**Plasmodium falciparum** sexual differentiation in malaria patients is associated with host factors and GDV1-dependent genes

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**Plasmodium** sexual differentiation is required for malaria transmission, yet much remains unknown about its regulation. Here, we quantify early gametocyte-committed ring (gc-ring) stage, *P. falciparum* parasites in 260 uncomplicated malaria patient blood samples 10 days before maturation to transmissible stage V gametocytes using a gametocyte conversion assay (GCA). Seventy six percent of the samples have gc-rings, but the ratio of gametocyte to asexual-committed rings (GCR) varies widely (0–78%). GCR correlates positively with parasitemia and is negatively influenced by fever, not hematocrit, age or leukocyte counts. Higher expression levels of GDV1-dependent genes, ap2-g, msrp1 and gexp5, as well as a gdv1 allele encoding H217 are associated with high GCR, while high plasma lysophosphatidylcholine levels are associated with low GCR in the second study year. The results provide a view of sexual differentiation in the field and suggest key regulatory roles for clinical factors and *gdv1* in gametocytogenesis in vivo.
Controlling malaria transmission is key to global malaria eradication efforts and continues to be highlighted as a critical research area by the recent Malaria Eradication (MaelERA) refresh panel. Human to human malaria transmission via a mosquito requires the production of sexual stage parasites, called gametocytes, which are produced during the erythrocytic cycle. After erythrocyte invasion by a merozoite, the parasite replicates producing new merozoites that will either continue asexual multiplication or develop into gametocytes during the next erythrocytic cycle. The regulation of this balance between asexual propagation and sexual differentiation is key to understanding the dynamics of malaria transmission and pathology in human hosts. After a *P. falciparum* gametocyte-committed merozoite invades an erythrocyte, it differentiates over ~10–12 days from a ring stage parasite through 5 distinct morphologic stages (I–V) into a single mature male or female gametocyte. For malaria transmission a male and a female gametocyte must be taken up in a blood meal by a mosquito, fertilize and begin differentiation into sporozoites that can be spread to another human host.

In the human host, immature stage I–IV *P. falciparum* gametocytes sequester and cannot be detected in peripheral blood samples, making it difficult to monitor sexual differentiation in the field until mature, transmissible stage V gametocytes are released into the circulation 8–10 days later. This delay in the ability to monitor early gametocyte formation makes it challenging to define the factors that influence gametocytogenesis in patients before they become infectious. Studies of stage V gametocyte prevalence in the field have clearly demonstrated wide variability between individuals, but it is hard to know if this is due to gametocyte production or survival. An association between stage V gametocyte levels and low hematoctrit has been reported, but whether these factors are causal is difficult to determine.

In culture, both asexual and sexual differentiation are intrinsic parts of the life cycle with each successive erythrocytic cycle producing a mixture of schizonts with merozoites destined for either gametocyte production or asexual replication suggesting an underlying stochastic or constitutive control mechanism. However, differences in sexual differentiation rates between *P. falciparum* strains can range from 0 to 20% suggesting parasite genetics play a role and variation within strains also indicates modulation by host factors such as lypo-phosphatidylincholine (LysPC) levels. Again, the underlying regulatory mechanisms remain unknown. Two genes have been reported to be required for *P. falciparum* gametocyte production in vitro, *gametocyte development protein 1* (*gdv1*, PF3D7_0935400) and the *apetela-2* transcription factor (*ap2-g*, PF3D7_1222600). The activity of *gdv1* was originally demonstrated by genetic complementation of a gametocyte-deficient line (Gadelt) that had a spontaneous deletion in the *gdv1* locus on chromosome 9. *Gdv1* deletions are frequently associated with the loss of gametocyte production in lab strains, while overexpression increases gametocyte production. Field populations also exhibit high *gdv1* allelic divergence that has been suggested to vary with transmission patterns. Transcription factor *ap2-g* was also identified as the target of a mutation in a gametocyte-deficient parasite line in both *P. falciparum* and *P. berghei*. Notably, AP2-G protein has been shown to enhance its own transcription as well as to be epigenetically repressed by heterochromatin protein 1 (HP1, PF3D7_1220900) in schizonts producing asexually committed merozoites. Recently, episomal expression of GDV1-tagged with green fluorescent protein (GFP) and a ligand stabilized-degradation domain (DD) in a parasite line with an intact *gdv1* locus has been shown to increase gametocyte production. The GFP-DD-tagged GDV1 also associated with HP1 and localized to heterochromatin sites throughout the genome, including the *ap2-g* locus suggesting that GDV1 and HP1 interact to regulate gametocyte production.

In the work reported here, we developed a gametocyte conversion assay (GCA) to quantify gametocyte-committed ring stage parasites in human blood samples, which allows direct comparison of sexual differentiation rates with clinical characteristics and gene expression levels. Gametocytes were detected in 76% of the samples from uncomplicated malaria patients and there was a wide range of asexual- to gametocyte-committed ring ratios (GCR). GCR was positively associated with parasitemia and negatively influenced by temperature. RNA levels of *ap2-g*, *AP2-G*-dependent gene, *msrp1* (PF3D7_1335000) and *AP2-G*-independent gametocyte-specific gene, *gexp5* (PF3D7_0936600) were also higher in high GCR samples. To more carefully analyze the regulation of gametocytogenesis, we generated a parasite line that allowed tight control of gametocyte production by tagging the endogenous *gdv1* gene with GFP and a ligand-dependent degradation domain (*Pfgdv1.gfp.dd*). In the absence of Shield 1 ligand (*Shld1, Pfgdv1.gfp.dd* gametocyte production was markedly decreased. Using this clonal *Pfgdv1.gfp.dd* line to perform GDV1 protein dosing experiments, we demonstrate that GDV1 acts as a ‘molecular rheostat’ during schizogony to control gametocyte production and gene expression. Together the data suggest that in humans there is a low basal level of GCR that is modulated by an interplay of parasitism, body temperature, and *P. falciparum* *gdv1*.

**Results**

**Gametocyte-committed ring quantification in patient samples.** To directly assess gc-rings in patient blood samples, we developed an ex vivo GCA to screen blood from uncomplicated malaria patients (0.5–13 years old) presenting at Ewing Health Center in Cape Coast, Ghana during two malaria seasons, July–August 2016 and 2017 (Fig. 1 and Supplementary Table 1). Briefly, one aliquot of a subject’s blood sample is used to quantify white blood cells (WBC) and hematocrit, another is used for RNA and DNA isolation, while the remaining material is cultured ex vivo in the presence of n-acetyl glucosamine (NAG) to block further asexual replication. The cultures are followed by Giemsa-stained smears over the next 8 days for gametocyte development. Only 1 sample in 2016 and none in 2017 had microscopically detectable stage V gametocytes on a thick blood smear made on Day 0 and by D4 stage V gametocytes were not detected in the ex vivo smear. Schizonts are also not observed in the NAG-treated ex vivo cultures indicating NAG had successfully blocked asexual development. Morphologically distinct stage II gametocytes are occasionally observed on D3 of ex vivo culture and became the predominant stage by D4. The parasites continue to progress to a mixture of III–V on D8, which is similar to the in vitro time course of standard lab-adapted cultures (Fig. 1b). Most of the gametocytes mature from stage II to III–V with a median difference between the D4 stage II–III and the D8 stage III–V gametocytemia of 0 (interquartile range –0.02 to 0.008, Fig. 1d), which is comparably to that seen in standard in vitro culture. GCR is calculated by dividing the D4 stage II and III gametocytemia or the D8 stage III–V gametocytemia by the total ring stage parasitemia on Day 0 (D0P).

As an internal check of the GCR calculations we compared the D4 and D8 gametocyte conversion rates for each sample and found they are significantly positively correlated both by Pearson and regression analysis (R² = 0.73, p < 0.00001) providing confidence in the counting method used to identify gametocytes (Fig. 1e). The GCR distribution for both collection years is also similar with a total of 74% and 79% of the ex vivo samples in 2016.
and 2017, respectively, containing detectable gametocytes. While 20% of the samples both years have GCR above 4%, the median GCR is only 0.6% in 2016 and 0.8% in 2017 consistent with high gametocyte formation in only a small number of individuals (Fig. 1c).

The D0 blood samples are also assessed for the presence of mature gametocytes using RT-qPCR to determine Pf25 transcript levels. As with the GCR a majority of the population have low levels of circulating stage V gametocytes and there is a large range of transcript levels. As with the GCR a bivariate association longitudinally in asymptomatic patients that do not have to be treated.

**Host/parasite factors modulating in vivo gametocytogenesis.** The relationship between patient age, temperature, parasitemia, WBCs levels, and hematocrit on D0 and gametocyte production and conversion on D4 or D8 is evaluated first by Pearson correlation (Fig. 2a) to identify significant bivariate associations. Before analysis gametocytemia, GCR and D0P are log transformed to normalize the distribution as assessed by histogram and normal quantile plots36. As previously reported37, D0 parasitemia is positively correlated with temperature and WBCs levels and negatively correlated with age. Age is also negatively correlated with temperature and WBCs and positively correlated with hematocrit. This pattern is consistent with the age dependent development of partial immunity that reduces parasite growth resulting in lower D0P and a decrease in associated symptoms,
including elevated temperature and WBC counts and decreased hematocrit. An age dependent change in the pyrogenic threshold has previously been reported for malaria \(^{38,39}\) and could contribute to the negative correlation between age and temperature. Although stage V gametocyte carriage has been negatively associated with the negative correlation between age and temperature. 

The D0P and D4 and D8 GC and GCR were normalized by natural log transformation (ln) prior to statistical analysis. The Pearson correlation coefficient is shown and probability is indicated by \(p \leq 0.05\) (*), \(p \leq 0.01\) (**), \(p \leq 0.001\) (***)). **Fig. 2** Summary of the statistical tests used to evaluate the association of D4 and D8 GCR with clinical parameters. The complete table for the bivariate correlation is shown in **a**, the multiple linear regression is in **Supplementary Table 2** and the interaction model is in **Supplementary Table 3**. The coefficients are shown with asterisks indicating \(p\) values as described for **a, c, d**. Graphical representation of the negative influence of temperature on the positive relationship between the D0P and GCR. The natural log of the D4 (\(n = 260\)) or D8 (\(n = 238\)) GCR are plotted against the natural log of the D0 parasitemia (D0P). Data and trend lines from subjects with temperatures \(< 38.5\) °C (the median D0 temperature) are shown in red dots and \(\geq 38.5\) °C are shown in black crosses. 

To further investigate the relationship between GCR and D0P and D0 temperature, we tested for an interaction between natural log normalized D0P and D0 temperatures by regression analysis using a continuous temperature model (Supplementary Table 3).
The results indicate a significant negative interaction between D0P and temperature on D4 as well as D8 GCR (D4GCR $p = 0.016$ and D8GCR $p = 0.002$) and also D4 and D8 gametocytemia (D4G $p = 0.004$ and D8G $p = 0.009$). The statistical analysis is summarized in Fig. 2b and the complete analysis is shown in Supplementary data (Supplementary Tables 2 & 3). The final model indicates that there is a positive correlation between D0P and GCR for patients with lower temperatures, but this association decreases as temperature increases. The negative influence of temperature on the association between D0P and GCR is demonstrated graphically by comparing the slopes of the trend lines for the lnD0P versus lnGCR plots for patients with temperatures < or ≥ the median temperature of 38.5 °C (Fig. 2c and d).

Metabolic analysis of host plasma. Both asexual parasitemia and temperature have been reported to be associated with serum lysocephatidylcholine (LysoPC) levels in malaria patients and LysoPC levels have recently been implicated in gametocytogenesis in vitro. To focus the initial analysis, we first compared individuals with high and low and undetectable GCR. Forty plasma samples (Supplementary Table 4) with a range of D0P (>35%), 20 (10 from each year) that have consistently high GCR (D4 GCR > 4.9%) and 20 (10 from each year) with low to undetectable GCR were selected for metabolomic analysis using the Biocrates AbsoluteIDQ® p180 kit and liquid chromatography tandem mass spectroscopy (LC-MS/MS). On first analysis there is no significant difference between the mean of the 13 LysoPC isomers in the high and low GCR samples. However, there is a significant difference between the mean total LysoPC isoforms levels in 2016 and 2017 (Mann–Whitney test, $p = 0.0035$) although the mean levels in either year are not different from uninfected control plasma and no other clinical parameters, including parasitemia, WBC, Age, Hb levels or temperature differed between uninfected control plasma and no other clinical parameters, including parasitemia, WBC, Age, Hb levels or temperature differed between the 2016 and 2017 patients in the cohorts (Supplementary Fig. 2a and b). Interestingly, in the 2017 cohort there is also a significant difference between the total LysoPC levels in the high and low GCR cohorts that is not observed in the 2016 cohort (Fig. 3a, Supplementary Fig. 2A, C&D). Notably, 7 of the 8 samples with total LysoPC levels above a threshold of 170 µM had no detectable gametocyte-committed rings. Receiver operating characteristic analysis of LysoPC levels and D4 GCR in the 2017 high and low GCR cohorts has an area under the curve of 0.780 ($p = 0.034$) suggestive of good overall accuracy (Fig. 3b). An optimal level of LysoPC at 170 µM had a sensitivity of 90% and a predictive false positive rate of 30% for predicting low D4 GCR. When the metabolomics analysis is extended to the remaining 153 metabolites detected in the Biocrates AbsoluteIDQ® p180 analysis no significant differences (FDR < 0.05) are detected between the average concentration of the metabolites in the high and low GCR samples in 2016, 2017 or in the combined data set. When the mean concentrations of the metabolites in the 2017 high and low cohorts are plotted against each other the slope of the line is 1.098 with an R$^2$ coefficient of 0.994 (Fig. 3c) indicating overall similarity in the two cohorts. Notably, none of the 153 metabolites falls significantly above or below the trend line indicating similar concentrations in both cohorts.

Early gametocyte gene expression is associated with high GCR. We then sought to evaluate potential in vivo molecular markers for early P. falciparum gametocytes in field samples using the same 40 samples used in the metabolomics study that included 20 with high and 20 with low GCR (Supplementary Table 4). In addition to gavl and ap2-g, we selected one ap2-g-dependent gene, msrp1, and one ap2-g-independent early gametocyte gene, gexp5 to evaluate in the D0 blood samples using 18s rRNA as well as a ring stage-specific gene, skeleton-binding protein (shb1, PF3D7_0501300 [https://plasmodb.org/plasmo/app/record/gene/PF3D7_0501300]) as constitutive controls for parasitemia (Fig. 4a & b). Ring-stage PfPgdv1.gfp.dd parasites grown in the absence of Shld1 to inhibit gametocyte production are used as the reference group to calculate relative abundance for the high and low GCR samples. Ap2-g, msrp1, gexp5 transcript levels are all significantly higher in the high GCR samples than the low GCR samples using either 18s rRNA or Shb1 as the control, while there is no difference in gavl RNA levels in the two groups. Pf525 transcript levels are also tested to evaluate stage V gametocyte levels in the high and low GCR samples and found not to differ (Fig. 4c). This work is the first demonstration that the in vivo expression levels of these gene correlate with ex vivo sexual differentiation, providing support for their use as biomarkers in the field and indicating that the changes in gene expression underlying gametocytogenesis are similar in vivo and in vitro.

Inducible regulation of gametocyte production. We next wanted to extend our analysis to the regulation of these genes during the transition from schizogony to gc-rings. However, due to sequestration of mature intraerythrocytic asexual parasites, schizonts are not accessible in human peripheral blood and therefore cannot be studied directly in patient samples. Given the similar
upregulation of these genes in gc-rings in vivo and in vitro, we decided to begin to study this in vitro using a transgenic parasite line (Pfgdv1.gfp.dd) that allowed inducible gametocyte production. In this parasite line the endogenous gdv1 gene in strain NF54 parasites is tagged with green fluorescent protein (gfp) followed by a Shld1 ligand-dependent FKBP-derived destabilization domain allowing GDV1 protein-dosing experiments by varying Shld1 concentrations. Two independently transformed lines (Pfgdv1.gfp.dd, T1 and T2) were tested for chromosomal integration (Supplementary Fig. 3A & B), GFP expression (Fig. 5a), and gdv1.gfp.dd transcript (Supplementary Fig. 3C) as well as gametocyte production in the presence and absence of Shld1 (Fig. 5c). As anticipated, gdv1.gfp.dd transcript levels are insensitive to Shld1, while Shld1 is required for GFP expression confirming successful integration into the gdv1 locus and Shld1-dependent GDV1.GFP-DD expression. The perinuclear pattern of GFP expression in schizonts is the same as that observed previously in parasites transformed with a plasmid directing episcopal expression of GFP-or HA epitope-tagged GDV124 demonstrating that protein localization is not affected by the addition of the DD at the C-terminus. GFP expression is first observed as DNA replication began, ~36 h post invasion, which is consistent with peak gdv1 transcript levels (www.Plasmodb.org42)43 (Supplementary Fig. 4). The GFP signal is observed by microscopy in all schizonts and remained high for 8 h before dissipating during merozoite segmentation and GFP expression is not observed in the newly formed ring stage parasites. Schizogony is monitored by flow cytometry using DNA stain SYTO 59, but the GDV1.GFP signal is not strong enough to be detected above the background green autofluorescence that also increased during schizogony. As expected Shld1 stabilization of GDV1.GFP-DD is also required for wild type levels of gametocyte production in both Pfgdv1.gfp.dd clonal lines (Fig. 5b & c). In the absence of Shld1 only an occasional gametocyte is observed, while exposure to Shld1 for only the last 12 h of the asexual cycle (36–48 h post RBC invasion) is sufficient to generate merozoites that are fully competent to invade red blood cells (RBCs) and differentiate over the next 10–12 days through the 5 morphologically distinct stages of gametocyte development (I–V) (Fig. 6).

GDV1 regulates ap2-g as well as ap2-g-independent genes. The ability to control gametocyte production in a clonal parasite line using Shld1 allowed direct analysis of the initial stages of sexual differentiation without contaminating subpopulations of older gametocytes present in wild type P. falciparum cultures. The expression of a range of stage-specific and constitutive transcripts were compared in MACS/sorbitol synchronized cultures that were grown in the presence or absence of Shld1 (Fig. 7 and Supplementary Fig. 5). RNA obtained during schizogony (36 ± 2 h post-invasion [hpi]) and 24 h later, during the subsequent ring stage, was tested for gametocyte-associated transcripts, ap2-g, msrp1, gexp5, Pfs16 (PF3D7_0406200)24,44 Pfge3 (PF3D7_1477700)45 and ap2-g3 (PF3D7_1317200)46,47, which was reported in a piggyBac screen for gametocytogenesis related genes in P. falciparum and has recently been shown to be upstream of ap2-g expression in the rodent malaria P. yoelii. In addition, 18s rRNA (PF3D7_0725600) and two asexual ring specific genes, kahrp (PF3D7_0202000)14 and sbp148 were tested as well as hpl-specific primers and conserved primers that amplify multiple var31 genes which are regulated by hpl (Supplementary Table 5).

In schizonts from Shld1-treated vs untreated Pfgdv1.gfp.dd.T1 cultures we found a significant increase only in mRNA corresponding to ap2-g, and msrp1 (Fig. 7b & Supplementary Fig. 5a), which is not observed in parental NF54 parasites (Fig. 7d and Supplementary Fig. 5b). This Shld1-dependent increase is maintained in RNA harvested 24 h later from the ring stage Pfgdv1.gfp.dd.T1 parasites (Fig. 7c and Supplementary Fig. 5a), while there continued to be no Shld1-dependent difference in NF54 parasites (Fig. 7e and Supplementary Fig. 5b). Transcripts for gexp5 as well as an additional early gametocyte gene, Pfs16, are also found to be significantly up-regulated in ring
stage parasites from the Shld1-treated Pfgdv1.gfp.dd.T1 group. At this early time point Pfg3 RNA levels are not significantly elevated. As expected there is no Shld1-dependent change in transcript levels for 18s rRNA, kahrp, or sbp1, which have not been associated with early gametocytes, but are included as controls for parasite stage. Additionally, RNA levels for gdl1, and var genes are not affected by Shld1 treatment, indicating that ligand-mediated stabilization of GDV1 is not required for expression of these genes. Ap2-g3 and hp1 transcript levels are also not affected by Shld1 treatment during schizogony, but in the subsequent ring stage RNA levels are slightly lower for hp1 and slightly higher for ap2-g3.

The expression of ap2-g, msrp1, hp1 and ap-g3 in Pfgdv1.gfp.dd. T1 parasites was further investigated by isolating RNA from schizonts when gdl1 transcript levels are increasing (~1–3 nuclei, 36 ± 2 hpi) and then again 10 h later during late schizogony when ap2-g3 levels increase and during the subsequent ring stage another 14 h later. Consistent with the previous experiment, in the presence of Shld1 transcript levels for ap2-g and msrp1 levels increase while in rings hp1 transcripts decrease and ap2-g3 transcripts increase slightly (Fig. 8a–d). Notably, even in the absence of Shld1 and in a gametocyte-deficient line (3D7Gdef) that lacks the gdl1 locus24 ap2-g and msrp1 transcript levels increase significantly during schizogony suggesting that GDV1 is not required to initiate transcription (Fig. 8a–b).

Next we performed a GDV1 protein-dosing experiment by varying Shld1 concentrations (0–1500 nM) to evaluate the role of GDV1 protein levels on ap2-g, msrp1 and gexp5 expression levels. An anti-GFP monoclonal antibody (mAb) is used to assess GDV1 levels in Pfgdv1.gfp.dd schizonts following 14 h treatment with different Shld1 concentrations and demonstrates a dose-dependent increase (Fig. 9a & b). GDV1.GFP.DD migrates as two bands and can only be extracted by adding 8 M Urea and 5% SDS, indicating it tightly associates with an insoluble complex, possibly the nuclear envelope. The estimated molecular weight of the anti-GFP positive upper bands from 5 independent experiments is 113 ± 2.8 kDa (mean ± s.d.), which is consistent with the predicted molecular weight of the full length chimeric protein (111.8 kDa) suggesting the lower band (100 ± 1.7 kDa) is processed, but this needs to be further tested.

To test for a dose-dependent effect of Shld1 on transcript levels, RNA was collected from late schizonts (46 ± 2 hpi) and 14 h later from the resulting ring stage parasites. Transcripts for ap2-g, msrp1 and gexp5 genes, as well as gametocyte production (Fig. 9c–e & Supplementary Fig. 6) increase in a dose dependent manner with a plateau in RNA levels at the concentration of Shld1 normally used to stabilize GDV1 in culture (500 nM). The similar Shld1 dose response curve for gametocyte production and RNA levels suggests that these transcripts might be potential markers for early gametocytes. We test this correlation directly using the data obtained over the course of our various different Shld1 experiments (Fig. 9f–h). The $R^2$ values for correlation between gametocyte conversion and the RNA levels of each of the 3 genes and are > 0.44 in ring stage parasites. In particular, ap2-g levels in ring stage parasites and gametocyte conversion have an $R^2$ of 0.92. In total, these data suggest the capacity of GDV1 protein levels to modulate gametocyte formation and transcription levels of downstream genes.

Gdv1 allele frequency in high and low GCR samples. The ability of GDV1 to act as a rheostat for gametocyte production coupled

**Fig. 5** Shld1-dependent GDV1-GFP expression and gametocyte production in Pfgdv1.gfp.dd. parasites. a Representative fluorescence microscopy images of unixed Pfgdv1.gfp.dd.T1 and T2 parasites cultured in the presence (On) or absence (Off) of 500 nM Shld1. Pfgdv1.gfp.dd parasites were re-suspended in PBS containing bisbenzimide (5 µg/ml) and visualized using a Zeiss Axiovert 200 fluorescence microscope at ×1000 magnification using AxioVision v4.3.0 101 software. b, c Shld1-dependent Pfgdv1.gfp.dd gametocyte production. Parental NF54 (wt) and Pfgdv1.gfp.dd clones T1 and T2 were set up at 1% parasitemia and maintained in the presence (+) or absence (−) of 500 nM Shld1 for 12 days. NAG (50 mM) was included in the media days 5–12. Parasitemia as determined using Giemsa-stained smears made on day 5 a and day 12 b. The mean ± s.e.m. of three independent experiments is plotted.
with prior field work that identified a SNP in gdv1 (Chr9:1378602) whose population frequency varied with transmission intensity prompted us to evaluate this SNP in patient samples in the high and low GCR cohorts that were used for metabolic analysis (Supplementary Table 4). Dried blood spots for DNA analysis were available for all twenty 2017 samples and all but one of the twenty 2016 samples. The high and low GCR samples have significantly different gdv1 allele distributions (Fisher exact test, $p = 0.022$) (Fig. 10), while there is no difference in msp2 allele frequencies in the two cohorts (Fig. 10). The frequency of the H217 GDV1 allele previously associated with continual malaria transmission in the Republic of Guinea is higher in the low GCR cohort (n = 19, 76%), while the frequency of the P217 allele, GDV1-dependent gene expression and, possibly, LysoPC levels. The GDV1-dependent genes are also strong candidates for sensitive molecular markers that could be used to identify symptomatic and asymptomatic gc-ring carriers to ensure treatment before the production of circulating transmissible stage V gametocytes.

The GCA assay developed in this work is a valuable first step toward evaluating gametocytogenesis in the field. The strong concordance of the GCR values calculated in the 2016 and 2017 malaria seasons as well as on days 4 and 8 demonstrate the reproducibility of the gametocyte counts and the ability of the stage II/III gametocytes detected on D4 to continue to mature to D8. The results are consistent with the circulation of gc-rings prior to sequestration in the bone marrow. Although we cannot rule out a subpopulation of sequestered gc-rings that never circulate, the circulating gc-rings provide a window to assess gametocytogenesis in blood samples within 24 h of the production of gametocyte-committed merozoites. This early detection of gc-rings allows much closer association with host and clinical parameters that may contribute to the balance generating capacity 10 days before the circulation of transmissible stage V gametocytes. The results indicate that gc-rings circulate in most patient’s peripheral blood, however the levels vary dramatically, from 78% to no detectable sexually committed rings. This pattern is consistent with a low basal level of sexual differentiation that is modulated by D0 parasitemia, patient temperature, gdv1 allele, GDV1-dependent gene expression and, possibly, LysoPC levels. The GDV1-dependent genes are also strong candidates for sensitive molecular markers that could be used to identify symptomatic and asymptomatic gc-ring carriers to ensure treatment before the production of circulating transmissible stage V gametocytes.

Discussion

Here we report the first direct comparison of the presence of gametocyte-committed ring stage parasites in human blood samples with clinical parameters and parasite transcript levels. The GCA allowed detection and quantification of gametocyte generating capacity 10 days before the circulation of transmissible stage V gametocytes. The results indicate that gc-rings circulate in most patient’s peripheral blood, however the levels vary dramatically, from 78% to no detectable sexually committed rings. This pattern is consistent with a low basal level of sexual differentiation that is modulated by D0 parasitemia, patient temperature, gdv1 allele, GDV1-dependent gene expression and, possibly, LysoPC levels. The GDV1-dependent genes are also strong candidates for sensitive molecular markers that could be used to identify symptomatic and asymptomatic gc-ring carriers to ensure treatment before the production of circulating transmissible stage V gametocytes.
between asexual and sexual differentiation. The further development of sensitive molecular gc-ring markers coupled with the existing stage V gametocyte markers (Pfs25 and mget, P3D7_146990)50, should make it possible to track gametocyte maturation over the next 10–12 day in asymptomatic parasite carriers. Such longitudinal analysis was not possible in this study of symptomatic malaria patients because they had to be treated. At enrollment most of the study population had RT-qPCR detectable circulating stage V gametocytes as well as gc-rings and there was a wide range of levels for both stages. However, there was no significant correlation between the number of stage V gametocytes in a D0 sample and its GCR suggesting that 10–12 days earlier their GCR was different. As mentioned above the only way to directly study the relationship between GCR and stage V gametocyte production is a longitudinal study monitoring asymptomatic individuals for the progression of gc-rings to stage V gametocytes two weeks later. We anticipate that young children at the beginning of the malaria season before the development of immunity would have a direct correlation between gc-ring markers/GCR, and stage V gametocytes 2 weeks later, which would provide in vivo validation for the GCR assay and gc-ring markers.

The positive association we found between D0P and GCR is consistent with previous in vitro data demonstrating that sexual differentiation increases with high parasitemia51,52, but attempts to identity a consistent density dependent trigger have been challenging53. Recently, LysoPC-depleted culture media has been shown to increase conversion rate in vitro54, but was not examined in humans. In the 40 samples we tested, the total plasma LysoPC levels ranged between (40–275 μM) and varied significantly between collection years. In 2017, high LysoPC levels were associated with low GCR, which is consistent with high plasma LysoPC having a negative influence on gametocyte production. The reason for the difference in LysoPC levels in the 2016 and 2017 plasma samples is unclear. As there was no significant difference in the other clinical parameters between the two years, it is possible that different plasma storage conditions in 2016 and 2017 could have been responsible. However, LysoPC levels have also been shown to be influenced by body weight55,56 and infection; decreasing during severe malaria541, African trypanosomiasis55, and sepsis56 so alternative explanations cannot be ruled out completely.

In contrast to low D0 parasitemia51 and high LysoPC levels54, high temperatures have not previously been specifically
Shld1 minus ring stage expression levels were used for For the 3D7.Gdef line, a one-way ANOVA with Dunnett compare expression between stages and conditions in the analyses a Bonferroni post-hoc test to correct for the analysis of 4 genes was used and probability is indicated, chromosome 9 deletion24. Expression levels in early schizonts in the absence of Shld1 are used as the reference for been shown to decrease parasite growth and viability58,59. The inverse relationship between D0P and temperature also ses39 and this may contribute to the relative increase in mature associated with a decrease in sexual differentiation57, but have been shown to decrease parasite growth and viability58,59. The negative influence of high D0 temperature on the positive relationship between D0 parasitemia and GCR was consistently observed on D4 and D8, but the effect was more pronounced on D4. This difference in the influence of temperature could be due to a temperature-induced delay in growth decreasing the number of gametocytes that reached stage II by D4. Stage II is the first morphologically distinct gametocyte stage so a delay would reduce the D4 gametocyte count, but could have less effect on the D8 counts which included stages III–V. An inverse relationship of D0 parasitemia and fever on GCR suggests that for a given parasitemia gametocyte production would be higher in asymptomatic individuals, which is consistent with recent work showing a positive correlation between parasite density and stage V gametocytes in asymptomatic infections50 and a negative correlation in a meta-analysis of symptomatic cases40. This inverse relationship between D0P and temperature also suggests that sexual differentiation would increase as an individual’s pyrogenic threshold to Plasmodium infection increases39 and this may contribute to the relative increase in mature gametocytes to asexual parasites that has been observed with age16.

In addition to the negative effect of temperature on sexual differentiation, another unexpected finding was the lack of correlation between high GCR and low hematocrit. Low hematocrit has previously been reported to be associated with an increase in circulating stage V gametocytes in patient blood smears15,17,18, The lack of correlation between GCR and hematocrit suggests that low hematocrit is not the major independent driver of sexual differentiation in this study. It is possible that the low hematocrit previously associated with circulating stage V gametocytes could have been due to the prolonged infection required for enough gametocytes to mature 10 days and be released into the circulation as stage Vs for detection by Giemsa-stained blood smear. Such differences between the factors associated with gc-rings and circulating stage V gametocytes highlight the need to evaluate sexual differentiation longitudinally at 2 week intervals in asymptomatic individuals to assess both gc-rings and the resulting stage V gametocytes that circulate 2 weeks later.

Sexual differentiation in vivo was also associated with an increase in RNA levels of three genes shown to be dependent on GDV1 protein levels in vitro, as well as the H217 GDV1 allele previously reported to be over represented in a region with limited seasonal malaria transmission29. Together, this data is consistent with GDV1 playing a key role in gametocytogenesis in vivo and the H217 GDV1 allele inducing sexual differentiation more effectively than the P217 allele in the field. In contrast to histidine, proline restricts the flexibility of the peptide chain and could alter protein structure potentially affecting function60, but this needs to be further evaluated by more detailed genetic studies. A recent field study extending the analysis of the gdv1 locus to additional West Africa locations found a structural dimorphism downstream from the gdv1 coding region that also demonstrated strong geographical divergence, but was not clearly linked to transmission intensity61. In contrast, an association between

Fig. 8 Gene expression patterns in the absence of GDV1. The fold change (2−ΔΔCt) of ap2-g a, msrp1 b, hp1 c, and ap2-g3 d RNA levels was tested in early schizonts (36 ± 2 hpi) (light grey), late schizonts (46 ± 2 hpi) (medium grey) and 12 ± 2 h later in ring stage parasites (dark grey) in Pfgdv1.gfp.dd.T1 parasites in the absence (Off) and presence (On) of Shld1 (a–d) as well as the clonal 3D7_Gdel parasite line that lacks the gdv1 locus due to a spontaneous chromosome 9 deletion24. Expression levels in early schizonts in the absence of Shld1 are used as the reference for the analysis for ap2-g a and msrp1 b, while the Shld1 minus ring stage expression levels were used for hp1 c and ap2-g3 d. The data are from 2 independent experiments performed in duplicate. To compare expression between stages and conditions in the Pfgdv1.gfp.dd parasite line a two-way ANOVA with Sidak’s multiple comparisons test was used. For the 3D7_Gdel line, a one-way ANOVA with Dunnett’s multiple comparisons test was performed to compare expression between stages. For both analyses a Bonferroni post-hoc test to correct for the analysis of 4 genes was used and probability is indicated, p ≤ 0.05 (‘*’), p ≤ 0.01 (‘**’), p ≤ 0.001 (‘***’).
low malaria transmission and high expression levels of gametocyte-associated genes, including ap2-g, has recently been reported in a publication comparing the transcriptomes of parasites isolated in areas with different patterns of malaria transmission. However, they did not directly assess gametocyte production or gdv1 allele. Clearly further work is needed to integrate these intriguing findings.

To extend our molecular analysis of the regulation of gametocytophogenesis, we used PfPgdv1.gfp.dd parasites that allow inducible gametocyte production by Shld1-dependent protection of GDV1 expression. GDV1 was required only during schizogony for the induction of gametocytes. Since schizonts are sequestered in vivo and not present in human blood samples we assessed regulation in vitro and found ap2-g and msrp1, not gexp5, transcript levels were significantly increased during schizogony in the presence of GDV1. However, close analysis of the in vitro data during schizogony in the PfPgdv1.gdf.dd line and a gdv1 deficient line (Gdel) indicates that, although ap2-g RNA levels are lower in the absence of GDV1, ap2-g transcripts still increase significantly during the transition from early to late schizonts. This low but significant GDV1-independent increase in ap2-g transcript levels suggests GDV1 is required to augment ap2-g mRNA levels during schizogony, rather than being directly involved in initiating transcription or required to release repression by displacing or preventing HP1 binding to H3K9Me3. The ability of Shld1-mediated stabilization of GDV1 to enhance ap2-g RNA levels not var gene expression, both of which are repressed by HP1, also argues against a direct effect of GDV1 on all HP1 repression sites. Based on recent data from P. yoelii it is possible that another ap2 transcription factor, ap2-g3, is required for this initial ap2-g expression and here we show that ap2-g3 levels are not affected during schizogony by GDV1 levels. It is possible that ap2-g3 initiates ap2-g transcription, while GDV1 is required to augment transcription or stabilize RNA levels, which is an established regulatory mechanism in P. falciparum. Future functional studies will include clinically relevant parasitemias, temperatures, LysopPC levels and gdv1 alleles in vitro to better define the signals influencing gametocyte production.

One of the major limitations of this study is that all the volunteers were recruited from a single clinic in an area of high seasonal transmission with a low levels of microscopically detectable stage V gametocytes. Reproducibility in different regions with different malaria transmission patterns and a wider range of ages is needed to validate the clinical parameters.
and gene expression profiles found to be associated with gc-ring stage parasites. To extend the study to include lower parasitemias and remote areas without access to tissue culture facilities or trained staff to maintain and quantify the ex vivo cultures, robust molecular markers are needed to identify gc-rings. Such markers will also allow the direct comparison of gc-rings. Such markers will also allow the direct comparison of gc-rings with and without 

**Methods**

**Study site and Population.** Blood samples were collected from children attending the Ewim Health Center in Cape Coast, Ghana[66]. Briefly, children (≤13 yrs) with *P. falciparum* parasitemia ranging between 1,000 and 250,000 per μl of blood based on the WHO standardized protocol[67] were recruited into the study and their axillary temperature recorded.

**Ethics Statement.** The study was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research and the Ghana Health Services and reviewed by MIDM, NIAID, NIH. Before recruitment each parent/guardian was informed of the objectives, methods, anticipated potential hazards of the study. The parents/guardians were encouraged to ask questions about any aspect of the study that was unclear to them and informed about their liberty to withdraw their children at any time without penalty. Children were enrolled only after written parental consent had been obtained. All patient information is treated as confidential.

**Sample collection.** Blood samples were obtained from consented children prior to drug treatment (D0). For each subject, blood was collected into acid citrate-dextrose solution (ACD) (2.0 ml) for parasite cultures, a tube containing ethylenediaminetetraacetic acid (EDTA) (0.5 ml) (BD Biosciences, San Jose, CA) for hematological analysis and in 2016 a PAXgene RNA blood tube (2.5 ml) (BD Biosciences) for RNA isolation. A Unit 3090 Plus (Urit Medical Electronics, Guilin, Guangxi 541004, P.R. China) was used to assess the hematological parameters, including the WBC count and Urit-12 hemoglobin meter (Urit Medical Electronics) was used to determine hemoglobin levels according to manufacturers’ instructions. Immediately after removing the samples for the hematological indices and making a thick blood smear, the EDTA sample was centrifuged and the plasma stored at −20°C for immunological tests. The thick blood smear was stained with Giemsa and used to quantify asexual and sexual stage parasites against 200 WBCs. In 2017 the cell pellet was resuspended in NucleoZOL and stored frozen until RNA analysis. All study participants were given a standard curative dose of artemether-lumefantrine (20/120 mg/kg) or artemether-amodiaquine (4/10 mg/kg) and scheduled for a follow up visit 7 days later.

**Ex vivo analysis of gametocyte commitment.** The samples collected in ACD tubes were centrifuged for five minutes at 2000 rpm to harvest plasma. The cells were washed twice with 5 ml of sterile RPMI 1640 media without serum or AlbuMax II (Thermo Fisher Scientific, Waltham, MA) and centrifuged at 2000 rpm for five minutes to remove white blood cells and the buffy coat. The red blood cell pellet was resuspended to a 3% hematocrit in RPMI 1640 media supplemented with 2% inactivated human serum and 0.5% AlbuMax II. Aliquots of the resuspended RBCs (3 ml) were added into 2 separate wells of a 12 well plate. NAG was added and then the plate was incubated at 37°C in an atmosphere of 5% CO2/95% O2/90% N2 for 8 days with daily media changes and thin smear preparation but without RBC supplementation. On the 8th day, the cultures were harvested and the parasites preserved in NucleoZOL (Macherey-Nagel). D0, D4 and D8 NucleoZOL (Macherey-Nagel) thick blood smears were Giemsa-stained and the distinct parasite stages observed by microscope (>1000) were counted. A total of 2000 RBCs were counted to determine the D0 ring stage parasitemia, while 20,000–30,000 RBCs were counted per smear from each of the duplicate D4 and D8 cultures. A sample was classified as gametocyte-deficient if no gametocyte was observed in 30,000 RBCs. Gametocyte conversion rates were calculated for each sample by dividing the average D4 stage II–III gametocytemia or the average D8 stage III–V gametocytemia from the two NAG-treated ex vivo cultures by the D0 parasitemia. For quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) analysis of the D0 blood sample, RNA was purified using the PAXgene RNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol and converted to cDNA for RT-qPCR as described below in the Quantitative reverse transcriptase-polymerase chain reaction section. The gene specific primers were designed using Primer3 version 4.1.0 and are listed in Supplementary Table 5 and the 2−ΔΔCT method for each gene was calculated according to the manufacturer’s instructions. By sequencing the plasmid concentration was

and was associated positively with D0 parasitemia and negatively with the ratio of gametocyte- to asexual-committed rings varied widely and was associated positively with D0 parasitemia and negatively influenced by patient temperature. GDV1 allele H277 and transcript levels of three GDV1-regulated genes, ap2-g, msrp1 and gexp5 were significantly higher in individuals with high ratios of gametocyte- to asexual-committed rings, while in the 2017 plasma samples high levels of LysoPC were associated with low levels of gametocyte production. In vitro, GDV1 was found to play a critical role in the regulation of the balance between asexual and sexual development during schizogony, prior to the production of gametocyte-committed rings. The combined findings support consistent low-level gametocytogenesis in the human host that is modulated by parasitemia and fever as well as GDV1 levels and allele.

**Fig. 10** GDV1 allele H277 is over represented in high GCR patient samples. (A) The frequency of the gdv1 alleles encoding H (black) and P (white) or (B) msrp2 FC27 (dark grey) and 3D7 (light grey) family alleles in the high (H) (n = 20) and low (L) GCR (n = 19) samples are plotted. Significance was assessed using a Fisher Exact test. The D4 GCR (%) of each of the samples with gdv1 allele H277 (H, black dot) or P217 (P, open circle) or msrp2 allele FC27 (dark grey dot) or 3D7 (light grey dot) is plotted with the mean and s.d. indicated. The Kruskal–Wallis test followed a Dunn’s multiple comparison test was used to compare the D4 GCR of the samples with distinct gdv1 or msrp2 alleles (Prism GraphPad v7.05). Probability is indicated, p > 0.05 (ns) and p ≤ 0.01(**)”
quantified by absorbance at 260 using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and the plasmid copy number per microliter was determined using an online treatment tool (https://www.genecopoeia.com/cnDNA.html). The plasmid was serially diluted and used as a qPCR template to generate a standard curve correlating Pf25 Cτ to transcript number. The results were used to calculate the number of Pf25 transcripts per micro liter of blood in each sample (Fig. 1f). The number of Pf25 transcripts/µl was then divided by the number of ring stage parasites per microliter of blood measured from the Giemsa-stained D0 thick smear to determine the % sexual stage parasitemia (Fig. 1g).

**Plasma metabolic analysis.** Plasma from 40 samples with D0 parasitemia > 0.35%, 10 with high GCR (5–66%) and 10 with undetectable to low GCR (0–0.3%) from each year (Supplementary Table 4) and three uninfected control samples were selected for evaluation using the AbsoluteIDQ p180 assay (Biocrates, Innsbruck, Austria). For WRC,GCR, the Cτ签订了 sexual stage parasitemia at the time of RNA collection.

**Parasite transformations.** Gdhv1 was tagged in frame with green fluorescent protein (GFP) followed by the FKBP-destabilization domain (DD) to allow ligand regulated protein degradation and track protein expression. To generate the transformation vector the 3′ end of gdhv1 was amplified using polymerase chain reaction (PCR) and synthetic oligonucleotides corresponding to gdhv1 bp 901–930 with a 5′ XhoI site and gdhv1 bp 1762–1797 flanked by Avrl (Supplementary Table 5). The PCR amplicon and p1605-GK-FBP-int plasmid[26] were digested with XhoI and Avrl. Prior to ligation with T4 ligase the digested plasmid was treated with alkaline phosphatase for 1 h. Electrocumponent DHCP E. coli were transformed with the ligation reaction and grown overnight on LB plates containing ampicillin (100 µg/ml). Ampicillin-resistant colonies were selected, screened for insert by restriction enzyme digestion and sequenced before being used for parasite transformation. Established protocols[27] were used for transformation. Successful transformants were obtained by two independent transformations. After 3 weeks off drug, WR99210 (Jacobus Pharmaceuticals, Plainsboro, NJ) was reapplied with or without WR99210 (10 ± 2 hpi) and parasites were MACs/sorbitol synchronized as the reference. The efficiency of the transformants was tested by serial dilution and ranged from 87 to 111%.

**GDV1 protein-dosing.** Pgdv1-gfp.dd parasite were MACs/orbitol synchronized and Shld1 concentrations ranging from 0 to 1500 nM added at ring stage. For the immunoblot schizonts harvested 14 h after treatment, with 0.05% saponin to remove the RBGs and then examined in 8 μm areas/50 SDs. The immunoblot was probed with anti-GFP mAb (#11-814-460-001, Roche, Indianapolis, IN) followed by horseradish peroxidase-labeled anti-mouse IgG and visualized using the Pierce SuperSignal West Dura kit (Thermo Fisher Scientific) and an ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ). The blot was then re-probed with rabbit anti-Histone 3 (anti-H3) antibody (#Ab1791, Abcam, Cambridge, MA), followed by alkaline phosphatase labeled secondary Ig and visualized using 5-bromo-4-chloro-3-indolylphosphate BCIP) and aluminin (NIFT) (Thermo Fisher Scientific). The density of the upper and lower bands was determined after background subtraction and normalization to the anti-histone 3 antibody signal using Image J software. For expression profiling, Shld1 was added to ring stage parasites and harvested 24 ± 2 hpi. For proteomic analysis, parasites were MACs/orbitol synchronized and grown in Nucleofor for RNA preparation. In two independent cultures parasites were grown in the absence of Shld1 as the reference. The efficiency of the transformants was tested by serial dilution and ranged from 87 to 111%.

**Genetic diversity.** Genomic DNA was extracted from two 3 mm punches of dried blood spot (D0) using QIAamp DNA Mini Kit (Qiagen), eluted in 120 µl of distilled sterile water and used to analyze gdhv1 and msp2 genotypes. For gdhv1 a 613 bp polymorphic region was PCR amplified using primers Msp2.Outer.M2-OR66 (Supplementary Table 5) and reaction conditions described above for Msp2 a nested PCR was used to discriminate the two major alleles (PCR and SSCP). For msp2 the polymorphic region was PCR amplified using family specific primers, msp2Outer.M2-OF and msp2Outer.M2-OR (Supplementary Table 5) and reaction conditions described above for gdhv1. Next, 0.5 µl of the outer PCR product was used as a template in a PCR with primer msp2 3D7/1C7.2F1 and either msp2 27C5.3Frev or msp2 27D5.3Frev. PCR products were separated using 2% ethidium bromide-stained agarose gels and visualized under UV illumination.

**Statistical analysis.** STA V14.0 was used for the Pearson correlation and ANOVA analysis, as well as the independent regression analysis to evaluate the association of the clinical parameters and the in transformed parasite data (D0 parasitemia, gametocytemia and gametocyte conversion rates on D4 and D8)[1]. To determine whether the parameters were normally distributed, histograms and normal Q-Q plots were used to determine whether the transformation was necessary. The relative quantity (2ΔΔCτ) was used to determine whether the parameters were normally distributed, histograms and normal Q-Q plots were used to determine whether the transformations used were appropriate. The metabolites are regulated protein degradation and track protein expression. To generate the transformation vector the 3′ end of gdhv1 was amplified using polymerase chain reaction (PCR) and synthetic oligonucleotides corresponding to gdhv1 bp 901–930 with a 5′ XhoI site and gdhv1 bp 1762–1797 flanked by Avrl (Supplementary Table 5). The PCR amplicon and p1605-GK-FBP-int plasmid[26] were digested with XhoI and Avrl. Prior to ligation with T4 ligase the digested plasmid was treated with alkaline phosphatase for 1 h. Electrocumponent DHCP E. coli were transformed with the ligation reaction and grown overnight on LB plates containing ampicillin (100 µg/ml). Ampicillin-resistant colonies were selected, screened for insert by restriction enzyme digestion and sequenced before being used for parasite transformation. Established protocols[27] were used for transformation. Successful transformants were obtained by two independent transformations. After 3 weeks off drug, WR99210 (Jacobus Pharmaceuticals, Plainsboro, NJ) was reapplied and the parasites were cloned by limiting dilution. The presence of a single-crossover chromosomal integration was assessed by PCR amplification using primers listed in Supplementary Table 5.
used to evaluate the frequency of gdv1 and map2 alleles in the 2016 and 2017 high and low GCR samples and the Kruskal–Wallis test followed a Dunn’s multiple comparison test was used to compare the GCR of the gdv1 and map2 alleles (Prism GraphPad v7.05). The four samples, two from each cohort, with both gdv1 alleles and the 11 samples, 5 from the high and 6 from the low GCR cohort, with both map2 alleles were included in the analysis. All statistical tests were two sided and P values > 0.05 were considered non-significant (ns), P values ≤ 0.05 = *, P values ≤ 0.01 = **, P values ≤ 0.001 = ***. P values ≤ 0.0001 = ****.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All data generated during this study are included in this published article and its supplementary information files. The raw data files are available on request. PlasmoDB.org accession numbers, ap2g, PF3D7_1226600; ap2-g3, PF3D7_1317200; arginyl-RNA synthetase PF3D7_1218600; gdv1, PF3D7_0954500; Pfg1, PF3D7_1477700; gdp5, PF3D7_0966600; hp1, PF3D7_1220900; kahrp, PF3D7_0202000; msrp1, PF3D7_1350000; Pj16, PF3D7_0406200; Pj25, PF3D7_1031000; Sbp1, PF3D7_0513000; 18s rRNA, PF3D7_0725600.

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