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Plasmodium falciparum K13 expression associated with parasite clearance during artemisinin-based combination therapy

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Background: Delayed parasite clearance and, consequently, reduced efficacy of artemisinin-based combination therapies have been linked with *Plasmodium falciparum K13* gene SNPs in Southeast Asia. In Africa, significantly prolonged clearance has not yet been observed and the presently restricted variation in parasite clearance cannot be explained by K13 polymorphisms.

Objectives: Our aim was to study the *in vivo pfK13* transcriptional response in patients treated with artemether–lumefantrine and explore whether the *pfk13* transcripts can explain the patients' parasite clearance outcomes.

Patients and methods: A total of 47 Tanzanian children with microscopically confirmed uncomplicated *P. falciparum* malaria were hospitalized and received artemether–lumefantrine treatment (clinical trial ID: NCT00336375). RNA was extracted from venous blood samples collected before treatment initiation and at five more timepoints after treatment. cDNA was synthesized and *pfk13* transcripts measured by real-time PCR.

Results: A wide range of *pfk13* transcript variation was observed throughout all timepoints after artemether–lumefantrine treatment. Taking parasite clearance data together with the *pfk13* transcripts profile, we observed a negative correlation inferring that *pfk13* down-regulation is associated with longer parasite clearance time.

Conclusions: The findings suggest that a reduced PfK13 transcriptional response may represent a first step towards artemisinin tolerance/resistance.

Introduction

Sustained artemisinin combination therapy (ACT) efficacy is dependent on the actions of both the long half-life component and the artemisinin derivative.

In Southeast Asia, a change in the parasite response to artemisinins has been observed, essentially characterized by a significant increase in infection clearance times.[1,2] This phenotype has also been associated with clinical failure after ACT treatment.[1,3,4] Of particular concern is the possibility that the reduction of artemisinin efficacy may also spread to Africa, where 90% of all malaria deaths occur, jeopardizing the United Nations sustainable developmental goal of 'ending the malaria epidemic' by 2030.[5]

Increased parasite clearance (PC) times have been firmly associated with sequence variation in the gene coding for the cytoplasmic K13 propeller-like protein (PF3D7_1343700).[6] Genetically modified *K13* loci confirmed the role of K13 propeller mutations in artemisinin resistance. Intriguingly, it was also observed that the levels of artemisinin resistance exhibited by strains with genetically modified K13 were lower compared with those of clinical isolates carrying the same mutations,[7] indicating the existence of other determinants beyond the known mutations. This is supported by field reports of artemisinin resistance independent of K13 mutations, emphasizing the need for an understanding of resistance factors beyond K13 mutations.[8] One possibility barely studied is the influence of *in vivo* K13 expression levels, prior to treatment

and after artemisinin impact. In this work, we have studied the *in vivo pfK13* expression in Tanzanian patients treated with artemether–lumefantrine and explore its association with patients' PC parameter outcomes.

Methods

Study site and sample collection

The trial under study, described in detail elsewhere, was conducted at the Fukayosi Primary Health Care Centre, Bagamoyo District, Tanzania[9] in accordance with the Declaration of Helsinki and Good Clinical Practice. It was granted ethics clearance by the National Institute for Medical Research, Dar es Salaam, Tanzania, and the Regional Ethics Committee, Stockholm, Sweden. Informed consent was obtained from parents/guardians of all children enrolled [Clinical Trials (US), identifier NCT00336375]. Briefly, 50 children with microscopically confirmed uncomplicated *Plasmodium falciparum* malaria were hospitalized and received artemether–lumefantrine treatment in six doses over 3 days. Venous blood samples were collected before treatment initiation (0 h) and at nine more timepoints (2, 4, 8, 16, 24, 36, 48, 60 and 72 h) after initiation of treatment. Parasite densities were determined by Giemsa staining, and PC was measured and documented as the proportion of patients with positive microscopy.[9]

Molecular analysis

RNA was extracted from venous blood of the 50 enrolled patients using an ABIPRISM H6100 Nucleic Acid PrepStation (Applied Biosystems, Fresno, CA, USA). Not expecting to find parasitic RNA 24 h post-treatment, total RNA (human and parasite) quality and quantity were measured using the AgilentRNA6000Pico total RNA assay in an Agilent 2100 Bioanalyser™ (Agilent, Santa Clara, CA, USA) and standardized prior to cDNA synthesis for the first six timepoints [0 h (before treatment) and 2, 4, 8, 16 and 24 h after treatment initiation]. Three out of 50 patients sampled had low total RNA quality and therefore were excluded from further transcript analysis.

Quantitative PCR was performed in triplicate with custom MGB TaqMan® probes for the *K13* gene, in an ABIPRISM® 7900HT Sequence Detection System (Applied Biosystems). The housekeeping gene serine-tRNA ligase (PF3D7_0717700), shown to be transcribed stably throughout different intraerythrocytic stages,[10–13] was used as an endogenous control (normalizer).

Oligonucleotides were as follows: K13 6-FAM, NFQ probe, 5′-ACGCCAGCATTGTTG-3′; PF3D7_0717700 VIC, TAMRA probe, 5′-TGAAAC TATAGAATCAAAAAGGTTACCACTCAAATACGCT)-3′; K13 primers, fw 5′-GTG GATTTGATGGTGTAGAATATTTAAATTCGA-3′, rev 5′-GCTTTTTTGGTAGACATAG GTGTACACA-3′; and PF3D7_0717700 primers, fw 5′-CCTCAGAACAACC ATTATGTGCTT-3′, rev 5′-TGTGCCCCTGCTTCTTTTCTA-3′. Amplification conditions were 40 cycles of 95°C (15 s) + 60°C (1 min). For each patient, *pfK13* transcript fold change in expression was calculated by the $2^{-\Delta\Delta Ct}$ method[14] in which the threshold cycle number (Ct) was normalized to the Ct of the housekeeping gene (PF3D7_0717700) for all timepoints (ΔCt) and ΔΔCt calculated using the ΔCt from timepoint 0 h (before treatment) as a calibrator. Spearman correlation was applied to assess linear relationships between *pfk13* expression variation throughout time and between PC parameters. Patient data were stratified in two groups defined as *pfK13* transcript expression of more or less than 1-fold change (1-fold change represents no *pfK13* transcript difference after treatment; ΔΔCt=0; $2^{\circ}=1$). The Mann–Whitney test was used on the patient and PC data grouped with more or less than 1-fold change of *pfk13* transcripts. The Cox proportional hazards analysis (Kaplan–Meier curve) was used to assess the effect of *pfk13* transcripts on the $PC_{50\%}$ outcome. The analyses were done with GraphPad Prism version 7.00 software.

Sequencing of the *pfk13* resistant loci, encompassing the SNP PfK13 C580Y, was performed in all infections using the primers 5′-CAA ATATTGCTACTGAAACTATG-3′ and 5′-TGTGCATGAAAATAAATATTAAAGAAG-3′, and sequencing primer 5′-AGGTGGATTTGATGGTGTAGAA-3′.

Results

We obtained *pfk13* transcript expression data for the 47 infections analysed at the six timepoints under review, with the exception of the 24 h point, at which 3 of the 47 infections did not provide reliable *pfk13* transcript results.

After artemether–lumefantrine treatment initiation, a wide range of *pfk13* transcript fold change in expression was observed throughout all timepoints (Figure 1a). Using the *pfk13* expression levels of each infection before treatment initiation (0 h) to understand the fold change in expression after treatment initiation, the expression ranged from 0.2- to 4.1-fold after 2 h, 0.1- to 4.4-fold after 4 h, 0.1- to 8.3-fold after 8 h, 0.1- to 7.7-fold after 16 h and 0.04- to 6.1-fold after 24 h.

Strong correlations of *pfk13* expression were observed between all post-treatment timepoints (Figure 1b), with individual infection variation defined at the first timepoint after treatment (2 h) and maintained over time (Figure 1a; black connection lines). The data were not normally distributed (Shapiro–Wilk, W=0.47–0.66, $P<0.0001$), showing skewness for decreased expression of *pfk13* response (Figure 1a; yellow bar showing median values). This observed differential expression was not linked to genetic variability at the *pfk13* gene-resistant loci since all infections were found to be WT from amino acid 464 up to the 3′-terminus of the ORF.

By taking PC data (described in Table 1) together with the *pfk13* expression profile over time, we noticed a clear clade based on Spearman correlation inferring that *pfk13* down-regulation is associated with longer PC time (Figure 1b). To confirm this, infections were stratified into two groups defined by infections with increased or decreased expression of *pfk13* by more than or less than 1-fold, respectively (Figure 1a, red dashed line cut-off of 1 signifying no variation compared with before treatment initiation; $2^{-\Delta\Delta Ct}=2^{\circ}$). Reinforcing the negative correlation result (Figure 1b), after 2 h of treatment, a significant difference ($P<0.001$) in PC times was observed, with higher mean $PC_{50\%}$ values in the infections group with reduced *pfk13* expression. This significant difference was maintained for the $PC_{90\%}$, $PC_{95\%}$ and $PC_{99\%}$, but did not reach significance for the PC slope half-life (Table 1). To better understand the risk factor of the two groups (*pfk13*<1 and *pfk13* > 1) to predict the PC time, we performed a Kaplan–Meier survival analysis using the $PC_{50\%}$. Significant difference in survival time for $PC_{50\%}$ is observed between the two groups (median *pfk13*<1=7.1 h; *pfk13* > 1=1.9 h) (Figure 1c).

Discussion

SNPs at PfK13 have been identified as molecular markers of ACT resistance among Southeast Asian patients.[15] These have been found in <3% in Africa,[16] possibly explaining the lack of extreme cases of long PC time. Variation in this phenotype nevertheless exists and has been well documented.[17] The question remains of whether there are other factors beyond ORF variation that might explain clearance differences in Africa. In this molecular prospective approach, we explored for the first time the transcriptional profile of *pfk13* during artemether–lumefantrine therapy. Since transcriptional variation appears to be higher than genetic

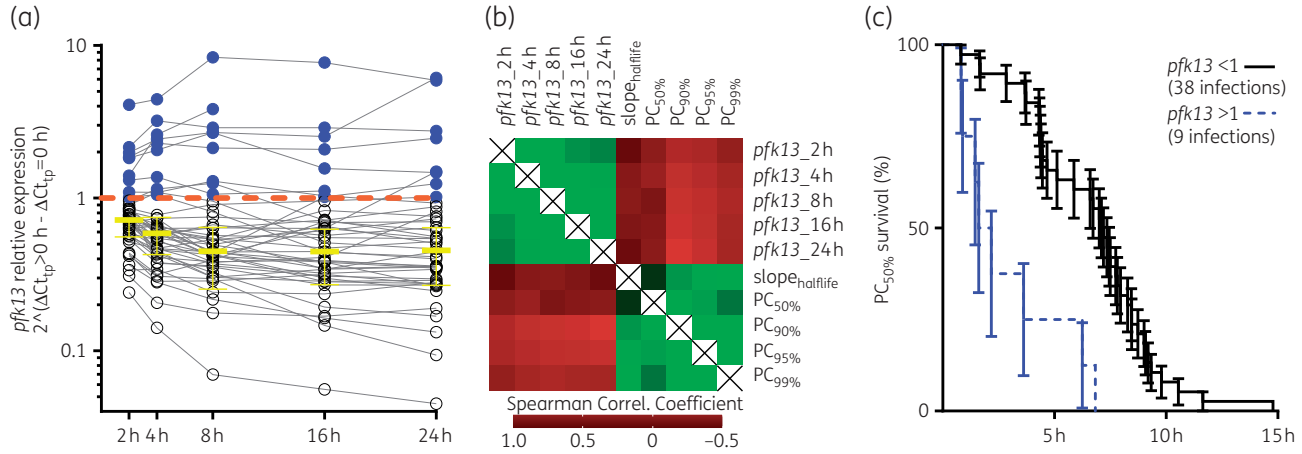

Figure 1. Changes in *pfk13* transcripts and PC in patients treated with artemether–lumefantrine. (a) *pfk13* relative expression by fold change after treatment compared with before treatment for every analysed patient sample over time. Connection lines demark individual patient samples over time. The dashed red line delineates the threshold for *pfk13* up-regulation (blue circles, >1-fold) and down-regulation (open circles, <1-fold). Median *pfk13* expression by timepoint is represented with a yellow dash, and standard error of the 47 samples. *pfk13* expression fold change was calculated by the $2^{-\Delta\Delta Ct}$ method normalized with the housekeeping gene PF3D7_0717700 and calibrated with paired infection data before treatment initiation (time 0 h). (b) The heat map shows the Spearman correlation coefficients of pairwise comparisons of the *pfk13* relative expression at different time-points and the PC parameters of matching patients. (c) Kaplan–Meier survival for $PC_{50\%}$ in the two groups defined in (a). χ^2 [Log-rank (Mantel–Cox) test]: 17.7; $P < 0.0001$. $PC_{50\%}$ median survival: *pfk13*<1=7.1 h; *pfk13* > 1=1.9 h. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 1. Characteristics of the studied population and differential *pfk13* expression

	Total (n=47)			*pfk13*<1[a] (n=38)		*pfk13* > 1[a] (n=9)		
	mean	SD	range	mean	SD	mean	SD	*P* value
Age (months)	50.0	30.0	12–119	50.7	28.6	46.9	37.1	0.510
Weight (kg)	14.3	5.5	8–30	14.3	5.4	14.0	6.4	0.474
Parasite density (Pf/mm^3 blood)	60 171	51 805	2120–200 400	61 744	50 076	53 529	61 420	0.397
Temperature (°C)	37.9	1.0	36.2–40.8	38.0	1.1	37.7	0.5	0.995
Slope half-life	2.5	1.1	0.5–5.2	2.6	1.1	2.1	1.1	0.343
$PC_{50\%}$ (h)	6.0	3.2	0.8–14.8	6.7	2.9	3.0	2.4	0.0008
$PC_{90\%}$ (h)	11.6	4.8	2.1–21.8	12.6	4.3	7.3	4.8	0.007
$PC_{95\%}$ (h)	14.1	5.7	2.6–26.2	15.2	5.2	9.4	5.8	0.016
$PC_{99\%}$ (h)	19.9	8.0	3.9–37.8	21.2	7.5	14.4	8.3	0.052

[a]*pfk13* expression profile after 2 h treatment. *pfk13* > 1 represents the infection group with fold change in relative expression above 1, and *pfk13*<1 represents the infection group with fold change in relative expression below 1. Pf, *P. falciparum*.

variation in our study, we have hypothesized that it might have an important role in the early stage of development of drug resistance.

Previously, a large *ex vivo* transcriptomic study of *P. falciparum* isolates from Southeast Asian and African patients, collected before treatment and encompassing normal and delayed PC time, revealed high variation in PfK13 expression, although not correlated with artemisinin resistance.[18] Our prospective study describing *pfk13* expression during treatment also showed a large range of transcription levels, with 20 times the dynamic range observed after 2 h of treatment. Interestingly, the intrinsic transcription features of each individual infection prevailed during the 24 h of study (Figure 1a).

The K13 propeller domain encodes six Kelch motifs theoretically playing a role in protein degradation via polyubiquitination or involved in cell response to oxidative stress. The C580Y mutation was shown to decrease affinity for a protein substrate, thereby increasing its steady-state levels by reducing ubiquitination and proteasomal degradation.[19] Parasites responding with decreased expression of *pfk13* could therefore act as a similar outcome, translated into a slow phenotype and manifested as longer PC times. This hypothesis could explain the clear negative correlation observed between the clearance parameters of the infections herein analysed and their *pfk13* transcriptional response (Figure 1b).

The difference between the *pfk13* gene expression groups could also be explained by the different ring developmental stage at the

sample collection time, as recently described by Gibbons *et al*.[20] These authors explored the transcriptional profiling of a recombinant *K13* strain, with increased susceptibility to artemisinins, revealing down-regulation of *pfk13* expression at early rings and up-regulation at the early trophozoite stage. They could distinguish this short period of stage development through a full transcriptomic approach and not by microscopic observation of morphological changes, constraining the validation of this hypothesis in our study.

A Kaplan–Meier survival analysis using the $PC_{50\%}$ for the two groups (*pfk13*<1 and *pfk13*>1) revealed a significant difference in survival time ($PC_{50\%}$ medians: *pfk13*$<1=7.1$ h; *pfk13*$>1=1.9$ h) (Figure 1c), demonstrating that there is a prognostic value of the K13 transcriptional profile to predict the PC time.

A larger study with full transcriptomic approach would be needed to dissect this phenotypic clearance outcome more comprehensively.

Our study thus revealed the *pfk13* transcripts as a potential additional factor relevant for *in vivo* PC outcome of ACT, at least in the scenario of artemether–lumefantrine managing PfK13 WT African parasites.

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Transparency declarations

None to declare.

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