

# Impairment of Invasion and Maturation and Decreased Selectivity of *Plasmodium falciparum* in G6PD Viangchan and Mahidol Variants

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**Background.** Protection against *Plasmodium falciparum* is observed in a population deficient in glucose-6-phosphate dehydrogenase (G6PD), particularly in African and Mediterranean regions. However, such protection remains unknown among G6PD-deficient individuals in Southeast Asia.

**Methods.** In this study, we assessed the invasion and maturation of *P. falciparum* K1 in a culture of erythrocytes isolated from Thai subjects carrying Viangchan (871G > A) and Mahidol (487G > A).

**Results.** We found that the parasites lost their ability to invade hemizygous and homozygous G6PD-deficient erythrocytes of Viangchan and Mahidol variants in the second and third cycles of intraerythrocytic development. It is interesting to note that *P. falciparum* parasites selectively grew in erythrocytes from hemi- and homozygous genotypes with normal G6PD activity. Moreover, externalization of phosphatidylserine upon *P. falciparum* infection was significantly increased only in Viangchan hemizygous variant cells.

**Conclusions.** This study is the first to show that blockage of invasion in long-term culture and potentially enhanced removal of parasitized erythrocytes were observed for the first time in erythrocytes from Viangchan and Mahidol G6PD-deficient individuals.

**Keywords.** glucose-6-phosphate dehydrogenase (G6PD); malaria; *Plasmodium falciparum*.

Glucose-6-phosphate dehydrogenase (G6PD) is critical for cells to survive under oxidative stress [1]. Glucose-6-phosphate dehydrogenase deficiency is the most common inherited enzymopathy, affecting ~400 million people worldwide [2]. More than 200 mutations in G6PD-coding regions cause various degrees of deficiency [2]. Because of X-chromosome linkage, hemizygous males and homozygous females are markedly deficient in G6PD activity, whereas the distribution of G6PD activity in populations of heterozygous females is widely distributed from levels close to those observed in hemizygous males/heterozygous females to those observed in normal phenotypes. Although most individuals with G6PD deficiency

are asymptomatic, each is vulnerable to neonatal jaundice and acute hemolytic anemia when exposed to drugs or infection [1].

Geographically, the prevalence of G6PD deficiency overlaps with malaria-endemic areas [3, 4]. Accordingly, a protective role of G6PD deficiency against malaria has been proposed, even though the findings are controversial. A study of children in Mali reported a similar protection associated with both genotypes but not heterozygous females [5]. Despite a reduction in the risk of cerebral malaria with heterozygous mutant *G6PD* alleles, an increased risk of severe malarial anemia has been reported [6, 7]. In addition, a study of the *G6PD* c0.202T allele in children in Kilifi County, Kenya, showed a protective effect against severe malaria in females carrying heterozygous variants but not in hemizygous males or homozygous females [8]. A meta-analysis of G6PD deficiency revealed potential protective effects against uncomplicated malaria in African heterozygotes but not in Asian heterozygotes [9, 10]. Another meta-analysis of *G6PD* Mediterranean variants revealed a protective effect against symptomatic *Plasmodium vivax* malaria in both hemizygous males and homozygous females and, to a lesser extent, in heterozygous females in the Pashtun ethnic group in Afghanistan [9]. In mainland Southeast Asia, *G6PD* Mahidol and Viangchan variants are the 2 most common mutations [11]. Epidemiological studies of both variants reported protective effects against *P. vivax* in male hemizygotes and female heterozygotes but not against *falciparum*

Received 30 April 2021; editorial decision 16 September 2021; accepted 20 September 2021; published online 24 September 2021.

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The Journal of Infectious Diseases® 2022;225:1238–47

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malaria [12, 13, 14, 15]. Despite these discrepancies, G6PD deficiency likely confers protection against malaria, particularly in female heterozygotes/homozygotes, male hemizygotes, or both.

Many studies have investigated the mechanisms underlying the protective effect of G6PD deficiency. In the first cycle of intraerythrocytic development, G6PD-deficient erythrocytes were refractory to parasite growth [16, 17, 18, 19]. However, serial passages of *Plasmodium falciparum* in G6PD-deficient erythrocytes revealed parasite growth adaptation [19]. Moreover, membrane damage in ring-stage parasites grown in G6PD-deficient erythrocytes enhanced phagocytosis [20]. These studies of African and Mediterranean genotypes demonstrate that invasion blockage, growth arrest, and loss of host cell membrane integrity likely contribute to protection against malaria. However, the protective role of G6PD deficiency remains largely unknown in Southeast Asia. To investigate whether the protective effect of G6PD deficiency is relevant to invasion and growth retardation of parasites, *P. falciparum* strain K1 parasites were cultured with erythrocytes from individuals carrying G6PD Mahidol and Viangchan variants. To our knowledge, this is the first study to investigate protective effects for all allelic types: hemizygotes, heterozygotes, and homozygotes. Furthermore, selective invasion of parasites and membrane changes in G6PD-deficient erythrocytes were examined.

## METHODS

### Ethics Statement

The research protocol was approved by the Ethical Review Committee for Research Involving Human Subjects in Research, Chulalongkorn University, in accordance with the International Conference on Harmonization-Good Clinical Practice ([ICH-GCP] COA. No. 156/2011).

### Sample Collection

Venous blood samples of normal G6PD (n = 15), Viangchan (871G > A) G6PD (n = 15; hemizygote = 5, heterozygote = 5, homozygote = 5), and Mahidol (487G > A) G6PD (n = 15; hemizygote = 5, heterozygote = 5, homozygote = 5) participants were obtained using ethylenediamine tetraacetic acid (EDTA) and acid-citrate-dextrose ([ACD] Sigma, St. Louis, MO)-containing vacutainers for genotyping and culture, respectively. Hemoglobin abnormalities were examined using either hemoglobin electrophoresis on a cellulose acetate membrane or an automatic high-performance liquid chromatography system. The hemoglobin concentration was measured using an automated cell counter (ADVIA 120 System; Siemens Healthineers). All subjects had normal hemoglobin levels and no hemoglobinopathies.

### Deoxyribonucleic Acid Extraction and Identification of *G6PD* Mutations

Deoxyribonucleic acid (DNA) was extracted using a Qiaquick Blood DNA extraction kit (QIAGEN) and subjected to polymerase chain reaction (PCR)-restriction fragment length polymorphism as previously described [21]. To identify less frequent *G6PD* mutations,

amplification of exons 6, 9, 11, 12, and 13 was performed by PCR, and primer pairs were designed using Primer 3 software. Fluorescence-based cycle labeling of amplicons was performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The labeled products were analyzed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). All mutations and polymorphisms were confirmed using both reverse and forward primers. Sequencing results were confirmed by Sanger Sequencing and Fragment Analysis Software Trials (Thermo Fisher Scientific) [21].

### Measurement of Hemoglobin Concentration and Glucose-6-Phosphate Dehydrogenase Activity

The ACD-preserved blood samples were stored at 4°C and subjected to G6PD activity measurement within 24 hours postcollection following the manufacturer's instructions (BIOLABO). In brief, leukocytes were removed, and erythrocytes were lysed at 4°C. After enzymatic reaction for 5 minutes, nicotinamide adenine dinucleotide (NADH) in the hemolysate was measured using 340-nm visible light spectrophotometry (Randox Daytona, IMED). The spectrometric measurement was performed at 37°C within 5 minutes. The rate of NAD phosphate (NADPH) production was measured as the change in absorbance ( $\Delta$ Abs) per minute. The hemoglobin concentration was quantified using the cyanmethemoglobin method. The G6PD activity and hemoglobin concentration were determined in duplicate. The G6PD enzyme activity was calculated, and the result is expressed in international units (IU) per gram of hemoglobin (IU/gHb) using the following formula according to the manufacturer's instructions. Levels of G6PD deficiency were established according to World Health Organization guidelines, and G6PD activity less than 1.5 IU/gHb was regarded as indicating a deficiency [22]. Moreover, the normal range of G6PD activity for the Thai population was 7.9–16.3 IU/gHb. Thus, a value of 7.9 IU/gHb was set as a cutoff for normal and heterozygous G6PD deficiency.

$$\text{G6PD (IU/gHb)} = \frac{(\Delta\text{Abs}/\text{min}) \times 5000}{\text{Hb(g/dL)}}$$

### *Plasmodium falciparum* K1 Strain Culture

Cell culture was performed within 24 hours as described previously [23]. The malaria culture medium consisted of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5.96 g/L HEPES, 2 g/L NaHCO<sub>3</sub>, 80 µg/mL gentamicin, and 10% heat-inactivated AB human serum. The parasite culture was maintained in 5% hematocrit and 5% CO<sub>2</sub> at 37°C. Parasite development was assessed using Giemsa staining of thin blood smears and observed under a light microscope [24, 25].

### Parasite Invasion and Maturation Assays

A highly synchronous culture of parasites was prepared according to previous reports [26]. In brief, parasites were cultured to obtain mostly ring-stage trophozoites and were mixed with 5% D-sorbitol. Ninety-five percent synchronicity of ring-stage parasites was

acceptable. After 20–24 hours of culture, schizonts were separated using Percoll gradients [27]. The purified schizonts were adjusted to 1% parasitemia with freshly prepared normal or G6PD-deficient erythrocytes at a final hematocrit of 5%. *Plasmodium* invasion and maturation were assessed according to a previous report, with some modifications [20, 28] (Figure 3A). In brief, invasion of erythrocytes was assessed at 12, 60, and 108 hours postinoculum (hpi) of purified schizonts (Figure 3A); maturation was assessed at 36, 84, and 132 hours. For the comparison among blood samples, first-cycle invasion is the ratio of ring parasitemia at 10–12 hpi to the inoculated schizont parasitemia; the second-cycle invasion is the ratio of ring parasitemia at 60–62 hpi to the trophozoite parasitemia at 36–38 hpi; and the third-cycle invasion is the ratio of ring parasitemia at 106–108 hpi to the trophozoite parasitemia at 82–84 hpi. The following ratios were calculated for the maturation assay: (1) trophozoite parasitemia at 36–38 hpi/ring parasitemia at 10–12 hpi was set as first-cycle maturation; (2) trophozoite parasitemia at 82–84 hpi/ring parasitemia at 60–62 hpi was set as second-cycle maturation; and (3) trophozoite parasitemia at 132–134 hpi/ring parasitemia at 106–108 hpi was set as third-cycle maturation. Thin blood films were prepared to evaluate parasitemia (100 fields of 100× objective lenses) and parasite development at each time point. The developmental stages of *P falciparum* were morphologically distinguished based on criteria described in previous reports [24, 25].

#### Measurement of Intracellular Glucose-6-Phosphate Dehydrogenase Activity and Parasitemia

Intracellular G6PD activity was examined using nitro blue tetrazolium chloride (Sigma-Aldrich, Hamminkeln, Germany), with NADH being reduced to dark-colored formazan [29, 30]. Thus, normal G6PD-containing erythrocytes contain dark-purple granules, hereinafter called G6PD-positive cells (GPCs). In contrast, G6PD-deficient erythrocytes, called G6PD-negative cells (GNCs), remain unstained. Parasitized erythrocytes among GPCs and GNCs were counted. The selectivity of parasite invasion is expressed as a selectivity index (SI): a ratio of the number of malaria parasites in GPCs to that in GNCs among 1000 cells of GSCs plus GNCs. The procedure and calculation of the SI were adapted from Nantakomol et al [31]:

$$SI = \frac{\text{Number of } P \text{ falciparum} - \text{infected GPCs}}{\text{Number of } P \text{ falciparum} - \text{GNCs}}$$

#### Measurement of Phosphatidylserine-Externalizing Erythrocytes

Erythrocytes were incubated with FITC-conjugated annexin-V (Becton Dickinson Biosciences), a specific marker for phosphatidylserine (PS), and phycoerythrin (PE)-conjugated glycophorin A (Becton Dickinson Biosciences), as described previously [32]. At 96 hours postinfection, the samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences). The mean fluorescence intensity of externalized PS was assessed using FlowJo version 1.0.

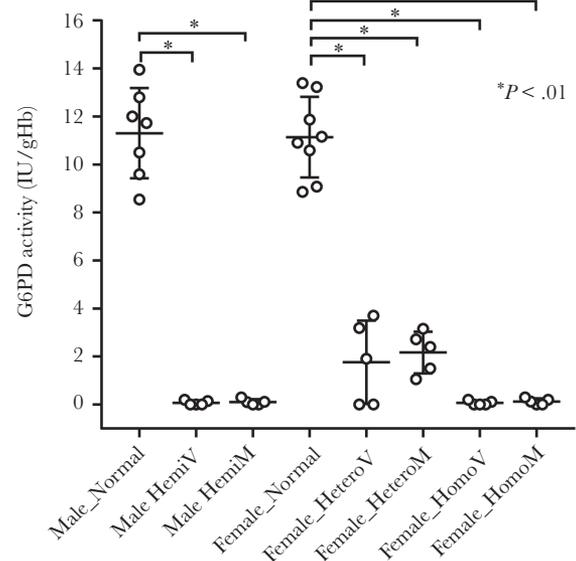
#### Statistical Analysis

Data are presented as the means ± standard deviation. GraphPad Prism 7 was used for data display and statistical analyses. Differences were evaluated using Student's *t* test (Mann-Whitney test). A *P* value less than .05 was considered statistically significant.

## RESULTS

#### Glucose-6-Phosphate Dehydrogenase Activity in the Studied Subjects

No mutations in exons 6, 9, 11, 12, or 13 were identified molecularly. Both females (*n* = 28) and males (*n* = 17) expressed Viangchan (33% male and 67% female) and Mahidol (33% male and 67% female) *G6PD* variants (Supplementary Table 1). There was no difference in G6PD activity between wild-type G6PD male and female subjects (*P* = .95) (Figure 1). However, G6PD activity was significantly decreased in erythrocytes of males carrying hemizygous Viangchan and Mahidol variants. Likewise, erythrocytes of female heterozygotes and homozygotes of both variant types exhibited lower enzymatic activity than wild-type cells (*P* < .05) (Figure 1). The G6PD-deficient erythrocytes of female heterozygotes exhibited higher G6PD activity than male hemizygotes, which lack normal G6PD erythrocytes. Severe G6PD deficiency was observed in both female homozygotes and male hemizygotes.



**Figure 1.** Glucose-6-phosphate dehydrogenase (G6PD) activity of the studied subjects. Scatter plot of G6PD activity in each genotype of *G6PD*. Each circle represents an individual subject. For each column, horizontal bars indicate the mean, and vertical bars indicate the standard deviation. Stars indicate a statistically significant difference in the values of the 2 groups. HemiM, Hemizygous Mahidol; HemiV, Hemizygous Viangchan; HeteroM, Heterozygous Mahidol; HeteroV, Heterozygous Viangchan; HomoM, Homozygous Mahidol; HomoV, Homozygous Viangchan.

### Parasitemia in Normal and Glucose-6-Phosphate Dehydrogenase-Deficient Erythrocytes

To determine the cycle numbers in which *P. falciparum* could develop in G6PD-deficient erythrocytes, highly synchronous schizonts were cultured in erythrocytes with various degrees of G6PD activity. At 12 hpi, parasitemia was not different between cultures of normal and G6PD-deficient erythrocytes (Figure 2A). At the second and third cycles of culture, parasitemia in G6PD-deficient erythrocytes decreased significantly compared with that in normal erythrocytes, regardless of the mutation type or zygosity ( $P < .01$ ) (Figure 2A, middle and right panels).

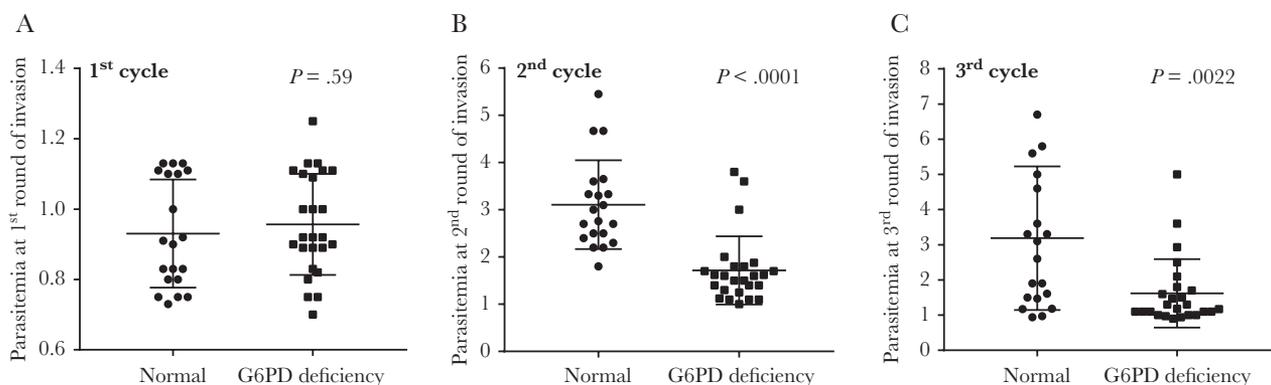
### Invasion and Maturation of the Parasite in Glucose-6-Phosphate Dehydrogenase (G6PD)-Normal and G6PD-Deficient Erythrocytes

To assess the ability of *P. falciparum* to invade and mature in G6PD-deficient erythrocytes, parasites were cultured for 3 cycles. There was no statistically significant difference in the invasion or maturation rates of *P. falciparum* in G6PD-deficient erythrocytes in the first cycle (left panel in Figure 3B and C). Intriguingly, a decline in the invasion rate of parasites cultured with cells from males hemizygous and females homozygous for the Viangchan and Mahidol variants was observed in the second cycle (Figure 3B, middle panel with  $P < .01$ ), although the parasites were able to develop into trophozoites, with a tendency toward an increase in maturation in Mahidol hemizygote cells (Figure 3C, middle panel). The observed decrease in the invasion rate continued at a higher rate in the third cycle of development in erythrocytes from hemizygous males and females homozygous for the Viangchan and Mahidol variants (Figure 3B, right panel). The third invasion rates in cells from females carrying heterozygous Viangchan and Mahidol variants were not different compared with those in cells from females carrying wild-type alleles. It is interesting to note that the invasion rate was likely decreased in female homozygotes, but the difference was not statistically significant. Moreover, an increasing trend with regard to maturation was detected in some cells from males hemizygous for the Viangchan and Mahidol

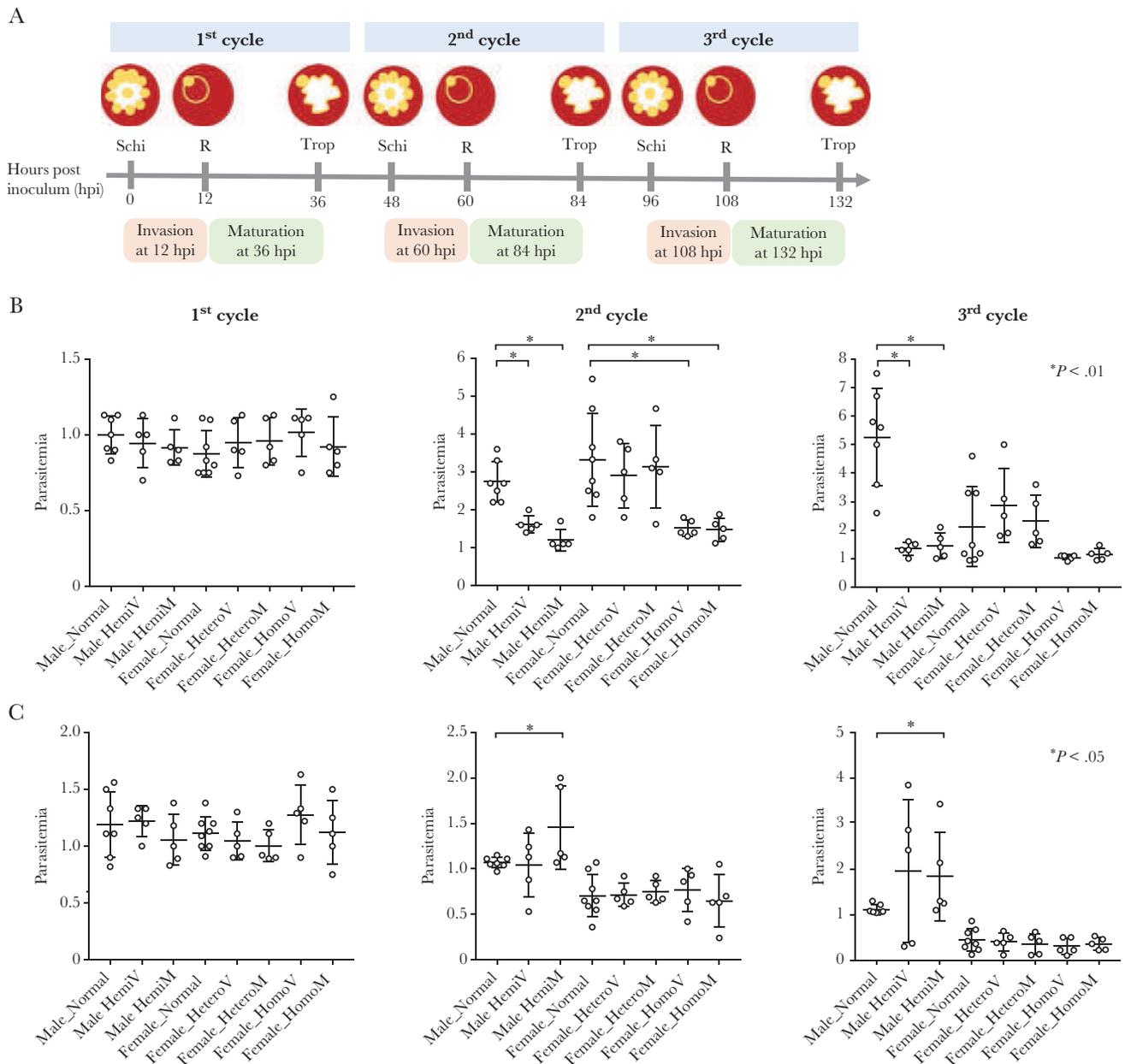
variants, but a similar tendency was not detected in all females (Figure 3B, right panel). All results are also shown in Table 1.

### Selectivity of Parasite Growth in Different Erythrocyte Phenotypes

To examine the preferential invasion and growth of parasites, we enumerated the number of trophozoites living in normal and G6PD-deficient erythrocytes. At 96 hours postculture with purified schizonts, the GPCs (arrowheads in Figure 4A) appeared dark blue, whereas the GNCs (arrows in Figure 4A) were unstained. In agreement with Figure 2A, the percentages of parasitized GNCs were significantly lower than those of parasitized GPCs in male hemizygous Viangchan and Mahidol and female homozygous Viangchan and Mahidol variants. However, there was no difference in the proportion of parasitized GNCs and GPCs of female heterozygous Viangchan and Mahidol variants (Supplementary Table 2). There was no difference in the average SI of cells from males and females carrying wild-type G6PD. Thus, we pooled individual data for statistical analysis. The average SI of hemizygous Viangchan and Mahidol variants was significantly higher than that of wild-type G6PD erythrocytes ( $P = .0079$ ) (Table 2, Figure 4B). In contrast, cells from females harboring heterozygous Viangchan and Mahidol variants showed SI values similar to those of wild types. It is significant that the SIs of cells from homozygous females were higher than those of cells from heterozygous females ( $P = .0079$ ). Moreover, between Viangchan and Mahidol variants, the average SIs of cells from hemizygous males and homozygous females were not different; however, these values were still 3-fold higher than those of wild-types ( $P < .01$ ). Of note, there were no significant differences in the SI of Viangchan and Mahidol variants for each genotype. Next, we examined the correlation between G6PD activity and SI in a quantitative manner, and SI correlated conversely with G6PD activity, regardless of sex (Figure 4C). Cells



**Figure 2.** Parasitemia in normal and glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes. At 10 to 12 hours post-schizont culture, Giemsa-stained, parasitized erythrocytes were enumerated under a microscope. In each scatter plot, dark dots represent parasitemia according to the level of G6PD activity, namely, normal and deficient, according to the World Health Organization classification. Both Mahidol and Viangchan G6PD deficiency variants were included. *Plasmodium falciparum* strain K1 was cultured for 3 cycles of development. (A) The first round of invasion. (B) The second round of invasion. (C) The third round of invasion. For each column, horizontal bars indicate the mean, and vertical bars indicate the standard deviation.



**Figure 3.** Invasion and maturation rates in 3 cycles of intraerythrocytic development. (A) Diagram showing the time points at which invasion and maturation were assessed. (B and C) Scatter plots of invasion and maturation rates. Dots represent the parasitemia of individual subjects and are grouped according to genotype. For each genotype, horizontal bars indicate the mean, and vertical bars indicate the standard deviation. Stars indicate statistically significant differences in the values of the 2 groups. HemiM, Hemizygous Mahidol; HemiV, Hemizygous Viangchan; HeteroM, Heterozygous Mahidol; HeteroV, Heterozygous Viangchan; HomoM, Homozygous Mahidol; HomoV, Homozygous Viangchan; Schi, Schizont; Trop, Trophozoite.

from individuals with G6PD activity less than 0.3 IU/gHb, involving hemizygous and heterozygous variants, were likely to have an SI greater than 2.0.

**Phosphatidylserine Externalization on Glucose-6-Phosphate Dehydrogenase-Deficient or Normal Erythrocytes After *Plasmodium falciparum* K1 Infection**

Enhanced clearance of parasitized erythrocytes causes low parasitemia [33, 34]. Indeed, erythrocytes are regularly eliminated from blood circulation via recognition of PS externalization by macrophages of the reticuloendothelial system [35,

36]. Hence, we hypothesized that *P falciparum* infection in G6PD-deficient erythrocytes may induce PS externalization to a higher degree than that occurring in normal erythrocytes. To ensure that the 96-hour culture period is suitable for assessing the effect of G6PD activity on PS externalization, we first examined the stability of G6PD activity at 0 and 96 hours postinfection. The level of G6PD activity remained stable in the culture (Supplementary Figure 1A). Moreover, the level of PS exposure on the 96-hour cultured infected erythrocytes significantly increased compared with that at the time point

**Table 1. Percentage of Parasite Invasion and Maturation in G6PD-Normal and G6PD-Deficient Erythrocytes**

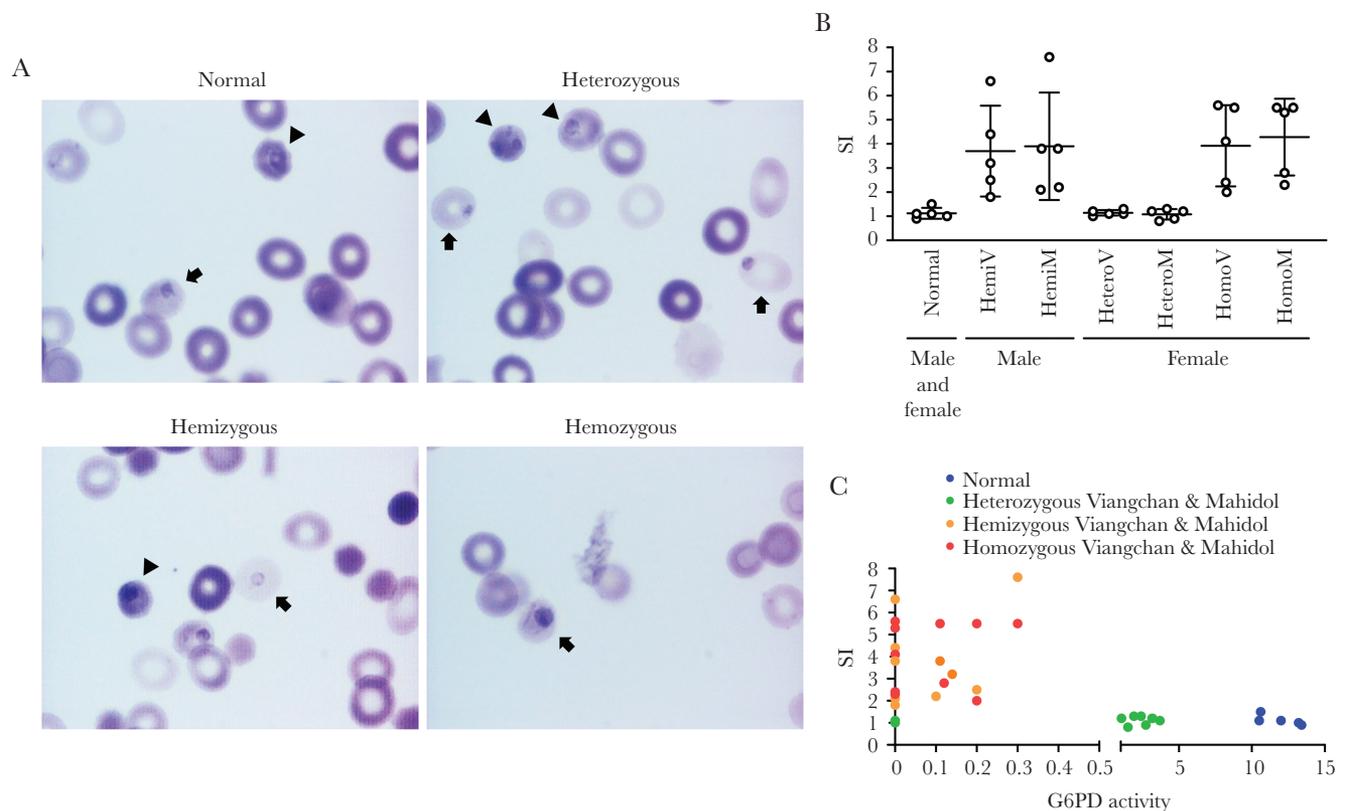
|                         | First Cycle |             | Second Cycle |             | Third                    |                            |
|-------------------------|-------------|-------------|--------------|-------------|--------------------------|----------------------------|
|                         | Invasion    | Maturation  | Invasion     | Maturation  | Invasion                 | Maturation                 |
| G6PD normal (n = 15)    | 0.48 ± 0.02 | 0.51 ± 0.08 | 0.91 ± 0.13  | 0.58 ± 0.28 | 1.95 ± 0.08              | 0.61 ± 0.07                |
| G6PD Viangchan (n = 13) | 0.45 ± 0.08 | 0.44 ± 0.04 | 0.78 ± 0.03  | 0.46 ± 0.30 | 0.75 <sup>a</sup> ± 0.04 | 0.21 <sup>a,b</sup> ± 0.01 |
| G6PD Mahidol (n = 12)   | 0.42 ± 0.03 | 0.49 ± 0.01 | 0.80 ± 0.06  | 0.50 ± 0.10 | 0.72 <sup>a</sup> ± 0.02 | 0.18 <sup>a,b</sup> ± 0.05 |

<sup>a</sup>Statistical significance of comparison among groups ( $P < .05$ ).

<sup>b</sup>Statistical significance of comparison among cycle of development ( $P < .05$ ).

before infection (Supplementary Figure 1B and C). Thus, we analyzed PS exposure at 96 hours of culture in a quantitative manner using flow cytometry. In this analysis, the mean fluorescence intensity indirectly indicates the PS level. In Figure 5A, the basal level of PS was  $102.3 \pm 27.1$  in infected erythrocytes with normal G6PD. Among all genotypes, a significant increase in PS was detected in cells from hemizygous

males carrying the Viangchan variant ( $261 \pm 119$ ) compared with wild types ( $P = .016$ ). Furthermore, the PS of Viangchan homozygotes ( $122 \pm 6.9$ ) was slightly higher than that of heterozygotes ( $103 \pm 8.6$ ) ( $P = .008$ ), indicating dependence on G6PD deficiency. Moreover, plotting PS intensity values against G6PD activity revealed an increasing PS trend ( $>150$ ) in some hemizygous and homozygous individuals with G6PD activity



**Figure 4.** Selectivity index (SI) of *Plasmodium falciparum*. (A) Representative images of the intracellular glucose-6-phosphate dehydrogenase (G6PD) assay. Normal G6PD-containing erythrocytes (G6PD-positive cells [GPCs]) have dark-purple granules (arrowheads); G6PD-deficient erythrocytes (G6PD-negative cells [GNCs]) remain unstained (arrows). *Plasmodium falciparum* inside erythrocytes appears as dense dark purple granules. Mahidol G6PD deficiency variants (heterozygote, hemizygote, and homozygote) are shown. (B) Scatter plot of the SI. The SI is the ratio of the number of malaria parasites in GPCs and that in GNCs among 1000 cells of GSCs plus GNCs. Each dot represents an individual subject. For each column, horizontal bars indicate the mean, and vertical bars indicate the standard deviation. Stars indicate statistically significant differences in the values of the 2 groups. (C) Scatter plot of the SI and G6PD activity. Blue dots represent normal G6PD. Green dots represent Viangchan and Mahidol heterozygous *G6PD* variants. Orange dots represent Viangchan and Mahidol hemizygous *G6PD* variants. Red dots represent Viangchan and Mahidol homozygous *G6PD* variants. HemiM, Hemizygous Mahidol; HemiV, Hemizygous Viangchan; HeteroM, Heterozygous Mahidol; HeteroV, Heterozygous Viangchan; HomoM, Homozygous Mahidol; HomoV, Homozygous Viangchan.

**Table 2. The Selectivity Index of the Plasmodium falciparum K1 Strain with G6PD-Deficient Erythrocytes**

| Selectivity index  | Normal   | Hemizygous |          | Heterozygous |           | Homozygous |          |
|--------------------|----------|------------|----------|--------------|-----------|------------|----------|
|                    |          | Viangchan  | Mahidol  | Viangchan    | Mahidol   | Viangchan  | Mahidol  |
| Median             | 1.1      | 3.2        | 3.8      | 1.1          | 1.2       | 4.1        | 5.3      |
| 25%–75% percentile | 0.95–1.3 | 2.15–5.5   | 2.15–5.7 | 1.05–1.25    | 0.85–1.25 | 2.2–5.55   | 2.55–5.5 |
| Mean               | 1.12     | 3.7        | 3.9      | 1.14         | 1.08      | 3.92       | 4.28     |
| Standard deviation | 0.23     | 1.88       | 2.23     | 0.11         | 0.22      | 1.68       | 1.59     |

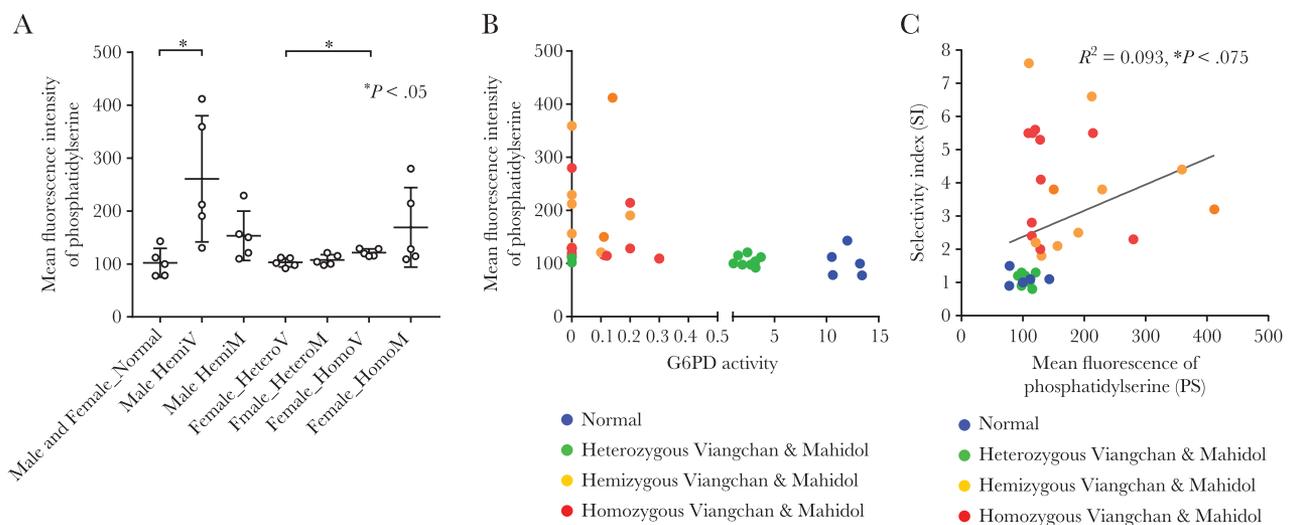
less than 0.3 IU/gHb. Nonetheless, no correlation between the level of PS and SI was observed ( $R^2 = 0.093$ ,  $P < .075$ ). These data suggest that decreased G6PD activity accelerates PS externalization.

**DISCUSSION**

Geographical overlap of malaria-endemic areas with G6PD deficiency leads to a selective advantage hypothesis, in which G6PD-deficient individuals are protected against malaria. Despite many epidemiological studies of African and Mediterranean variants, the underlying mechanisms remain unclear. Most attempts have provided an explanation for G6PD African and Mediterranean variants. To our knowledge, this is the first study to include all genotypes of Viangchan and Mahidol variants, the most common G6PD deficiency in Southeast Asia [11]. In summary, a reduction in parasitemia in subsequent cycles of intraerythrocytic development suggests a defect in invasion and/or maturation of *P falciparum*. Nitro blue tetrazolium chloride-based staining suggests that *P falciparum* parasites selectively develop inside erythrocytes with

normal G6PD activity. In this study, G6PD-mediated protection was revealed by extending the culture longer than a previous study [20]. First, parasites selectively grow in erythrocytes with normal G6PD activity. Second, accelerated PS externalization upon infection may enhance the removal of parasitized G6PD-deficient erythrocytes, likely reducing the risk of severe *P falciparum* malaria, as reported in epidemiological studies [37, 5, 6, 7].

In agreement, the G6PD activity of erythrocytes from hemizygous males carrying the Mahidol and Viangchan variants was similar to that in a previous report [38], suggesting that the subjects recruited were suitable as a reference population for an in vitro study. Roth et al [17] provided evidence of the protective effect of African A<sup>-12,13</sup> and Mediterranean G6PD deficiency during the first cycle of development in vitro. In addition, Cappadoro et al [20] found that 2-cycle development of *P falciparum* in Mediterranean G6PD-deficient erythrocytes exhibited indistinguishable invasion and growth, suggesting no protective effect of Mediterranean G6PD deficiency in male hemizygotes. In this study, we extended the culture of *P falciparum* to a third cycle and recruited not only male hemizygotes



**Figure 5.** Phosphatidylserine (PS) externalization. (A) Scatter plot of the mean fluorescence intensity of PS. Each dot represents an individual subject grouped according to genotype. For each column, horizontal bars indicate the mean, and vertical bars indicate the standard deviation. Stars indicate statistically significant differences in the values of the 2 groups. (B) Scatter plot of the mean fluorescence intensity of PS and glucose-6-phosphate dehydrogenase (G6PD) activity. Blue dots represent normal G6PD. Green dots represent Viangchan and Mahidol heterozygous *G6PD* variants. Orange dots represent Viangchan and Mahidol hemizygous *G6PD* variants. Red dots represent Viangchan and Mahidol homozygous *G6PD* variants. (C) Regression analysis of the selectivity index (SI) and mean fluorescence intensity of phosphatidylserine. HemiM, Hemizygous Mahidol; HemiV, Hemizygous Viangchan; HeteroM, Heterozygous Mahidol; HeteroV, Heterozygous Viangchan; HomoM, Homozygous Mahidol; HomoV, Homozygous Viangchan.

but also female heterozygotes and homozygotes for Mahidol and Viangchan variants. We found that the invasion rate of *P falciparum* in the second and third cycles of intraerythrocytic development declined slightly in cultures with normal erythrocytes but decreased markedly in those with G6PD-deficient erythrocytes, particularly when using cells from severely deficient hemizygotes and homozygotes. The discrepancy may be due to differences in parasite strain, variant type, or developmental cycle number. In contrast to invasion, maturation of *P falciparum* in the second and third cycles increased slightly in cultures of hemizygous Viangchan and Mahidol variant cells, indicating growth adaptation similar to *Plasmodium* survival after serial passages in G6PD-deficient erythrocytes [19].

Human erythrocytes rely on NADPH to cope with oxidative stress, and G6PD is the only source of NADH. With time, G6PD activity declines without replenishment, causing the accumulation of oxidants and clearance by macrophages [39, 40]. *Plasmodium falciparum* reportedly increases PS externalization, resulting in adherence of parasitized cells to macrophages and endothelial cells [41]. In addition, membrane damage in ring-stage parasites grown in G6PD-deficient erythrocytes enhanced phagocytosis [20]. Consistent with these findings, we observed a significant increase in PS exposure on *P falciparum* grown in Viangchan hemizygous erythrocytes, with a tendency toward an increase for cells from Viangchan homozygotes. However, there are limitations that need to be addressed. First, the G6PD activity of *Plasmodium* trophozoites replenished the loss of host G6PD [20]. This may explain parasite survival in G6PD-deficient host cells in the second and third cycles of development, particularly in the harsh microenvironment caused by hemizygous Viangchan and Mahidol variant cells. Second, similar to other hemoglobinopathies [28], enhanced phagocytosis of *P falciparum*-infected erythrocytes deficient in G6PD has been proposed to explain low parasitemia [20]. An increase in PS exposure by G6PD-deficient erythrocytes may support this proposal, but further phagocytic analysis is needed. Third, merozoites invade the host via Band3, and a loss-of-function of Band3 is enhanced by oxidative stress [42, 43, 44]. Thus, the expression of Band3 may provide an explanation. Finally, formazan-based detection of intracellular G6PD is not able to distinguish between live and dead parasites, confounding the selection index of this study. Fluorescence dyes capable of directly detecting NADH and living and dead parasites would allow accurate estimation.

## CONCLUSIONS

In conclusion, our data provide laboratory-based evidence to support the hypothesis that Viangchan and Mahidol variant G6PD deficiency protects against *P falciparum*, likely through reduced invasion, growth retardation, and loss of cell membrane integrity.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Supplementary Figure 1.** Evaluation of the 96-hour culture for assessing the phosphatidylserine (PS) externalization of normal G6PD erythrocytes. A. Stability of G6PD activity at 0 and 96 hours postinfection. Each filled circle is from 3 independent experiments. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance. B. Microscopic images indicate PS exposure at 0 and 96 hours postinfection. Cells were incubated with FITC-conjugated Annexin V following the manufacturer's instructions. After washing, the cells were subjected to confocal microscopic examination. More than 1000 cells were examined under the microscope. Representative images were from 3 independent experiments. Noncircular shaped, green dots were excluded from analysis. Scale bars = 50  $\mu\text{m}$ . C. Scatter plot of PS exposure at 0 and 96 hours postinfection. Filled circles of each panel represent individual cells of 3 independent experiments. The fluorescence intensity of FITC in individual cells was analyzed using ImageJ and indicated PS exposure. The Mann-Whitney test was performed to assess statistical significance. A *P* value less than 0.05 was considered statistically significant.

## Notes

**Acknowledgments.** We thank the staff of the Oxidation in Red Cell Disorders Research Unit, Department of Clinical Microscopy, Faculty of Allied Health Sciences, Chulalongkorn University, and the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

**Financial support.** This research was funded by Thailand Research Fund through the Royal Golden Jubilee PhD program (PHD/0116/2560), Chulalongkorn University and Thailand Science Research Innovation (Grant Number RTA6280006). K. K. is supported by the Research Career Development Grant of the Thailand Science Research and Innovation and Mahidol University (Grant Number RSA6280102).

**Potential conflicts of interest.** All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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