Prevalence of G6PD deficiency and associated haematological parameters in children from Botswana

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ABSTRACT

Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency is commonly seen in malaria endemic areas as it is known to confer a selective advantage against malaria. Recently, we reported a high proportion of asymptomatic reservoir of \textit{Plasmodium vivax} in Botswana, that calls for intervention with primaquine to achieve radical cure of \textit{vivax} malaria. Considering that individuals with this enzyme deficiency are at risk of haemolysis following primaquine treatment, assessment of the population for the relative frequency of G6PD deficiency is imperative. Samples from 3019 children from all the districts of Botswana were successfully genotyped for polymorphisms at positions 202 and 376 of the G6PD gene. Haematological parameters were also measured. The overall population allele frequency (based on the hemizygous male frequency) was 2.30\% (95\% CI, 1.77–2.83), while the overall frequency of G6PD-deficient genotypes A- (hemizygote and homozygote genotypes only) was 1.26\% (95\% CI, 0.86–1.66). G6PD deficiency is spread in Botswana according to the historical prevalence of malaria with a North-West to South-East decreasing gradient trend. There was no association between G6PD status and \textit{P. vivax} infection. G6PD A- form was found to be associated with decreased RBC count and haemoglobin levels without a known cause or illness. In conclusion, we report for the first time the prevalence of G6PD deficiency in Botswana which is relevant for strategies in the malaria elimination campaign. Further work to examine the activities of the enzyme in the Botswana population at risk for malaria is warranted.

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1. Introduction

Malaria is the most dangerous parasitic disease worldwide, with half of the world’s population living in malaria risk areas. Most of the cases are due to *Plasmodium falciparum* (Pf) especially in Sub-Saharan Africa, and *Plasmodium vivax* (Pv) in Asia. In recent years there has been an overall reduction in the malaria prevalence worldwide (World Health Organization, 2017) such that several countries, including eight from Africa, are in the elimination phase in conformity with the new Global Technical Strategy for Malaria 2016–2030 (World Health Organization, 2015a).

Botswana is a semi-arid, landlocked country of Southern Africa. Historically, malaria was known to be endemic (stable) in the Northern and North-Eastern corridors while to the South, Central and West it was largely epidemic (unstable). One recent retrospective study estimated that the average parasite rate in the Northern districts in the early 1960s was about 70% in children aged 1 to 14 years (Craig et al., 2007), declining with latitude to 0–2% in the Southern districts of Botswana. The reported prevalence was due to a nation-wide vector control program intervention which started in the mid 1940s (Mabaso et al., 2004). This activity has been sustained in addition to extensive distribution of long-lasting insecticide-treated mosquito nets (Simon et al., 2013; Chihanga et al., 2016) and drug treatment of uncomplicated cases with artemether-lumefantrine, which is the first line artemisinin combination therapy in Botswana. However, following abundant rains, cases with artemether-lumefantrine, which is the first line artemisinin combination therapy in Botswana. However, following abundant rains, malaria outbreaks still occur (see for example: http://www.moh.gov.bw/press%20release/MALARIA%20PRESS%20RELEASE.pdf; http://www.moh.gov.bw/press%20release/awareness_malaria.pdf).

Recently, as part of the Malaria Indicator Survey (MIS) (Botswana Ministry of Health, 2012), clusters of Pf infection were identified in the Eastern, Southern and Central districts (Howes et al., 2015; Motshoge et al., 2016) with a national prevalence being around 5% in asymptomatic children. This finding has brought a new paradigm shift in the strategy towards elimination, driving a focus towards containment of the Pf clusters. The only drug recommended for the radical cure of Pf (by clearing liver hypnozoites) and killing Pf gametocytes to prevent transmission by the vector is primaquine (PQ). Unfortunately the drug cannot be administered freely without knowledge of the Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency status of the subjects, as G6PD deficient individuals stand a risk of drug induced hemolysis. G6PD deficiency is considered the most common enzymopathy in humans, affecting about 5% of the world’s population and making it a significant public health problem (Cappellini and Fiorelli, 2008). The *G6PD* gene is highly polymorphic. The most common form of *G6PD* worldwide is the B form (wild-type enzyme), which is characterized by alleles G and A at the 202 and 376 nucleotides of the (202G-376A), respectively. In Africa up to 40% of the population carries a different variant, the G6PD A form (WHO Working Group, 1989) characterized by a 202G-376G allele with 15% reduction in enzyme activity (Battistuzzi et al., 1977). When there is an added mutation at the 202 nucleotide (G > A), the double mutant (202A-376G) is referred to as G6PD A- variant, with approximately 12% of enzyme activity compared to the B allele (Beutler et al., 1989). The G6PD variants are selected for in malaria endemic populations due to the protective role they confer against severe *Pf* malaria (Sirugo et al., 2014; Luzzatto et al., 2016) and possibly Pv, as previously described for the Southeast Asian and Mediterranean G6PD variants (Louicharoen et al., 2009; Leslie et al., 2010).

The gene for G6PD is located on the X-chromosome, and G6PD deficiency is inherited in a sex-linked fashion, being fully expressed in hemizygous males and homozygous females, but in only a proportion of female heterozygotes (Beutler et al., 1962; Luzzatto, 2006). In any female from the fetal stage onward only one X-chromosome in each cell is active, the other being inactive (Beutler et al., 1962). The pattern, referred to as X-chromosome inactivation determines that a heterozygous female for G6PD deficiency carries a double population of red cells, some with and some without the enzyme deficiency, so that heterozygous females may be phenotypically normal, mildly to moderate reduced, and grossly deficient.

Exposure to oxidative stress factors including PQ, infections and fava beans (Calabrò et al., 1989; Reading et al., 2016; Chu et al., 2017), can elicit acute haemolysis in G6PD-deficient female heterozygotes, homozygotes or male hemizygotes. Considering that PQ is essential for the radical cure of *Pv* infections and for blocking the transmission of *Pf* gametocytes, the use of PQ represents an ethical challenge as those who could benefit the most may also be at greatest risk of harm (Baird and Surjadijaja, 2011; Manjuran et al., 2015).

We report here for the first time, the G6PD status and haematological indices of subjects who also participated in the MIS study.

2. Materials and methods

2.1. Study sites, sample collection and genotyping

The Malaria Indicator Survey was conducted in May 2012 in Botswana (Botswana Ministry of Health, 2012; Motshoge et al., 2016) on asymptomatic children recruited from schools, welfare clinics and health posts. The study was approved by the Human Research and Development Division of the Botswana Ministry of Health [PPME-13/18/2013] and the Institutional Review Board of the University of Pennsylvania [protocol number 820378]. Research permit was obtained from the University of Botswana where the molecular work was done. Informed consent was obtained individually from the parents or legal guardian of the subjects prior to enrolment.

Study sites (see list of districts in Table 1 and geographical location in Fig. 1) were selected with the support of Statistics Botswana from 15 districts in Botswana to be representative of the population. The actual study included 3019 subjects aged between 2 and 12 years.

A 2.5 to 3 ml venous blood was collected into EDTA tubes. Genomic DNA was isolated from red blood cell pellets using the QiAamp DNA Blood mini kit (Qiagen Inc., Valencia, CA) or Quick-gDNA™ Blood Mini Prep (Zymo Research, USA).

G6PD status was determined by PCR (polymerase chain reaction) for two single nucleotide polymorphisms in the G6PD gene (202G > A and 376A > G, rs1050828 and rs1050829, respectively). Primers, as described by (Mombo et al., 2003), were used to amplify a 919 bp and a 585 bp fragments of the G6PD gene for 202 and 376 polymorphisms, respectively. Subsequently, RFLP (restriction fragment length polymorphism) method using the restriction enzymes NlaIII and FokI for 202 and 376 alleles, respectively, was used to determine the genetic polymorphisms (Mombo et al., 2003). Initially, three 376 and the three 202 different genotypes from males and females subjects were sequenced to ascertain the presence of the indicated alleles. For each run, positive controls from sequenced samples and negative controls were included with all PCR series and analysed in parallel with the samples.


In order to estimate the true G6PD deficiency at the population level, both hemizygous A- males and homozygous A- females were considered and counted (see Table 1), with the caveat that this would represent the minimum of the true prevalence of G6PD deficiency as some heterozygous females will have a homozygote-like phenotype. For this reason it is also possible to use the male hemizygous frequency of deficiency as the overall population-level allele frequency (this avoids issues of homozygote inclusion, and also the issue of the sex ratio in the sample and how that influences the results).
Table 1
G6PD genotypes and frequencies in 15 health districts of Botswana.

<table>
<thead>
<tr>
<th>Districts</th>
<th>DNA n</th>
<th>G6PD B' n (%)</th>
<th>G6PD A' n (%)</th>
<th>G6PD A' homo-n (%)</th>
<th>G6PD A' hetero-n (%)</th>
<th>Male + Homo-Female n (%)</th>
<th>Hardy-Weinberg test for 376 polymorphism&lt;sup&gt;⁎&lt;/sup&gt;</th>
<th>Hardy-Weinberg test for 202 polymorphism&lt;sup&gt;⁎&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okavango</td>
<td>178</td>
<td>60 (74.07)</td>
<td>15 (18.52)</td>
<td>6 (7.41)</td>
<td>55 (56.7)</td>
<td>3 (3.09)</td>
<td>27 (27.83)</td>
<td>12 (12.37)</td>
</tr>
<tr>
<td>Ngami</td>
<td>200</td>
<td>79 (85.87)</td>
<td>12 (13.04)</td>
<td>1 (1.09)</td>
<td>75 (69.44)</td>
<td>2 (1.85)</td>
<td>22 (20.37)</td>
<td>1 (0.93)</td>
</tr>
<tr>
<td>Chobe</td>
<td>86</td>
<td>41 (97.62)</td>
<td>0 (0.00)</td>
<td>1 (2.38)</td>
<td>41 (67.21)</td>
<td>1 (1.64)</td>
<td>14 (22.95)</td>
<td>5 (8.20)</td>
</tr>
<tr>
<td>Boteti</td>
<td>172</td>
<td>70 (92.11)</td>
<td>4 (5.26)</td>
<td>2 (2.63)</td>
<td>70 (72.92)</td>
<td>2 (2.08)</td>
<td>16 (16.67)</td>
<td>1 (1.04)</td>
</tr>
<tr>
<td>Tutume</td>
<td>278</td>
<td>114 (85.07)</td>
<td>10 (14.05)</td>
<td>4 (6.48)</td>
<td>105 (72.92)</td>
<td>4 (2.78)</td>
<td>25 (17.36)</td>
<td>10 (6.94)</td>
</tr>
<tr>
<td>North East</td>
<td>138</td>
<td>64 (83.11)</td>
<td>10 (12.99)</td>
<td>3 (3.90)</td>
<td>41 (67.21)</td>
<td>1 (1.64)</td>
<td>14 (22.95)</td>
<td>5 (8.20)</td>
</tr>
<tr>
<td>Francistown</td>
<td>167</td>
<td>61 (8.59)</td>
<td>5 (7.04)</td>
<td>5 (7.04)</td>
<td>72 (75.00)</td>
<td>0 (0.00)</td>
<td>19 (19.79)</td>
<td>5 (5.21)</td>
</tr>
<tr>
<td>Bobirwa</td>
<td>103</td>
<td>39 (90.70)</td>
<td>3 (6.98)</td>
<td>1 (2.32)</td>
<td>44 (73.33)</td>
<td>0 (0.00)</td>
<td>13 (21.67)</td>
<td>3 (5.00)</td>
</tr>
<tr>
<td>Ghanzi</td>
<td>147</td>
<td>70 (89.74)</td>
<td>4 (5.13)</td>
<td>4 (5.13)</td>
<td>51 (73.91)</td>
<td>2 (2.90)</td>
<td>15 (21.74)</td>
<td>1 (1.45)</td>
</tr>
<tr>
<td>Senowe/Palapye</td>
<td>208</td>
<td>102 (99.03)</td>
<td>1 (0.97)</td>
<td>0 (0.00)</td>
<td>94 (89.52)</td>
<td>1 (0.95)</td>
<td>8 (7.62)</td>
<td>1 (0.95)</td>
</tr>
<tr>
<td>Kweneng West</td>
<td>320</td>
<td>140 (90.32)</td>
<td>15 (9.68)</td>
<td>0 (0.00)</td>
<td>131 (76.61)</td>
<td>6 (3.51)</td>
<td>33 (19.30)</td>
<td>1 (0.58)</td>
</tr>
<tr>
<td>Kweneng East</td>
<td>414</td>
<td>158 (88.27)</td>
<td>20 (11.17)</td>
<td>1 (0.56)</td>
<td>167 (71.06)</td>
<td>10 (4.26)</td>
<td>51 (21.70)</td>
<td>5 (2.13)</td>
</tr>
<tr>
<td>Kgatleng</td>
<td>331</td>
<td>144 (91.14)</td>
<td>12 (7.59)</td>
<td>2 (1.27)</td>
<td>124 (71.78)</td>
<td>7 (4.05)</td>
<td>36 (20.81)</td>
<td>6 (3.47)</td>
</tr>
<tr>
<td>South East</td>
<td>133</td>
<td>63 (92.65)</td>
<td>4 (5.88)</td>
<td>1 (1.47)</td>
<td>56 (86.15)</td>
<td>1 (1.54)</td>
<td>6 (9.23)</td>
<td>2 (3.08)</td>
</tr>
<tr>
<td>GoodHope</td>
<td>138</td>
<td>72 (93.51)</td>
<td>5 (6.49)</td>
<td>0 (0.00)</td>
<td>45 (73.77)</td>
<td>2 (3.28)</td>
<td>13 (21.31)</td>
<td>1 (1.64)</td>
</tr>
<tr>
<td>Total</td>
<td>3019</td>
<td>1277 (89.05)</td>
<td>124 (8.65)</td>
<td>23 (2.30)</td>
<td>1164 (73.44)</td>
<td>33 (2.30)</td>
<td>306 (19.30)</td>
<td>69 (43.5, 95%)</td>
</tr>
</tbody>
</table>

a G6PD composite genotypes were assigned as follows:
B: 202G/376A<sup>♂</sup> - 202GG/376AA<sup>♀</sup>.
A: 202G/376G<sup>♂</sup> - 202GG/376AG<sup>♀</sup> (heterozygous) - 202GG/376GG<sup>♀</sup> (homozygous).
A-: 202A/376G<sup>♂</sup> - 202AA/376GG<sup>♀</sup> (homozygous) - 202GA/376GG<sup>♀</sup> (heterozygous).

- The H-W equilibrium (only in female individuals) was tested with chi-square statistic. However, when genotypic classes had expected values of < 5, we run the Monte-Carlo permutation test. This consists in reshuffling the observed numbers of alleles among individuals at random a large number of times (10,000 iterations), and deriving an empirical probability from the genotypic distributions obtained in each replicate.

- P value = 0.033.
- P value = 0.044.
- P value = 0.045.
- Allele frequency for 376A = 0.849; allele frequency for 376G = 0.151. Expected genotype numbers are: 1161.5 - 411.9 - 36.5, for AA, AG and GG, respectively. There is a significant defective number of heterozygous genotypes (P value = 0.045).
- Allele frequency for 202G = 0.975; allele frequency for 202A = 0.025. Expected genotype numbers are: 1529.0 - 79.9 - 1.0, for GG, GA and AA, respectively. There is a significant defective number of heterozygous genotypes (P value = 0.024).
2.3. Statistical analysis

Percentages were used to describe categorical variables like G6PD status and genetic variables such as molecular Pf, Pv and P. malariae (Pm) positivity, these last data is already published (Motshoge et al., 2016). Plasmodium spp. molecular detection was carried out through a modified Snounou et al. (1993) protocol. Evaluation of Hardy-Weinberg equilibrium at 202 and 376 loci was performed only on female subjects using the HWSIM software (freely available at http://krunch.med.yale.edu/hwsim/) and Monte-Carlo permutation test performed after Hardy-Weinberg equilibrium at 202 and 376 loci was performed only on female subjects using the HWSIM software (freely available at http://krunch.med.yale.edu/hwsim/). The results indicated are the same of Table 1. The 15 districts of sampling are listed in a North-West, East to South-East direction, according to the districts are summarised in Table 1. The 15 districts of sampling are listed in a North-West, East to South-East direction, according to the historic Pf malaria exposure and the actual malaria prevalence in the country (Craig et al., 2007). In Table 1 we listed the G6PD deficiency prevalence by district with only subjects with A- mutations (hemizygous males and homozygous females combined) being considered fully deficient. However, it is also useful to look at the hemizygous males frequency to have a better estimate of the real level of deficiency in the population. A higher prevalence of deficiency (in hemizygous male being 7.41%) was reported in Okavango district from where most of the confirmed Pf malaria case of Botswana are reported (Moakofhi et al., 2018), while the other malaria endemic districts (Ngami, Chobe, Boteti, Tutume and Bobirwa) showed a lower prevalence (in hemizygous male ranging from 1.09% to 4.48%). Districts that occasionally experience malaria outbreaks (North-East, Francis-town and Ghanzi) are associated with an important prevalence of G6PD deficiency (in hemizygous male ranging from 3.90% to 7.04%). Finally, southern districts showed less G6PD deficiency, with very low rates (in hemizygous male ranging from 0.00% to 1.47%). Chi square test for trend confirmed a statistical significant association between the North-West to South-East geographical direction and prevalence of G6PD deficiency among the districts (see Table 2). G6PD 202 genotypes frequencies were in agreement with Hardy-Weinberg in all districts, while for G6PD 376 genotypes two districts showed a heterozygote defect (Table 1). Multiple red blood cell indices (RBC count, Hb, MCV, and RDW) differed significantly between G6PD groups. Post hoc analyses revealed that those of G6PD type A- had lower RBC counts than those of type A or B (4.34 × 10^6 μL^{-1} vs 4.53 × 10^6 μL^{-1}, P = 0.009, and 4.34 × 10^6 μL^{-1} vs 4.60 × 10^6 μL^{-1}, P = 0.0002) and G6PD type A had lower RBC counts than those of type B (4.53 × 10^6 μL^{-1} vs 4.60 × 10^6 μL^{-1}, P = 0.007). Those of G6PD type A- also had greater MCV than those of type B (83.4 fL vs 80.8 fL, P = 0.04). Prevalence of anaemia (as defined by haemoglobin < 11 g/dL for those aged 24–59 months and < 11.5 g/dL for those aged 5–11 years) was 22.0% in the cohort. A non-statistically significant association among G6PD status and Pv positivity was found (see Table 4). We also performed the analysis in female subjects only, and the results were not noticeably different. The comparison was done on 2855 isolates with a Pv molecular parasite prevalence of 5.04%.

### Table 2

<table>
<thead>
<tr>
<th>Chi-square test for trend</th>
<th>Degrees of freedom</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Pearson’s Chi-square</td>
<td>34.87</td>
<td>14</td>
</tr>
<tr>
<td>Chi-square for slope (linear trend)</td>
<td>13.44</td>
<td>1</td>
</tr>
<tr>
<td>Chi-square for non-linearity</td>
<td>21.43</td>
<td>13</td>
</tr>
</tbody>
</table>

The test measured the association between the North-West to South-East geographical direction and the G6PD deficiency prevalence (measured as hemizygous male frequency) by district.
heterozygotes could be a result of physical mixing of populations with genotype frequencies (Table 1). The reason for the observed decreasing trend, following malaria endemicity trends in the past (Craig et al., 2007). In general, a blanket role out of PQ in the country could put a slight increase in RBC elimination rate.

Finally, we support the use of PQ as gametocytocidal for Pf at the dosage recommended by WHO (World Health Organization, 2015a,b; Bancone et al., 2016), even though most of the Pf infections occur in the Northern districts where G6PD deficiency is relatively higher. However, we raise some concern regarding the prescription of PQ for the management of relapsing Pv malaria (hypnozoite stage in the liver) as the 14 days prophylactic treatment may put the patient at high risk of severe haemolysis. Therefore, our recommendation to the National Malaria Program in Botswana is to adopt a point-of-care test for the detection of G6PD enzymopathy status (Luzzatto, 2012; Versluys et al., 2009). Slightly increased MCV could be explained by increased number of reticulocytes in G6PD deficient subjects which is probably due by a slight increase in RBC elimination rate.

4. Discussion

Botswana revised its malaria treatment guidelines in 2015 to include PQ although the G6PD status of the population was unknown. The only estimate of deficiency prevalence in Botswana is found in the Malaria Atlas Project (Howes et al., 2013). Our results, which is a GWAS study on 2315 individuals and associated with rs1050828 SNP, demonstrate disease protection since we did not recruit symptomatic malaria cases. The subjects of the current study were asymptomatic and febrile (Motshoge et al., 2016). That said, our data did not show any evidence of G6PD deficiency conferring protection against Pf infection (see Table 4).

It has been reported that the G6PD A- form is associated with decreased RBC count and haemoglobin levels (Lo et al., 2011), which was also noted in this study (see Table 3). The differences in RBC count (as well as haemoglobin level) were statistically significant, although likely not associated with any differences in clinical phenotype. Interestingly, the decrease of RBC count and increase of MCV was also found in a GWAS study on 2315 individuals and associated with rs1050828 SNP, namely the 202G > A variation (Ding et al., 2013). Other published reports are in line with these findings (Ajlani, 2000; Domingos et al., 2015). Slightly increased MCV could be explained by increased number of reticulocytes in G6PD deficient subjects which is probably due by a slight increase in RBC elimination rate.

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Fisher’s exact test P = 0.06.

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Botswana revised its malaria treatment guidelines in 2015 to include PQ although the G6PD status of the population was unknown. The only estimate of deficiency prevalence in Botswana is found in the Malaria Atlas Project (Howes et al., 2013). Our results, which is a GWAS study on 2315 individuals and associated with rs1050828 SNP, demonstrates disease protection since we did not recruit symptomatic malaria cases. The subjects of the current study were asymptomatic and febrile (Motshoge et al., 2016). That said, our data did not show any evidence of G6PD deficiency conferring protection against Pf infection (see Table 4).

It has been reported that the G6PD A- form is associated with decreased RBC count and haemoglobin levels (Lo et al., 2011), which was also noted in this study (see Table 3). The differences in RBC count (as well as haemoglobin level) were statistically significant, although likely not associated with any differences in clinical phenotype. Interestingly, the decrease of RBC count and increase of MCV was also found in a GWAS study on 2315 individuals and associated with rs1050828 SNP, namely the 202G > A variation (Ding et al., 2013). Other published reports are in line with these findings (Ajlani, 2000; Domingos et al., 2015). Slightly increased MCV could be explained by increased number of reticulocytes in G6PD deficient subjects which is probably due by a slight increase in RBC elimination rate.

Finally, we support the use of PQ as gametocytocidal for Pf at the dosage recommended by WHO (World Health Organization, 2015a,b; Bancone et al., 2016), even though most of the Pf infections occur in the Northern districts where G6PD deficiency is relatively higher. However, we raise some concern regarding the prescription of PQ for the management of relapsing Pv malaria (hypnozoite stage in the liver) as the 14 days prophylactic treatment may put the patient at high risk of severe haemolysis. Therefore, our recommendation to the National Malaria Program in Botswana is to adopt a point-of-care test for the detection of G6PD enzymopathy status (Luzzatto, 2012; Versluys et al., 2009). Slightly increased MCV could be explained by increased number of reticulocytes in G6PD deficient subjects which is probably due by a slight increase in RBC elimination rate.

5. Conclusion

In Botswana, G6PD deficiency predominates in the geographical area known to be endemic for malaria (North-West) and decreases...
gradually with the latitude to its lowest frequency in the South-East. G6PD deficient phenotype A- is associated to very mild anaemia. Therefore G6PD screening before PQ treatment could be necessary in some districts of Botswana where G6PD deficiency prevalence is high. Although genetic analysis provides a reliable method of detecting known G6PD variants, the equipment and technique is not widely available for routine screening in developing countries. Thus, we recommend that the National Malaria Programme carry out further studies to determine the reliability of various rapid qualitative and quantitative assays in this population before PQ-based radical cure of 
Pv can be implemented (Ley et al., 2017). Another valuable recommendation would be to focus future G6PD studies on 
Pv patients, as these individuals are the subgroup requiring the radical cure treatment, thus at risk of severe haemolysis if G6PD carriers.

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