falciparum* isolates that were successfully analysed by amplicon-based sequencing and RT-qPCR, compared to those which were not.

Red lines represent the median and T bars de interquartile range. Mann Whitney U test were used for the statistical comparison. House-keeping (*PF08\_0085*) gene amplified (HK+) and non-amplified (HK-); Samples successfully sequenced (Seq) and non-sequenced (Non-Seq) by the next generation Sequencing platform.

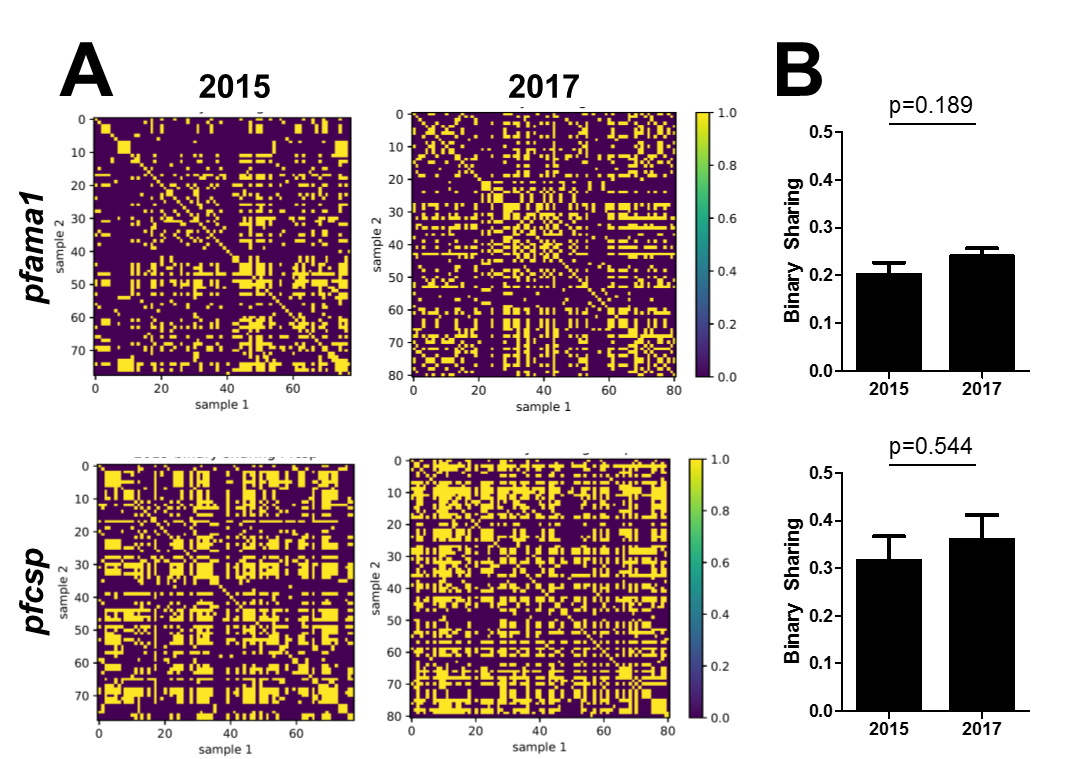


**Figure s2.** Correlation and agreement (Bland-Altman plots of the difference versus average) for COI assessed using *pfama1*, *pfcsp* and *pfmsp1/2*.

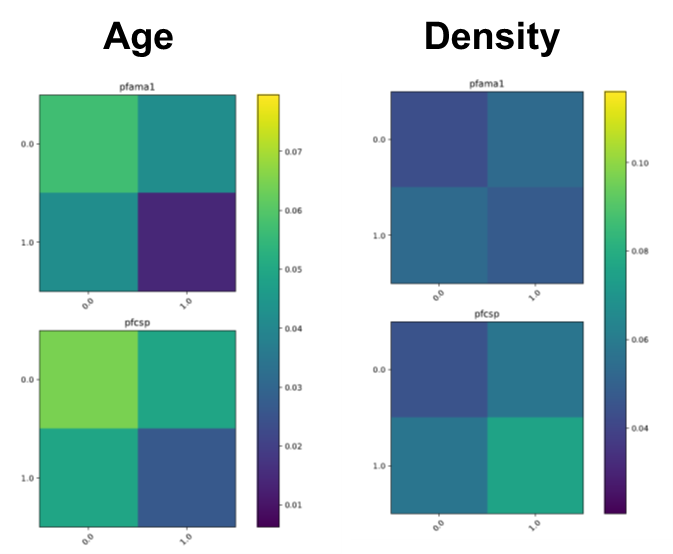


**Figure s3.** Matrix showing the values of binary sharing between sample pairs for *pfama1* (top) and *pfcsp* (bottom) in 2015 (left) and 2017 (right) (**A**). Histograms showing the distributions of all these values for *pfama1* (left) and *pfcsp* (right) for 2015 and 2017 (**B**).

Binary sharing gives a binary output defined by whether a pair of samples have any haplotype in common.

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**Figure s4.** Genetic relatedness between different populations using Pearson correlation coefficient. Comparison of populations by age and density.

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**Table s1.** Gene specific primer sequences with overhangs (in lowercase) used in next-generation sequencing where overhangs can act as annealing site in the indexing PCR.

Indexing primer contains sequence to anneal to the overhangs, 8-base indices and adapter sequences for the final PCR product to bind to the sequencing flow cell.

|  |  |
| --- | --- |
| **Primers** | **Sequences** |
| *pfcsp\_F* | tcgtcggcagcgtcagatgtgtataagagacagACAATCAAGGTAATGGACAAGG |
| *pfcsp\_R* | gtctcgtgggctcggagatgtgtataagagacagACGACATTAAACACACTGGAAC |
| *pfama1\_F* | tcgtcggcagcgtcagatgtgtataagagacagATATAGACTTCGATCAGGGAAATGT |
| *pfama1\_R* | R - gtctcgtgggctcggagatgtgtataagagacagGGACCATTATTTTCTTGAGCTGC |
| Index\_1\_F | AATGATACGGCGACCACCGAGATCTACACaaaagggaTCGTCGGCAGCGT |
| Index\_1\_R | CAAGCAGAAGACGGCATACGAGATtcccttttGTCTCGTGGGCTCGGAGA |
| Index\_2\_F | AATGATACGGCGACCACCGAGATCTACACaaacagccTCGTCGGCAGCGT |
| Index\_2\_R | CAAGCAGAAGACGGCATACGAGATggctgtttGTCTCGTGGGCTCGGAGA |
| Index\_3\_F | AATGATACGGCGACCACCGAGATCTACACaaactcgcTCGTCGGCAGCGT |
| Index\_3\_R | CAAGCAGAAGACGGCATACGAGATgcgagtttGTCTCGTGGGCTCGGAGA |
| Index\_4\_F | AATGATACGGCGACCACCGAGATCTACACaaagacacTCGTCGGCAGCGT |
| Index\_4\_R | CAAGCAGAAGACGGCATACGAGATgtgtctttGTCTCGTGGGCTCGGAGA |
| Index\_5\_F | AATGATACGGCGACCACCGAGATCTACACtttcctacTCGTCGGCAGCGT |
| Index\_5\_R | CAAGCAGAAGACGGCATACGAGATgtaggaaaGTCTCGTGGGCTCGGAGA |
| Index\_6\_F | AATGATACGGCGACCACCGAGATCTACACtggttacaTCGTCGGCAGCGT |
| Index\_6\_R | CAAGCAGAAGACGGCATACGAGATtgtaaccaGTCTCGTGGGCTCGGAGA |
| Index\_7\_F | AATGATACGGCGACCACCGAGATCTACACtgcaccatTCGTCGGCAGCGT |
| Index\_7\_R | CAAGCAGAAGACGGCATACGAGATatggtgcaGTCTCGTGGGCTCGGAGA |
| Index\_8\_F | AATGATACGGCGACCACCGAGATCTACACtctgagttTCGTCGGCAGCGT |
| Index\_8\_R | CAAGCAGAAGACGGCATACGAGATaactcagaGTCTCGTGGGCTCGGAGA |
| Index\_9\_F | AATGATACGGCGACCACCGAGATCTACACacacatggTCGTCGGCAGCGT |
| Index\_9\_R | CAAGCAGAAGACGGCATACGAGATccatgtgtGTCTCGTGGGCTCGGAGA |
| Index\_10\_F | AATGATACGGCGACCACCGAGATCTACACacgtcttcTCGTCGGCAGCGT |
| Index\_10\_R | CAAGCAGAAGACGGCATACGAGATgaagacgtGTCTCGTGGGCTCGGAGA |
| Index\_11\_F | AATGATACGGCGACCACCGAGATCTACACaccacacaTCGTCGGCAGCGT |
| Index\_11\_R | CAAGCAGAAGACGGCATACGAGATtgtgtggtGTCTCGTGGGCTCGGAGA |
| Index\_12\_F | AATGATACGGCGACCACCGAGATCTACACagagacacTCGTCGGCAGCGT |
| Index\_12\_R | CAAGCAGAAGACGGCATACGAGATgtgtctctGTCTCGTGGGCTCGGAGA |
| Index\_13\_F | AATGATACGGCGACCACCGAGATCTACACagcagtctTCGTCGGCAGCGT |
| Index\_13\_R | CAAGCAGAAGACGGCATACGAGATagactgctGTCTCGTGGGCTCGGAGA |
| Index\_14\_F | AATGATACGGCGACCACCGAGATCTACACaggctagaTCGTCGGCAGCGT |
| Index\_14\_R | CAAGCAGAAGACGGCATACGAGATtctagcctGTCTCGTGGGCTCGGAGA |
| Index\_15\_F | AATGATACGGCGACCACCGAGATCTACACatcgtgtgTCGTCGGCAGCGT |
| Index\_15\_R | CAAGCAGAAGACGGCATACGAGATcacacgatGTCTCGTGGGCTCGGAGA |
| Index\_16\_F | AATGATACGGCGACCACCGAGATCTACACatgaccgcTCGTCGGCAGCGT |
| Index\_17\_F | AATGATACGGCGACCACCGAGATCTACACatgggaagTCGTCGGCAGCGT |
| Index\_18\_F | AATGATACGGCGACCACCGAGATCTACACcacaggtgTCGTCGGCAGCGT |
| Index\_19\_F | AATGATACGGCGACCACCGAGATCTACACcagtactaTCGTCGGCAGCGT |
| Index\_20\_F | AATGATACGGCGACCACCGAGATCTACACcatgcttgTCGTCGGCAGCGT |
| Index\_21\_F | AATGATACGGCGACCACCGAGATCTACACccacagaaTCGTCGGCAGCGT |
| Index\_22\_F | AATGATACGGCGACCACCGAGATCTACACcccttgctTCGTCGGCAGCGT |
| Index\_23\_F | AATGATACGGCGACCACCGAGATCTACACcctaatacTCGTCGGCAGCGT |

**Table s2.** Proportions of laboratory strains gDNAs with known polymorphisms prepared to assess sequence quality and performance characteristics.

Mixtures were created using stocks with 4 different *pfama1* and *pfcsp* genotypes in the region amplified for deep sequencing. This control DNAs were mixed to reach a final concentration of 1 ng/µl. Set-1 and Set-2 are replica of each other containing gDNA (quantified through Qubit analysis) of *Pf* laboratory strains in different proportions.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **ng** | | | |  |  | ***Coverage*** | | | |  | ***Detected*** | |
| **Ratios in mixturesa** | **HB3** | **DD2** | **7G8** | **3D7** | **COI** | **MAF (%)** | ***pfcsp*** | | ***pfama1*** | |  | ***pfcsp*** | ***pfama1*** |
|  |  |  |  |  |  |  | **Set 1** | **Set 2** | **Set 1** | **Set 2** |  |  |  |
| 1 | 1 |  |  |  | 1 | 0 | 11298 | 7717 | 5941 | 12030 |  | Yes | Yes |
| 1:2 | 0.5 | 0.5 |  |  | 2 | 50 | 10034 | 8208 | 5527 | 11586 |  | Set-2 | Yes |
| 1:3 | 0.33 | 0.33 | 0.33 |  | 3 | 33.30 | 10250 | 7791 | 6545 | 12854 |  | Yes | Yesc |
| 1:4 | 0.25 | 0.25 | 0.25 | 0.25 | 4b | 25 | - | - | 3663 | 5240 |  | - | Yes |
| 1:10 | 0.9 | 0.1 |  |  | 2 | 10 | 9910 | 7213 | 3242 | 7177 |  | Yes | Yes |
| 1:100 | 0.99 | 0.01 |  |  | 2 | 1 | 10557 | 7424 | 2377 | 6791 |  | Yes | No |
| 1:1000 | 0.999 | 0.001 |  |  | 2 | 0.10 | 9151 | 6957 | 4016 | 6777 |  | Yes | No |
| 1:1500 | 0.9993 | 0.0007 |  |  | 2 | 0.07 | 7405 | 6408 | 2959 | 7139 |  | Set-1 | No |
| 1:3000 | 0.9997 | 0.0003 |  |  | 2 | 0.03 | 11396 | 4061 | 7123 | 11956 |  | No | No |
| 1:4000 | 0.99975 | 0.00025 |  |  | 2 | 0.025 | 11930 | 6219 | 6021 | 12732 |  | No | No |
| 1:5000 | 0.9998 | 0.0002 |  |  | 2 | 0.02 | 12613 | 7934 | 6465 | 10663 |  | No | No |
| 1:10000 | 0.9999 | 0.0001 |  |  | 2 | 0.01 | 11238 | 8390 | 10412 | 12578 |  | No | No |
| *a, F*inal conc. 1ng make-up with major strain*; b, only used for pfama1 gene, c, found COI=2 instead of COI=3. COI = complexity of infection; MAF = minor allele frequency.* | | | | | | | | | | | | | |

**Table s3.** Slope, efficiency and limit of detection (LOD) of the RT-qPCR.

The table shows for each target: slope, efficiency (E) and limit of detection (LOD). The amplification efficiency (E) of the qPCR assays is estimated on the basis of the equation *E = (10−1/slope − 1) × 100.*The LOD is the lowest concentration of target gene (copies/20 µl of qPCR reaction) that can be detected by a given qPCR assay. LOD was calculated testing in triplicate serial dilutions of 3D7 gDNA obtained from in vitro cultures of known parasitemia assessed by microscopy. Dilutions without amplification (no Ct detected) were considered negative.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Slope** | **Efficiency** | **LOD\*** |
| ***pfs25*** | -3.38 | 97.60 | 2.5 c/20ul |
| ***pfs230*** | -4.67 | 63.79 | 6.25 c/20ul |
| ***pfap2g*** | -4.70 | 63.25 | 25 c/20ul |
| ***pfgexp02*** | -3.92 | 79.89 | 0.25 c/20ul |
| ***pfuce*** | -3.32 | 99.90 | 2.5 c/20ul |
| \* Copies of target gene per 20 µL of qPCR reaction | | | |
| uce: ubiquitin-conjugating enzyme | | |  |

**Table s4.** Main characteristics of all participants with *P. falciparum* infection detected by real-time quantitative PCR. P value provides the statistical significance of the comparison between all study participants with a *P. falciparum* infection and those from whom samples were successfully analyzed (Table 1) by RT-qPCR (gametocyte-specific markers) and next-generation sequencing (NGS).

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | **Nov 2015** | **May 2015** | **May 2017** | **RT-qPCR** | |  | **NGS** | |
|  |  | **(n=168)a** | **(n=101)b** | **(n=139)c** | **pa** | **pb** |  | **pc** | **pd** |
| Study Area, n (%)1 | |  |  |  |  |  |  |  |  |
|  | Magude Sede | 107 (64) | 70 (69) | 77 (55) | 0.924 | 0.861 |  | 0.941 | 0.964 |
|  | Motaze | 27 (16) | 22 (22) | 24 (17) |  |
|  | Panjane | 22 (13) | 8 (8) | 11 (8) |  |
|  | Mahele | 4 (2) | 0 (0) | 12 (9) |  |
|  | Mapulanguene | 8 (5) | 1(1) | 15 (11) |  |
| Age, Mean (SD)2 | | 17.1 (15.4) | 11.8 (16.9) | 13.3 (17.2) | 0.209 | 0.263 |  | 0.118 | 0.438 |
| Gender, n (%)1 | |  |  |  |  |  |  |  |  |
|  | Female | 90 (54) | 56 (55) | 71 (52) | 0.226 | 0.876 |  | 0.674 | 0.779 |
|  | Male | 78 (46) | 45 (45) | 65 (48) |  |
| Parasite density (qPCR), GM (SD)3 | | 18.7 (51.9) | 71.3 (221.9) | 67.4 (208,1) | 0.098 | <0.001 |  | <0.01 | 0.042 |
| GM, Geometric mean; SD, Standard deviation | | |  |  |  |  |  |  |  |
| 1, Fisher's test; 2, Student`s test | | | |  |  |  |  |  |  |
| a, May 2015; b, May 2017; c, Nov 2015, d, May 2017 | | | | | | | | | |

**Table s5.** Performance of the amplicon deep sequencing.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | **2015 (n=78)** | |  | **2017 (n=81)** | |  | **All (n=159)** | |
|  |  | ***pfcsp*** | ***pfama1*** |  | ***pfcsp*** | ***pfama1*** |  | ***pfcsp*** | ***pfama1*** |
| **Initial reads/sample: mean (SD)** | | 18353 (12362) | |  | 19599 (7789) | |  | 18988 (10275) | |
| **Number of reads/sample after demultiplexing: mean (SD)** | | 9591 (7508) | 8258 (6002) |  | 8372 (4452) | 10659 (4228) |  | 8970 (6155) | 9481 (5297) |
| **Number of reads/sample after merging: mean (SD)a** |  | 8724 (7137) | 5983 (6427) |  | 7234 (4339) | 6449 (4250) |  | 7965 (5908) | 6221 (5416) |
| **Total singleton identified: n, (%)** | | 6785 (1.0) | 11219 (2.4) |  | 6331 (1.1) | 9102 (1.7) |  | 13116 (1.0) | 20321 (2.1) |
| **Total Chimers identified: n, (%)** | | 3565 (0.5) | 7616 (1.6) |  | 3457 (0.6) | 3271 (0.6) |  | 7022 (0.6) | 10887 (1.1) |
| **Total indels identified: n, (%)** |  | 947 (0.1) | 192 (0.1) |  | 1171 (0.2) | 119 (0.0) |  | 2118 (0.2) | 311 (0.0) |
| **Number of distinct haplotypes** |  | 49 | 43 |  | 40 | 23 |  | 57 | 45 |

a, p=0.589 and 0.112 for the comparison (t Student’s test) of number of reads in 2015 and 2017 for *pfama1* and *pfcsp*, respectively.

**Table s6.** *P. falciparum* gametocyte-specific transcripts and genetic metrics of diversity and relatedness by study area.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | | **Magude Sede** | **Motaze** | **Panjane** | **Mahele** | **Mapulanguene** | **p** |
| ***Pf* genetic markers** | | | |  |  |  |  |  |  |
|  | **Monoclonal: n. %** | | | | | | | | |
|  |  | ***pfama1*** | **No** | 50 (54) | 15 (52) | 10 (56) | 2 (25) | 6 (50) | 0.638 |
|  |  | **Yes** | 42 (46) | 14 (48) | 8 (44) | 6 (75) | 6 (50) |
|  |  | ***pfcsp*** | **No** | 59 (64) | 19 (66) | 14 (78) | 5 (63) | 10 (83) | 0.614 |
|  |  | **Yes** | 33 (36) | 10 (34) | 4 (22) | 3 (38) | 2 (17) |
|  | **Rare haplotypes. n (%)** | | | | | | | | |
|  |  | ***pfama1*** | **No** | 87 (95) | 28 (97) | 16 (89) | 8 (100) | 11 (92) | 0.672 |
|  |  | **Yes** | 5 (5) | 1 (3) | 2 (11) | 0 (0) | 1 (8) |
|  |  | ***pfcsp*** | **No** | 83 (90) | 27 (93) | 15 (83) | 7 (88) | 10 (83) | 0.643 |
|  |  | **Yes** | 9 (10) | 2 (7) | 3 (17) | 1 (13) | 2 (17) |
|  | **COI. Mean (SD)** | | | | | | | | |
|  |  | ***pfama1*** |  | 2.59 (2.22) | 1.86 (1.13) | 1.89 (1.08) | 1.38 (0.74) | 1.67 (0.89) | 0.092 |
|  |  | ***pfcsp*** |  | 2.82 (2.22) | 2.72 (1.75) | 3.67 (3.12) | 3.63 (3.70) | 4.50 (3.55) | 0.139 |
|  | **Shannon. Mean (SD)** | | | | | | | | |
|  |  | ***pfama1*** |  | 0.32 (0.51) | 0.17 (0.29) | 0.17 (0.29) | 0.06 (0.15) | 0.19 (0.40) | 0.260 |
|  |  | ***pfcsp*** |  | 0.37 (0.52) | 0.28 (0.36) | 0.25 (0.34) | 0.27 (0.41) | 0.51 (0.56) | 0.555 |
|  | **Shared haplotypes. Mean (SD)** | | | | | | | | |
|  |  | ***pfama1*** |  | 0.06 (0.05) | 0.08 (0.06) | 0.07 (0.05) | 0.08 (0.06) | 0.07 (0.05) | 0.368 |
|  |  | ***pfcsp*** |  | 0.06 (0.06) | 0.07 (0.06) | 0.04 (0.04) | 0.06 (0.06) | 0.06 (0.06) | 0.390 |
|  |  |  |  |  |  |  |  |  |  |
| **Gametocyte-specific trasncription** | | | | |  |  |  |  |  |
|  | **Prevalence of transcript detection. n (%)** | | | | | | | | |
|  |  | ***pfs25*** | **Neg** | 6 (9) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0.588 |
|  |  | **Pos** | 59 (91) | 23 (100) | 11 (100) | 3 (100) | 5 (100) |
|  |  | ***pfs230*** | **Neg** | 29 (45) | 7 (30) | 5 (45) | 0 (0) | 0 (0) | 0.163 |
|  |  | **Pos** | 36 (55) | 16 (70) | 6 (55) | 3 (100) | 5 (100) |
|  |  | ***pfap2g*** | **Neg** | 19 (29) | 5 (22) | 3 (27) | 0 (0) | 0 (0) | 0.655 |
|  |  | **Pos** | 46 (71) | 18 (78) | 8 (73) | 3 (100) | 5 (100) |
|  |  | ***pfgexp02*** | **Neg** | 26 (40) | 4 (17) | 3 (27) | 0 (0) | 0 (0) | 0.106 |
|  |  | **Pos** | 39 (60) | 19 (83) | 8 (73) | 3 (100) | 5 (100) |
|  | **Relative transcript levels. Mean (SD)** | | | | | | | | |
|  |  | ***pfs25*** |  | 3.22 (8.54) | 1.52 (3.40) | 2.96 (4.79) | 4.30 (5.27) | 14.66 (34.17) | 0.415 |
|  |  | ***pfs230*** |  | 0.26 (0.57) | 0.32 (0.66) | 0.23 (0.43) | 0.64 (0.83) | 1.04 (1.03) | 0.618 |
|  |  | ***pfap2G*** |  | 1.67 (3.04) | 1.55 (2.81) | 1.49 (2.45) | 1.42 (0.72) | 8.90 (10.29) | 0.344 |
|  |  | ***pfgexp02*** |  | 0.24 (1.08) | 1.26 (4.41) | 0.91 (3.62) | 2.71 (1.22) | 5.72 (5.44) | 0.232 |

**Table s7.** Proportions and means of *P. falciparum* genetic diversity metrics.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Monoclonal (n, %)** | | |  | **COI (mean, SD)** | | |  | **Shannon (mean. SD)** | | |  | **Rare haplotypes (n. %)** | | |
|  | **Period (2015: n=78; 2017: n=81)** | | | | | | | | | | | | | | |
|  | **Pre-MDA** | **Post-MDA** | **p** |  | **Pre-MDA** | **Post-MDA** | **p** |  | **Pre-MDA** | **Post.MDA** | **p** |  | **Pre-MDA** | **Post-MDA** | **p** |
| ***pfama1*** | 31 (40) | 45 (56) | 0.057 |  | 2.59 (2.17) | 1.91 (1.43) | 0.021 |  | 0.36 (0.48) | 0.15 (0.37) | 0.002 |  | 8 (10) | 1 (1) | 0.016 |
| ***pfcsp*** | 23 (29) | 29 (36) | 0.405 |  | 3.00 (2.06) | 3.12 (2.85) | 0.756 |  | 0.43 (0.49) | 0.26 (0.44) | 0.018 |  | 13 (17) | 4 (5) | 0.021 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | **Age (≤15 years: n=122; >15 years: n=37)** | | | | | | | | | | | | | | |
|  | **≤15y** | **>15y** | **p** |  | **≤15y** | **>15y** | **p** |  | **≤15y** | **>15y** | **p** |  | **≤15y** | **>15y** | **p** |
| ***pfama1*** | 57 (47) | 19 (51) | 0.708 |  | 2.31 (1.91) | 2.03 (1.69) | 0.416 |  | 0.27 (0.45) | 0.20 (0.38) | 0.456 |  | 7 (6) | 2 (5) | 1 |
| ***pfcsp*** | 34 (28) | 18 (49) | 0.027 |  | 3.26 (2.55) | 2.41 (2.18) | 0.067 |  | 0.36 (0.49) | 0.27 (0.39) | 0.301 |  | 11 (9) | 6 (16) | 0.23 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | **Parasite density by qPCR (≤200 parasites/µL: n=89; >200 parasites/µL: n=70)** | | | | | | | | | | | | | | |
|  | **≤200 p/µL** | **>200 p/µL** | **p** |  | **≤200 p/µL** | **>200 p/µL** | **p** |  | **≤200 p/µL** | **>200 p/µL** | **p** |  | **≤200 p/µL** | **>200 p/µL** | **p** |
| ***pfama1*** | 44 (49) | 32 (46) | 0.749 |  | 1.94 (1.35) | 2.63 (2.30) | 0.021 |  | 0.19 (0.37) | 0.33 (0.50) | 0.040 |  | 4 (4) | 5 (7) | 0.508 |
| ***pfcsp*** | 33 (37) | 19 (27) | 0.234 |  | 2.96 (2.73) | 3.20 (2.15) | 0.540 |  | 0.30 (0.44) | 0.40 (0.51) | 0.187 |  | 9 (10) | 8 (11) | 0.802 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | **Gender (Females: n=79, Males: n=80)** | | | | | | | | | | | | | | |
|  | **Fem** | **Male** | **p** |  | **Fem** | **Male** | **p** |  | **Fem** | **Male** | **p** |  | **Fem** | **Male** | **p** |
| ***pfama1*** | 37 (47) | 39 (49) | 0.874 |  | 2.18 (1.71) | 2.31 (2.00) | 0.648 |  | 0.24 (0.40) | 0.26 (0.47) | 0.706 |  | 4 (5) | 5 (6) | 1 |
| ***pfcsp*** | 28 (35) | 24 (30) | 0.502 |  | 2.86 (2.45) | 3.26 (2.53) | 0.311 |  | 0.27 (0.40) | 0.41 (0.53) | 0.063 |  | 7 (9) | 10 (13) | 0.609 |

**Table s8.** Univariate and multivariate regression analysis of *Pf* diversity metrics by study variables.

The diversity metrics are included as dependent variables in each of the regression models and period (baseline: 2017), age (baseline: ≤15), parasite densities (baseline: ≤200 parasites/µL) and gender (baseline: female). The coefficients express relative (in the proportion of monoclonal infections or the detection of rare haplotypes) and absolute differences (number of concurrent infections and Shannon index values) using baseline categories as reference. Multivariate models included period, parasite densities and age.

**A. Univariate**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | **Monoclonal\*** | | |  | **COI\*\*** | | |  | **Shannon\*\*\*** | | |  | **Rare haplotypes\*** | | |
|  | **VARIABLE** | **Coef** | | **95%CI** | **p** |  | **Coef** | **95%CI** | **p** |  | **Coef** | **95%CI** | **p** |  | **Coef** | **95%CI** | **p** |
| ***pfama1*** | |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | **Period** | 1.9 | | 1.01-3.56 | **0.047** |  | 0.74 | 0.58-0.94 | **0.012** |  | 0.8 | 0.70-0.92 | **0.002** |  | 0.11 | 0.01-0.90 | **0.039** |
|  | **Age** | 1.2 | | 0.58-2.51 | 0.622 |  | 0.88 | 0.66-1.17 | 0.374 |  | 0.94 | 0.80-1.11 | 0.456 |  | 0.94 | 0.19-4.73 | 0.939 |
|  | **Density** | 0.86 | | 0.46-1.61 | 0.641 |  | 1.35 | 1.07-1.71 | **0.012** |  | 1.15 | 1.01-1.32 | **0.040** |  | 1.63 | 0.42-6.33 | 0.477 |
|  | **Gender** | 1.08 | | 0.58-2.01 | 0.809 |  | 1.06 | 0.84-1.35 | 0.620 |  | 1.03 | 0.90-1.18 | 0.706 |  | 1.25 | 0.32-4.84 | 0.747 |
| ***pfcsp*** | |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | **Period** | 1.33 | | 0.69-2.60 | 0.397 |  | 1.04 | 0.82-1.31 | 0.734 |  | 0.84 | 0.72-0.97 | **0.018** |  | 0.26 | 0.08-0.84 | **0.024** |
|  | **Age** | 2.45 | | 1.15-5.22 | **0.002** |  | 0.74 | 0.55-0.98 | **0.038** |  | 0.91 | 0.77-1.09 | 0.301 |  | 1.95 | 0.67-5.70 | 0.221 |
|  | **Density** | 0.63 | | 0.32-1.25 | 0.186 |  | 1.08 | 0.86-1.37 | 0.504 |  | 1.11 | 0.95-1.28 | 0.187 |  | 1.15 | 0.42-3.14 | 0.790 |
|  | **Gender** | 0.78 | | 0.40-1.52 | 0.465 |  | 1.14 | 0.90-1.44 | 0.268 |  | 1.15 | 0.99-1.33 | 0.063 |  | 1.47 | 0.53-4.08 | 0.460 |
| \*Logistic regression; \*\*Negative binomial regression; \*\*\* Linear regression | | | | | | | | | | | | | | | | | |

**B. Multivariate**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | **Monoclonal\*** | | |  | **COI\*\*** | | |  | **Shannon\*\*\*** | | |  | | **Rare haplotypes\*** | | |
|  | **VARIABLE** | | **Coef** | **95%CI** | **p** |  | **Coef** | **95%CI** | **p** |  | **Coef** | **95%CI** | **p** |  | | **Coef** | **95%CI** | **p** |
| ***pfama1*** | |  |  |  |  |  |  |  |  |  |  |  |  |  | |  |  |  |
|  | **Period** | | 2.00 | 1.05-3.81 | **0.035** |  | -0.32 | -0.55--0.08 | **0.008** |  | -0.23 | -0.37--0.10 | **0.001** |  | | 0.10 | 0.01-0.85 | **0.035** |
|  | **Age** | | 1.34 | 0.60-2.98 | 0.471 |  | -0.08 | -0.38-0.22 | 0.593 |  | -0.06 | -0.22-0.11 | 0.492 |  | | 0.85 | 0.14-5.04 | 0.859 |
|  | **Density** | | 0.89 | 0.45-1.73 | 0.728 |  | 0.29 | 0.04-0.53 | **0.022** |  | 0.14 | 0.00-0.28 | **0.046** |  | | 1.76 | 0.40-7.84 | 0.457 |
| ***pfcsp*** | |  |  |  |  |  |  |  |  |  |  |  |  |  | |  |  |  |
|  | **Period** | | 1.60 | 0.79-3.22 | 0.191 |  | 0.00 | -0.23-0.24 | 0.968 |  | -0.20 | -0.34--0.05 | **0.009** |  | | 0.28 | 0.08-0.90 | **0.032** |
|  | **Age** | | 2.51 | 1.11-5.69 | **0.028** |  | -0.30 | -0.60-0.00 | 0.053 |  | -0.10 | -0.29-0.08 | 0.274 |  | | 1.88 | 0.56-6.25 | 0.305 |
|  | **Density** | | 0.78 | 0.38-1.62 | 0.506 |  | 0.01 | -0.23-0.25 | 0.935 |  | 0.08 | -0.07-0.24 | 0.277 |  | | 1.55 | 0.50-4.80 | 0.450 |
| \* Logistic regression; \*\* Negative binomial regression; \*\* Linear regression | | | | | | | | | | | | | | |  |  |  |  |

**Table s9.** Genetic similarity in 2015 and 2017 using different metrics. p value obtained from a t Student’s test.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | ***pfama1*** | | | ***pfcsp*** | | |
|  | ***2015*** | ***2017*** | ***pa*** | ***2015*** | ***2017*** | ***pa*** |
| **Metric** | Mean (SD) | Mean (SD) | p | Mean (error) | Mean (error) | p |
| **Binary sharing** | 0.202 (0.034) | 0.241 (0.032) | 0.402 | 0.316 (0.045) | 0.360 (0.049) | 0.509 |
| **Jaccard distance** | 0.949 (0.006) | 0.901 (0.008) | <0.001 | 0.925 (0.010) | 0.908 (0.011) | 0.261 |
| **Pearson correlation** | 0.023 (0.008) | 0.074 (0.013) | 0.001 | 0.055 (0.013) | 0.060 (0.015) | 0.783 |
| a, t Student's test | |  |  |  |  |  |

**Table s10.** Distribution of *P. falciparum* isolates with transcripts detectable by RT-qPCR (proportion) and relative copy numbers (RCN) by study variable. GM: Geometric mean; SD: Standard deviation.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Proportion** | | | | |  | **RCN** | | | | |
|  | **2015** | | **2017** | |  |  | **2015** | | **2017** | |  |
|  | **n=50** | | **n=57** | |  |  | **n=50** | | **n=57** | |  |
|  | **n** | **%** | **n** | **%** | **p** |  | **GM** | **SD** | **GM** | **SD** | **p** |
| ***pfs25*** | 47 | *94* | 54 | *95* | 1.000 |  | 1.85 | *4.26* | 4.41 | *11.13* | 0.067 |
| ***pfs230*** | 26 | *52* | 40 | *70* | 0.073 |  | 0.21 | *0.42* | 0.39 | *0.84* | 0.109 |
| ***pfap2g*** | 32 | *64* | 48 | *84* | 0.025 |  | 1.12 | *1.84* | 2.58 | *4.64* | 0.014 |
| ***pfgexp02*** | 28 | *56* | 46 | *81* | 0.007 |  | 0.16 | *0.68* | 1.33 | *4.96* | 0.007 |
|  |  |  |  |  |  |  |  |  |  |  |  |
|  | **Age** | | | |  |  | **Age** | | | |  |
|  | **≤15 years** | | **>15 years** | |  |  | **≤15 years** | | **>15 years** | |
|  | **n=90** | | **n=17** | |  |  | **n=90** | | **n=17** | |
|  | **n** | **%** | **n** | **%** | **p** |  | **GM** | **SD** | **GM** | **SD** | **p** |
| ***pfs25*** | 84 | *93* | 17 | *100* | 0.587 |  | 2.57 | *6.56* | 5.94 | *10.34* | 0.198 |
| ***pfs230*** | 58 | *64* | 8 | *47* | 0.187 |  | 0.31 | *0.67* | 0.20 | *0.37* | 0.405 |
| ***pfap2g*** | 73 | *81* | 7 | *41* | 0.001 |  | 2.12 | *3.59* | 0.64 | *1.17* | 0.010 |
| ***pfgexp02*** | 66 | *73* | 8 | *47* | 0.045 |  | 0.66 | *2.67* | 0.10 | *0.44* | 0.081 |
|  |  |  |  |  |  |  |  |  |  |  |  |
|  | **Gender** | | | |  |  | **Gender** | | | |  |
|  | **Female** | | **Male** | |  |  | **Female** | | **Male** | |
|  | **n=51** | | **n=56** | |  |  | **n=51** | | **n=56** | |
|  | **n** | **%** | **n** | **%** | **p** |  | **GM** | **SD** | **GM** | **SD** | **p** |
| ***pfs25*** | 47 | *92* | 54 | *96* | 0.421 |  | 2.97 | *8.05* | 2.91 | *6.43* | 0.962 |
| ***pfs230*** | 28 | *55* | 38 | *68* | 0.232 |  | 0.20 | *0.44* | 0.41 | *0.81* | 0.075 |
| ***pfap2g*** | 35 | *69* | 45 | *80* | 0.186 |  | 1.14 | *2.08* | 2.57 | *4.22* | 0.017 |
| ***pfgexp02*** | 33 | *65* | 41 | *73* | 0.404 |  | 0.30 | *1.24* | 0.77 | *3.16* | 0.239 |
|  |  |  |  |  |  |  |  |  |  |  |  |
|  | **Density by qPCR** | | | |  |  | **Density by qPCR** | | | |  |
|  | **≤200 p/L** | | **>200 p/L** | |  |  | **≤200 p/L** | | **>200 p/L** | |
|  | **n=44** | | **n=63** | |  |  | **n=44** | | **n=63** | |
|  | **n** | **%** | **n** | **%** | **p** |  | **GM** | **SD** | **GM** | **SD** | **p** |
| ***pfs25*** | 43 | *98* | 58 | *92* | 0.397 |  | 5.42 | *10.58* | 1.92 | *5.13* | 0.030 |
| ***pfs230*** | 21 | *48* | 45 | *71* | 0.016 |  | 0.25 | *0.51* | 0.32 | *0.70* | 0.556 |
| ***pfap2g*** | 18 | *41* | 62 | *98* | <0.001 |  | 0.79 | *1.60* | 3.03 | *4.03* | <0.001 |
| ***pfgexp02*** | 18 | *41* | 56 | *89* | <0.001 |  | 0.05 | *0.23* | 2.34 | *7.39* | <0.001 |
|  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |
| *\* Took antimalarial in the last 30 days* | | | | |  |  |  |  |  |  |  |

**Table s11.** Univariate and multivariate regression analysis of *P. falciparum* isolates with transcripts detectable by RT-qPCR and relative copy numbers (RCN) by study variable.

The transcript variables (detection of transcripts in logistic models and RCN in linear models) are included as dependent variables in each of the regression models and period (baseline: 2017), age (baseline: ≤15), parasite densities (baseline: ≤200 parasites/µL) and gender (baseline: female). The coefficients express relative (in the proportion of monoclonal infections or the detection of rare haplotypes) and absolute differences (number of concurrent infections and Shannon index values) using baseline categories as reference. Multivariate models included period, parasite densities and age.

**A. Univariate**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | ***pfs25*** | | | | ***pfs230*** | | |  | ***pfap2g*** | | |  | ***pfgexp02*** | | |
|  |  | **Proportion** | | | | | | | | | | | | | | |
|  |  | **Coef** | **95%CI** | **p** |  | **Coef** | **95%CI** | **p** |  | **Coef** | **95%CI** | **p** |  | **Coef** | **95%CI** | **p** |
| **Period** | | 1.15 | 0.22-5.97 | 0.869 |  | 2.17 | 0.98-4.80 | 0.055 |  | 3 | 1.20-7.50 | **0.019** |  | 3.29 | 1.39-7.79 | **0.007** |
| **Age** | | NA | NA | 0.587 |  | 0.49 | 0.17-1.40 | 0.182 |  | 0.16 | 0.05-0.49 | **0.001** |  | 0.32 | 0.11-0.93 | **0.037** |
| **Gender** | | 2.3 | 0.40-13.12 | 0.349 |  | 1.73 | 0.79-3.81 | 0.170 |  | 1.87 | 0.77-4.53 | 0.166 |  | 1.49 | 0.65-3.40 | 0.342 |
| **Density** | | 0.27 | 0.03-2.39 | 0.239 |  | 2.74 | 1.22-6.13 | **0.014** |  | 89.6 | 11.36-706.23 | **<0.001** |  | 11.6 | 4.30-31.07 | **<0.001** |
|  |  | **Relative copy numbers (RCN)** | | | | | | | | | | | | | | |
| **Period** | | 2.38 | 0.95-5.98 | 0.067 |  | 1.92 | 0.87-4.25 | 0.109 |  | 2.31 | 1.20-4.45 | **0.014** |  | 8.49 | 1.86-38.89 | **0.007** |
| **Age** | | 2.31 | 0.65-8.18 | 0.198 |  | 0.63 | 0.21-1.87 | 0.405 |  | 0.3 | 0.12-0.73 | **0.010** |  | 0.15 | 0.02-1.24 | 0.081 |
| **Gender** | | 0.98 | 0.38-2.49 | 0.962 |  | 2.07 | 0.94-4.55 | 0.075 |  | 2.25 | 1.17-4.34 | **0.017** |  | 2.57 | 0.54-12.28 | 0.239 |
| **Density** | | 0.35 | 0.14-0.89 | **0.030** |  | 1.28 | 0.57-2.88 | 0.556 |  | 3.82 | 2.03-7.21 | **<0.001** |  | 44.6 | 10.75-185.06 | **<0.001** |
| ND, Not determined | | | | | | | | | | | | | | | | |

**B. Multivariate**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | ***pfs25*** | | | | ***pfs230*** | | |  | ***pfap2g*** | | |  | ***pfgexp02*** | | |
|  |  |  | **Coef** | **95%CI** | **p** |  | **Coef** | **95%CI** | **p** |  | **Coef** | **95%CI** | **p** |  | **Coef** | **95%CI** | **p** |
|  |  | **Proportion** | | | | | | | | | | | | | | | |
| **Period** | |  | 1.16 | 0.22-6.13 | 0.862 |  | 2.08 | 0.92-4.72 | 0.079 |  | 3.92 | 1.10-13.94 | **0.035** |  | 3.77 | 1.35-10.49 | **0.011** |
| **Age** | |  | NA |  |  |  | 0.64 | 0.21-2.00 | 0.444 |  | 0.25 | 0.05-1.29 | 0.099 |  | 0.60 | 0.15-2.31 | 0.454 |
| **Density** | |  | 0.34 | 0.04-3.06 | 0.337 |  | 2.42 | 1.04-5.64 | **0.040** |  | 82.58 | 10.04-679.51 | **<0.001** |  | 11.33 | 3.89-33.06 | **<0.001** |
|  | | **Relative copy numbers (RCN)** | | | | | | | | | | | | | | | |
| **Period** | |  | 2.62 | 1.05-6.55 | **0.039** |  | 1.90 | 0.85-4.29 | 0.118 |  | 2.08 | 1.12-3.86 | **0.020** |  | 6.16 | 1.53-24.80 | **0.011** |
| **Age** | |  | 1.61 | 0.44-5.84 | 0.466 |  | 0.65 | 0.21-2.04 | 0.460 |  | 0.45 | 0.19-1.06 | 0.068 |  | 0.51 | 0.07-3.63 | 0.498 |
| **Density** | |  | 0.35 | 0.14-0.93 | **0.035** |  | 1.11 | 0.47-2.60 | 0.813 |  | 3.05 | 1.60-5.83 | **<0.001** |  | 33.01 | 7.64-142.54 | **<0.001** |

### **References**

1. Mayor A, Serra-Casas E, Bardaji A, et al. Sub-microscopic infections and long-term recrudescence of Plasmodium falciparum in Mozambican pregnant women. *Malar J* 2009; **8**: 9.

2. Mayor A, Bardaji A, Macete E, et al. Changing Trends in P. falciparum Burden, Immunity, and Disease in Pregnancy. *N Engl J Med* 2015; **373**(17): 1607-17.

3. Taylor SM, Mayor A, Mombo-Ngoma G, et al. A quality control program within a clinical trial Consortium for PCR protocols to detect Plasmodium species. *Journal of clinical microbiology* 2014; **52**(6): 2144-9.

4. Mayor A, Hafiz A, Bassat Q, et al. Association of severe malaria outcomes with platelet-mediated clumping and adhesion to a novel host receptor. *PLoS One* 2011; **6**(4): e19422.

5. Aguilar R, Magallon-Tejada A, Achtman AH, et al. Molecular evidence for the localization of Plasmodium falciparum immature gametocytes in bone marrow. *Blood* 2014; **123**(7): 959-66.

6. Schneider P, Reece SE, van Schaijk BC, et al. Quantification of female and male Plasmodium falciparum gametocytes by reverse transcriptase quantitative PCR. *Mol Biochem Parasitol* 2015; **199**(1-2): 29-33.

7. Portugaliza HP, Llora-Batlle O, Rosanas-Urgell A, Cortes A. Reporter lines based on the gexp02 promoter enable early quantification of sexual conversion rates in the malaria parasite Plasmodium falciparum. *Sci Rep* 2019; **9**(1): 14595.

8. Gupta H, Galatas B, Matambisso G, et al. Differential expression of var subgroups and PfSir2a genes in afebrile Plasmodium falciparum malaria: a matched case-control study. *Malar J* 2019; **18**(1): 326.