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Comparative analysis of peripheral whole blood transcriptome from asymptomatic carriers reveals upregulation of subsets of surface proteins implicated in *Plasmodium falciparum* phenotypic plasticity

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A B S T R A C T

The molecular mechanism underlying *Plasmodium falciparum's* persistence in the asymptomatic phase of infection remains largely unknown. However, large-scale shifts in the parasites' gene expression during asymptomatic infections may enhance phenotypic plasticity, maximizing their fitness and leading to the persistence of the asymptomatic infections. To uncover these mechanisms, we aimed to identify parasite genetic factors implicated in asymptomatic infections through whole transcriptome analysis. We analyzed publicly available transcriptome datasets containing asymptomatic malaria (ASM), uncomplicated malaria (SM), and malaria-naïve (NSM) samples from 35 subjects for differentially expressed genes (DEGs) and long noncoding RNAs. Our analysis identified 755 and 1773 DEGs in ASM vs SM and NSM, respectively. These DEGs revealed sets of genes coding for proteins of unknown functions (PUFs) upregulated in ASM vs SM and ASM, suggesting their role in underlying fundamental molecular mechanisms during asymptomatic infections. Upregulated genes in ASM vs SM revealed a subset of 24 clonal variant genes (CVGs) involved in host-parasite and symbiotic interactions and modulation of the symbiont of host erythrocyte aggregation pathways. Moreover, we identified 237 differentially expressed noncoding RNAs in ASM vs SM, of which 11 were found to interact with CVGs, suggesting their possible role in regulating the expression of CVGs. Our results suggest that *P. falciparum* utilizes phenotypic plasticity as an adaptive mechanism during asymptomatic infections by upregulating clonal variant genes, with long noncoding RNAs possibly playing a crucial role in their regulation. Thus, our study provides insights into the parasites' genetic factors that confer a fitness advantage during asymptomatic infections.

1. Background

Malaria remains a major global health concern, affecting most tropical and subtropical regions. A total of 247 million cases and 619,000 deaths were reported in 2022 [1]. About 95 % of the reported cases occurred in sub-Saharan Africa. The World Health Organization aims to reduce malaria incidence and mortality rates by 90 % and eradicate and prevent malaria in malaria-free countries by 2030 [2].

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This global target can be achieved by accurately detecting and treating symptomatic infections and asymptomatic carriers [3]. Individuals acquiring *Plasmodium* species infection from a mosquito bite are prone to display symptoms consistent with classical case presentation for malaria within 8–14 days of the bite [4,5]. While the period of onset of symptoms may vary, studies have noted that some individuals with detectable *Plasmodium* species infection delay showing symptoms whatsoever for an unforeseeable duration [6,7]. Notably, while individuals with symptomatic infections readily present at the health facilities and get treated to deter transmission, the asymptomatic individuals, despite accounting for a large proportion of malaria infections [5,8], do not visit health facilities and hence remain obscure to treatment and control interventions. Thus, they maintain active transmission of malaria infections in a local setting [9–12].

Harboring *Plasmodium* parasites without portraying symptoms has been attributed to acquired immunity after several exposures to malaria infections, anti-parasite immunity, and a factor of balance between a pro and anti-inflammatory response that appears to be inherent in parasite modulation of the host [13–15]. However, the biology of asymptomatic infections remains poorly understood, particularly with regard to the parasite factors that contribute to the establishment of these infections, with almost no data available on the transcriptome, functional genomics, proteome, and phenotypic description of the parasites [16]. Moreover, the current knowledge of *Plasmodium* parasite biology is based on clinical malaria infections and clonal laboratory-cultured parasites, which may not accurately reflect the characteristics of asymptomatic infections. Besides, a longitudinal analysis of the implications of asymptomatic malaria infections revealed that such infections rarely progress to clinical malaria [17]. Notably, the persistence of the parasites during asymptomatic infections without host immune system clearance suggests a complex interaction between host and parasite factors.

The interactions between hosts and parasites involve complex molecular mechanisms, including adhesive interactions of infected erythrocytes that govern parasite invasion [18–21], activation of specific host immune responses, and epigenetic regulation [22]. These molecular interactions are mediated by specific parasite ligands expressed on the surface of infected red blood cells [23]. Furthermore, these interactions facilitate *Plasmodium*'s adaptation to changing environments, particularly through epigenetic regulation that modulates gene expression patterns in response to environmental changes. The involvement of epigenetics gives rise to new phenotypes, allowing the parasite to effectively adjust to the host conditions [24]. Epigenetic mechanisms involve DNA methylation, histone modification, and regulation by noncoding RNAs. Noncoding RNAs have emerged as pivotal players in epigenetic control, influencing gene expression at both the transcriptional and post-transcriptional levels [25,26].

In *P. falciparum*, noncoding RNAs have been implicated in regulating the monoallelic expression of virulent (*var*) genes, crucial for immune evasion and establishing chronic disease [27–30]. The activation and interference of antisense lncRNAs mediate the expression switching of the *var* genes, and the activation of a specific *var* gene depends on the expression of its corresponding antisense lncRNA, while interference with the lncRNA transcript results in the silencing of the gene [31–33]. In addition, ncRNAs also regulate clonal variant gene expression, highlighting their significant role in *Plasmodium*'s phenotypic plasticity [34]. Clonal variant genes are involved in host-parasite interactions [35,36] and confer phenotypic plasticity [37]. It is evident that transcriptional variations play a significant role in plasticity. Nevertheless, the parasite factors and the molecular mechanisms underlying the persistence of the parasites in asymptomatic phase infections remain elusive.

We hypothesized that large-scale changes in gene expression of *P. falciparum* parasites during asymptomatic infections confer phenotypic plasticity, enabling the parasite to persist in the host and respond to the host's immune pressure. We compared the transcriptomes of *P. falciparum* isolated from asymptomatic, symptomatic, and naïve malaria infections to investigate this hypothesis. Our analysis provides valuable insight into the transcriptional changes and genetic factors that confer a fitness advantage to the parasite during asymptomatic infection and contribute to a deeper understanding of asymptomatic infections.

2. Materials and methods

This was a retrospective comparative study that aimed to identify genetic factors that confer a fitness advantage to *P. falciparum* during asymptomatic infection. We compared publicly available transcriptome datasets containing three different sample types: uncomplicated malaria, naïve malaria, and asymptomatic malaria. Symptomatic samples were collected from consenting participants enrolled in healthcare facilities in Timika, Papua Province, Indonesia. Asymptomatic and naïve malaria samples were obtained from children in health facilities in Mali, with written informed consent from parents or guardians.

2.1. Genes expression data acquisition

The National Center for Bioinformatic Information's Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) was searched for malaria transcriptome datasets following these criteria: (I) The dataset had to be generated from blood samples obtained directly from the field, not from culture-adapted parasites, to reflect the behavior of *Plasmodium falciparum* in its natural environment; (II) The dataset had to be RNA sequencing data, not from microarray because RNAseq can detect novel, unannotated genes compared to standard microarray technology; (III) The dataset had to be isolated from asymptomatic malaria carriers or symptomatic or naïve malaria individuals. The datasets were identified and retrieved from the GEO database as raw data using the sratoolkit 3.0 under GEO Series accession number GSE148125 for asymptomatic and naïve malaria transcriptome datasets [38] and accession number PRJEB21707 for uncomplicated symptomatic malaria transcriptome dataset [39]. Briefly, the GSE148125 was generated from *P. falciparum* parasites isolated whole blood samples collected from Malian children with persistent subclinical malaria during the asymptomatic infections in the dry season (n = 11) as well as children of the same age presenting with their first clinical malaria during the wet season (n = 12), naïve malaria infections. The PRJEB21707 was generated from venous blood samples drawn from individuals presenting with uncomplicated malaria in one of the healthcare facilities in Timika, Papua Province, Indonesia (n = 12). The sample size largely depended on RNA sequencing data availability but was sufficient enough to yield an 80 % statistical power to detect significant differences in gene expression since previous studies have used smaller sample sizes. For both datasets, the erythrocytes were isolated from the blood sample and frozen in an RNA-stabilizing preservative; libraries of 100 bp were prepared and sequenced on 2500-HT Hiseq (Illumina) using Rapid run chemistry (Illumina) as described elsewhere [38,39].

2.2. Quality assessment and trimming of the sequencing reads

The raw RNA fastq files were assessed for quality and adapter contamination using FastQC v 0.11.9 software. The adaptor sequences ligated to the reads were then trimmed using the Trimmomatic v0.39 [40]. Reads with ambiguous sequence content ("N") exceeding 5 % were also removed. The processed reads were aligned to the *P. falciparum* 3D7 reference genome (GCF_000002765.5) from NCBI using HISAT2 with default settings [41]. The output sam files were sorted and converted to binary bam output using Samtools. The processed bam files obtained gene-level counts using the Rsubread Featurecounts software version 1.6.1 [42]. Following the gene-level quantification, the DESeq2 [43] package in R, available through the Bioconductor project, was used to identify differentially expressed genes using default parameters, allowing for control of the fold change cut-offs, log2 fold-change shrinkage,

and custom *P*-value. Genes with a test statistics *P*-value, FDR (adjusted for False Detection Rate) [44] of <0.05, and those with log2 fold-change (log2FC) of at least ± 2 between the studied groups were considered differentially expressed and retained for further analysis.

2.3. Gene set expression and pathway analysis

Functional gene enrichment analysis was performed in ClusterProfiler v 4.6.0 [45], a package in the R statistical analysis tool. Gene ontology terms were assigned to differentially expressed genes, and pathway gene enrichment analysis was performed on Kyoto Encyclopedia and Genomes (KEGG) (with a *P*-value <0.05).

2.4. Extraction of PfEMP1 encoding and regulator genes

The genomic coordinates of the *P. falciparum* var genes and the regulator proteins' coding genes were obtained from the NCBI archive and used to prepare a bed file. The bed file was used to extract all reads from the sorted bam files that aligned to the genomic coordinates using bed tools intersect [46]. The obtained reads files were then assembled using the Cufflinks v2.2.1. The assembled transcripts were merged using Cuffmerge v1.0.0, and the obtained gtf file was used to assign genomic features to the extracted reads in Featurecounts. DESeq2 package was used to perform differential gene expression in R to determine the transcription patterns of *var* genes between the groups. To investigate the *var* gene regulation, we explored the expression profile of genes coding for the *var* genes regulator proteins, including KAHRP, KAHSP40, HSP70-x, Mauler's cleft proteins (SBP1), and REX1.

2.5. Long noncoding RNA characterization

The aligned reads were assembled and merged using StringTie (version 1.0.1) with the parameters (-f 0.01 -c 0.01, -m). The transcripts with lengths less than 200 nt and <2 exons were discarded. The remaining transcripts were annotated using the gffCompare software version 0.11.2 [47], followed by the use of custom scripts to select all transcripts with class codes "x," "i," "u," "e," and "o" while discarding all those that were unwanted. The remaining transcripts' protein-coding ability was predicted using the CPC, PLEK, and FEEnc software [48–50]. The transcripts without protein-coding ability were employed in the remainder of the study.

2.6. Analysis of differentially expressed long noncoding RNA

The R package DESeq2 was utilized to perform the differentially expressed analysis [43]. The fold changes of lncRNAs were calculated via log2FC. The lncRNAs exhibiting a fold change ≥ 2 and FDR <0.05 were considered the DE-lncRNAs. In order to investigate the role of lncRNAs in the regulation of CVGs, the interaction of the top 15 upregulated lncRNAs with the clonally variant genes was performed using the LncTar software.

3. Results

3.1. Summary of the datasets

After intensively searching the SRA database, we identified two RNA seq datasets appropriate for this study. One dataset was generated from venous blood samples drawn from individuals presenting with uncomplicated and severe malaria in one of the healthcare facilities in Timika, Papua Province, Indonesia; however; in this study, we only utilized 12 samples obtained from uncomplicated malaria. The other dataset was generated from *P. falciparum* parasites obtained from 11 Malian children with persistent subclinical malaria during the asymptomatic infections in the dry season as well as 12 children of the same age presenting with their first clinical malaria during the wet season as naïve malaria infections. All datasets were generated from *P. falciparum* isolated from naturally infected individuals. However, since the datasets were generated from different sequencing machines and time, downstream analysis was subject to batch effect.

3.2. Quality control characteristics of the datasets

The filtered reads were aligned to the *P. falciparum* 3D7 GCF_000002765.5 reference genome, yielding an average value of 8.4 million per sample. A total of 295.7 of 823.5 million reads (35.91 %) mapped to unique regions of the *Plasmodium* reference genome; 21.5 million reads for asymptomatic, 261.4 million reads for symptomatic, and 71 million reads for naïve asymptomatic malaria. Generally, the proportion of the reads mapping to the reference genome per sample ranged from 1.7 % to 91.1 %, with asymptomatic samples being the least mapped, with a mean of 1.95 million reads per sample and only 7.2 % of the total mapped reads (Table 1, Table S1).

3.3. Comparative analysis of DEGs between asymptomatic malaria carriers versus symptomatic and naïve malaria-infected individuals

We identified 844 and 1553 genes significantly upregulated in parasites isolated from asymptomatic carriers ($n = 11$) compared to symptomatic ($n = 12$) and naïve symptomatic individuals ($n = 12$), respectively (Fig. 1b and 1d). Moreover, 111 and 220 genes were downregulated in asymptomatic compared to symptomatic and naïve symptomatic individuals, respectively (Fig. 1b and d). We identified the upregulation of several uncharacterized genes and the downregulation of several genes belonging to the *Plasmodium* helical interspersed subtelomeric (*phist*) family (Table 2). The top 10 upregulated genes in the blood transcriptome of asymptomatic compared to naïve symptomatic malaria encoded conserved proteins of unknown functions, kinases, histone-lysine N-methyltransferase, H3 lysine-4 specific, and DNA repair and recombination elements (Table 2). Further, the differential gene expression analysis revealed that the *phist* genes were the most differentially expressed with the highest log2FC values (Tables 2 and 3). The hierarchical clustering of the significantly expressed genes is represented in a heatmap (Fig. S1) (see Table 4).

Enrichment analyses of the upregulated genes revealed the biological functions involved in host-parasite interaction.

Enrichment analysis of the 955 DEGs identified activated biological processes involved in symbiotic interactions, interaction with the host, and inter-species interaction between organisms; cellular processes involved in modulation of symbiont of host erythrocyte aggregation, translocation of molecules into the host, translocation of peptides into the host, and modulation of host cell processes. The genes involved in host-parasite interactions included members of the multi-family genes *rifin*, *stevor*, and Maurer's proteins, as well as the putative plasmepsin VIII (Table 3).

The significantly downregulated pathways included the infected host cell surface knob formation and other biological processes involved in methylation, rRNA processing, and metabolism. Moreover, the ontology analysis identified ten activated and ten suppressed of the top 20 enriched pathways in asymptomatic compared to naïve symptomatic individuals (ASM vs. NSM). We observed upregulation of the pathways

Table 1
Sequencing data summary statistics.

Variable	Total reads (in a million reads)	Mapped reads	% of mapped reads	Total number of samples
Asymptomatic	446.9	21.5	4.82	11
Symptomatic	261.4	203.2	77.74	12
Naïve symptomatic	115.3	71	61.58	12
Total	823.5	295.7	35.9	35

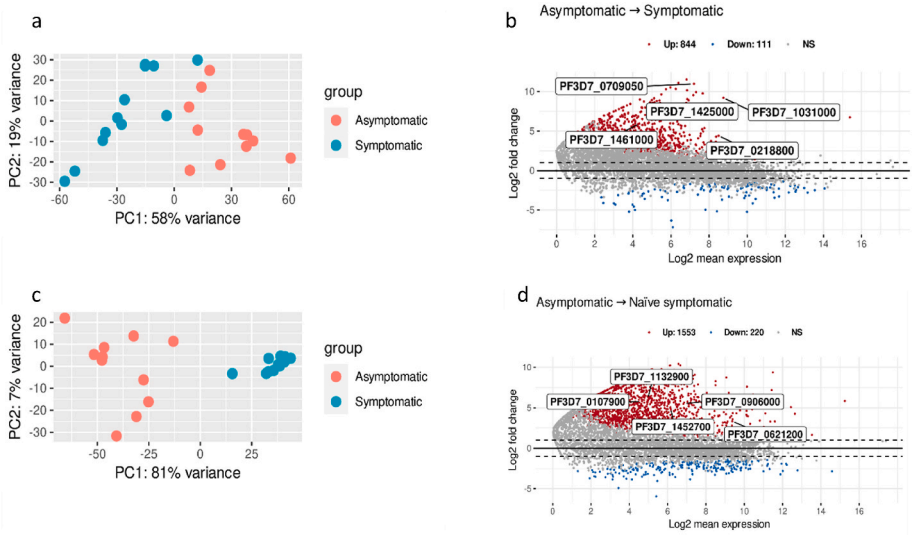

Fig. 1. Differential gene expression between asymptomatic and symptomatic malaria: (**a**) Principal component analysis showing clustering of the samples in asymptomatic vs uncomplicated malaria; (**b**) MA plot showing results for the RNA-Seq experiment as a function of fold change ± 2 and mean expression in asymptomatic vs complicated malaria. The X-axis represents the log2 mean expression, and the Y-axis represents the log2 transformed fold change. Red points represent significantly upregulated genes, while the blue points represent downregulated genes. The gray points represent non-significant differentially expressed genes; (**c**) Principal component analysis showing clustering of samples in asymptomatic vs. naïve symptomatic malaria; (**d**) MA plot representing the RNA-Seq results of asymptomatic vs naïve symptomatic malaria. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

involved in rRNA processing and material transportation within and outside the cell and of the apicoplast: GO:0020011. Plastid: GO:0009536, Mitochondrion: GO:0005739, and supramolecular complex: GO:0099080 were among the top 20 significantly enriched pathways (Fig. 2b).

The two comparisons (ASM vs. SM and ASM vs. NSM) commonly enriched six GO terms. Five terms, rRNA processing, rRNA metabolic process, small-subunit proteasome, translocation of peptides into the host, and translocation of molecules into the host, were enriched in ASM vs. SM while downregulated in ASM vs. NSM. However, the supramolecular GO:0099080 was downregulated in both comparisons (Fig. 2b).

Our network analysis of the 955 differentially expressed genes in asymptomatic malaria compared to symptomatic malaria showed highly interconnected clusters reflecting biologically interconnected proteins (Fig. 3). Furthermore, we identified specific genes implicated in the biological processes involved in symbiotic interaction, interaction with the host, and inter-species interactions between organisms (Table 3, Fig. S2). These observations appear to suggest that these genes play a crucial role in the molecular interactions between *P. falciparum* parasites and the host organism.

The KEGG pathway analysis was performed on the 955 and 1773 differentially expressed genes in ASM vs. SM and ASM vs. NSM, respectively. The analysis identified key pathways enriched in ASM vs. SM (Fig. 4a) and ASM vs. NSM (Fig. 4b). In asymptomatic vs. symptomatic, two pathways (malaria and DNA replication) were activated out of the 15 top most enriched pathways. In asymptomatic vs. naïve malaria, five of the top 15 enriched pathways were suppressed (Fig. 4b).

3.4. Regulation of PfEMP1 genes

We did not find significant differences in the expression of *var* groups (Fig. S3). We, however, identified seven enriched genes (Fig. 5a), five downregulated and two upregulated. Five enriched transcripts were identified as novel transcripts by Cufflinks (CUFF.42, CUFF.50, CUFF.75, CUFF.219, and CUFF 203). Notably, we identified the downregulation of the *Plasmodium* Histone 3 (PF3D7_0610400) implicated in epigenetic regulation of the *var* genes gene expression.

3.5. Identification and characterization of long noncoding RNA

Our analysis identified 237 statistically significant differentially expressed long noncoding RNA at the FDR<0.05 and foldchange value of ±2 relative to the normalized count's *P*-value. Of these genes, 216 (18 %) were upregulated, and 21 (1.5 %) were downregulated. The principal component analysis of differentially expressed lncRNAs accounted for a 41 % variation between the asymptomatic and symptomatic groups (Fig. 6b). The down-regulated and upregulated lncRNAs are shown in (Fig. 6c). The proportion of DE lncRNAs was higher compared to the down-regulated DE lncRNAs. Cluster analysis of the differential expressed lncRNAs is depicted in a heatmap (Fig. 6a)

Interaction of long noncoding RNA with identified differentially expressed genes.

We found 11 long noncoding RNA interacting with the target mRNAs (Table 5). These long noncoding RNAs interacted with multiple mRNAs, for instance, four noncoding RNAs: MSTRG.3150, MSTRG.3921, MSTRG.2879, and MSTRG.1293 were found to interact with *Pfmc-2tm*Maurer's cleft two transmembrane proteins but also interacted with other mRNAs coding for the *stevor* and *rifin* proteins.

4. Discussion

Here, we present the variation in transcriptional patterns of *P. falciparum* parasites and provide evidence that clonally variant gene expression could be mediating the establishment of asymptomatic malaria infections. Our study design and analysis elucidated transcription patterns of *P. falciparum* in asymptomatic infections by analyzing whole transcriptome data of parasites isolated from individuals presenting with asymptomatic malaria (ASM), uncomplicated malaria clinical infections (SM), and malaria-naïve individuals. The results showed significant variation in the transcription patterns of *P. falciparum* parasites during asymptomatic infections compared to uncomplicated and naïve malaria infections. Most of the transcriptional differences between parasites isolated from asymptomatic infection compared to naïve and symptomatic infections occurred in clonal variant genes (CVGs) such as *stevor, phist, rif,* and genes coding for proteins of unknown functions. Particularly, we identified specific multi-family genes encoding stevor,

Table 2
Top 10 upregulated genes in asymptomatic compared to symptomatic and naïve symptomatic malaria.

Asymptomatic vs. Symptomatic		Asymptomatic vs. Naïve symptomatic.	
Gene ID	Description	Gene ID	Description
PF3D7_0930000	procollagen lysine 5-dioxygenase, putative	PF3D7_0930000	procollagen lysine 5-dioxygenase, putative
PF3D7_1471700	conserved *Plasmodium* protein, unknown function	PF3D7_0904200	conserved *Plasmodium* protein, unknown function
PF3D7_0709050	small nucleolar RNA	PF3D7_1471700	conserved *Plasmodium* protein, unknown function
PF3D7_1221400	inner membrane complex protein 1h, putative	PF3D7_0803400	DNA repair and recombination protein RAD54, putative
PF3D7_0518800	secreted ookinete protein, putative	PF3D7_1221000	histone-lysine N-methyltransferase, H3 lysine-4 specific
PF3D7_1141900	inner membrane complex protein 1b, putative	PF3D7_0407800	conserved *Plasmodium* protein, unknown function
PF3D7_1471600	conserved *Plasmodium* protein, unknown function	PF3D7_0504500	conserved *Plasmodium* protein, unknown function
PF3D7_0825800	conserved *Plasmodium* protein, unknown function	PF3D7_0607700	conserved *Plasmodium* protein, unknown function
PF3D7_1020200	conserved *Plasmodium* protein, unknown function	PF3D7_0515400	conserved *Plasmodium* protein, unknown function
PF3D7_0502300	conserved *Plasmodium* protein, unknown function	PF3D7_1371700	serine/threonine protein kinase, FIKK family

Table 3
Genes implicated in the parasite-host interaction.

Gene ID	Description
PF3D7_1101700	Pfmc-2tmMaurer's cleft two transmembrane protein
PF3D7_0701600	Pfmc-2tmMaurer's cleft two transmembrane protein
PF3D7_0101300	Pfmc-2tmMaurer's cleft two transmembrane protein
PF3D7_1000600	Rifin
PF3D7_1207300	conserved Plasmodium protein, unknown function
PF3D7_1100800	Pfmc-2tmMaurer's cleft two transmembrane protein
PF3D7_1247800	dipeptidyl aminopeptidase 2
PF3D7_0114100	Pfmc-2tmMaurer's cleft two transmembrane protein
PF3D7_0832000	Stevor
PF3D7_1039700	Pfmc-2tmMaurer's cleft two transmembrane protein
PF3D7_1254100	Stevor
PF3D7_0832900	Stevor
PF3D7_0100600	Rifin
PF3D7_0324100	Pfmc-2tmMaurer's cleft two transmembrane protein
PF3D7_0800400	Rifin
PF3D7_1372800	Stevor
PF3D7_0305600	AP endonuclease (DNA-[apurinic or apyrimidinic site] lyase), putative
PF3D7_1250100	osmiophilic body protein
PF3D7_0732700	Rifin
PF3D7_0300400	Stevor
PF3D7_0425700	Rifin
PF3D7_0300500	Rifin
PF3D7_0316000	microneme associated antigen
PF3D7_1465700	plasmepsin VIII

Table 4
Top 15 Upregulated long noncoding RNAs in Asymptomatic malaria compared to symptomatic malaria

Gene ID	baseMean	log2FC	lfcSE	stat	*P*-value	*P* adj
MSTRG.1486	123.26	10.73	0.66	14.85	6.94E-50	4.99E-47
MSTRG.1358	35.32	8.69	1.04	7.42	1.16E-13	9.28E-12
MSTRG.1673	10.62	8.06	1.53	4.61	4.10E-06	6.14E-05
MSTRG.3921	740.48	7.67	0.36	18.14	1.47E-73	2.11E-70
MSTRG.3150	6.57	7.48	0.87	7.48	7.26E-14	6.53E-12
MSTRG.2123	57.63	7.42	0.76	8.41	4.01E-17	6.41E-15
MSTRG.5302	20.32	7.36	1.52	4.18	2.95E-05	0.0003534
MSTRG.1293	30.67	7.31	0.88	7.19	6.33E-13	4.34E-11
MSTRG.2879	47.22	6.70	0.99	5.75	8.74E-09	2.73E-07
MSTRG.3083	30.16	6.66	0.84	6.73	1.74E-11	8.92E-10
MSTRG.4288	5.45	6.60	0.80	6.99	2.63E-12	1.40E-10
MSTRG.179	4.19	6.55	1.12	4.93	8.19E-07	1.47E-05
MSTRG.4565	16.91	6.53	0.76	7.27	3.51E-13	2.65E-11
MSTRG.829	18.8704	6.40	0.58	9.32	1.17E-20	2.41E-18
MSTRG.4325	5.6036	6.31	0.82	6.49	8.17E-11	3.92E-09

rifin, and the *Pfmc*-2tm Maurer's cleft surface proteins implicated in host-parasite interactions that were differentially expressed in parasites isolated from asymptomatic compared to those from symptomatic infections, suggesting their role in *P. falciparum* adaptation. These results provide a snapshot of the role of transcriptional variability in the adaption of *P. falciparum* parasites to the fluctuating host environment. Remarkably, the high expression of genes coding for uncharacterized proteins suggests that these genes may play a significant role in establishing asymptomatic infections and thus call for a critical assessment of their functions. Additionally, we identified upregulated lncRNA with log2FC > 5 interacting with the clonal variant genes, suggesting their possible role in epigenetic reprogramming and regulation of CVGs.

Our results revealed the upregulation of a subset of clonal variant genes in asymptomatic compared to symptomatic malaria, suggesting the significance of these genes in *P. falciparum* plasticity and resistance to stress, increasing parasite fitness in asymptomatic infection. Transcriptional variability of clonal variant genes is an intrinsic property of *P. falciparum* and has been implicated in host-parasite protein interactions mediating parasite adaption to changing environments beyond immune evasion [35]. Through such transcriptional variations, new phenotypic variants arise in response to environmental changes, resulting in heterogeneous parasites with a fitness advantage [51,52]. While, *P. falciparum* clonal variant genes can be grouped into those showing hypervariability between *Plasmodium* isolates and which are driven by immune evasion (*rif, stevor, var, pfmc*-2tm) and those conserved across *Plasmodium* isolates that are implicated in the functional diversification of the parasites (including; *fikk, extramp, phist, dnaj, cs*, among others), [35,53]. We, observed upregulation of multigene families showing hypervariability, including *stevor*, *rifin*, and *pfmc*-2tm, and downregulation of a subset of *phist* genes. This observation suggests that transcriptional variation of CVGs during asymptomatic infection is driven by immune evasion rather than functional diversification.

The regulatory mechanisms controlling the activation and suppression state of the clonal variant genes do not primarily depend on the

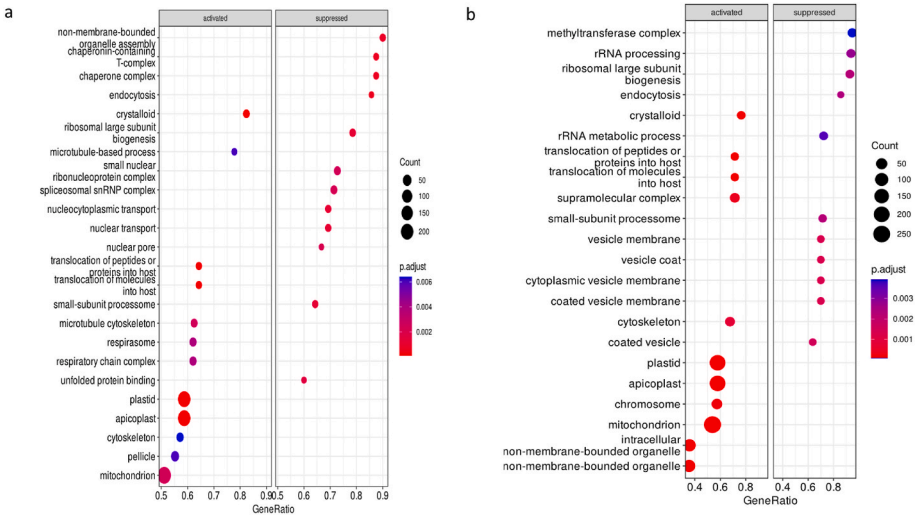

Fig. 2. Scatterplots of the enriched gene ontology terms: (**a**) the biological process, cellular component, and molecular function categories of the differentially expressed genes in asymptomatic compared to symptomatic malaria; (**b**) ontology terms enriched in asymptomatic vs. naïve symptomatic. The size of the dot represents the level of expression. The color of the vertical bar represents the significance level as a function of the adjusted *P*-value (*P*adjust). The deeper the red color, the more the significance level. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

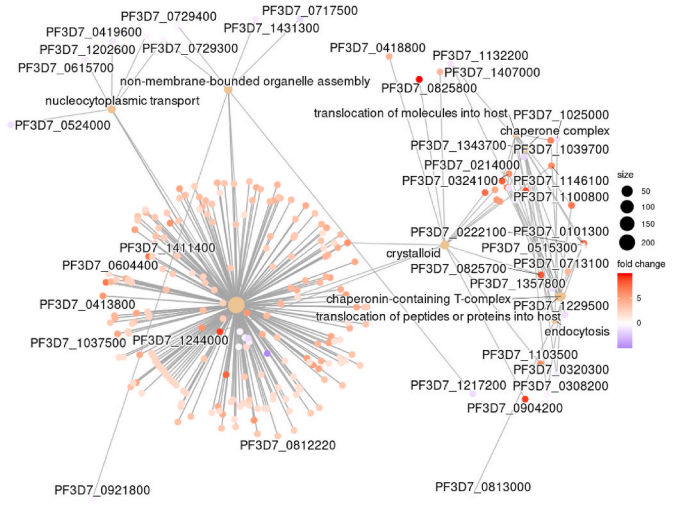

Fig. 3. Network analysis showing the genes involved in enriched gene ontology pathways. The color of the vertical bar depicts gene expression as a function of foldchange, while the size of the dot represents the number of genes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

DNA sequence of these genes but rather on epigenetic and heterochromatin formation [52]. Given that the epigenetic state of the genome is influenced by environmental cues [54], we suggest that the epigenetic mechanisms can modulate the activation and repression of these CVGs in response to the fluctuating host environment. While epigenetic mechanisms remains hallmark in in gene expression, the long noncoding RNAs are emerging as important epigenetic regulators [55,56] categorically in regulating host and pathogen immunological processes and functions [57–59], thus making them a good target for understanding disease etiology. This study identified several lncRNA upregulated in parasites isolated from asymptomatic individuals compared to symptomatic individuals by investigating the interaction of the top 15 differential expressed long noncoding RNA with mRNA transcripts implicated in host-parasite interactions. The interaction of the 11 long noncoding RNAs with CVG genes was also identified (Table 5). These findings suggest a possible significant role of lncRNA in regulating CVG gene expression. However, in-depth immunoprecipitation assays are required to give insight into the role of the lncRNA in epigenetic regulation and establishment of asymptomatic infections.

Accurate detection and quantification of asymptomatic malaria infections, whether microscopic or submicroscopic, is crucial for transmission-blocking and community intervention programs [60]. Currently, asymptomatic infection detection relies on quantitative real-time PCR (qRT-PCR) [55], which, although sensitive, is relatively expensive. This necessitates the development of rapid and cost-effective molecular diagnostic tools. Our data demonstrates the expression of two transcripts: procollagen lysine 5-dioxygenase, putative (PF3D7_0930000), and PF3D7_1471700, a protein of unknown function. These transcripts rank among the top 10 upregulated in both comparisons and have been previously identified as gametocyte-enriched transcripts [61]. They serve as potential biomarkers for developing molecular diagnostic tools for identifying asymptomatic infections.

The ontology analysis of the upregulated genes revealed several GO terms related to the plastid, kinase activity, signaling cellular response to DNA damage stimulus, apicoplast microtubule cytoskeleton, supramolecular complex, mitochondrion, and crystalloid. The enrichment analysis of the CVGs genes revealed biological processes implicated in host-parasite interactions, including symbiotic interactions, interspecies interaction between organisms, and the translocation of molecules into the host (Fig. 3), suggesting their significant role in establishing symbiotic host-parasite interactions. These findings concur with findings by Almelli and coworkers reporting similar protein profiles in asymptomatic individuals in Cameroon [62]. KEGG analysis identified suppression of metabolic pathways involved in carbon metabolism, fatty acid biosynthesis, and metabolism, suggesting suppression of several intermediates of glycolysis and pentose-phosphate pathways. Suppression of the central-carbon metabolism intermediates as an adaptive property of *P. falciparum* in isoleucine-starved conditions [63]. Given that the same metabolic pathways are suppressed during asymptomatic infections, we suggest that the parasite could alter its metabolism pathways similarly to establish asymptomatic infections. However, this needs further research.

Since subsets of *var* genes encoding *Plasmodium falciparum* erythrocyte membrane protein 1 (*PfEMP1*) have been associated with different forms of clinical malaria, given their significant role in virulence, we investigated whether there is a subset of the *PfEMP1* coding genes upregulated in asymptomatic infections compared to uncomplicated

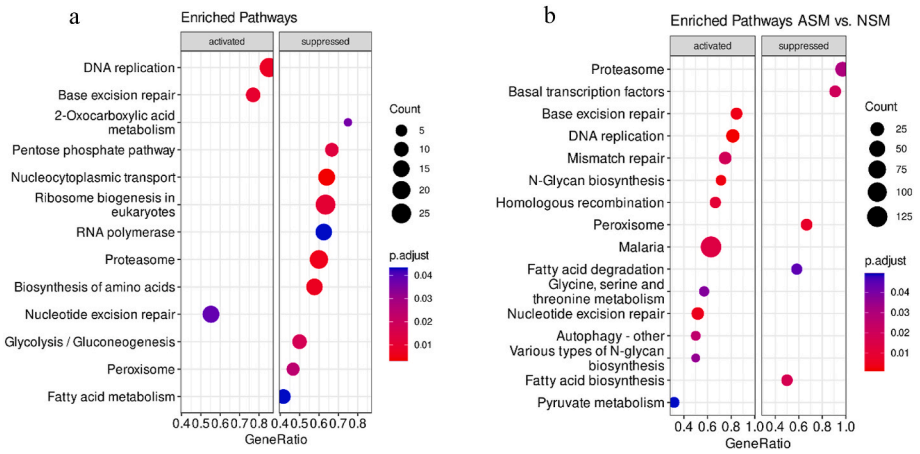

Fig. 4. Enriched KEGG pathways asymptomatic in malaria compared to symptomatic and naïve symptomatic malaria: (**a**) shows enriched pathways in asymptomatic vs. naïve symptomatic malaria, while Figure (**b**) is asymptomatic vs. symptomatic.

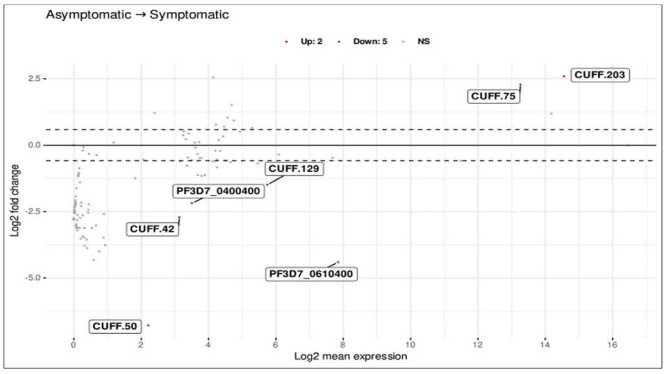

Fig. 5. An MA plot showing the significantly enriched genes in asymptomatic malaria compared to symptomatic Malaria at FDR <0.05 and log2FC values of ±1.5.

clinical malaria. We further evaluated the expression of regulator genes of the *var* switching process. We did not find significant variation in the expression of the *var* genes, with only a few uncharacterized transcripts found to be differentially expressed: CUFF.42, CUFF.50, CFF.75, CUFF.129, and CUFF.203. However, to this end, we reported down-regulation of the *P. falciparum* putative nuclear pore protein nucleoporin, NUP116/NSP116 (PF3D7_1473700), and the Histone 3 (H3) factors that regulate *P. falciparum* var switching. The process of *var* switching is not primarily dependent on transcriptional factors such as *cis*-acting elements but rather epigenetic regulation, which relies on heterochromatin remodeling and localization of a locus within the nucleus. This transcription occurs at an undefined perinuclear site away from the 59 repressed gene clusters [64]. The nucleoporin plays a significant role in chromatin organization and transcriptional regulation of genes [65]; however, there is a paucity of information on the role of these proteins in gene regulation in *P. falciparum*. A recent study investigating the role of perinuclear PfNup116 in *var* monoallelic expression using PfNup116 antibodies concluded that the expression is independent of the perinuclear pore [66]; thus, the role of Nup 116 in *P. falciparum* gene regulation remains elusive.

Our study was not without limitations. First, the sample size was small, and there were few RNAseq data on parasites isolated from asymptomatic malaria infections. Secondly, the datasets were generated from different areas and time points and thus were subjected to batch effect. Despite these limitations, the study provides important insights into transcriptional variations of *P. falciparum*, conferring fitness advantage to the parasites during asymptomatic infections. While the parasite may largely be using this fitness advantage to remain undetected in asymptomatic infections, the study did not exhaustively examine the role of host immunological factors that could maximize parasite fitness advantage and influence the asymptomatic phenotype. To additionally inform of the immunological mechanisms involved in maintaining asymptomatic infections within the human host, future research should include dual transcriptome profiling of both the parasite and the host to enhance understanding of immunological mechanisms behind asymptomatic infections. Validating the observed gene expression profile at a protein level using protein expression is crucial.

5. Conclusions

Overall, this study reveals upregulation of a subset of *P. falciparum* clonal variant genes during asymptomatic infection compared to uncomplicated and naïve malaria infection. This is an important observation suggesting that adaptation through clonally variant gene expression could be fundamental in establishing *P. falciparum* asymptomatic malaria infections. Also, given the upregulation of several genes encoding proteins of unknown function, suggest the significant role of these proteins in establishing asymptomatic infections. Hence, necessitating evaluation of these genes to elucidate their potential for biomarker development, as vaccines target and to understand asymptomatic infections better. However, in this study, we do not report host transcription patterns during asymptomatic infection. Therefore, we recommend dual sequencing analysis to fully elucidate host-parasite interactions, and genetic factors implicated in asymptomatic malaria infections. Generally, this study provides insight into how the *P. falciparum* parasites adapt to establish asymptomatic infections and provides new avenues for investigating asymptomatic infections in the front of renewed efforts to eradicate malaria in future.

Disclaimer

Materials have been reviewed by the Walter Reed Army Institute of Research (WRAIR), and there is no objection to its presentation and/or publication. The opinions or assertions contained herein are private views of the author and are not construed as official or reflect the true views of the Department of Army or the Department of Defense. The investigators have adhered to the policies for the protection of human subjects as prescribed in AR 70–25.

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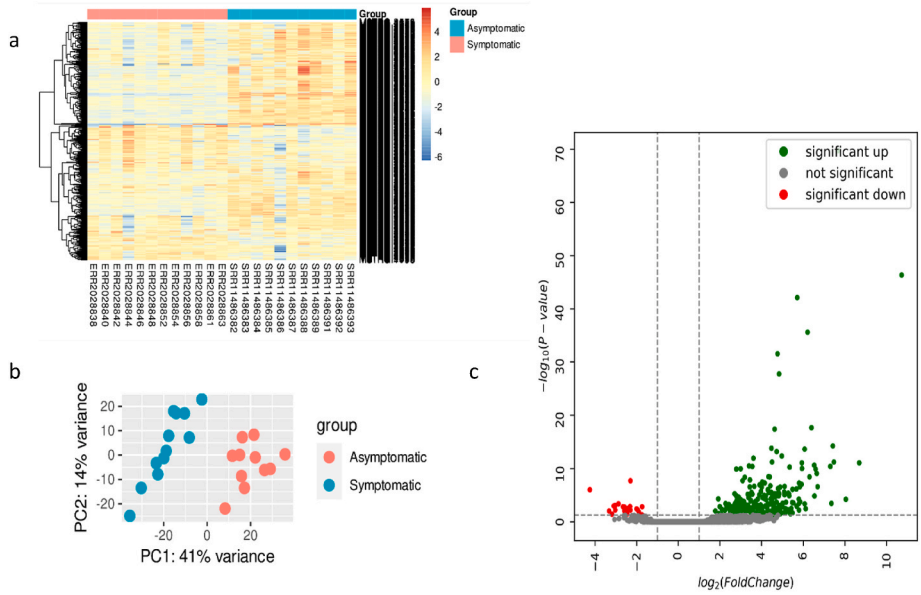

Fig. 6. Differential lncRNA expression of parasites isolated from asymptomatic malaria carriers and individuals presenting with uncomplicated symptomatic malaria: (**a**) Heatmap depicting hierarchical clustering of the significantly expressed lncRNA transcripts. Each row represents different annotated genes, while the horizontal bar at the top shows the different groups studied. The color of each vertical bar represents the expression level of specific differentially expressed genes from non-detected (yellow) to upregulated (in red) and to downregulated (in blue); (**b**) Principal component analysis showing clustering of the samples; (**c**) Volcano plot showing results for the RNA-Seq experiment as a function of fold change. Red points represent significantly upregulated genes, while green points represent downregulated genes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 5
The interaction of the Top 20 DE lncRNAs with genes implicated in the host-parasite interactions that are enriched in asymptomatic malaria.

Noncoding RNA	Target	Gene symbol	dG	ndG
MSTRG.1358	apyrimidinic_site_lyase_putative	PF3D7_0305600	−6.98	−0.17
MSTRG.3921	Rifin	PF3D7_1000600	−3.66	−0.31
MSTRG.3921	Pfmc_2TM	PF3D7_1100800	−4.72	−0.16
MSTRG.3921	Rifin	PF3D7_0100600	−4.30	−0.33
MSTRG.1293	Pfmc_2TM	PF3D7_0701600	−6.18	−0.13
MSTRG.1293	Pfmc-2TM	PF3D7_1100800	−6.93	−0.14
MSTRG.1293	Microneme associated antigen	PF3D7_0316000	−5.14	−0.17
MSTRG.5302	Microneme associated antigen	PF3D7_0316000	−6.71	−0.11
MSTRG.3150	Pfmc-2TM	PF3D7_1101700	−3.99	−0.14
MSTRG.3150	Pfmc-2TM	PF3D7_0701600	−3.99	−0.14
MSTRG.3150	Pfmc-2TM	PF3D7_0101300	−3.99	−0.14
MSTRG.3150	Pfmc-2TM	PF3D7_1100800	−4.31	−0.15
MSTRG.3150	Pfmc-2TM	PF3D7_0114100	−3.99	−0.14
MSTRG.3150	Pfmc-2TM	PF3D7_1039700	−3.99	−0.14
MSTRG.3150	Pfmc-2TM	PF3D7_0324100	−3.99	−0.14
MSTRG.3150	Rifin	PF3D7_0732700	−3.84	−0.10
MSTRG.3150	Rifin	PF3D7_0300500	−3.84	−0.10
MSTRG.4325	Stevor	PF3D7_0832900	−2.61	−0.17
MSTRG.4325	Rifin	PF3D7_0100600	−2.47	−1.24
MSTRG.4325	Apyrimidinic site lyase putative	PF3D7_0305600	−3.26	−1.63
MSTRG.4325	Stevor	PF3D7_0300400	−1.69	−0.84
MSTRG.4325	Rifin	PF3D7_0425700	−3.26	−1.63
MSTRG.4325	Microneme associated antigen	PF3D7_0316000	−3.96	−0.14
MSTRG.829	Dipeptidyl aminopeptidase 2	PF3D7_1247800	−2.80	−0.40
MSTRG.829	Apyrimidinic site lyase putative	PF3D7_0305600	−2.71	−0.13
MSTRG.829	Plasmepsin VIII putative	PF3D7_1465700	−4.59	−0.13
MSTRG.2879	Pfmc-2TM	PF3D7_0701600	−8.50	−0.41
MSTRG.2879	Pfmc-2TM	PF3D7_0101300	−8.50	−0.40
MSTRG.2879	Rifin	PF3D7_1000600	−7.31	−0.11
MSTRG.2879	LIMP protein	PF3D7_1207300	−9.08	−0.11
MSTRG.2879	Pfmc-2TM	PF3D7_0114100	−8.50	−0.40
MSTRG.4288	Stevor	PF3D7_1372800	−3.66	−0.11
MSTRG.4565	Plasmepsin VIII putative	PF3D7_1465700	−2.99	−0.13
MSTRG.3083	LIMP protein	PF3D7_1207300	−7.07	−0.14
MSTRG.3083	Stevor	PF3D7_0832900	−2.19	−0.36
MSTRG.3083	Rifin	PF3D7_0100600	−4.35	−0.12
MSTRG.3083	Stevor	PF3D7_1372800	−2.19	−0.37

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

Data used was obtained from NCBI sequence read archive

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101596.

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