Clin. Lab. 2024;70:XXX-XXX

ORIGINAL ARTICLE

Strategic Retesting to Reduce False Positive SARS-CoV-2 PCR Test Results

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SUMMARY

Background: **Nucleic acid amplification testing is the gold standard for SARS-CoV-2 diagnostics, although it may produce a certain number of false positive results. There has not been much published about the characteristics of false positive results. In this study, based on retesting, specimens that initially tested positive for SARS-CoV-2 were classified as true or false positive groups to characterize the distribution of cycle threshold (CT) values for N1 and N2 targets and number of targets detected for each group.**
Methods: **Specimens that were positive for N-gene on retesting and accompanied with S-gene were identified as true positives (true positive based on retesting, rTP), while specimens that retested negative were classified as false positives (false positive based on retesting, rFP).**
Results: **Of the specimens retested, 85/127 (66.9%) were rFP, 16/47 (34.0%) specimens with both N1 and N2 targets initially detected were rFP, and the CT values for each target was higher in rFP than in rTP. ROC curve analysis showed that optimal cutoff values of CT to differentiate between rTP and rFP were 34.8 for N1 and 33.0 for N2. With the optimal cutoff values of CT for each target, out of the 24 specimens that were positive for both N1 and N2 targets and classified as rTP, 23 (95.8%) were correctly identified as true positives. rFP specimens had a single N1 target in 52/61 (85.2%) and a single N2 target in 17/19 (89.5%). Notably, no true positive results were obtained from any specimens with only N2 target detected.**
Conclusions: **These results suggest that retesting should be performed for positive results with a CT value greater than optimal cutoff value for each target or with a single N1 target amplified, considering the possibility of a false positive. This may provide guidance on indications to perform retesting to minimize the number of false positives.**
(Clin. Lab. 2024;70:xx-xx. DOI: 10.7754/Clin.Lab.2023.231214)

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KEYWORDS

false positive, retesting, COVID-19, SARS-CoV-2, NAAT, direct PCR

INTRODUCTION

COVID-19, which emerged in Wuhan, China, in December, 2019, has rapidly spread, leading to a global pandemic [1,2]. Laboratories use nucleic acid amplification test (NAAT), antigen test, and antibody test for diagnosis of COVID-19. NAAT, in particular, is con-

Manuscript accepted December 19, 2023

sidered the gold standard for SARS-CoV-2 diagnostics [3,4].

Because NAATs for the detection of SARS-CoV-2 RNA are highly sensitive, laboratories should exercise caution with potential false positive results [5]. Furthermore, because false positive results lead to adverse consequences for various societies surrounding patients as well as the patients themselves, positive results need to be carefully interpreted when differentiating false positives from true positives [6]. Generally, a positive result with a high cycle threshold (CT) value can be seen in the early or convalescent stages of infection with low viral load, but it may also represent a false positive result [7,8]. It may be difficult to discriminate false from true positive results with a high CT in the absence of clinical history [9,10].

In our hospital laboratory, when specimens with high CT values cause the difficulty of distinguishing true from false positives, the same specimens are retested to ensure reproducibility of the initial results [11,12]. In the present study, the distribution of CT values and characteristics of the detection pattern of target genes in positive SARS-CoV-2 NAAT test results were retrospectively examined to clarify the utility of retesting for positive specimens.

MATERIALS AND METHODS

Sample collection

The specimens consist of 127 positive specimens which, by review of the electronic medical records, were considered to have never been infected with COVID-19 in the past, and were taken out of the 6,560 specimens that performed NAAT with the Ampdirect™ 2019-nCoV detection kit (Shimadzu Corporation, Kyoto, Japan) from September to November, 2022. Among the 127 positive specimens, 24 were nasopharyngeal mucus specimens, and 103 were saliva specimens. Before the initial testing, all specimens were transferred to separate sample tubes. The nasopharyngeal mucus specimens, collected with a sterile swab and suspended in 1 mL of 0.9% sterile saline, were transferred to a separate sample tube. The saliva specimens were diluted 4-fold with Dulbecco's buffered saline in a separate sample tube and then centrifuged at 3,000 g for 1 minute. For retesting, two samples were prepared: the initial specimen and the re-prepared specimen. To process the reprepared sample, nasopharyngeal mucus specimens were transferred from the initial specimens to a new separate sample tube, and saliva specimens were diluted 4-fold with Dulbecco's buffered saline in a new separate sample tube; this was followed by centrifugation at 3,000 g for 1 minute.

PCR

Routine NAAT for SARS-CoV-2 RNA was done using the Ampdirect™ 2019-nCoV detection kit (Shimadzu Corporation, Kyoto, Japan). An assay of the Ampdirect™ 2019-nCoV detection kit (Shimadzu Corporation, Kyoto, Japan) was performed according to the manufacturer's instructions. RNA extraction was mixed with 5 μL of sample and 5 μL of 2019-nCoV Sample Treatment Reagent and was preheated at 90℃ for 5 minutes. RT-PCR reaction solutions were mixed at a ratio of 6.5 μL of 2019-nCoV Reagent A, 6.5 μL of 2019-nCoV Reagent B, and 2 μL of 2019-nCoV Reagent C per test. Reagent B contains primers and probes due to the detecting Nucleocapsid (N)1 and N2 gene regions of SARS-CoV-2 genomic RNA and internal control (IC). RT-PCR mixture was performed with 25 μL, 10 μL of RNA extract plus 15 μL of the PCR reaction solution. RT-PCR analysis was run on reverse transcription at 1 cycle of 42℃ for 600 seconds, preincubation at 1 cycle of 95℃ for 60 seconds, 2 step amplifications at 45 cycles of 95℃ for 5 seconds and 60℃ for 30 seconds. Detection of the IC, the N1 gene, and the N2 gene was utilized Cy5 (excitation: 650 nm, emission: 670 nm), FAM (excitation: 495 nm, emission: 520 nm), and ROX (excitation: 575 nm, emission: 600 nm), respectively. Real-time PCR system used the cobas z480 system (Roche Diagnostics K.K, Tokyo, Japan). For assessment of the resulting, IC was amplificated under 40 cycles and was considered positive when either N1 gene, or N2 gene, or both of the genes were amplified under 40 cycles. However, even if the IC was not amplificated, the specimens were considered positive, if both the N1 gene and the N2 gene were amplificated.

Spike (S) gene of SARS-CoV-2 was detected using SARS-CoV-2 Direct Detection RT-qPCR core Kit (TAKARA-Bio Inc, Shiga, Japan) and Primer/Probe L452R (SARS-CoV-2) ver.2 (TAKARA-Bio Inc, Shiga, Japan). This reagent can be confirmed SARS-CoV-2 in both the detection systems of the 452L wild type and the L452R mutant type. RNA extraction and purification was performed using QIAamp Viral RNA Mini kit (QIAGEN K.K, Tokyo, Japan). RT-PCR reaction solutions were mixed at a ratio of 15 μL of RT-qPCR mix, 3 μL of Primer/Probe L452R ver. 2 (10x), and 6 μL of RNase free water per test. RT-PCR mixture was performed with 30 μL, 6 μL of RNA extract plus 24 μL of PCR reaction solution. RT-PCR analysis was run on reverse transcription at 1 cycle of 52℃ for 600 seconds, preincubation at 1 cycle of 95℃ for 15 seconds, 2 step amplifications at 45 cycles of 95℃ for 5 seconds and 60℃ for 30 seconds. Detection of the 452L wild type and the L452R mutant type was utilized FAM (excitation: 495 nm, emission: 520 nm), Cy5 (excitation: 650 nm, emission: 670 nm), respectively. Real-time PCR system used the cobas z480 system (Roche Diagnostics K. K, Tokyo, Japan). Detection of SARS-CoV-2 RNA was considered positive when either the 452L wild type or the L452R mutant type under 40 cycles.

Verification of SARS-CoV-2 RNA

Positive specimens from the retesting were confirmed SARS-CoV-2 RNA using PCR of S gene.

Definition of judgment

Specimens that performed retesting determined true positive (true positive based on retesting, rTP), false positive (false positive based on retesting, rFP), and the Indeterminate groups as follow. The rTP was positive on the initial test and on either the two samples that were retested or on one of the two samples that were retested, and that confirmed S gene by PCR. The rFP was positive on the initial test, however, the two retested samples were both negative. The Indeterminate groups initially tested positive, and either both of the two retested specimens or at least one of the two specimens tested positive. However, S gene was not detected by PCR.

Evaluation of CT value for retested specimens

For the specimens that detected both the N1 gene and the N2 gene in the initial test, that detected single N1 gene in the initial test, and that detected single N2 gene in the initial test, significant differences for CT value of rTP and rFP were calculated by Mann-Whitney U test, as well as a significance level of $p < 0.05$. For the specimens that detected both the N1 gene and the N2 gene in the initial test and that detected single N1 gene in the initial test, cutoff value of CT value distinguishing between rTP and rFP were calculated receiver operating characteristic (ROC) curve.

RESULTS

Out of the 6,560 specimens that had been tested using NAAT for SARS-CoV-2 RNA, 127 specimens were retested. Of those, 103/5,023 (2.1%) specimens were Saliva and 24/1,537 (1.6%) specimens were nasopharyngeal mucus. Among the specimens subjected to retesting, the rates of rTP and rFP were 28/127 specimens (22.0%) and 85/127 specimens (66.9%), respectively. In saliva specimens, 23/103 specimens (22.3%) were rTP and 73/103 specimens (70.9%) were rFP. In nasopharyngeal mucus specimens, 5/24 specimens (20.8%) were rTP and 12/24 specimens (50.0%) were rFP (Table 1).

Of those tested, 24/47 specimens (51.1%) with both N1 and N2 targets detected were rTP, 16/47 specimens (34.0%) were rFP, and 7/47 specimens (14.9%) were the Indeterminate groups. 4/64 specimens (6.6%) with only N1 target detected were rTP, 52/64 specimens (85.2%) were rFP, and 5/64 specimens (8.2%) were the Indeterminate groups. 17/19 specimens (89.5%) with only N2 target detected were rFP, 2/19 specimens (10.5%) were the Indeterminate groups, and no rTP results were detected (Figure 1).

In the initial testing, the mean N1 CT values in specimens with both N1 and N2 genes detected were 28.6 (range: 21 - 36) (Group A) for rTP, and 36.1 (range: 31 - 38) (Group C) (vs. Group A, $p < 0.001$) for rFP. The means N2 gene CT values in specimens detected both N1 and N2 genes were 27.8 (range: 20 - 35) (Group E) for rTP, and 35.2 (range: 31 - 39) (Group F) (vs. Group F, $p < 0.001$) for rFP (Figure 2). In initial testing, the mean N1 gene CT values in specimens with only N1 gene detected were 35.3 (range: 33 - 37) (Group B) for rTP, and 37.2 (range: 32 - 40) (Group D) (vs. Group B, $p = 0.048$) for rFP. In initial testing, the mean N2 gene CT value in specimens with only N2 gene detected were 37.6 (range: 33 - 40) (Group G) (Figure 2).

For the specimens that detected both the N1 and N2 genes in the initial test, the cutoff value of CT values and area under curve (AUC) distinguishing between rTP and rFP were 34.8, 0.94 in the N1 gene, respectively (Figure 3: (a)), and 33.0, 0.88 in the N2 gene, respectively (Figure 3: (b)). For the specimens that detected only N1 gene in the initial test, cutoff values of CT value and AUC distinguishing between rTP and rFP were 37.2, 0.76 in the N1 gene, respectively (Figure 3: (c)).

DISCUSSION

Of 6,560 specimens tested for SARS-CoV-2, 127 newly positive SARS-CoV-2 specimens were studied. Using the original extracted and re-extracted RNA samples, two individual retestings were performed on 127 initially positive specimens prior to reporting the result to the ordering physician, identifying 85 false positive results (66.9%) (Table 1). These results indicate that retesting of positive specimens is a relatively simple and useful means to detect false positives [13].

Specimens that retested for the N-gene target were subsequently tested using PCR targeting the S-gene target to be classified into true positive (rTP), false positive (rFP), and indeterminate groups. Significant findings were obtained when the distribution of CT values and detection pattern of targets for each group were examined.

Of the 47 specimens that were positive for both N1 and N2 genes, 24 specimens (51.1%) were classified as rTP, whereas 16 specimens (34.0%) were determined to be rFP (Figure 1). When CT values were evaluated for specimens with both N1 and N2 targets detected, the mean CT values for rFP groups (C, 36.1; F, 35.2) were higher than those for rTP groups (A, 28.6; E, 27.8), respectively (Figure 2). Considering that CT values may be useful for distinguishing true positives from false positives, ROC curve analysis was performed to define optimal cutoff values of CT for each target (Figure 3). With the optimal cutoff values of 34.8 for N1 and 33.0 for N2, of 24 specimens that were positive for both N1 and N2 targets and classified as rTP, 23 (95.8%) were correctly identified as true positives. This result suggests that the use of cutoff values obtained may be possible in differentiating true positives from false positives when both N1 and N2 gene targets are detected. In contrast, of specimens with CT values below the cutoff for both N1 and N2, three specimens were classified as indeterminate results, and one specimen was classified as rFP. Among three indeterminate specimens, the N

Table 1. SARS-CoV-2 PCR test results of initial testing and retesting in saliva and nasopharyngeal specimens.

Specimen type	Saliva		Nasopharyngeal		Total	
	number	(%)	number	(%)	number	(%)
Initial test results						
Positive [a]	121	(2.4)	510	(33.2)	631	(9.6)
Negative	4,799	(95.5)	1,003	(65.3)	5,802	(88.4)
Subject to retesting [b]	103	(2.1)	24	(1.6)	127	(1.9)
Total	5,023	(100)	1,537	(100)	6560	(100)
Retest results						
rTP [c]	23	(22.3)	5	(20.8)	28	(22.0)
rFP [d]	73	(70.9)	12	(50.0)	85	(66.9)
Indeterminate [e]	7	(6.8)	7	(29.2)	14	(11.0)
Total	103	(100)	24	(100)	127	(100)

[a] - All specimens that initially tested positive for SARS-CoV-2.
[b] - Newly positive SARS-CoV-2 specimens from patients without previous positive results and known exposure to individuals with COVID-19 infection.
[c] - Classified as true positives based on retesting (i.e., specimens that retested positive for N gene and were also positive for S gene (L452)).
[d] - Classified as false positives based on retesting (i.e., specimens that retested negative for N gene).
[e] - Classified as indeterminate results based on retesting (i.e., specimens that retested positive for N gene but were negative for S gene (L452)).

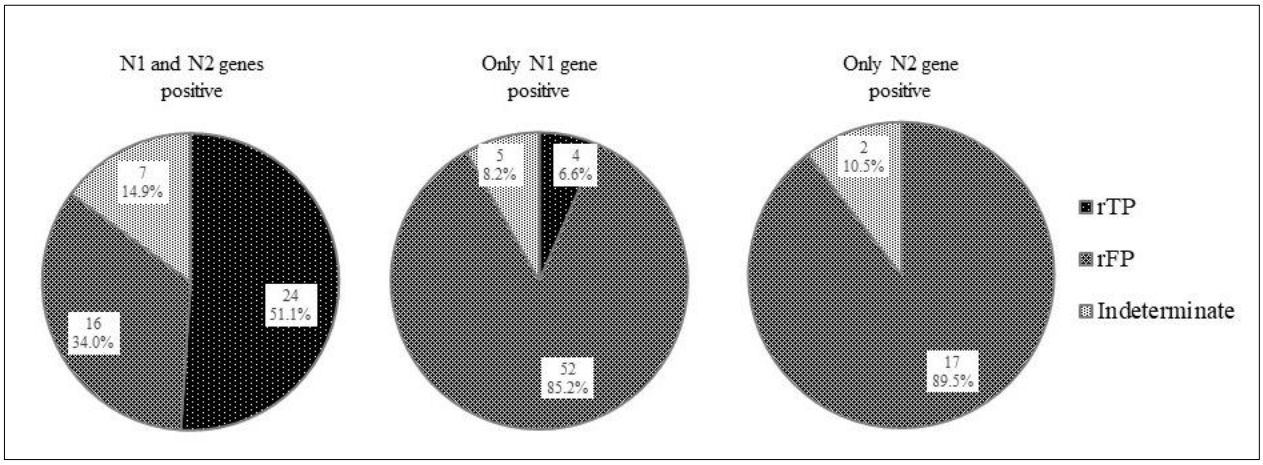

Figure 1. Pie chart representing the amounts of true positives based on retesting (rTP), false positives based on retesting (rFP), and indeterminate results in 127 initially positive specimens with only one or two genes detected.

rTP - specimens that retested positive for the N gene and were also positive for the S gene (L452) were classified as true positives, rFP – specimens that retested negative were classified as false positives. Indeterminate - specimens that retested positive for the N gene but were negative for the S gene (L452) were classified as indeterminate.

gene was reproducibly detected but the S gene was not detected, suggesting the presence of viral RNA fragments [14,15]. The cause of one rFP result may be due to PCR contamination [16].

Next, of the 61 specimens with only N1 target detected, 52 specimens (85.2%) were classified as rFP, while 4 specimens (6.6%) were determined to be rTP (Figure 1). Focusing on the CT values of specimens with only N1

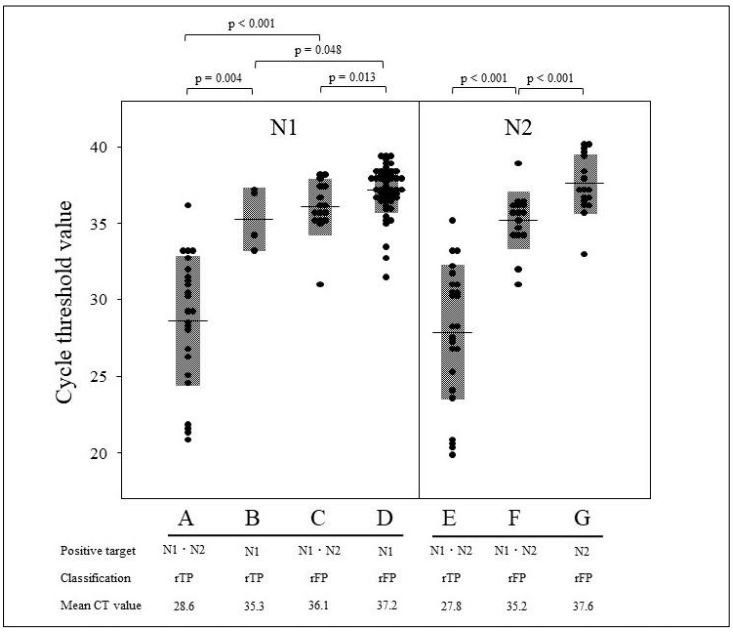

Figure 2. Distribution of cycle threshold values from N1 and N2 amplifications of 127 initially positive specimens by genes detected and classification.

CT - cycle threshold, rTP - classified as true positives based on retesting, rFP - classified as false positives based on retesting, A - specimens that were positive for both N1 and N2 targets and were classified as rTP, B - specimens that were positive for only N1 target and were classified as rTP, C - specimens that were positive for both N1 and N2 targets and were classified as rFP, D - specimens that were positive for only N1 target and were classified as rFP, E - specimens that were positive for both N1 and N2 targets and were classified as rTP, F - specimens that were positive for both N1 and N2 targets and were classified as rFP, G - specimens that were positive for only N2 target and were classified as rFP.

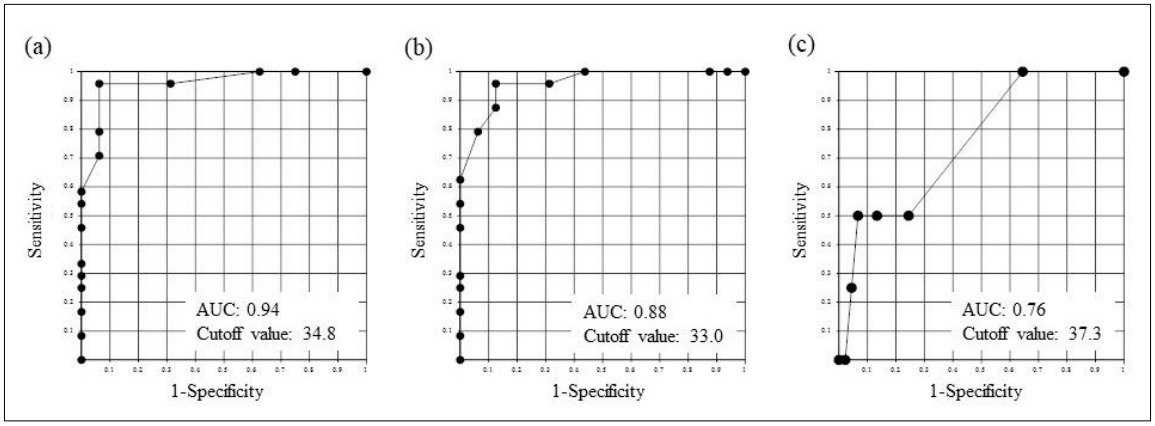

Figure 3. Receiver operating characteristic curves for PCR cycle threshold values.
(a) - Curve for A vs. C, (b) - Curve for E vs. F, (c) - Curve for B vs. D, CT - cycle threshold, rTP - classified as true positives based on retesting, rFP - classified as false positives based on retesting, A - N1 CT values of specimens that were positive for both N1 and N2 targets, and were classified as rTP, B - N1 CT values of specimens that were positive for only N1 target, and were classified as rTP, C - N1 CT values of specimens that were positive for both N1 and N2 targets, and were classified as rFP, D - N1 CT values of specimens that were positive for only N1 target, and were classified as rFP, E - N2 CT values of specimens that were positive for both N1 and N2 targets, and were classified as rTP, F - N2 CT values of specimens that were positive for both N1 and N2 targets, and were classified as rFP, AUC - area under the curve.

target detected (B and D), the mean N1 CT values for rTP specimens with a single N1 target detected (B, 35.3) were higher than those for rTP specimens with both N1 and N2 targets detected (A, 28.6), reflecting a lower viral load in specimens of the B group (Figure 2) [9,10]. Considering that only N1 was detected in these specimens, there may be a difference in detection sensitivity between the N1 and N2 PCR assays [17,18]. Furthermore, the mean N1 CT values for rFP specimens with only N1 target detected (D, 37.2) were higher than those for rTP specimens with a single N1 target detected (B, 35.3) (Figure 2). The difference in mean CT values between the B and D groups might be due to the difference between specific amplification of genetic material from the virus and non-specific amplification associated with primer-probe sets [19]. Although a significant difference was observed in the mean CT values between the B and D groups, ROC curve analysis showed that the AUC was lower in specimens with only N1 detected (B versus D, 0.76) than in specimens with both N1 and N2 detected (A versus C, 0.94), suggesting lower ability to discriminate between the B and D groups (Figure 3). These results suggest that specimens with a single N1 target detected should be retested to distinguish between true positives and false positives [13].

Lastly, of the 19 specimens with only N2 target detected, 17 specimens (89.5%) were classified as rFP, whereas no specimens were classified as rTP (Figure 1). Focusing on the CT values of specimens with a single N2 target detected (F and G), the mean N2 CT values for rFP specimens with a single N2 target detected (G, 37.6) were higher than those for rFP specimens with both N1 and N2 targets detected (F, 35.2) (Figure 2). Considering the difference in CT values between the G and F groups, false positive results of the G group might be attributed to non-specific amplification with primer-probe sets [19,20], while the false positive signal of the F group was likely caused by contamination with minute levels of positive samples or positive controls [16]. Furthermore, N2-gene positive/N1-gene negative result combinations are less likely to occur in true positive specimens where viral RNA is present because detection of N2 is less sensitive than N1. This could explain why specimens with only N2 detected are non-specific reactions. These results suggest that retesting may be omitted because true positive test results are unlikely in case of specimens with a single N2-gene detected [21, 22]. However, because the sample size where the N2 target alone was detected was not large enough in this study, further studies with larger sample sizes are needed to validate these findings.

CONCLUSION

In the present study, the utility of retesting by direct RT-PCR for SARS-CoV-2, the CT values for discriminating between true positives and false positives, and the characteristics of the detection pattern of targets that require retesting were clarified. Retesting of positive specimens is considered the simplest means to ensure the accuracy of NAAT. In the case of an initial test result even with both N1 and N2 targets detected, with a CT value greater than 34.8 for the N1 gene and greater than 33.0 for the N2 gene, or with a single N1 gene detected, retesting is recommended to prevent false positive results with SARS-CoV-2 PCR tests.

Acknowledgment:
The authors would like to thank all the staff of the Department of Clinical Laboratory, Kindai University Hospital for their assistance.

Ethical Considerations:
This study was approved by the Ethics Committee of Kindai University, School of Medicine (approval number: R04-183).

Declaration of Interest:
The authors have no conflicts of interest relevant to this article.

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