

# Diagnostic impact of high serum midkine level in patients with gastric cancer

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## Abstract

**Aim:** We evaluated the diagnostic impact of serum midkine (s-MK) levels in patients with gastric cancer using a monoclonal antibody enzyme-linked immunosorbent assay system (ELISA) to detect s-MK levels.

**Methods:** Serum samples were obtained from 131 patients with gastric cancer including stage I (n = 71), stage II (n = 28), stage III (n = 16), and stage IV (n = 16) before surgery. Serum samples were analyzed using ELISA to detect soluble midkine. A cut-off value was fixed at 421 pg/mL, and the sample divided into two groups: a high s-MK group and a low s-MK group. Clinicopathological factors and prognosis were compared between these two groups using univariate and multivariate analyses. Comparison of two groups was analyzed by Fisher's exact probability test. Statistical significance was considered at  $P < 0.05$ .

**Results:** High s-MK was significantly associated with high carcinoembryonic antigen (CEA) ( $P < 0.01$ ). Positive rate of s-MK was higher than the positive rates of CEA in patients with stage I/II gastric cancer. Combination with CEA + CA19-9 + s-MK increased the positive rates of patients with stage I/II gastric cancer. No other clinicopathological factors were associated with s-MK. Although the high s-MK group showed worse overall survival than the low s-MK group, the difference was not statistically significant.

**Conclusion:** s-MK level is increased even during early-stage gastric cancer. Combined with s-MK, the positive rate of CEA + CA19-9 was increased in patients with stage I/II gastric cancer.

## KEYWORDS

cancer antigen 19-9, carcinoembryonic antigen, enzyme-linked immunosorbent assay, gastric cancer, midkine

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## 1 | INTRODUCTION

Although surgical management has improved<sup>1</sup> and new chemotherapeutic modalities have been introduced,<sup>2</sup> the clinical outcomes of patients with advanced gastric cancer are not satisfactory.<sup>3</sup> Various serum biomarkers for early diagnosis in the field of gastrointestinal cancers have been reported.<sup>4</sup> One example is midkine, expressed at higher levels in various solid tumors than in adjacent normal tissues,<sup>5</sup> even during early-stage disease.<sup>6-8</sup>

Serum midkine (s-MK) levels reflect MK expression levels in cancer tissues because MK is a secreted cytokine. Based on meta-analysis, Jing et al<sup>9</sup> reported that s-MK level was an effective means of diagnosing cancer other than gastric cancer. In previous studies,<sup>10,11</sup> polyclonal antibodies were used in enzyme-linked immunosorbent assay (ELISA). Polyclonal antibody can bind to multiple epitopes; however, monoclonal antibody can bind to one specific epitope only. Generally, polyclonal antibody ELISA shows a relatively high positive rate but low specificity with high coefficient of variation. Indeed, the coefficient of variation of polyclonal antibody ELISA was 9.5%<sup>12</sup> and the coefficient of variation of monoclonal antibody ELISA was 2.4%-4.16%.<sup>13,14</sup> Recently, an ELISA using monoclonal antibodies against midkine was developed.<sup>13</sup> We evaluated the diagnostic abilities of this assay system using monoclonal antibodies for malignant mesothelioma<sup>15</sup> and head and neck cancer.<sup>16</sup> Only two reports used polyclonal antibody ELISA to evaluate s-MK levels in patients with gastric cancer.<sup>10,11</sup> However, clinicopathological significance of the s-MK levels was not evaluated in those studies.

Therefore, in the present study, we used monoclonal antibody ELISA<sup>13</sup> to evaluate the diagnostic impact of s-MK level. In addition, we evaluated the clinicopathological and prognostic significance of s-MK levels in patients with gastric cancer.

## 2 | SUBJECTS AND METHODS

### 2.1 | Subjects

Between 2010 and 2014, 131 patients with primary gastric adenocarcinoma treated with gastrectomy at the Department of Gastroenterological Surgery, Toho University Hospital were enrolled in this study. Excluded were patients who were treated with neoadjuvant therapies. Because the Gastric Cancer Treatment Guidelines recommend neoadjuvant chemotherapy only for some cases with multiple lymph node metastases, we currently do not carry out neoadjuvant chemotherapy as a standard treatment option. All the patients were followed up until the end of 2017, or to their death. All patients underwent gastrectomy with standard lymphadenectomy except those with stage IV. Informed consent was obtained from all the subjects. Informed consent was obtained from all the patients, and the Ethics Committee of Toho University School of Medicine approved the study (nos. 22-112 and 22-047).

### 2.2 | Serum sampling and enzyme immunoassay for serum midkine

After obtaining written informed consent, sera were obtained before surgery by venipuncture and immediately centrifuged at 3000 g for 5 minutes. The serum was frozen at -80°C until the assay was carried out. Repeated thawing and freezing of the samples was avoided. A commercially available enzyme-linked immunosorbent assay kit for midkine (Cellmid, Sydney, NSW, Australia) was used to measure s-MK levels.<sup>13</sup> We determined s-MK levels in the collected samples according to the manufacturer's protocol.<sup>16</sup> Absorbance was measured with a microplate reader (Epoch 2; BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 450 nm and was analyzed using Gen5 software (BioTek Instruments, Inc.). Data of healthy volunteers were provided by the company.<sup>13</sup> However, characteristics of healthy controls, such as age, smoking, and chronic diseases, are unknown. Mean value obtained from 99 healthy volunteers was 208 pg/mL, with a standard deviation (SD) of 107 pg/mL, according to the manufacturer's protocol data. Therefore, the cut-off value was fixed as mean + 2 SD to be 421 pg/mL as previously described.<sup>15</sup>

### 2.3 | Carcinoembryonic antigen and CA19-9 assay

Carcinoembryonic antigen (CEA) levels were measured with a CEA-2 enzyme immune assay (EIA) kit (Elecsys CEAll; Roche Diagnostics K.K., Tokyo, Japan) following the manufacturer's instructions with a cut-off value of 5.0 ng/mL.<sup>17</sup> Cancer antigen 19-9 (CA19-9) levels were measured with a CA19-9 EIA kit (Elecsys CA19-9; Roche Diagnostics K.K.) with a cut-off value of 37 U/mL.<sup>17</sup>

### 2.4 | Statistical analyses

Data are expressed as means plus standard deviations. A paired *t*-test was used to make between-group comparisons. Kaplan-Meier product limit method was used to calculate the survival probabilities. Log-rank test was used to test the differences between the groups. Fisher's exact probability test was applied to determine the significance of differences between the two groups. Cox's proportional hazards model was used to assess the influence of each clinicopathological variable on survival. EZR statistical software was used to carry out all the statistical analyses.<sup>18</sup> Statistical significance was defined as  $P < 0.05$ .

## 3 | RESULTS

Subjects were 87 males (66%) and 44 females (34%) with a median age of 72 years (range: 35-92 years) and were classified according to the TNM/UICC guidelines as follows: 71 stage I; 28 stage II; 16 stage III; and 16 stage IV. Patients with stage IV disease showed distant lymph node metastasis ( $n = 3$ ), liver metastasis ( $n = 1$ ), cancer cells

on peritoneal cytology ( $n = 6$ ), and peritoneal metastasis ( $n = 6$ ). As a result of passage disturbance or bleeding, these cases were treated with gastrectomy. For the entire population, the median follow-up period was 39 months.

### 3.1 | Comparison of serum midkine levels between gastric cancer patients and healthy controls

Serum midkine levels of gastric cancer patients ( $344 \pm 565$  pg/mL) were significantly higher than those of the healthy controls ( $208 \pm 107$  pg/mL;  $P = 0.02$ ). Based on the cut-off value of 421 pg/mL (mean + 2 SD in healthy controls), the positive rate of healthy and gastric cancer was 5% and 21%, respectively. Sensitivity was 21%, specificity was 95%, positive predictive value was 85% negative predictive value was 48%, and accuracy was 53%.

### 3.2 | Comparison of serum midkine levels according to clinicopathological factors of patients with gastric cancer

Table 1 shows the comparison of s-MK levels according to clinicopathological characteristics of patients with gastric cancer, including CEA and CA19-9. CEA was significantly associated with high s-MK. Tumor depth was slightly associated with s-MK. Gender, age, CA19-9, lymph node metastasis, distant metastasis, and differentiation were not associated with s-MK. Multivariate analysis showed that CEA was an independent risk factor ( $P = 0.01$ ) (Table 1). Smoking was significantly associated with CEA and s-MK. However, chronic inflammatory diseases, such as collagen disease, chronic hepatitis, empyema arthritis, chronic obstructive pulmonary disease (COPD), and diabetes mellitus, were not associated with CEA (Table S1) and s-MK. Various inflammatory blood laboratory data, such as C-reactive protein (CRP), white blood cells, and neutrophils, were not associated with s-MK (Table S2).

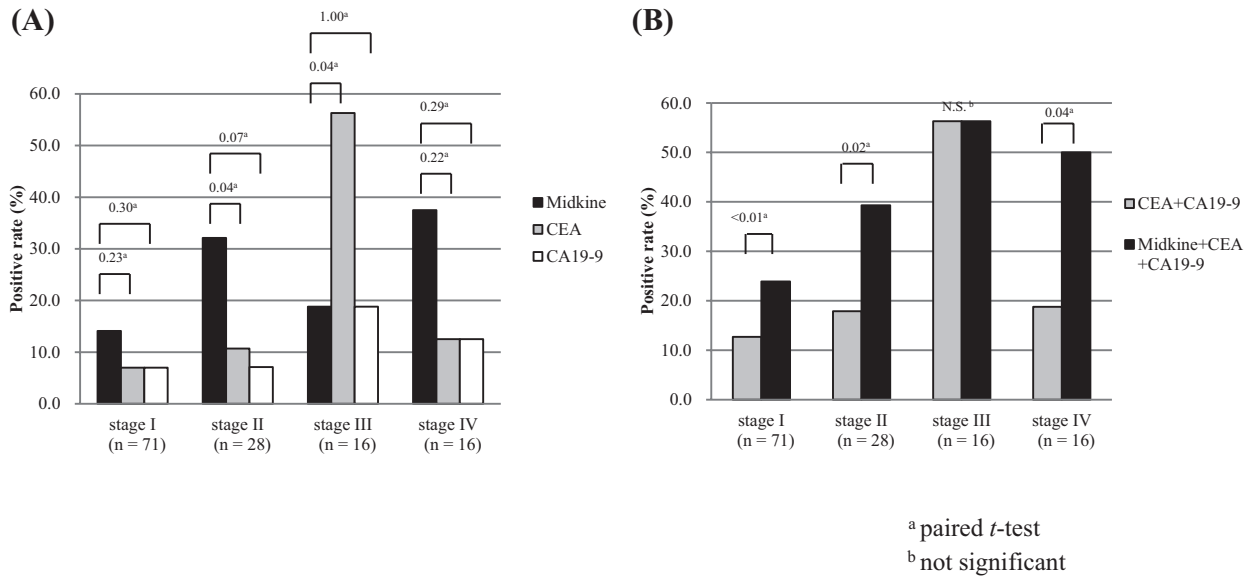
**TABLE 1** Comparison of positive rates of high serum midkine levels according to clinicopathological characteristics of patients with gastric cancer

Variables	Fisher's exact probability test			Logistic regression analysis		
	s-MK $\leq 421$ pg/mL ( $n = 103$ )	s-MK $> 421$ pg/mL ( $n = 28$ )	P-value	Odds ratio	95% CI	P-value
Gender						
Male	67	20	0.65			
Female	36	8				
Age (y)						
$\leq 65$	37	6	0.18	1.88	0.66-5.36	0.24
$> 65$	66	22				
CEA (ng/mL) <sup>a</sup>						
$\leq 5$	92	19	$< 0.01$	4.02	1.38-11.8	0.01
$> 5$	10	9				
CA19-9 (U/mL) <sup>b</sup>						
$\leq 37$	90	25	1			
$> 37$	10	3				
Tumor depth						
T1	49	8	0.09	1.59	0.59-4.35	0.36
T2-T4	54	20				
Lymph node metastasis						
No metastasis	61	15	0.67			
Metastasis	42	13				
Distant metastasis						
M0	93	23	0.31	1.91	0.51-7.2	0.34
M1	10	5				
Differentiation						
Differentiated	51	18	0.20	0.64	0.25-1.63	0.35
Undifferentiated	52	10				

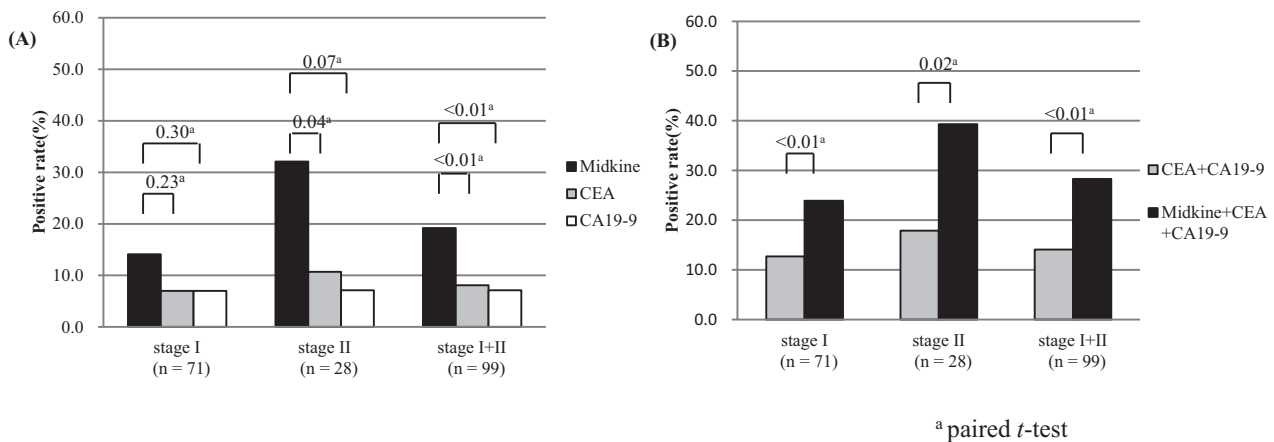
<sup>a</sup>Value of one case was lost.

<sup>b</sup>Values of three cases were lost.

CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; s-MK, serum midkine.



**FIGURE 1** Positive rates of serum tumor markers in gastric cancer. (A), Comparison of positive rates of serum tumor markers. (B), Comparison of positive rates between carcinoembryonic antigen (CEA)/cancer antigen 19-9 (CA19-9) and CEA/CA19-9/midkine

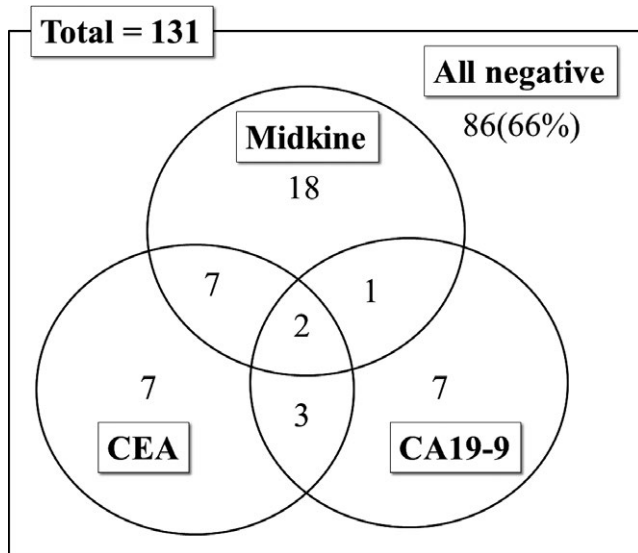


**FIGURE 2** Positive rates of serum tumor markers among stage I, II, and I + II gastric cancer. (A), Comparison of positive rates of serum tumor markers. (B), Comparison of positive rates between carcinoembryonic antigen (CEA)/cancer antigen 19-9 (CA19-9) and CEA/CA19-9/midkine

### 3.3 | Positive rate of each tumor marker according to each TNM stage in gastric cancer

Positive rates of s-MK at each TNM stage were as follows: 10 of 71 patients with stage I (14.1%), nine of 28 patients with stage II (32.1%), three of 16 patients with stage III (18.8%), and six of 16 patients with stage IV (37.5%) (Figure 1A). The positive rate of s-MK was higher than CEA and CA19-9 in patients with stage I/II disease. However, positive s-MK rates were lower than CEA in patients with stage III disease. For patients with stage I, stage II, and stage IV disease, overall positive rates were significantly higher in CEA/CA19-9 combined with s-MK than those observed with CEA/CA19-9 without s-MK (Figure 1B). Figure 2 clearly shows that positive rates of

s-MK in patients with stage I/II disease were higher than those of CEA, particularly in patients with stage I/II gastric cancer. Figure 3 shows the relationship between s-MK, CEA, and CA19-9 in patients with gastric cancer. Of the 131 patients, 18 patients were detected by s-MK only (Figure 3). In contrast, seven cases each were detected by CEA only or by CA19-9 only. The positive rate of s-MK was higher than that of both CEA and CA19-9. Number of s-MK positive cases ( $n = 28$ ) was significantly greater than that of CEA positive cases ( $n = 19$ ) ( $P < 0.01$ ). The positive rate of combination of s-MK + CEA was 29% ( $n = 38$ ) and of combination of s-MK + CA19-9 was also 29% ( $n = 38$ ). These combinatory positive rates were significantly higher than those of CEA + CA19-9 (21%) ( $P < 0.01$ ) (Table S3). Among s-MK positive cases, 17 were differentiated type and 11



**FIGURE 3** Relationship between positive serum tumor marker findings in patients with gastric cancer. CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen

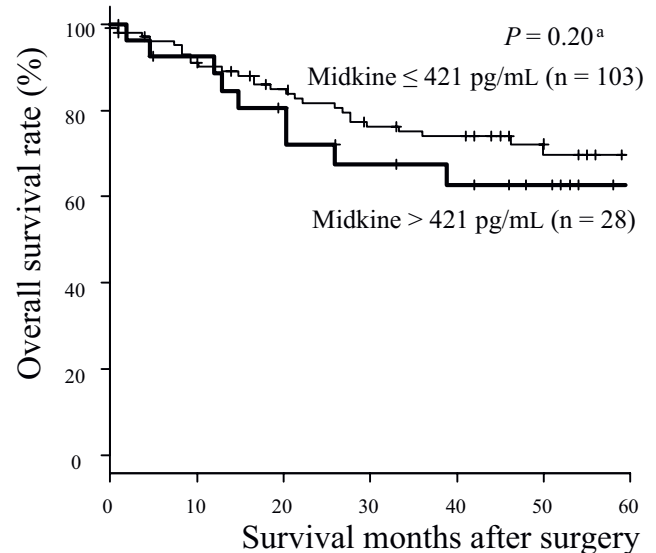
were undifferentiated type. Among CEA positive cases, 12 were differentiated type and five were undifferentiated type. Among cancer antigen 19-9 (CA 19-9) positive cases, 10 were differentiated type and three were undifferentiated type. We could not find any significant association between each tumor marker and pathological factors. Moreover, no difference was observed between each tumor marker in the infiltrative pattern, lymphatic invasion, and venous invasion.

### 3.4 | Univariate and multivariate analysis of risk factors on survival

Patients with high s-MK showed worse overall survival than those with low s-MK. However, the difference was not statistically significant (Figure 4). The left panel of Table 2 shows the univariate analysis to determine the prognostic impact of clinicopathological factors. Gender, tumor depth, lymphatic metastasis, and distant metastasis were significantly correlated with survival by log-rank test. In the multivariate analysis, gender, tumor depth, and presence of distant metastasis were significantly associated with poor survival; however, s-MK was not found to be a significant prognostic factor (Table 2, right panel).

## 4 | DISCUSSION

We found that positive s-MK rate in patients with gastric cancer was significantly higher than that observed in healthy subjects. Although s-MK was slightly associated with tumor depth, this association was not statistically significant. Although the high s-MK group showed worse overall survival, the difference was not statistically significant. Of the 131 patients, 18 (14%) patients were detected by s-MK



<sup>a</sup> Log-rank test

**FIGURE 4** Comparison of overall survival between the high and low midkine groups

only. Overall positive rates of CEA + s-MK or CA19-9 + s-MK were higher than the positive rate of CEA + CA19-9.

Carcinoembryonic antigen was the only factor to be associated with s-MK. Although tumor depth was slightly associated with s-MK by univariate analysis, it did not emerge as an independent factor during the multivariate analysis. Such a discrepancy can be explained partly by the interaction between tumor depth and CEA. Because the positive rates of s-MK in patients with stage I/II disease were higher than the CEA, particularly in patients with stage I/II gastric cancer, s-MK may be useful. CEA is a glycoprotein present in gastrointestinal cancer, whereas s-MK is a member of growth factors or cytokines; therefore, we believe that it cannot be replaced with CEA. Indeed, as shown in Figure 3, 19 cases were positive for s-MK but negative for CEA. Conversely, 10 cases were positive for CEA but negative for s-MK. Therefore, we thought that combining s-MK with CEA/CA19-9 increased the overall sensitivity.

Although the difference of positive rates between polyclonal and monoclonal MK antibody is important, previous studies have not shown details of pathological data.<sup>10,11</sup> Therefore, it was difficult to compare sensitivity and specificity between the two types of ELISA. Moreover, previous polyclonal antibody ELISA is currently unavailable. Our argument was that we were using monoclonal antibodies to pursue high specificity.

Similar to previous reports on mesothelioma<sup>15</sup> and head and neck carcinoma,<sup>16</sup> there was no clear association between tumor progression and s-MK positive rate. Although the number of stage III/IV patients in our present study was not sufficient, we could not find significantly higher positive rates in stage III/IV patients than in stage I/II patients. Such a tendency was also observed in previous reports,<sup>15,16</sup> which may be explained by the hypothesis that the biological characteristic of cancer cells to express MK at the early phase of carcinogenesis remained unchanged during tumor progression.

	Univariate P-value <sup>a</sup>	Multivariate P-value <sup>c</sup>		
		Hazard ratio	95% CI <sup>b</sup>	P-value
Gender				
Male/Female	0.05	2.33	1.01-5.26	0.04
Age (y)				
>65/≤65	0.29			
s-MK (pg/mL)				
>421/≤421	0.2	1.19	0.57-2.48	0.65
CEA (ng/mL)				
>5/≤5	0.94			
CA19-9 (U/mL)				
>37/≤37	0.21			
Tumor depth				
T2-4/T1	<0.01	4.80	1.58-14.6	<0.01
Lymph node metastasis				
N+/N-	<0.01	1.23	0.52-2.93	0.63
Distant metastasis				
M1/M0	<0.01	2.76	1.21-6.29	0.02
Differentiation				
Undiff. <sup>d</sup> /diff. <sup>d</sup>	0.53			

<sup>a</sup>Log-rank test.

<sup>b</sup>Adjusted 95% confidence interval.

<sup>c</sup>Cox proportional hazard model.

<sup>d</sup>Differentiation.

CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; s-MK, serum midkine.

Unfortunately, the reason behind the s-MK positive rate being relatively low, and lower than the CEA positive rate in stage III, was unclear. Although high s-MK (>400 pg/mL) was reported to be a poor prognostic factor in patients with non-small cell lung cancer,<sup>19</sup> we could not confirm such a prognostic impact of s-MK in gastric cancer.

One of the limitations of the present study involved the assessment of MK immunoreactivity in the tumor tissues. Yamashita et al<sup>16</sup> showed that s-MK levels were positively associated with immunohistochemical staining scores in patients with head and neck squamous cell carcinoma. Moreover, Xia et al<sup>19</sup> showed that the immunoreactivity of MK was significantly associated with s-MK in patients with lung cancer. Actually, their study included 91 patients with adenocarcinoma among the 153 with lung cancer. Such an association may be present in patients with gastric cancer.

In conclusion, s-MK levels increased, even during early stages of gastric cancer. In combination with s-MK, the positive rate of CEA + CA19-9 increased in the patients with stage I/II gastric cancer. Although high s-MK seemed to be a poor prognostic factor for patient survival, the difference was not statistically significant.

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**TABLE 2** Univariate and multivariate analysis of risk factors for overall survival

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## CONFLICTS OF INTEREST

Author H.S. owns stock of Cellmid Limited. Other authors declare no conflicts of interest associated with the present study.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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