

Minocycline Susceptibility and *tetB* Gene in Carbapenem-Resistant *Acinetobacter baumannii* in Taiwan

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Purpose: In this study, we evaluated the minocycline susceptibility rate in carbapenem-resistant *Acinetobacter baumannii* (CRAB) clinical strains, and the association between *tetB* carriage and minocycline susceptibility in CRAB.

Patients and Methods: A total of 100 genetically unrelated CRAB clinical strains from bloodstream infection were randomly collected from a medical center in Taiwan. An argument for a new minocycline susceptibility breakpoint of 1 mg/L was suggested based on pharmacokinetic (PK) and pharmacodynamic (PD) studies. Strains with minocycline minimum inhibitory concentrations (MICs) of >1 mg/L were classified as PK-PD non-susceptible. *TetB* carriage was detected by polymerase chain reaction (PCR).

Results: Fifty-five (55%) CRAB strains were susceptible to minocycline according to the Clinical and Laboratory Standards Institute (CLSI) criteria, among which 98.2% (54/55) were PK-PD non-susceptible. The minocycline MIC_{50/90} was 4/16 mg/L. Ninety-seven (97%) strains carried *tetB*. All of the *tetB*-positive strains and 66.7% (2/3) of the *tetB*-negative strains were PK-PD non-susceptible. By statistical analysis, *tetB* carriage was significantly correlated with PK-PD non-susceptibility ($P = 0.03$) and a higher minocycline MIC ($P = 0.02$). The sensitivity and specificity of the *tetB* PCR for predicting PK-PD non-susceptibility were 98% and 100%, respectively.

Conclusion: At our institute, most CRAB strains were PK-PD non-susceptible and most carried *tetB* gene. Recognizing the minocycline MIC and *tetB* status may be essential when using minocycline to treat CRAB-related infections.

Keywords: CRAB, minocycline PK-PD non-susceptibility, multidrug resistance, efflux

Introduction

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) represents an escalating global public health threat.¹ A substantial proportion of the CRAB isolates are also extensively drug-resistant (XDR).²⁻⁴ The therapeutic options available for CRAB infections are limited, and the most effective and safe regimens remain unknown.⁵ Minocycline, a semisynthetic tetracycline derivative, is proven to exert in vitro antibacterial activity against *A. baumannii*.⁶ Clinical experiences suggest that minocycline is an attractive therapeutic option for drug-resistant *A. baumannii* infections.^{7,8} Among the XDR *A. baumannii* complex, minocycline is the second-most active agent after colistin, with a susceptibility rate of 67.3–72.7%.^{4,9} However, debates regarding the minocycline susceptibility breakpoint for *A. baumannii* remain. The Clinical and Laboratory Standards Institute (CLSI) minocycline breakpoint for *A. baumannii* is 4 mg/L. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has not defined a minocycline breakpoint for *A. baumannii*. Many experts have suggested the need for clinical validation or re-evaluation of the CLSI breakpoint.^{10,11} Although favorable clinical experiences are reported in using minocycline at dose of 400 mg daily to treat *A. baumannii* infections, the cases are relatively few and most regimens employ minocycline combined a second or third agent rather than monotherapy.¹² An in vitro pharmacodynamic (PD) study using a pharmacokinetic (PK) model demonstrates the emergence of resistance in *A. baumannii* strains with minocycline MICs of 3 or 4 mg/L under

exposure to 400 mg/day minocycline.⁶ Based on the PK and PD studies,^{6,13} Tsakris et al have estimated a new susceptibility breakpoint of 1 mg/L for *A. baumannii* when daily dose of 400 mg minocycline is administered.¹¹

Minocycline resistance in *A. baumannii* is mainly mediated by the narrow-spectrum efflux pump, TetB, which belongs to the major facilitator superfamily.¹⁴ *A. baumannii* strains lacking the *tetB* gene reportedly have lower minocycline minimum inhibitory concentrations (MICs) than do those carrying *tetB*.^{15,16} The prevalence of *tetB* in *A. baumannii* has increased over recent years, and the minocycline MICs for *tetB*-carried *A. baumannii* have also increased.^{15–17} This phenomenon may be achieved by acquiring pre-existing resistance determinants via the mobile genetic elements, followed by amplification in response to selection, as is commonly observed in multi-drug resistant *A. baumannii*.¹⁸ For example, insertion sequence is found to play a role in spreading *tetB*.¹⁹ Furthermore, *tetB* is found in AbaR-type genomic antibiotic resistance islands or transposons, by which that import *tetB* and other antibiotic resistance genes into the globally disseminated *A. baumannii* clones.^{20–22} Another important factor may involve the clonal spread of *tetB*-carried *A. baumannii*.^{19,23} The AdeABC efflux pump, belonging to the resistance-nodulation-cell division efflux system, is associated with the development of tigecycline resistance. Minocycline is reportedly a weaker substrate of AdeABC pumps than tigecycline.²⁴ Thus, tigecycline-resistant *A. baumannii* isolates may remain susceptible to minocycline.^{14,24} *TetB* testing is considered a valuable tool for gene-based susceptibility evaluation.¹⁶ A global study shows that the presence of *tetB* is highly associated with MICs above the CLSI susceptibility breakpoint of 4 mg/L, and the lack of *tetB* gene could be a marker of CLSI-defined minocycline susceptibility in *A. baumannii*.¹⁶

In Taiwan, the minocycline susceptibility rate is 85% among all species of *A. baumannii* complex isolates, including *A. baumannii*, *A. nosocomialis* and *A. pittii*.²⁵ The minocycline susceptibility rate of CRAB, the bacterium of critical-priority, is unknown. Furthermore, the extent of *tetB* carriage is also unknown. In this study, we evaluated the minocycline susceptibility rate and the prevalence rate of *tetB* carriage in CRAB. We also analyzed the possible association involving *tetB* carriage and the minocycline susceptibility.

Materials and Methods

Study Samples and Species Identification

This study was conducted at the National Taiwan University Hospital (NTUH), Taipei, a 2600-bed medical center located in Taipei City, Taiwan. Clinical *A. baumannii* isolates from patients with bloodstream infections (BSIs) were collected between 2014 and 2017 as previously described.²⁶ Only the first episode was included if the patient had experienced multiple episodes of *A. baumannii* BSIs, and only one isolate was included for each episode. Between 2014 and 2016, the *A. baumannii* complex isolates primarily identified by conventional biochemical methods were collected for species identification according to the 16S–23S ribosomal RNA gene intergenic spacer region, as previously described.²⁷ Isolates of *A. nosocomialis* and *A. pittii* were excluded.²⁸ In 2017, the *A. baumannii* isolates were identified directly by the hospital microbiological laboratories using matrix-assisted laser desorption ionization-time of flight mass spectrometry Bruker Biotyper system (MicroFlex LT; Bruker Daltonik GmbH, Bremen, Germany), which was showed to be a rapid, simple, and reliable method to discriminate *A. baumannii* from other *A. pittii* and *A. nosocomialis*.²⁹ Among the *A. baumannii* isolates collected, those preliminarily identified as carbapenem-resistant by the hospital clinical laboratory were collected for further microbiological studies. Imipenem or meropenem resistance was confirmed by antimicrobial susceptibility testing as described below. This study was approved by the Research Ethics Committee of NTUH (NTUH 201008047R). The isolates were collected as part of routine clinical practice, and the requirement for informed consent was waived by the ethics committee.

Pulsed-Field Gel Electrophoresis

Randomly selected CRAB isolates were subjected to pulsed-field gel electrophoresis (PFGE) to determine the clonal relatedness.³⁰ Strains with relatedness $\geq 80\%$ were defined as PFGE-related strains. A total of 100 PFGE-unrelated CRAB strains were collected for further minocycline antimicrobial susceptibility testing and *tetB* gene detection.

Antimicrobial Susceptibility Testing

The MICs of amikacin, ampicillin/sulbactam, ceftazidime, cefepime, ciprofloxacin, levofloxacin, imipenem and meropenem for *A. baumannii* strains were determined using Sensititre GNX3F (Trek Diagnostics, West Sussex, UK). The MICs of minocycline,

tigecycline, and colistin were determined using broth microdilution. The MIC results were interpreted according to the CLSI recommendations and/or the EUCAST criteria for all antimicrobial agents.^{31,32} The CLSI minocycline breakpoints for *A. baumannii* were as follows: MIC ≤ 4 mg/L for susceptibility, 8 mg/L for intermediate resistance, and ≥ 16 mg/L for resistance. EUCAST did not issue minocycline breakpoints for *A. baumannii*. Strains with minocycline MICs of >1 mg/L were classified as PK-PD non-susceptible.¹¹ For tigecycline, the non-species-related PK-PD breakpoint defined by EUCAST as ≤ 0.5 mg/L for susceptibility was used.

Detection of *tetB* Gene

tetB carriage was detected using polymerase chain reaction (PCR). The primers used for amplification of the *tetB* gene were as previously described.¹⁶ *tetB*_F (5'-TAC GTG AAT TTA TTG CTT CGG-3') and *tetB*_R (5'-ATA CAG CAT CCA AAG CGC AC-3'). PCR was performed using TaKaRa TaqTM (TaKaRa Beijing, China) under the following conditions: 95°C for 5 min; followed by 35 cycles of 95°C for 20s, 55°C for 20s and 72°C for 1 min, with a final extension step of 72°C for 5 min. The PCR products were analyzed by a 1% agarose gel electrophoresis.

Statistical Analysis

A two-sample Student's *t*-test of proportions was used to compare the susceptibility rates to the different antimicrobial agents. The rank-sum test was used to compare the MICs between the two groups. Fisher's exact test was used to compare categorical variables between the two groups. Two-sided *P* values <0.05 were considered to be statistically significant. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the *tetB* testing in predicting minocycline susceptibility were determined. Data were analyzed using Stata software (version 14; StataCorp, College Station, TX, USA).

Results

Antimicrobial Susceptibility Testing

A total of 100 genetically unrelated CRAB clinical strains were subjected to antimicrobial susceptibility testing for minocycline, colistin, tigecycline, and other antimicrobial agents (Table 1). The colistin MIC_{50/90} was 0.25/1 mg/L. The tigecycline MIC_{50/90} was 2/4 mg/L, and none of the CRAB strains was susceptible to tigecycline with MIC of ≤ 0.5 mg/L. The minocycline MIC_{50/90} was 4/16 mg/L. The minocycline susceptibility rate of the 100 CRAB strains was 55%, which as interpreted according to the CLSI criteria. Among the 55 CLSI-defined minocycline-susceptible CRAB strains, 54 strains (98.2%) were PK-PD non-susceptible (MICs >1 mg/L). The susceptibility rate to sulbactam, ceftazidime, cefepime and piperacillin–tazobactam for all 100 strains from 0% to 4%, and susceptibility rate to amikacin was 13%. Ninety-nine strains (99%) were PK-PD non-susceptible (MICs >1 mg/L). None of the strains exhibited a minocycline MIC of ≤ 0.5 mg/L, and only one strain (1%) had a minocycline MIC of 1 mg/L.

TetB Carriage and Correlation with Susceptibility Testing

There was a high positive rate of *tetB* carriage in CRAB. Of the 100 tested strains, 97 (97%) were positive for *tetB* using PCR. The median minocycline MICs were 4 mg/L (range, 2–16 mg/L) for *tetB*-positive strains and 2 mg/L (range, 1–4 mg/L) for *tetB*-negative strains. By statistical analysis, *tetB* carriage was significantly correlated with a higher minocycline MIC ($P = 0.02$). The distribution of minocycline MICs of *tetB*-positive and -negative strains presented a modest degree of separation (Figure 1). All the three *tetB*-negative strains and 53.6% (52/97) of the 97 *tetB*-positive strains were susceptible to minocycline according to the CLSI criteria. *tetB* carriage was not significantly correlated with CLSI-defined minocycline non-susceptibility ($P = 0.25$). All of the *tetB*-positive strains and 66.7% (2/3) of the *tetB*-negative strains were PK-PD non-susceptible. *tetB* carriage was significantly correlated with minocycline PK-PD non-susceptibility (MIC >1 mg/L) ($P = 0.03$).

All of the 100 CRAB strains were resistant to tigecycline, with MICs of >0.5 mg/L. For *tetB*-positive strains, the median tigecycline MIC was 2 mg/L (range, 1–16 mg/L), and the MIC_{50/90} was 2/4 mg/L. For *tetB*-negative strains, the median tigecycline MIC was 2 mg/L (range, 2–16 mg/L). *tetB* carriage was not correlated with a higher tigecycline MIC

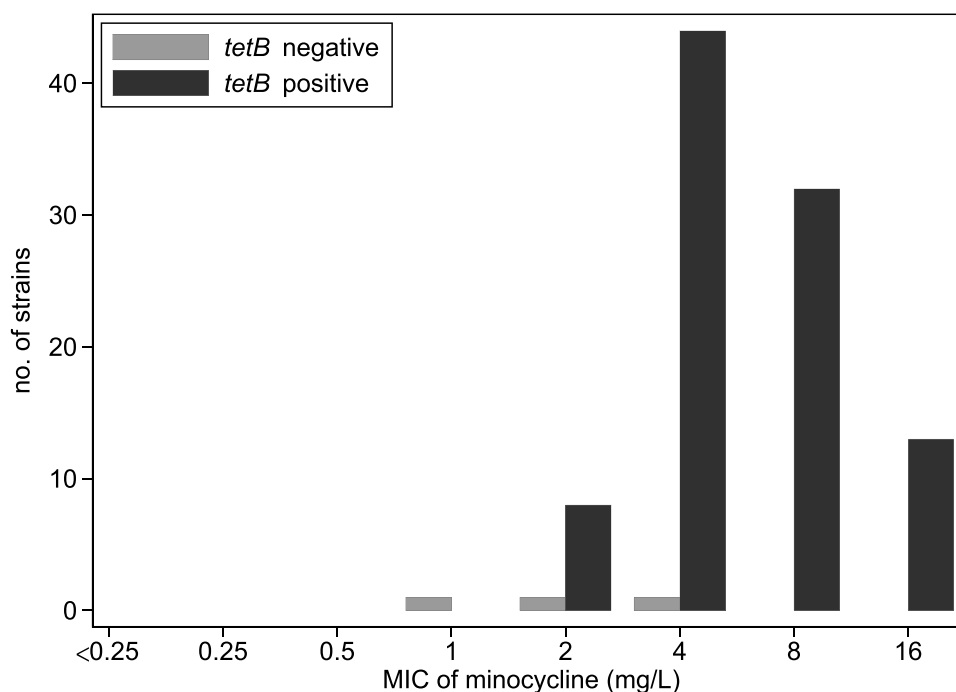
Table 1 In vitro Antimicrobial Activity of Antimicrobial Agents Against 100 Genetically Unrelated Carbapenem-Resistant *Acinetobacter baumannii* Strains

Antimicrobial Agent	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)	Range (mg/L)	CLSI Criteria ^a			EUCAST Criteria ^a	
				%S	%I	%R	%S	%R
Ampicillin–sulbactam	64/32	>64/32	16/8 to >64/32	0	4	96	–	–
Amikacin	>32	>32	≤4 to >32	13	0	87	11	89
Ceftazidime	>16	>16	4 to >16	1	0	99	–	–
Cefepime	>16	>16	4 to >16	4	13	83	–	–
Ciprofloxacin	>2	>2	>2	0	0	100	0	100
Levofloxacin	>8	>8	4 to >8	0	6	94	0	100
Piperacillin–tazobactam	>64	>64	64 to >64	0	2	98	–	–
Imipenem	>8	>8	>8	0	0	100	0	100
Meropenem	>8	>8	>8	0	0	100	0	100
Colistin	0.25	1	<0.25 to >16	0	91	9	91	9
Minocycline	4	16	1 to 16	55	32	13	–	–
Tigecycline	2	4	1 to 16	0	0	0	0	100

Note: ^aCLSI (2021) and EUCAST (2022) criteria.

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; MIC₅₀, 50% minimum inhibitory concentration; MIC₉₀, 90% minimum inhibitory concentration. S, susceptible; I, intermediate; R, resistant.

($P = 0.23$). The sensitivity and specificity of the *tetB* PCR for predicting CLSI-defined minocycline non-susceptibility (MIC >4 mg/L) were 100% and 5.5%, respectively, and the PPV and NPV were 46.4% and 100%, respectively. The sensitivity and specificity of the *tetB* PCR to predict minocycline PK-PD non-susceptibility (MIC >1 mg/L) were 98%

**Figure 1** Distribution of minocycline minimum inhibitory concentrations (MICs) of the 100 carbapenem-resistant *Acinetobacter baumannii* strains.

and 100%, respectively, and the PPV and NPV were 100% and 33.3%, respectively. The area under the receiver operating characteristic (ROC) curve of using *tetB* PCR to predict CLSI-defined minocycline non-susceptibility (MIC >4 mg/L) and minocycline PK-PD non-susceptibility (MIC >1 mg/L) were 0.5273 and 0.9899, respectively.

Discussion

Since safe and effective therapeutic options for CRAB infections are severely limited, characterization of the clinical isolates by phenotypic and molecular methods is important to provide information on the epidemiological characteristics of such pathogens.³³ In the present study, we tested the susceptibility of 100 genetically unrelated CRAB clinical strains to minocycline. Among the tested antimicrobial agents, minocycline was the most active agent against CRAB, according to the CLSI criteria. Fifty-five CRAB strains (55%) were susceptible to minocycline. In addition, among the 100 CRAB strains analyzed here, 99% were PK-PD non-susceptible, with minocycline MICs of >1 mg/L. Most of the tested strains (97%) carried *tetB* gene.

When the CLSI susceptibility breakpoint was applied, the susceptibility rate to minocycline was 55%. However, there remains ongoing debates regarding minocycline susceptibility breakpoints. Tsakris et al estimated the minocycline susceptibility breakpoint for *A. baumannii* using a Monte Carlo simulation based on PD data. The breakpoints were expected to be 0.5 mg/L and 1 mg/L when minocycline were administered at doses of 200 mg/day and 400 mg/day, respectively.¹¹ In the present study, the percentage (1%) of CRAB strains with relatively low minocycline MIC (≤ 1 mg/L) was much lower than those reported in other regions, ie, there were 15.9% US and 18.4% UK CRAB isolates with minocycline MICs of ≤ 0.5 mg/L, and 32.7% CRAB isolates with minocycline MICs of ≤ 1 mg/L in both regions.^{15,34}

The clinical efficacy and the optimal dose of minocycline for treating *A. baumannii* infections remain unclear. In infections caused by *A. baumannii* with minocycline MICs of 3 or 4 mg/L, clinical failure occurred in a substantial proportion of critically ill patients receiving minocycline at the dose of 200 mg/day.³⁵ Minocycline at the dose of 400 mg/day combined with a second or third active drug was suggested based on a few favorable clinical experiences.¹² An in vitro PD study,⁶ which revealed the emergence of resistance in *A. baumannii* strains with minocycline MICs of 3 or 4 mg/L under exposure to 400 mg/day minocycline,⁶ also favored combination therapy rather than minocycline monotherapy. A recent in vitro PD study suggested that a minocycline dose of 700 mg/day was the pharmacodynamically optimized dose against CRAB strains with minocycline MICs of 2 mg/L, when minocycline was used in combination with polymyxin B and sulbactam.³⁶ The high doses of minocycline, such as 700 mg or 800 mg/day, had been reported in the literature for non-infectious diseases. A Phase I study revealed that minocycline administered at a dose of 300 mg twice daily was the maximum-tolerated dose in healthy adult subjects.³⁷ Among the CRAB strains in the present study, there were only 1% with a relatively low minocycline MIC of ≤ 1 mg/L and 9% with minocycline MICs of 2 mg/L. Considering the high percentage of CRAB strains with minocycline MIC of >1 mg/L and the unresolved safety issues involving high-dose minocycline, the role of minocycline in treating CRAB-related infections in clinical settings needs be defined, and additional clinical studies are warranted.

In addition to the increasing prevalence of *tetB*-carried *A. baumannii* over recent years, the minocycline MICs for *tetB*-carried *A. baumannii* also increased.^{15–17} A substantial proportion of *A. baumannii* carried *tetB* in the USA and worldwide. In one report, 77.6% of the 107 US CRAB strains collected between 2009 and 2015 carried *tetB*. The minocycline MICs were relatively low (≤ 1 mg/L) for approximately 15% of the *tetB*-positive strains.¹⁵ Among clinical *A. baumannii* isolates collected worldwide between 1998 and 2015, 64.0% (165/258) carried *tetB*. There were almost all (99.4%, 164/165) of the *tetB*-positive isolates with minocycline MICs of ≥ 4 mg/L, and only 0.6% of the *tetB*-positive isolates with relatively a low minocycline MIC (≤ 1 mg/L).¹⁶ In the present study, there were 91.8% (89/97) of the 97 *tetB*-positive strains with minocycline MICs of ≥ 4 mg/L, and only 8.2% (8/97) with minocycline MICs of 2 mg/L. The minocycline MICs were >1 mg/L for all of the 97 *tetB*-positive CRAB strains. The distribution of minocycline MICs in our *tetB*-carried CRAB strains was similar to that observed in global *tetB*-carried *A. baumannii* isolates.¹⁶ In that study, the presence of *tetB* is highly associated with MICs above the CLSI-defined susceptibility breakpoint, as well as MICs above the PK-PD susceptibility breakpoint of 1 mg/L.¹⁶ However, the role of *tetB* testing in discriminating minocycline PK-PD susceptibility in CRAB should be interpreted with caution, as the number of *tetB*-negative CRAB strains in our study was insufficient to determine such

an association. Moreover, the application of *tetB* testing for predicting minocycline MICs might vary in different populations of *A. baumannii*, especially the minocycline MICs are relatively low for a substantial proportion of *tetB*-carried *A. baumannii*.^{15,38} One study exploring on the *tetB*-carried *A. baumannii* isolates with minocycline MICs of ≤ 1 mg/L found that there were minocycline-resistant subpopulations, which increased after incubation with minocycline. However, such subpopulations were not detected among isolates not carrying *tetB*.³⁸ That study implies the induction of the TetB efflux pump in *tetB*-carried isolates under exposure to minocycline. Detection of *tetB* status might still have a role in discriminating minocycline non-susceptibility or the likelihood of developing resistance during exposure to minocycline when minocycline is chosen as a therapeutic regimen for CRAB infections. In addition, the high prevalence of *tetB*-carried CRAB with minocycline MICs of >1 mg/L in our hospital also warrants further investigations.

This study had several strengths. First, we focused on CRAB, which ranked highest for research of any effective drug, for example, minocycline. Second, all CRAB strains were identified as genetically unrelated by PFGE. Therefore, our results regarding minocycline susceptibility for CRAB may be robustly representative. Third, we clarified whether the CRAB strains carried *tetB* to detect a potential relatively higher minocycline MIC. However, this study also had several limitations. First, we did not examine the *tetB* gene expression levels in our CRAB strains. Although *tetB* carriage is statistically correlated with minocycline MIC above the PK-PD susceptibility breakpoint of 1 mg/L and a higher minocycline MIC, antimicrobial resistance might result from differences in *tetB* gene expression.³⁹ The association between *tetB* gene expression levels and minocycline MIC in our CRAB strains warrants further investigation. Whether our CRAB strains express *tetB* constitutively,³⁹ and whether the mechanisms regulating *tetB* expression, such as Tet repressor protein encoded by *tetR*, differ in *A. baumannii* from other genus might also warrant exploration.⁴⁰ Furthermore, we did not assess whether other efflux pumps might have contributed to the relatively higher minocycline MICs detected in our CRAB strains. However, the TetB efflux pump has been reported to be the primary mechanism of minocycline resistance.⁴¹ Finally, this study reported the minocycline susceptibility rate of CRAB clinical strains in a single medical center. Nevertheless, we had highlighted the importance of obtaining the information regarding local minocycline MICs and *tetB* gene prevalence.

Conclusion

The findings of this study suggest that although minocycline was the most active agent against CRAB clinical strains according to the CLSI criteria at our institute, most of our CRAB strains might be classified as PK-PD non-susceptible with minocycline MICs of >1 mg/L. Most of the CRAB strains carried *tetB* gene. Recognizing the minocycline MIC and the *tetB* status may be essential when using minocycline to treat CRAB-related infections.

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Author Contributions

All authors made a significant contribution to the work reported, including in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all of these areas; took part in drafting, or critically reviewing the article; gave final approval of the version to be submitted; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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