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Characterization of inflammatory profiles and endothelial dysfunction in diabetic limb arterial occlusion

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Abstract. Our study aims to detect the changes of adiponectin (APN), endothelin 1 (ET)-1, nitric oxide (NO), cystatin C (cysC) in diabetic limb arterial occlusion (DLAO) patients and unravel their associations with endothelial function. Total 240 type 2 diabetes mellitus (T2DM) patients were divided into a DM group (n = 80, ankle brachial index (ABI) ≥ 0.9) and a DLAO group (n = 160, ABI < 0.9). ABI, flow-mediated dilation (FMD) and nitroglycerin-mediated dilation (NMD), serum APN, ET-1, NO, and cysC were compared. There were significant increases in cysC and ET-1, and significant decreases in APN, NO, FMD and NMD of DLAO patients compared to T2DM patients. Serum APN and NO were positively correlated with ABI, while serum cysC and ET-1 were negatively correlated with ABI. cysC, ET-1 and diastolic blood pressure (DBP) were independent predictors of the severity of DLAO. Serum APN was positively correlated with FMD, NMD and NO, but was negatively correlated with ET-1 and cysC. FMD and NMD were positively correlated with APN and NO, and negatively correlated with ET-1 and cysC. Our study deciphers opposite roles of APN, NO, cysC and ET-1 in the development of DLAO and maintaining endothelial function.

Key words: Ankle brachial indice — Flow-mediated dilation — Nitroglycerin-mediated dilation — Endothelial function — Inflammation — Diabetes

Abbreviations: ABI, ankle brachial index; APN, adiponectin; BMI, body mass index; cysC, cystatin C; DBP, diastolic blood pressure; DLAO, diabetic limb arterial occlusion; ET-1, endothelin 1; FBG, fasting blood glucose; FMD, flow-mediated dilation; HbA1c, glycosylated hemoglobin; HDL-C, high density lipoprotein cholesterol; hsCRP, hypersensitive C-reactive protein; IL-6, interleukin-6; LDL-C, low density lipoprotein cholesterol; NMD, nitroglycerin-mediated dilation; PAD, peripheral arterial disease; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglyceride; WHR, waist hip ratio.

Highlights

- DLAO patients had exacerbated inflammation and endothelial dysfunction.
- APN and NO protect against DLAO and endothelial dysfunction.
- cysC, and ET-1 promotes DLAO and endothelial dysfunction.
- Serum NO was a protective factor against DLAO.
- Serum APN was positively correlated with NO.
- Serum APN was negatively correlated with cysC and ET-1.

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Introduction

Peripheral arterial disease (PAD) characterized by progressive narrowing and occlusion of peripheral arteries, especially the lower extremity arteries, is a common manifestation of atherosclerosis and one major complication in patients with type 2 diabetes mellitus (T2DM) (Yang et al. 2017; Arora et al. 2019). Concomitant PAD in DM patients is defined as diabetic limb arterial occlusion (DLAO) as well (Hur et al. 2018). Prevalence of PAD is 20–50% in diabetic populations, compared to 10–26% in non-diabetic populations (Mishra 2021; Stoberock et al. 2021). DLAO patients are more susceptible to cardiovascular events, contributing to higher morbidity and mortality as well as an increased risk of lower extremity amputation (Hur et al. 2018; Akalu and Birhan 2020).

PAD in diabetic patients have complicated multifactorial pathophysiological mechanisms, involving excessive inflammatory response, overproduction of advanced glycation end products and reactive oxygen species, and dyslipidemia (Yang et al. 2017). Endothelial cells that line blood vessels and produce nitric oxide (NO) and prostacyclin are a critical regulator of vascular homeostasis (Bach 2015). Endothelial cells play an important role in the initiation of atherosclerosis by binding to inflammatory monocytes, which are further differentiated into macrophages and foam cells (Zhang et al. 2017). Substantial evidence shows that endothelial dysfunction is critical for the initiation and progression of diabetic vascular complications, such as PAD (Shi and Vanhoutte 2017; La Sala et al. 2019). Although it has been established that impairment of endothelial function is correlated with inflammation in the pathogenesis of diabetic vascular complications (Domingueti et al. 2016), the relationships between inflammatory factors and endothelial function in DLAO patients have not been fully elucidated.

Ankle brachial index (ABI) is a widely-accepted indicator for presence and severity of PAD in diabetics (Casey et al. 2020). ABI value of 1.00–1.30 is considered normal, while ABI ≤ 0.90 is used for diagnosis of PAD (Høyer et al. 2019). Flow-mediated dilation (FMD) and nitroglycerin-mediated dilation (NMD) are commonly used indicators of endothelial function, evaluating endothelial-dependent and-independent vasodilation, respectively (Allon et al. 2016; Wang et al. 2019). In the current study, we evaluated the associations of FMD and NMD with inflammatory biomarkers including adiponectin (APN), endothelin 1 (ET)-1, NO, and cystatin C (cysC) in the patients with diagnosed DLAO based on ABI value. Our study may contribute to a deeper understanding of the pathophysiological mechanisms of DLAO and lay a foundation for improving therapeutic outcomes.

Methods

Study design

This retrospective study included a total of 240 T2DM patients who were hospitalized in Shanghai Hospital of Integrated Traditional Chinese and Western Medicine from July 2017 to July 2020. Inclusion criteria were: a) age 50–85 years; b) diagnosis of T2DM based on diagnostic criteria of American Diabetes Association (2007) and c) ABI was measured and recorded. Exclusion criteria were: a) severe cardiac, hepatic, and renal insufficiency, hematologic diseases, and tumors; b) concomitant infections and fever; c) psychological disorder or disturbance of consciousness; d) diabetic foot ulcer; e) acute arterial occlusion or thrombosis of lower limbs and f) acute cardio-cerebrovascular disorder.

Patients were divided into DM group (patients with 0.9 ≤ ABI ≤ 1.3 and without medical history of myocardial infarction or cerebral infarction) and DLAO group (DLAO patients with ABI < 0.90). DLAO was diagnosed according to Clinical Diagnosis and Efficacy Criteria for Diabetic Limb Arterial Occlusion (Cui 2004). The DM group (n = 80) included 42 males and 38 females, while the DLAO group (n = 160) included 87 males and 73 females. Our research was approved by the Ethics Committee of Shanghai Hospital of Integrated Traditional Chinese and Western Medicine. Written informed consent was obtained from each participant prior to the research.

Clinical indices and anthropometric measurements

Anthropometric indices were measured in each participant before breakfast. Demographic data were collected from medical records. Waist and hip circumferences were measured following standard procedures for waist hip ratio (WHR) calculation (waist circumference/hip circumference). Body mass index (BMI) was calculated as body weight/height^2 (kg/m^2).

Fasting cubital venous blood samples were collected from each patient at 6:00 a.m. in the next morning for measurement of fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), APN, cysC, ET-1, NO, hypersensitive C-reactive protein, (hsCRP), and interleukin-6 (IL-6). Hitachi 7600 automatic biochemical analyzer (Hitachi Medical (Guangzhou) Co., Ltd.) was used for measurement of TC, TG, HDL-C and LDL-C. Glycosylated hemoglobin (HbA1c) was assayed using high performance liquid chromatography. cysC was detected using immunoturbidimetric assay. Fresh venous blood sample (4 ml) was centrifuged at 3000 r/min for 15 min at 2–8°C (centrifuge radius = 20 cm) within 30 min after blood collection. The supernatant was separated and stored at −80°C.

Serum APN, ET-1, and NO levels were measured using immunosorbent method with human ELISA kits (KND Biotechnology Company, Quanzhou, China). hsCRP and IL-6 were detected using scattering turbidimetry (CardioPhase hsCRP, Siemens, Germany), and chemiluminescence assay (Elecsys IL-6, Roche Diagnostics), respectively. ABI defined by the ankle/brachial blood pressure ratio, is a fast, simple and noninvasive reflector of atherosclerotic and arterial stiffness (Li et al. 2020). ABI < 0.90 is a widely accepted standard for diagnosis of PAD (Iribarren et al. 2018).

FMD and NMD were measured using high-frequency ultrasound according to standard guidelines (Kaczmarska et al. 2019). Briefly, patients were examined in the supine position, and the brachial artery was imaged in the longitudinal plane to capture baseline images (d0). A blood pressure cuff around the forearm was inflated to increase blood pressure to 250 mm Hg that was sustained for 4.5 min. Brachial artery diameter was measured 60–90 s after deflation of the cuff (d1). After a 25-min rest, patients were administrated sublingually with 0.5 mg nitroglycerin and brachial artery diameter was measured again 5 min later (d2). FMD and NMD were calculated using the following formula:

$$FMD = (d1 - d0)/d0 \times 100\%$$

$$NMD = (d2 - d0)/d0 \times 100\%$$

These measurements were performed in three consecutive cardiac cycles and the mean values were calculated.

Statistical analysis

Quantitative data in the normal distribution are represented by mean ± standard deviation (SD) and compared between groups using Student's *t* test. Data with skewed distribution

Table 1. Clinical characteristics of the patients in DM group and the DLAO group

Variable	DM (n = 80)	DLAO (n = 160)	χ^2/t	p
Age (year)	70.35 ± 9.03	69.66 ± 9.74	0.31	0.76
Sex			0.075	0.784
Male	42	87		
Female	38	73		
Duration of diabetes (year)	12.64 ± 7.61	13.68 ± 8.27	1.15	0.26
BMI (kg/m^2)	24.31 ± 3.4	24.46 ± 1.21	0.171	0.862
SBP (mmHg)	144.17 ± 9.43	149.29 ± 9.53	3.749	<0.01
DBP (mmHg)	81.03 ± 6.76	85.01 ± 6.37	4.117	<0.01
HR (times/min)	78.97 ± 9.31	80.61 ± 9.37	1.268	0.205
ABI	0.967 ± 0.095	0.338 ± 0.023	12.264	<0.01
WHR	0.930 ± 0.061	0.950 ± 0.041	2.829	<0.01
APN (μg/ml)	5.044 ± 0.33	4.626 ± 0.43	7.286	<0.01
cysC (mg/l)	1.051 ± 0.13	1.350 ± 0.22	9.603	<0.01
NO (μmol/l)	69.424 ± 4.46	65.872 ± 5.88	4.418	<0.01
ET-1 (pg/ml)	42.703 ± 4.35	46.275 ± 5.84	4.222	<0.01
FMD (%)	6.071 ± 0.84	4.471 ± 0.60	10.923	<0.01
NMD (%)	13.050 ± 1.34	10.990 ± 2.12	7.061	<0.01
HDL-C (mmol/l)	1.064 ± 0.28	1.157 ± 0.36	1.463	0.143
LDL-C (mmol/l)	2.553 ± 0.83	2.735 ± 0.88	1.264	0.206
TC (mmol/l)	4.480 ± 1.03	4.704 ± 0.90	1.822	0.068
TG (mmol/l)	1.588 ± 0.63	1.945 ± 0.66	4.152	<0.01
hsCRP (mg/l)	3.11 ± 1.85	6.88 ± 3.43	7.877	<0.01
IL-6 (pg/ml)	4.84 ± 1.58	11.71 ± 3.98	12.46	<0.01
FBG (mmol/l)	7.831 ± 2.78	8.204 ± 3.05	0.991	0.322
HbA1C (%)	8.76 ± 1.27	8.98 ± 1.46	0.22	0.83

DM, diabetes mellitus; DLAO, diabetic limb arterial occlusion; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; ABI, ankle brachial index; WHR, waist hip ratio; APN, adiponectin; cysC, cystatin C; NO, nitric oxide; ET-1, endothelin 1; FMD, flow-mediated dilation; NMD, nitroglycerin-mediated dilation; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein- cholesterol; TC, total cholesterol; TG, triglyceride; hsCRP, high sensitivity C-reactive protein; IL-6, interleukin 6; FBG, fasting blood glucose; HbA1c, haemoglobin A1c.

Table 2. Correlations of ABI value with clinical characteristics of DLAO patients

Variable	r	*p*
BMI (kg/m^2)	−0.235	0.003
SBP (mmHg)	0.118	0.136
DBP (mmHg)	0.133	0.094
HR (time/min)	0.029	0.714
WHR	0.145	0.067
APN (μg/ml)	0.964	<0.01
cysC (mg/l)	−0.647	<0.01
NO (μmol/l)	0.919	<0.01
ET-1 (pg/ml)	−0.919	<0.01
FMD (%)	0.884	<0.01
NMD (%)	0.635	<0.01
HDL-C (mmol/l)	0.538	<0.01
LDL-C (mmol/l)	−0.545	<0.01
TC (mmol/l)	−0.702	<0.01
TG (mmol/l)	−0.925	<0.01
hsCRP (mg/l)	−0.939	<0.01
IL-6 (pg/ml)	−0.939	<0.01
FBG (mmol/l)	−0.02	0.805
HbA1C (%)	0.128	0.102

For abbreviations, see Table 1.

were compared using rank sum test. Qualitative data were expressed as percentages and compared using chi-square test. Spearman correlation coefficients were calculated for correlation analysis. Logistic regression analysis was used to identify risk factors of DLAO. Significance was defined to be $p < 0.05$.

Results

Comparison of demographic and clinical characteristics of patients

Demographic and clinical characteristics of all patients were shown in Table 1. The DM group and the DLAO group were not significantly different in age, sex, diabetes duration, BMI, FBG, HR, HDL-C, LDL-C, TC and HbA1c ($p > 0.05$). The DLAO group had significantly higher systolic blood pressure (SBP), DBP and WHR than the DM group ($p < 0.01$). Significant decrease in APN and NO levels and significant increase in cysC and ET-1 were observed in the DLAO group compared to the DM group ($p < 0.01$). Regarding endothelial dysfunction, FMD and NMD values were obviously decreased in the DLAO group in comparison with those in the DM group ($p < 0.01$).

Correlation analysis between ABI and clinical characteristics of patients in DLAO

Spearman correlation analysis revealed that in the DLAO group, ABI was strongly positively correlated with serum APN and NO ($p < 0.01$, r = 0.964, 0.919), and negatively correlated with serum cysC and ET-1 levels ($p < 0.01$, r = −0.647, −0.919) (Table 2). Moreover, correlations of ABI with blood lipids were investigated in the DLAO patients. Significant positive correlation was observed for HDL-C ($p < 0.01$, r = 0.538), whereas significant negative correlation was observed for LDL-C, TC and TG ($p < 0.01$, r = −0.545, −0.702, −0.925) (Table 2).

Ordinal logistic regression analysis identified independent predictors of the severity of DLAO

Ordinal Logistic regression analysis was performed to identify factors associated with ABI. DLAO patients were stratified by severity based on ABI value: patients with $0.4 \leq ABI < 0.9$ was defined as mild DLAO ($n = 65$), patients with $0 < ABI < 0.4$ was defined as moderate DLAO ($n = 51$) and patients with ABI = 0 was regarded as severe DLAO ($n = 44$). With ABI as dependent variable, independent variables included BMI, cysC, ET-1, TC and DBP. As shown in Table 3, cysC (OR = 7.626×10^{-10}, 95%CI = 2.369×10^{-16}–0.002; $p = 0.006$), ET-1 (OR = 0.229, 95%CI = 0.113–0.467; $p < 0.001$) and DBP (OR = 1.253, 95%CI = 1.021–1.538; $p = 0.031$) were independent predictors of the severity of DLAO.

Table 3. Logistic regression analysis for ABI value in the DLAO group

Variable	β	OR	95%CI		*p*
			Upper	Lower	
cysC (mg/l)	−20.994	7.626×10^{-10}	0.002	2.369×10^{-16}	0.006
ET-1 (pg/ml)	−1.473	0.229	0.467	0.113	<0.001
BMI (kg/m^2)	−0.099	0.906	1.325	0.619	0.609
TC (mmol/l)	−0.244	0.784	2.767	0.222	0.705
DBP (mmHg)	0.226	1.253	1.538	1.021	0.031

β, regression coefficient; OR, odds ratio; 95%CI, 95% confidence interval. For more abbreviations, see Table 1.

Correlation analysis between APN and blood lipids, inflammatory factors and endothelial function in DLAO patients

Spearman correlation analysis was also performed to evaluate associations of APN with blood lipids, inflammatory factors and endothelial function in the DLAO group. Table 4 showed that APN was positively correlated with NO, FMD, NMD, and HDL-C ($p < 0.01$, r = 0.95, 0.893, 0.652, and 0.556), but was negatively correlated with cysC, ET-1, LDL-C, TC, TG, hsCRP, and IL-6 ($p < 0.01$, r = −0.91, −0.945, −0.568, −0.734, −0.965, −0.981, and −0.983).

We further analyzed relationships of endothelial function to inflammatory factors. Tables 5 and 6 showed that both FMD and NMD were positively correlated with APN ($p < 0.01$, r = 0.893, 0.652) and NO ($p < 0.01$, r = 0.866, 0.656), and were negatively correlated with cysC ($p < 0.01$, r = −0.798, −0.597), ET-1 ($p < 0.01$, r = −0.862, −0.652), and IL-6 ($p < 0.01$, r = −0.886, −0.66).

Discussion

DLAO is a common and serious complication of DM, and an essential cause of cardiovascular events, non-traumatic amputation and mortality (Nativel et al. 2018). Inflammation and endothelial dysfunction are two key pathogenic mechanisms of DLAO (Strain and Paldánius 2018). Our study characterized APN, NO, ET-1 and cysC in serum of DLAO patients, with a special emphasis on their relationships with endothelial function. We found that inflammation and endothelial dysfunction were exacerbated in DLAO patients compared to T2DM patients. Our study indicated that anti-inflammatory APN and NO inhibited the progression of DLAO and protected endothelial function, while pro-inflammatory cysC and ET-1 promoted the progression of DLAO and worsened endothelial dysfunction. Additionally, cysC, ET-1 and DBP were independent predictors of the severity of DLAO. Moreover, APN was positively correlated with NO, but negatively correlated with cysC and ET-1. Our study provides understanding on the biological roles of APN, NO, ET-1 and cysC and the relationships between inflammation and endothelial dysfunction in the pathophysiology of DLAO. It may have implications in prevention and treatment of DLAO.

APN secreted by adipocytes is a classic anti-inflammatory agent and plays a central role in insulin resistance/T2DM and cardiovascular disease (Fang and Judd 2018). ET-1, a potent vasoconstrictor produced by endothelial cells, exerts a pro-inflammatory effect, and strengthens oxidative stress and atherosclerosis in the biology of DM (Jain et al. 2019). Endothelial NO has strong anti-inflammatory property and suppresses activation of endothelial cells and macrophages in atherogenesis (Förstermann et al. 2017). cysC is a biomarker of renal function as well as inflammation (Muslimovic et al. 2015). cysC serum level is elevated in T2DM patients and associates with the progression of DM (Xu et al. 2020). The present study found that DLAO patients had higher BP, strengthened inflammatory response, and impaired endothelial function compared to T2DM patients. Consistently, it is the well-documented that inflammatory response and endothelial dysfunction are critical players in the development of lower-extremity PAD in T2DM patients (Du et al. 2015). Hypertension promotes atherosclerosis

Table 4. Correlations of APN value with clinical characteristics in the DLAO group

Variable	r	*p*
cysC (mg/l)	−0.91	<0.01
NO (μmol/l)	0.95	<0.01
ET-1 (pg/ml)	−0.945	<0.01
FMD (%)	0.893	<0.01
NMD (%)	0.652	<0.01
HDL-C (mmol/l)	0.556	<0.01
LDL-C (mmol/l)	−0.568	<0.01
TC (mmol/l)	−0.734	<0.01
TG (mmol/l)	−0.965	<0.01
hsCRP (mg/l)	−0.981	<0.01
IL-6 (pg/ml)	−0.983	<0.01

For abbreviations, see Table 1.

Table 5. Correlations of FMD with inflammatory factors in the DLAO group

Variable	r	*p*
APN (μg/ml)	0.893	<0.01
cysC (mg/l)	−0.798	<0.01
NO (μmol/l)	0.866	< 0.01
ET-1 (pg/ml)	−0.862	<0.01
hsCRP (mg/l)	0.153	0.55
IL-6 (pg/ml)	−0.886	<0.01

For abbreviations, see Table 1.

Table 6. Correlations of NMD with inflammatory factors in the DLAO group

Variable	r	*p*
APN (μg/ml)	0.652	<0.01
cysC (mg/ll)	−0.597	<0.01
NO (μmol/l)	0.656	<0.01
ET-1 (pg/ml)	−0.652	<0.01
hsCRP (mg/l)	0.065	0.41
IL-6 (pg/ml)	−0.66	<0.01

For abbreviations, see Table 1.

and has been established to be a high-risk factor for chronic diabetic vascular complications (Yamazaki et al. 2018). Our study indicates that hypertension is a risk factor of DLAO.

Serum APN level is correlated with occurrence of micro-vascular complications in T2DM patients (Wang et al. 2020). Blood lipids including TC, TG and HDL-C are associated with micro-vascular complications in T2DM patients (Yang et al. 2019). In agreement with the above reports, the current study found that serum APN, NO, and HDL-C were positively correlated with ABI value, while serum cysC, ET-1, LDL-C, TC and TG were negatively correlated with ABI value. It implies that APN, NO, and HDL-C prohibit the progression of PAD, while cysC, ET-1, LDL-C, TC and TG advance the progression of PAD. Dyslipidemia and inflammation are two important pathological mechanisms underlying the connection between DM and atherosclerosis (Poznyak et al. 2020). It can be speculated that inflammation and hyper-lipidemia promotes atherosclerosis in the progression of DLAO. Endothelial NO production is decreased in DM, fostering activation of endothelial cells, infiltration of macrophages and the development of atherosclerosis (Förstermann et al. 2017). Logistic regression analysis showed that serum NO is a protective factor against DLAO. It indicates that NO can inhibit the progression of DLAO and that stimulating endothelial production of NO is a potential therapeutic strategy.

APN stimulates fatty acid oxidation, increases glucose intake, protects against atherosclerosis and has vasculoprotective and angiogenic properties (Park et al. 2016; Achari and Jain 2017). APN stimulates endothelial production of NO, decreases inflammatory cytokines and mitigates oxidative stress (Chen et al. 2015). Consistently, we found that serum APN had positive correlations with HDL-C, FMD, NMD, and NO and negative correlations with LDL-C, TC, TG, hsCRP, and IL-6 in DLAO patients. Moreover, there is *in vivo* evidence that serum APN is negatively correlated with serum ET-1 in T2DM rats (Han et al. 2017). Serum cysC prohibits clearance of serum APN through the cysC-APN complex and compromises the beneficial effect of APN on vasculature (Matsumoto et al. 2017). In agreement with these studies, our study found that serum APN was negatively correlated with serum ET-1 and cysC in DLAO. It reveals that APN fights against inflammatory and protects endothelial function in DLAO and the underlying mechanisms possibly involve lipids metabolism, NO, hsCRP, IL-6, ET-1 and cysC.

Endothelial NO plays multifaceted roles in maintaining vascular homeostasis, and reduction of endothelial production of NO is a defined feature of endothelial dysfunction (Cyr et al. 2020). Hyperglycemia-induced ET-1 production is a contributor to endothelial dysfunction and vascular remodeling in diabetes (Padilla et al. 2018; Ouerd et al. 2021). cysC is related to vascular development and angiogenesis, exerting an inhibitory effect on proliferation, migration, and permeability of endothelial cells (Li et al. 2018). In the current study, we consistently found that APN and NO were positively correlated with FMD and NMD, whereas ET-1, cysC, and IL-6 were negatively correlated with FMD and NMD. These results suggest that APN and NO exert protective effects on endothelial function, while ET-1, cysC, and IL-6 impair endothelial function in the pathogenesis of DLAO.

Conclusion

Taken together, the retrospective study reveals suppressive roles of APN and NO, and promoting roles of cysC and ET-1 in the progression of PAD and endothelial dysfunction. APN has positive correlation with NO, and negative correlation with cysC and ET-1 in serum. cysC, ET-1 and DBP were independent predictors of the severity of DLAO. This study characterizes the inflammatory profiles of DLAO and paves way for design of promising therapeutic strategies. Further studies are warranted to validate our results and deepen the research on the molecular mechanisms of DLAO.

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Conflict of interest. The authors declare that they have no conflict of interest.

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