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ORIGINAL RESEARCH

Different Contributions of *embB* and *ubiA* Mutations to Variable Level of Ethambutol Resistance in *Mycobacterium tuberculosis* Isolates

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Objective: To explore the association between the variant mutations within *embB* and *ubiA*, and the degree of ethambutol (EMB) resistance of *Mycobacterium tuberculosis* (*M. tuberculosis*) isolates.

Methods: A total of 146 *M. tuberculosis* isolates were used to determine the minimum inhibitory concentrations (MICs) of EMB with a 96-well microplate-based assay. The mutations within *embB* and *ubiA* among these isolates were identified with DNA sequencing. Moreover, a multivariate regression model and a computer model were established to assess the effects of mutations on EMB resistance.

Results: Our data showed that overall 100 isolates exhibited 28 mutated patterns within the sequenced *embB* and *ubiA*. Statistical analysis indicated that *embB* mutations Met306Val, Met306Ile, Gly406Ala, and Gln497Arg, were strongly associated with EMB resistance. Of these mutations, Met306Val and Gln497Arg were significantly associated with high-level EMB resistance. Almost all multiple mutations occurred in high-level EMB-resistant isolates. Although the mutation within *ubiA* accompanied with *embB* mutation presented exclusively in EMB-resistant isolates, four single *ubiA* mutations (Ala39Glu, Ser173Ala, Trp175Cys, and Val283Leu) leading to protein instability were observed in EMB-susceptible isolates.

Conclusion: This study highlighted the complexity of EMB resistance. Some individual mutations and multiple mutations within *embB* and *ubiA* contributed to the different levels of EMB resistance.

Keywords: ethambutol, multidrug-resistant tuberculosis, *embB*, *ubiA*, mutations, minimum inhibitory concentration

Introduction

Ethambutol (EMB), an important first-line anti-tuberculosis drug, is routinely used along with other drugs for treating pan-sensitive tuberculosis (TB) and drug-resistant TB, including multidrug-resistant TB (MDR-TB). EMB inhibits arabinosyltransferase (the EmbCAB protein) encoded by the *embCAB* operon which is involved in the biosynthesis of arabinan,[1] a component of arabinogalactan in the cell wall of TB. Numerous reports have indicated that mutations in the *embCAB* operon, particularly the EMB resistance-determining region (ERDR) of the *embB* gene,[2–4] are mainly responsible for EMB resistance in TB. However, some studies also observed that TB clinical strains could resist EMB without any mutations within *embCAB*.[3,5] On the other hand, mutations in *embCAB* among EMB-susceptible strains were also found.[3,6] These suggested that additional mechanisms might be involved in EMB resistance.

Some reports demonstrated that mutations in another gene, *ubiA* (*Rv3806c*), appear to confer a high level EMB resistance.[7–9] The *ubiA* encodes DPPR (decaprenylphosphoryl-β-D-5-phosphoribose) synthase that is involved in the DPA pathway for cell wall synthesis. Mutations in *ubiA* contribute to an increase in DPA level.[10,11] Consequently, the increased intracellular DPA

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competitively binds to EmbCAB protein against EMB, resulting in a high-level of EMB resistance.[7] The *ubiA* mutations almost always occur in EMB-resistant strains that also contain *embB* mutations, and *ubiA* appears to have multiplicative effects with *embB* mutations on MICs.[7] Nevertheless, there were several studies showing the presence of *ubiA* mutations among EMB-susceptible isolates.[8,12]

In this study, we explored the prevalence of *embB* and *ubiA* mutations in 146 MDR-TB isolates from China and evaluated their associations with the different levels of EMB resistance. Moreover, mutations in *ubiA* on protein structure were also evaluated.

Materials and Methods

Mycobacterium tuberculosis Isolates

Overall 146 MDR-TB isolates were collected from 146 patients with pulmonary tuberculosis in China. H37Rv was used as a reference (ATCC 27294). All strains were cultured on Lowenstein-Jensen (L-J) medium and freshly subcultured before being used for MIC testing.

MIC Testing

The Minimum Inhibitory Concentration (MIC) of EMB was determined in vitro, using the Sensititre® plates (Thermo Fisher Scientific Inc., Cleveland, Ohio, USA), and all steps were performed according to the manufacturer's instructions. H37Rv (ATCC 27294) was used as a quality control and was tested with each batch of MIC testing. This control strain is susceptible to EMB with MICs ≤1 μg/mL in this study. According to previous studies,[12,13] the strain was considered susceptible if its MIC was ≤ 2 μg/mL, low-level resistant (LLR) if its MIC was > 2–< 5 μg/mL, and high level resistant (HLR) if the MIC was ≥ 5 μg/mL.

DNA Isolation, PCR, and DNA Sequencing

All isolates on the L-J slants were collected and inactivated by heating at 95°C for 20 minutes. Supernatants containing genomic DNA were collected by centrifugation and stored at −20°C for further use.

The hot region of the *embB* gene was amplified was amplified using the primers as previously described.[14,15] The fragments containing *ubiA* were amplified using the following primers: ubiA-F (5'-GTGAAGATGTG GTGACTCAACCTCCG-3') and ubiA-R (5'-AACAGCGGCCCCAACCGTTGCTATC-3'). All amplified products were purified, dried, and loaded onto an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). The sequences generated were compared with the H37Rv reference genome (GenBank accession number NC_000962) using BioEdit v7.05.3.

Statistical Analysis

The association of the *embB* and/or *ubiA* mutation with the EMB MICs was evaluated with a regression multivariate model. A *P* value of less than 0.01 was considered to be statistically significant. All statistical data were performed using SAS (version 9.3) software (SAS Institute, Cary, NC).

Effects of Mutations on UbiA

The wild type UbiA in *M. tuberculosis* was obtained from the AlphaFold Protein Structure Database (access ID: P9WFR5). The effects of single point mutations on UbiA protein stability were accessed with PremPS Server[16] (https://lilab.jysw.suda.edu.cn/research/PremPS/), using the wild type UbiA (P9WFR5) as a template.

Results

EMB MICs Results

The Results of MICs of the 146 MDR-TB isolates are summarized in Table 1. According to the MICs, 146 isolates were classified into one of three EMB MIC categories: susceptible (MIC ≤ 2 μg/mL), low-level resistant (MIC = 4 μg/mL), and high-level resistant (MIC ≥ 8 μg/mL), which included 41, 44, and 61 isolates, respectively.

Table 1 EMB MICs Distributions in All Mutated Isolates

Mutation		No. of isolates							MIC (μg/mL)	
		All	MIC (μg/mL)							
embB	*ubiA*		≤0.5	1	2	4	8	16	≥32	Median (IQR)	Range
Met306Ile		23			2	14	4	3		4 (4–8)	2–16
Met306Leu		3				1	2			8 (4–8)	4–8
Met306Val		26				1	17	8		8 (8–16)	4–16
Tyr319Cys		1				1				4	/
Asp328Gly		1				1				4	/
Asp328Tyr		1					1			8	/
Phe330Ile		2				2				4 (4–4)	4–4
Asp354Ala		2				2				4 (4–4)	4–4
Leu402Val		1					1			8	/
Gly406Ala		9				5	3	1		4 (4–8)	4–16
Gly406Asp		4				4				4 (4–4)	4–4
Gly406Ser		3				2		1		4 (4–16)	4–16
Gln497Arg		6				1	5			8 (8–8)	4–8
Gln497Pro		1					1			8	/
Ala505Val		1						1		16	/
Gly246Arg, Met306Val		1					1			8	/
Met306Val, Gly406Asp		1						1		16	/
Met306Val, Gln497His		2						1	1	24 (16–32)	16–32
Met306Ile, Gln497Pro		1						1		16	/
Asp300Gly	Ser173Ala	1					1			8	/
Met306Leu	Ala167Thr*	1					1			8	/
Gly406Ser	Met180Ile*	1				1				4	/
Gly406Ala	Ala237Thr*	1					1			8	/
Gln497Arg	Ala38Thr	1					1			8	/
Gln497Arg	Val55Leu*	1						1		16	/
Gln497Arg	Phe59Cys*	1					1			8	/
	Ala39Glu*	1			1					2	/
	Ser173Ala	1			1					2	/
	Trp175Cys	1			1					2	/
	Val283Leu*	1			1					2	/

Note: *Mutation not previously reported.

Mutations in *embB* and *ubiA*

DNA sequencing showed that a total of 100 clinical isolates, including six susceptible isolates, 35 low-level EMB-resistant isolates, and 59 high-level EMB-resistant isolates, carried at least one non-synonymous mutation in the sequenced *embB* and *ubiA* regions (Table 1). Among these mutations, 88 isolates (88.0%) harbored a single mutation, while 12 (12.0%) isolates harbored double mutations. However, there were still 11 isolates, including nine low-level EMB-resistant isolates and two high-level EMB-resistant isolates, which harbored no mutations in the sequenced *embB* and *ubiA*.

Overall 96 isolates (containing two susceptible isolates, 35 low-level EMB-resistant isolates, and 59 high-level EMB-resistant isolates) had mutations within *embB*. The most common mutation among all mutants observed in 58.0% (58/100) isolates was at codon 306, followed by codons 406 and 497, present in 19.0% (19/100) and 13.0% (13/100) mutated isolates, respectively. Met306 was replaced by Val (30 isolates), Ile (24 isolates), and Leu (4 isolates); Gly406 was replaced by Ala (10 isolates), Asp (5 isolates), and Ser (4 isolates); Gln497 was replaced by Arg (9 isolates), Pro (2 isolates), and His (2 isolates). In addition, other mutations were also identified in codons 246 (1 isolate), 300 (2 isolates), 319 (1 isolate), 328 (2 isolates), 330 (2 isolates), 354 (2 isolates), 402 (1 isolate), and 505 (1 isolate).

For the *ubiA* gene, non-synonymous mutations were observed in four EMB-susceptible isolates, one low-level EMB-resistant isolate, and six high-level EMB-resistant isolates (Table 1). These mutations consisted of ten unique changes, which are Ala38Thr, Ala39Glu, Val55Leu, Phe59Cys, Ala167Thr, Ser173Ala, Trp175Cys, Met180Ile, Ala237Thr, and Val283Leu. To our knowledge, mutations Ala39Glu, Val55Leu, Phe59Cys, Ala167Thr, Met180Ile, Ala237Thr, and Val283Leu, have not been described previously. Most mutations within *ubiA* were accompanied by the additional mutations of *embB*. Interestingly, all four single *ubiA* mutations (Ala39Glu, Ser173Ala, Trp175Cys, and Val283Leu) were observed in EMB-susceptible isolates (MICs = 2 μg/mL). Nevertheless, *ubiA* mutations with additional *embB* mutation were observed in only EMB-resistant isolates.

Association Between the Mutations and EMB MIC

Considering 12% of isolates carried more than one mutation, the association between mutations and EMB resistance was evaluated with multivariate regression (Table 2). In the multivariate model, the *embB* mutations Met306Val, Met306Ile, Gly406Ala, and Gln497Arg were associated with EMB resistance (P <0.01), with the Odds Ratio (OR) values of 83.742, 23.802, 30.405, and 27.268, respectively (Table 2). However, there were still two EMB-susceptible isolates (with MICs of 2 μg/mL) that harbored Met306Ile mutations.

Isolates with higher MICs were more likely to have mutations Met306Val and Gln497Arg, with the median MICs of 8 μg/mL. The multivariate model also indicated that these two mutations were significantly correlated with high-level EMB resistance (P <0.01), with the OR values of 163.45 and 45.091, respectively (Table 3).

Multiple mutations were observed in 12 isolates, 11 of which (83.3%) were classified into high-level EMB category. These isolates harbored at least one mutation within *embB*. All five isolates carrying double mutations within *embB*

Table 2 Logistic Regression Multivariate Model Results Between Mutations and EMB Resistance

Mutation		No. of isolates	Median MIC (IQR)	OR value	*P*
embB	***ubiA***				
Met306Val		30	8 (8–16)	83.742	<0.0001*
Met306Ile		24	4 (4–8)	23.802	<0.0001*
Gly406Ala		10	6 (4–8)	30.405	<0.0001*
Gln497Arg		9	8 (8–8)	27.268	0.0002*
Gly406Asp		5	4 (4–4)	11.589	0.0228
Met306Leu		4	8 (6–8)	11.589	0.0228
Gly406Ser		4	4 (4–10)	11.589	0.0228
Gln497Pro		2	12 (8–16)	2.411	0.5667
Gln497His		2	16 (16–32)	/	/
Phe330Ile		2	4 (4–4)	5.332	0.1987
Asp354Ala		2	4 (4–4)	5.332	0.1987
	Ser173Ala	2	5 (2–8)	2.334	1

Note: *Significant at the 0.01 threshold.

Table 3 Logistic Regression Multivariate Model Results Between *embB* Mutations and HLR

Mutation	OR value (95% CI)	*P*
Met306Val	163.45 (20.135–>999.999)	<0.0001*
Met306Ile	2.818 (0.973–8.164)	0.0562
Gly406Ala	5.636 (1.396–22.757)	0.0152
Gln497Arg	45.091 (5.12–397.099)	0.0006*

Note: *Significant at the 0.01 threshold.

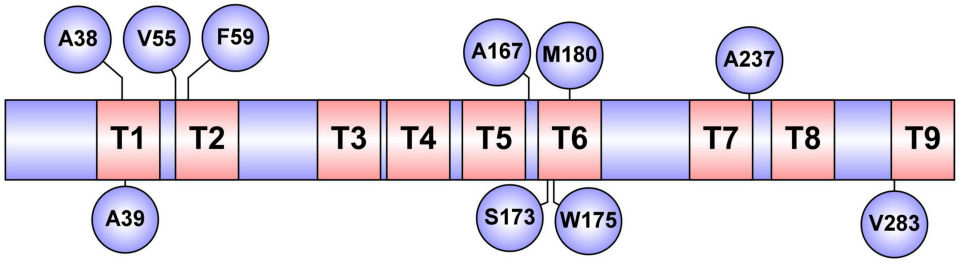

Figure 1 Schematic representation of mutated amino acid codons location of UbiA in *M. tuberculosis*. T1–T9 indicate transmembrane domains.

belonged into high-level EMB category. The remaining seven isolates harbored one mutation in *embB* and one mutation in *ubiA*. One of them had MICs of 4 μg/mL and classified into a low-level EMB category. However, none of the EMB-susceptible isolates carried multiple mutations, which were detected only in EMB-resistant isolates. The percentage of multiple mutations in high-level resistant isolates was > 6 times that in low-level resistant isolates (18.64% versus 2.86%).

Effects of Mutations on UbiA Stability

On the basis of computer modeling, the UbiA protein is an α-helical protein with nine transmembrane domains and no large carboxy-terminal region. Interestingly, most mutation sites mentioned above including the 38th, 39th, 55th, 59th, 173rd, 175th, 180th, 237th, and 283rd were all localized in the five transmembrane domains, namely T1, T2, T6, T7, and T9 of UbiA except for the mutation 167th localized in the extracytoplasmic loop between T5 and T6 (Figure 1). Using P9WFR5 as a template, five features were obtained by PremPS on-line tool for each mutated UbiA (Table 4). Of these features, ΔΔG is usually used to predict the stability of the protein caused by mutations. It is obtained by quantifying the change of unfolding Gibbs free energy (ΔG) of a protein after a single point mutation. Thus, positive and negative signs correspond to destabilizing and stabilizing mutations, respectively. According to the results, almost all (9 isolates) of the ten mutated UbiA had greater ΔGs than the wild type UbiA, indicating destabilizing effects of these point mutations. Additional computational models further predicted a damaging effect of single mutations A39E and W175C on the UbiA function (Figure 2).

Table 4 the Detailed Parameters for the *ubiA* Mutants

Mutation	PremPS (ΔΔG)	Location	PSSM	ΔCS	ΔOMH
Ala38Thr	1.16	SUR	0.3607	0.885	−0.0395
Ala39Glu	1.62	COR	0.6661	0.7211	0.2038
Val55Leu	−0.1	COR	−0.4394	0.7222	−0.2421
Phe59Cys	1.16	SUR	0.3014	0.715	0.8154
Ala167Thr	1.65	COR	0.3546	0.9498	−0.1052
Ser173Ala	0.87	SUR	−0.0458	0.9695	−0.0183
Trp175Cys	1.45	COR	0.4911	0.6669	−5.00E-04
Met180Ile	0.72	COR	−0.0062	0.5022	−0.1282
Ala237Thr	1.15	COR	−0.5092	1.3367	0.0391
Val283Leu	0.82	SUR	0.4192	0.4846	−0.2839

Notes: PremPS, Quantitative changes in unfolding Gibbs free energy (ΔΔG). Location, there are two values for location, "COR" indicates that the mutated amino acid is buried in the protein core, while "SUR" means the mutated residue is located on the surface of the protein. PSSM, Position-Specific Scoring Matrix created by PSI-BLAST; ΔCS, change of conservation after mutation calculated by PROVEAN method; ΔOMH, difference of hydrophobicity scale between mutant and wild-type residue type.

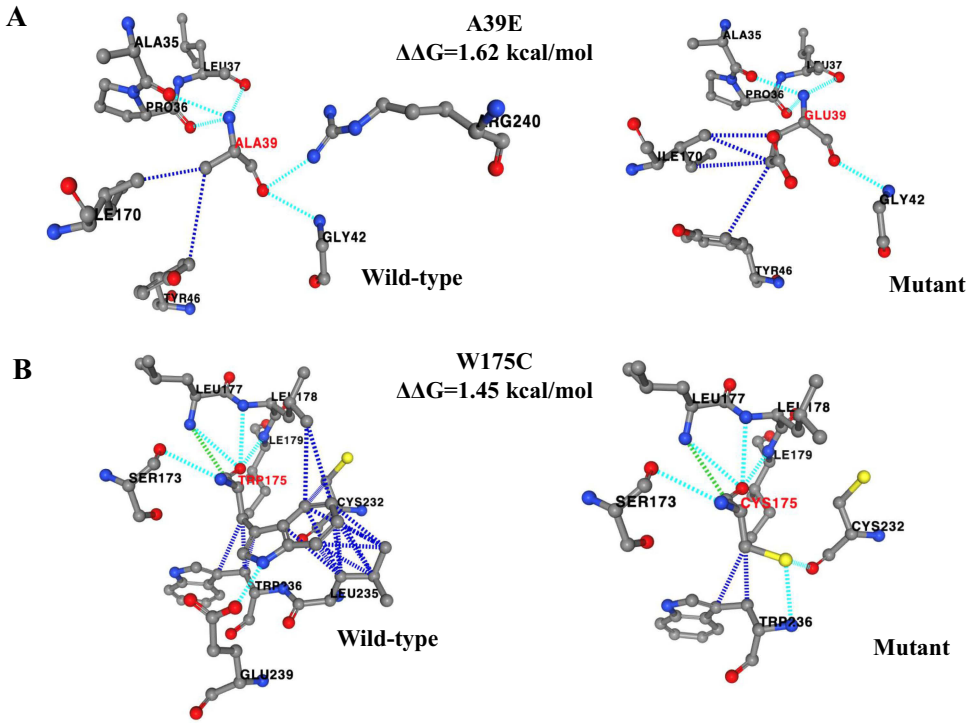

Figure 2 The impact of the A39E (**A**) and W175C (**B**) amino changes in the protein stability (ΔΔG) of UbiA predicted using the PremPS online tool. For clarity, the ribbon was hidden and only the non-covalent interactions affected by the substitutions are displayed. Dotted lines represent hydrophobic (blue), polar (light blue), and Van der Waals (green) interactions in the wild-type and mutant structures. Positive ΔΔG predicts a reduction in the stability of the resulted protein.

Discussion

It was well reported that mutations within *embB* were mainly conferring EMB resistance. Accordingly, our study showed that 89.5% (94/105) of EMB-resistant isolates and 4.9% (2/41) of EMB-susceptible isolates harbored the mutation within *embB*. A total of 11 distinct codons in *embB* were detected that had mutations causing amino acid changes. Most mutations occurred in *embB* codon 306, codon 406, and codon 497, resulting in three different amino acid changes. Mutations Met306Val, Met306Ile, Gly406Ala, and Gln497Arg were significantly associated with EMB resistance, consistent with the prior studies.[4,17] According to the latest World Health Organization (WHO) catalogof mutations and their association with drug resistance in TB, these four mutations belonged to group 1 (associated with resistance) for EMB,[17] and thus could be used as crucial markers for EMB resistance. It is notable that there were still two EMB-susceptible isolates harboring Met306Ile with MICs of 2 μg/mL, confirming the findings that *embB*306 mutations occurred in a few EMB-susceptible strains. Besides these four mutations, there were some other mutations, such as Gly406Asp, Met306Leu, and Gly406Ser, which were exclusively present in EMB-resistant isolates. Although these three mutations also belonged to group 1 (associated with resistance) for EMB,[17] they were not detected by multivariate analysis, possibly due to limited sample number in this study. Hence, further studies including more isolates with these mutations are required.

We also found that mutations Met306Val and Gln497Arg were strongly associated with high-level EMB resistance, similar to the reports that these two mutations were commonly observed in EMB high-level resistant isolates.[2,13,14] It is notable that isolates harboring single mutation *embB* Asp328Tyr, Leu402Val, or Ala505Val had MICs of ≥ 8 μg/mL and belonged to the EMB high-level group. However, the number of these mutated isolates was very scarce. Additional investigations that include a substantial panel of isolates with these mutations will be needed in the future.

Previous reports indicated that the mutated prevalence of *ubiA* among EMB-resistant isolates were varied with geographic location.[9] Our study showed that the prevalence of *ubiA* mutations among EMB-resistant isolates was 6.7%, comparable to those of other studies from China (8.3%),[12] Thailand (8.9%),[8] and South Korea (9.5%),[9] but significantly lower than those in North India (17.2%)[13] and Africa (45.5%).[9] Most mutated sites of UbiA located in the transmembrane domains.[9] Accordingly, our results showed that 90% (9/10) mutations occurred in the transmembrane domains of UbiA. An important finding from

sequencing of the *ubiA* in this study was the identification of some novel mutations. Moreover, we also observed four single mutations (Ala39Glu, Ser173Ala, Trp175Cys, and Val283Leu) in EMB-susceptible isolates. Some reports showed that *ubiA* mutations were always observed together with *embB* mutations in EMB-resistant isolates.[7,9] Of these four mutations, Trp175Cys was reported to occur exclusively in EMB-resistant isolates and confer EMB resistance.[11] Computer models also suggested these four mutations could reduce the protein stability. However, all four isolates harboring a single mutation within *ubiA* had a MIC of 2.0 μg/mL, which is close to the breakpoint MIC definition of EMB resistance. These results suggested that a single mutation occurring in *ubiA* likely confers a small increase in the MIC. Yet, these mutations may be important, as they represent the first, often pre-resistant step in the evolution of high-level EMB resistance.

Previous reports also suggested that *ubiA* usually mutated along with the *embB* mutations.[12,13] Accordingly, in the current study, 63.6% (7/11 isolates) of isolates carrying *ubiA* mutations combine with *embB* mutations. In accordance with prior reports,[7–9] our results demonstrated that almost all isolates carrying *ubiA* mutations together with *embB* mutations were classified into EMB high-level group. Multiple mutations within *embB* were also more likely to occur in isolates with high-level resistance, supporting the idea that EMB resistance is selected in a stepwise fashion, involving multiple mutations in one or several genes that interact to produce high-level MICs.[7]

Although the most common mutated region within *embB* and the whole *ubiA* were explored in our study, there were still 10.5% (11/105) of isolates that lacked a resistance-associated mutation. The EMB MICs for the 11 strains that lacked mutations ranged from 4 to 8 μg/mL, implying that resistance in these isolates might be involved in other mutations outside the sequenced region or other mechanisms like permeability and efflux pumps.[18] In addition, some mutations exclusively among EMB-resistant isolates do not prove that they confer or otherwise participate in resistance to this drug. Additional molecular genetics, biochemical, and enzymatic studies are required to prove that the mutations that we observed participate in the response of TB to EMB treatment.

Conclusion

In summary, we revealed the comprehensive profiles of mutations within *embB* and *ubiA*, and their associations with EMB resistance levels. These results will broaden our mechanistic understanding of EMB resistance in TB, which helps to develop molecular diagnosis and manage treatment decisions.

Ethical Approval

This study was approved by the Ethics Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. All patients participating in the study provided written consent. Every procedure involving human participants complied with the ethical standards of the Declaration of Helsinki.

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Disclosure

All authors have no competing interests in this work.

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