Declining Efficacy of Artemisinin Combination Therapy Against P. Falciparum Malaria on the Thai–Myanmar Border (2003–2013): The Role of Parasite Genetic Factors

Aung Pyae Phyo,1,2 Elizabeth A. Ashley,1,2 Tim J. C. Anderson,4 Zbynek Bozdech,5 Verena I. Carrara,1,3 Kanlaya Siriprawot,1 Shalini Nair,4 Marina McDevitt White,1 Jerzy Dzielenko,1 Claire Ling,1,2,3 Stephanie Proux,1 Kamonchanok Kongkahong,1 Athanee Jeeyapant,1 Charles J. Woodrow,2,3 Malika Imwong,1,2, Rose McGready,1,2 Khin Maung Lwin,1,2 Nicholas P. J. Day,2,3 Nicholas J. White,2,3 and Francois Nosten1,2

1Shoklo Malaria Research Unit, Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Mae Sot, Thailand; 2Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom; 3Mahidol Oxford Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; 4Department of Genetics, Texas Biomedical Research Institute, San Antonio; and 5Division of Molecular Genetics & Cell Biology, School of Biological Sciences, Nanyang Technological University, Singapore

Background. Deployment of mefloquine–artesunate (MAS3) on the Thailand–Myanmar border has led to a sustained reduction in falciparum malaria, although antimalarial efficacy has declined substantially in recent years. The role of Plasmodium falciparum K13 mutations (a marker of artemisinin resistance) in reducing treatment efficacy remains controversial.

Methods. Between 2003 and 2013, we studied the efficacy of MAS3 in 1005 patients with uncomplicated P. falciparum malaria in relation to molecular markers of resistance.

Results. Polymerase chain reaction (PCR)–adjusted cure rates declined from 100% in 2003 to 81.1% in 2013 as the proportions of isolates with multiple Pfmdr1 copies doubled from 32.4% to 64.7% and those with K13 mutations increased from 6.7% to 83.4%. K13 mutations conferring moderate artemisinin resistance (notably E252Q) predominated initially but were later overtaken by propeller mutations associated with slower parasite clearance (notably C580Y). Those infected with both multiple Pfmdr1 copy number and a K13 propeller mutation were 14 times more likely to fail treatment. The PCR-adjusted cure rate was 57.8% (95% confidence interval [CI], 45.4, 68.3) compared with 97.8% (95% CI, 93.3, 99.3) in patients with K13 wild type and Pfmdr1 single copy. K13 propeller mutation alone was a strong risk factor for recrudescence (P = .009). The combined population attributable fraction of recrudescence associated with K13 mutation and Pfmdr1 amplification was 82%.

Conclusions. The increasing prevalence of K13 mutations was the decisive factor for the recent and rapid decline in efficacy of artemisinin-based combination (MAS3) on the Thailand–Myanmar border.

Keywords. Plasmodium falciparum malaria; mefloquine–artesunate; Pfmdr1; K13 mutation; artemisinin resistance.

Received 24 March 2016; accepted 5 June 2016. Published online 16 June 2016.
Correspondence: A. P. Phyo, Shoklo Malaria Research Unit, PO Box 46, Mae Sot, Tak, Thailand 63110 (aungpyae@shoklo-unit.com).

Clinical Infectious Diseases® 2016;63(6):784–91
© The Author 2016. Published by Oxford University Press for the Infectious Diseases Society of America. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited. DOI: 10.1093/cid/ciw888
(DHA)–piperaquine in Cambodia [19]. An observational cohort study reported that treatment failure rates were higher in patients infected with parasites carrying the K13 C580Y mutation compared with the R539T mutation, but there were only 4 wild-type infections in that study. Two additional mutations (MAL13:1718319 and MAL10:688956), associated with parasite clearance rate in a genome wide association (GWAS) study [20], were in strong linkage disequilibrium with C580Y. The authors concluded that this triple mutant genotype might have contributed to the failures observed. A second study of 241 patients showed falciparum malaria recrudescence was associated with increased piperaquine in vitro 50% inhibitory concentrations and the presence of K13 mutations at 3 Cambodian sites [21] where isolates with multiple copies of Pfmdr1 are rare [21].

In contrast to Cambodia, *P. falciparum* malaria parasites along the Thailand–Myanmar border frequently have multiple copies of Pfmdr1 [4, 22]. The MAS3 combination has been deployed successfully as a first-line regimen for more than 15 years, but failure rates in recent years have risen. To determine the factors contributing to declining ACT efficacy, we studied 1005 falciparum malaria patients treated with MAS3 between 2003 and 2013.

**METHODS**

**Patient Recruitment**

The study was designed for the longitudinal monitoring of MAS3 efficacy in prospectively enrolled patients presenting to the clinics of the Shoklo Malaria Research Unit [23] with uncomplicated *P. falciparum* malaria, excluding pregnant women, patients with severe malaria [24] or >4% infected red blood cells, and those who had been treated with mefloquine in the previous 60 days. All patient treatments were supervised. Oral artesunate (Guilin Pharmaceutical Co., People’s Republic of China), was given at a dose of 4 mg/kg/day for 3 days. Mefloquine (Roche, Switzerland, or CIPLA, India) was given either as split doses of 15 mg/kg and 10 mg/kg or 8 mg/kg once a day for 3 days [25]. On the first treatment day (day 0) a full clinical examination was performed; parasitemia and hematocrit were measured from capillary blood. Blood smears were microscopically examined daily until negative. Patients were seen weekly for 6 weeks. At each visit symptoms were recorded and a capillary blood sample was obtained for malaria smear and hematocrit. Dried blood spots for parasite genotyping were collected on grade 3 MM Whatman paper (Whatman, United Kingdom) at the first visit and in case of recurrence. Recurrence was defined by the occurrence of parasitemia during follow-up due either to a recrudescence or a reinfection. In 2013 single-dose primaquine as a gametocytocide (0.25 mg/kg) was added routinely on the first day of treatment.

**Parasite Genotyping**

Parasite DNA was extracted and genotyped at 3 polymorphic loci (MSP1, MSP2, and GLURP) to distinguish recrudescence from reinfection [26]. We determined Pfmdr1 copy number using quantitative polymerase chain reaction (PCR) and K13 polymorphisms by direct sequencing of PCR products (see Supplementary Methods). Pfmdr1 and K13 sequencing were done retrospectively after patient recruitment was completed.

**Statistical Analyses**

Data were analyzed using Stata 14 (StataCorp, College Station, Texas). Normally distributed data were compared using Student t test and nonnormally distributed data were compared using the Mann–Whitney rank sum test. Categorical variables were assessed using χ2 tests. Analysis of variance was used to compare 3 or more groups. We used logistic regression to examine the association between each potential risk factor and used outcome and multiple logistic regression to analyze resulting risk factors. Survival time data were assessed using Cox regression. The population attributable fraction (PAF) for falciparum malaria recrudescence was calculated for K13 mutations and Pfmdr1 amplification individually and then for the marker combination using the formula 1 – (1 – PAFK13) × (1 – PAFPfmdr1). We censored patients with indeterminate genotypes, new infections with *P. falciparum*, or those lost to follow-up.

**Ethical Approval**

The Oxford Tropical Research Ethics Committee (OXTREC 562-15) and the faculty of Tropical Medicine, Mahidol University (MUTM 2015-019-01) gave ethical approval for the study.

**RESULTS**

**Patients**

Between January 2003 and December 2013, 1022 patients were recruited, and 1005 remained in the study (Figure 1). Overall, 290 (28.8%) patients did not complete the full 42 days but were included in survival analyses. There were significant changes in gender ratio, age, duration of symptoms, and admission temperatures over the recruitment period, with an increasing proportion of older febrile male patients over time (Supplementary Table 1). The proportion of patients with fever at admission (tympanic temperature ≥37.5°C) rose significantly from 26.2% in 2003 to 65.4% in 2013 (P < .005). There was also an increase in the median duration of symptoms before presentation from 2 days in 2003 to 3 days in 2013 (P = .04).

**Clinical Responses**

MAS3 was well tolerated and cleared the clinical symptoms rapidly. By 24 hours 84.3% (95% confidence interval [CI], 81.6, 86.7) and by 72 hours 98.0% (95% CI, 95.2, 99.1) of patients were afebrile. There was no change in fever clearance times over the study period. The mean fractional reduction in hematocrit from baseline to day 7 did not differ significantly by year (test for trend P = .08). Risk factors associated with anemia (hematocrit <30%) on admission were pretreatment gametocyteemia (adjusted odds ratio [aOR], 9.43; 95% CI, 5.45, 16.34; P < .0001), age <13 years (aOR 3.77; 95% CI, 2.17, 6.56;
and female gender (aOR 2.18; 95% CI, 1.28, 3.72; P = .004). No patient in the cohort developed severe malaria, and there were no deaths.

**K13 Sequencing**

K13 sequences from 699 (68.5%) admission parasite isolates and 112 (59.9%) recurrent isolates were analyzed (Supplementary Table 2). In the admission infections, 24 different nonsynonymous polymorphisms were detected, including 20 in the propeller region (Figure 2 and Supplementary Table 3). No sample had more than 1 mutation. The most frequent mutations were C580Y (10.4%) and E252Q (8.3%). The proportion of infections caused by isolates with any K13 polymorphism increased from 6.7% (1/15; 95% CI, .2, 31.9) in 2003 to 83.9% (52/62; 95% CI, 72.3, 92.0) in 2013 (P < .001; Figure 3). During the period 2005–2009, the E252Q mutation was most common, but from 2010 the K13 propeller mutations (notably C580Y) predominated (Figure 2A and 2B). More recurrent isolates had K13 mutations (81/112 [72.3%]) compared with admission isolates (314/699 [44.9%]; P < .0001). In the recurrent isolates, more recrudescent isolates also had K13 mutant alleles compared with reinfections (65/76 [85.5%] vs 12/24 [50%]; P < .001).

**Pfmdr1 Copy Number**

Pfmdr1 copy numbers were measured in 726 (71.4%) admission isolates and 65 (34.8%) recurrent isolates (Supplementary Table 3).
The proportion of infections caused by parasites with multiple (>1) \(\text{Pfmdr1}\) copies on admission doubled from 32.4% (95% CI, 17.4, 50.5) in 2003 to 64.7% (95% CI, 46.5, 80.3) in 2013 \((P = .031; \text{Figure 3})\). There was no significant difference in the distribution of \(\text{Pfmdr1}\) copy number with gender or age. Significantly more recurrent isolates had multiple copies of \(\text{Pfmdr1}\) compared with admission isolates \(377/726 (51.9\%\); \(P < .001\)). Among these recurrent isolates, more recrudescent isolates also had multiple copies of \(\text{Pfmdr1}\) compared with reinfections \(38/42 (90.5\%)\) vs \(8/13 (61.5\%\); \(P = .002\)).

Our study did not characterize the single nucleotide polymorphisms of the \(\text{Pfmdr1}\) gene because these relevant single-nucleotide polymorphisms (SNPs) are rare in Thailand [27] and mefloquine resistance is driven by copy number changes on a \(\text{Pfmdr1}\) wild-type background \([4, 28–30]\). Isolates carrying wild-type \(K13\) were associated with single-copy \(\text{Pfmdr1}\), while \(K13\) mutations were associated with amplified \(\text{Pfmdr1}\) (Fisher exact test, \(P < .001\)) (Supplementary Figure 1).

The success rate for genotyping of \(K13\) and \(\text{Pfmdr1}\) was lower in recurrent infections because of significantly lower parasitemia \((P = .0004)\) and thus much lower parasite DNA concentrations.

**Parasite Clearance**

Clearance data were available for 957 patients (95.2%). There was a significant increase in the proportion of patients who were parasitemic at day 3 \((P < .001, \text{test for trend}; \text{Table 1})\).
Multivariate analysis showed that mutation in the K13 gene was the strongest risk factor for day 3 positivity, with later year of treatment, higher parasitemia, higher hematocrit, and fever on admission (but not Pfmdr1 amplification) as independent risk factors (Table 2). K13 propeller mutations were stronger predictors of day 3 positivity, and the 3 most common propeller mutations (C580Y, N458Y, and R561H) were each significantly associated with day 3 positivity (Table 2).

Cure Rates

Of the 186 patients with recurrent *P. falciparum* infections, there were 117 (62.9%) recrudescences and 53 (28.5%) reinfections, with 2 indeterminate results, 1 amplification failure, and 13 missing samples. PCR-adjusted parasitological efficacy at day 42 remained above or close to 90% from 2003 to 2009 but declined sharply thereafter (test for trend, \( P < .001 \); Table 1, Figure 3). There was a similar trend for recurrence (PCR unadjusted) rate. The lowest cure rates were recorded in 2011 when PCR-adjusted and unadjusted cure rates fell to 43.6% and 50.9%, respectively.

Predictors for Recrudescence

In a Cox regression model, increased Pfmdr1 copy number and K13 mutation were significant independent predictors of *falciparum* malaria recrudescence (with a multiplicative effect when in combination) along with year of recruitment and age (Table 3). The risk of recrudescence was even higher for K13 propeller mutations as a group and for the 3 most common individual propeller mutations (C580Y, N458Y, and R561H; Table 3).

Cure rates were highest for infections with isolates with single-copy Pfmdr1 gene and wild-type K13 (97.8%) and lowest in patients with multiple copies of Pfmdr1 and any K13 propeller

### Table 2. Predictors for Persistent Day 3 Asexual Parasitemia Following Mefloquine–Artesunate Treatment

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Adjusted Odds Ratio</th>
<th>95% Confidence Interval</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit on admission</td>
<td>1.04</td>
<td>1.00, 1.09</td>
<td>.04</td>
</tr>
<tr>
<td>Year of recruitment</td>
<td>1.14</td>
<td>1.02, 1.27</td>
<td>.02</td>
</tr>
<tr>
<td>Log parasitemia on admission</td>
<td>1.42</td>
<td>1.20, 1.68</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Fever (threshold of 37.5°C on admission)</td>
<td>2.50</td>
<td>1.50, 4.18</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Any K13 mutant</td>
<td>6.59</td>
<td>3.53, 12.30</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Subsets of K13 mutants(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any K13 propeller mutant</td>
<td>9.60</td>
<td>4.86, 18.95</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Isolates with E252Q</td>
<td>2.25</td>
<td>.91, 5.58</td>
<td>.08</td>
</tr>
<tr>
<td>Isolates with C580Y</td>
<td>7.61</td>
<td>3.42, 16.95</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Isolates with N458Y</td>
<td>8.75</td>
<td>3.10, 24.75</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Isolates with R561H</td>
<td>14.98</td>
<td>5.35, 41.92</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

\(^a\) Compared with wild type.

### Table 3. Predictors for *Plasmodium falciparum* Recrudescence by Day 42 Following Mefloquine–Artesunate

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Adjusted Hazard Ratio</th>
<th>95% Confidence Interval</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.97</td>
<td>.95, .99</td>
<td>.001</td>
</tr>
<tr>
<td>Year of recruitment</td>
<td>1.20</td>
<td>1.10, 1.44</td>
<td>.02</td>
</tr>
<tr>
<td>Multiple Pfmdr1 copy number (&gt;1)</td>
<td>2.68</td>
<td>1.52, 4.75</td>
<td>.001</td>
</tr>
<tr>
<td>Any K13 mutant</td>
<td>3.84</td>
<td>1.77, 8.36</td>
<td>.001</td>
</tr>
<tr>
<td>Subsets of K13 mutants(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any K13 propeller mutant</td>
<td>4.76</td>
<td>2.11, 10.75</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>K13 E252Q</td>
<td>2.83</td>
<td>1.03, 7.75</td>
<td>.04</td>
</tr>
<tr>
<td>K13 C580Y</td>
<td>6.04</td>
<td>2.00, 12.75</td>
<td>.001</td>
</tr>
<tr>
<td>K13 R561H</td>
<td>5.88</td>
<td>2.24, 15.40</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>K13 N458Y</td>
<td>7.20</td>
<td>2.56, 20.24</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Compound genotypes(^b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple Pfmdr1 + wild type K13</td>
<td>3.27</td>
<td>.84, 12.65</td>
<td>.09</td>
</tr>
<tr>
<td>Single Pfmdr1 + K13 propeller mutant</td>
<td>5.73</td>
<td>1.54, 21.26</td>
<td>.009</td>
</tr>
<tr>
<td>Multiple Pfmdr1 + K13 propeller mutant</td>
<td>14.05</td>
<td>3.99, 49.48</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

\(^a\) Compared with wild type.

\(^b\) Compared with single Pfmdr1 copy number and wild-type K13.

SNP (57.8%; Table 4, Figures 4 and Supplementary Figure 2). Cure rates declined markedly in recent years as the prevalence of K13 mutations rose (Supplementary Figure 2).

The PAFs (equivalent to the percentage reduction in recrudescence infections that would occur if these mutations were not present) for K13 and Pfmdr1 amplification were 69.0 and 41.9%, respectively. The PAF for the 2 factors in combination was 82%. Hence these 2 factors alone explain almost entirely the increased ACT treatment failure rate observed in this population.

### Pre- and Post-treatment Gametocytemia

The proportions of patients presenting with pre-treatment gametocytes or with post-treatment gametocytemia and the associated risk factors are shown in the Supplementary Materials (Supplementary Tables 1, 1.1 and 1.2). There was no association...
between the presence of K13 propeller mutations (aOR 1.02; 95% CI, .47, 2.19) or multiple copies of Pfmdr1 (aOR 1.28; 95% CI, .75, 2.17) and gametocytemia, either at admission or during follow-up.

**DISCUSSION**

MAS3 for the treatment of uncomplicated *P. falciparum* malaria has had a remarkable therapeutic longevity and a dramatic impact on morbidity and mortality on the Thailand–Myanmar border. When artesunate was introduced in 1991, mefloquine was a failing drug, the incidence of falciparum malaria was rising, and most parasite isolates had multiple copies of Pfmdr1 associated with mefloquine resistance [4]. After deployment of this ACT in 1994, mefloquine recovered its efficacy, and less fit isolates with multiple Pfmdr1 copies were replaced by single copy–containing isolates [31]. High cure rates were then sustained for more than a decade [22, 32]. In 2006, 53% of patients presenting with uncomplicated falciparum malaria had infections with multiple copies of Pfmdr1 [22]. The rise in prevalence of *P. falciparum* parasites with K13 propeller mutations was the definitive event that led to the demise of MAS3. The temporal sequence of K13 selection is informative. The E252Q mutation (which is located on the stem region of the K13 gene; Figure 2A), associated with moderate slowing of parasite clearance [15], predominated before 2010. Then parasites with the E252Q mutation were progressively overtaken by parasites with K13 propeller mutations, conferring greater reductions in parasite clearance rates. The main propeller mutation was C580Y, a polymorphism common in Cambodia [14, 15]. The E252Q mutation was associated with a moderate increase in risk of treatment failure, suggesting that the selective advantage of the K13 mutation is proportional to the degree of parasite clearance prolongation. It is possible that genetic changes outside the K13 locus have contributed to the emergence of artemisinin resistance and the selection of K13 mutations [20]. It remains to be seen whether parasite genotypes with even slower clearance will become established while ACTs remain the mainstream of antimalarial treatment.

The large body of data presented here strongly support the hypothesis that there is a substantial impact of K13 mutations on treatment failure. K13 mutations in admission samples are associated with a failure rate of 21.5%. In combination with multiple copies of Pfmdr1, this rises to 42.2%. The sharp decline in MAS3 cure rates temporally corresponds with the emergence of K13 propeller mutant isolates on a long-standing genetic background of Pfmdr1 amplification. The proportion of treatment failures that can be attributed to K13 mutations and multiple copies of Pfmdr1 (ie, PAF) is 82%. Hence, these 2 factors alone explain the majority of increase in treatment failure observed. Other parasite genetic factors that our study was not designed to detect or changes in the demographics of the patient population may have contributed to the 18% of unexplained variation in treatment failure.

We highlight 3 additional features of particular interest. First, K13 mutations and Pfmdr1 amplification have a multiplicative (rather than an additive) effect on risk of treatment failure. Synergy between these 2 resistance determinants may help to explain why failure rate declined precipitously in 2009—parasites carrying both markers only became common as K13 mutations rose in frequency. Second, in contrast to the 2
Cambodian studies, we observed multiple K13 mutations in this study. These data demonstrate significant heterogeneity in the impact of different K13 mutations on treatment failure. A mutation outside the K13 propeller (E252Q) increased treatment failure relative to wild-type parasites, but the 3 common propeller mutations had a much greater effect (Table 3). These data suggest that surveillance for emergence of K13 mutations should be expanded to include K13 regions outside the propeller domain.

Third, 2 studies [15, 19] provide evidence that gametocyte carriage is elevated in parasites bearing K13 mutations, leading to the suggestion that such parasites may have a transmission advantage. In contrast, in this large study from a single location, we observed no association between gametocyte carriage and K13 mutations.

What are the implications of these data for the therapeutic life span of ACTs in Southeast Asia and beyond? The most widely deployed ACT is the coformulation of artemether and lumefantrine (AL), which has a resistance mechanism that is similar to that of mefloquine, involving Pfmdr1 amplification [29, 33, 34]. In most areas where AL is used, there is no evidence for resistance to either component. However, in Myanmar where AL has recently been introduced, K13 propeller mutants and Pfmdr1 amplification are widespread [28, 35–38]. The therapeutic lifetime of AL in Myanmar and other areas with a high prevalence of K13 mutations may be relatively short.

ACTs containing an alternative partner drug should provide relief in the short term. In 2012 the first-line treatment of uncomplicated P. falciparum malaria in our treatment centers on the Thailand–Myanmar border was changed from MAS3 to DHA–piperaquine, which is currently highly efficacious in this area but relies increasingly on the piperaquine component. The recent emergence of piperaquine resistance in Cambodia [39] and associated rising failure rates with DHA–piperaquine [19, 21, 40] also cast doubt over the long-term future of this combination. Alternatives are needed desperately. With new antimalarials still years from deployment, there is an urgent need to eliminate P. falciparum from the area before the recent and substantial gains in malaria control are reversed.

**Supplementary Data**

Supplementary materials are available at http://cid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copypedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

**Notes**

**Acknowledgments.** We thank the staff of Shoklo Malaria Research Unit and all the collaborators for their efforts and all the patients who participated to the study.

**Disclaimer.** The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.


**Financial support.** Funding for the Shoklo Malaria Research Unit came from the Wellcome Trust of Great Britain (grant B9RTO22). Work conducted at Texas Biomedical Research Institute was funded by the National Institutes for Health (grant R37 AI048071 to T. J. C. A.) and was conducted in facilities constructed with support from the Research Facilities Improvement Program (grant C06 RR013556) from the National Centre for Research Resources.

**Potential conflicts of interest.** N. J. W. is cochair of the World Health Organization antimalarial treatment guidelines committee. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**References**


