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Serum NGAL and Cystatin C Comparison With Urinary Albumin-to-Creatinine Ratio and Inflammatory Biomarkers as Early Predictors of Renal Dysfunction in Patients With Type 2 Diabetes

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Introduction: Diabetic nephropathy is associated with specific histological changes. Early detection of poor glomerular and tubular function can be achieved with biomarkers of diabetes. The aim of this study was to evaluate the accuracy of kidney dysfunction biomarkers in type 2 diabetes (T2D).

Methods: Patients with T2D were grouped according to their glycated hemoglobin level. Patients' urine and blood samples were taken to measure cystatin C (CysC), neutrophil gelatinase-associated lipocalin, beta-trace protein levels, and the first morning void albumin-to-creatinine ratio. Patients in the end stage of renal disease or receiving dialysis were not included. Receiver operating characteristic curves were generated, and the areas under the curve were compared with the performance of the biomarkers used to evaluate kidney dysfunction in T2D.

Results: Ninety patients with T2D were chosen. CysC was positively correlated with creatinine ($P < 0.001$), estimated glomerular filtration rate ($P < 0.001$), and urinary beta-trace protein ($P = 0.01$). The area under the curve was 0.635 for CysC, 0.621 for serum neutrophil gelatinase-associated lipocalin, and 0.660 for the albumin-to-creatinine ratio. A crude logistic regression model showed a positive association between serum CysC ($P = 0.01$) and serum neutrophil gelatinase-associated lipocalin ($P < 0.001$). A linear regression model showed a positive association between serum CysC, creatinine, and estimated glomerular filtration rate ($P < 0.001$) but did not show a positive association with glycated hemoglobin ($P = 0.892$).

Discussion: Neutrophil gelatinase-associated lipocalin and serum CysC were positively associated with the presence of renal dysfunction and had better performance on receiver operating characteristic analysis than the other markers evaluated in patients with T2D without kidney dysfunction.

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Diabetic nephropathy is a syndrome characterized by the presence of macroalbuminuria, a slow and progressive decline in glomerular filtration rate (GFR), elevated blood pressure, and cardiovascular mortality.[1]

Renal diabetes histopathologic changes include thickening of the glomerular basement membrane, mesangial matrix expansion, hyalinosis, and thickening of the afferent and efferent arterioles. The classic lesion described by Kimmestiel and Wilson in 1936 is characterized by the diffused and intercapillary proliferation of the mesangial matrix or nodular sclerosis. These changes are very common in type 1 diabetes, but for type 2 diabetes (T2D), there is a lack of scientific research comprehensively showing the progression of kidney disease; however, the changes are thought to be similar.[1,2]

Glomerular mesangial matrix expansion can accelerate the progression of diabetes by approximately 15 years, and its degree of increase is directly proportional to the extent of the decreases in GFR and the area of capillary filtration. Thus, the presence of albuminuria is closely related to the existence of

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glomerular basement membrane thickening and mesangial expansion.[3]

In an animal model using mice, an increase in kidney size above hyperfiltration was attributed to high net reabsorption in the proximal convoluted tubule, causing reduced tubular hydrostatic pressure and inhibiting fluid secretion at the end portion of the segment.[4]

An ideal renal function marker must have constant production, rapid diffusion in the extracellular space, free clearance, an absence of resorption and/or tubular secretion, an absence of deletion or extrarenal degradation, and the existence of accurate and reproducible tests without interference from other components.[5]

Many markers have been used to predict early-stage renal dysfunction in diabetic patients; however, the reference levels have not been established for these markers. The presence of microalbuminuria is a sign of the presence of diabetic kidney disease and marks the beginning of more intense therapy.

Thus, the primary objective of this study was to evaluate the accuracy of neutrophil gelatinase-associated lipocalin (NGAL) and serum cystatin C (CysC) as markers of renal dysfunction in T2D. The secondary objectives were to evaluate the performance of other urinary and inflammatory markers and to understand their interaction in patients with T2D.

METHODS

Study Population

The study population consisted of patients who had T2D for at least 5 years according to their medical records and were older than 21 years. The study was approved by the ABC Medical School ethics committee, and all individuals gave signed informed consent before inclusion.

Ineligible patients were those with chronic kidney disease with a cause other than T2D, those who were receiving dialysis, those who had undergone hospitalization for any reason within 30 days before sample collection, those who had cancer and were being treated, those who had AIDS and were taking immunosuppressive drugs, and those who had undergone kidney or renal and pancreatic transplants.

Laboratory Measurements

GFR Valuation

The Modification of Diet in Renal Disease simplified equation was used to calculate the estimated GFR (eGFR) in ml/min per 1.73 m^2.[6] Renal dysfunction was defined as eGFR $<$ 60 ml/min per 1.73 m^2 according to the Kidney Disease: Improving Global Outcomes chronic kidney disease definition.

Blood samples were taken to determine serum creatinine, NGAL, and CysC levels as glomerular markers of kidney function.

Serum creatinine was measured using the colorimetric method. The NGAL concentrations and CysC were determined using an automated enzyme-linked immunosorbent assay (IBL International, Hombrechtikon, Switzerland) with the aid of Labotech (Adaltis, Rome, Italy) enzyme-linked immunosorbent assay equipment.

Tubular Function Evaluation

To evaluate the presence of tubular dysfunction, the following urinary markers were measured: urinary NGAL, CysC, urinary gene expression of beta-trace protein (BTP), and urinary albumin-to-creatinine ratio (ACR). The cutoff values used in determining the normality of NGAL and CysC were defined according to each calibration reagent used. The ACR was considered to be normal if its value was less than 30 mg/g; microalbuminuria was indicated by levels between 30 and 300 mg/g, and macroalbuminuria was indicated by levels above 300 mg/g.

Glycemic Control Evaluation

Glycemic control assessment was performed using the values of fasting glucose and glycated hemoglobin (HbA1c). Values above 140 mg/dl for glucose and above 7% for HbA1c were considered abnormal.

Inflammatory Profile Evaluation

The inflammatory profile of each patient was obtained by measuring the serum levels of the following markers: ultrasensitive C-reactive protein, interleukin-6, tumor necrosis factor-α, homocysteine, serum beta-2 microglobulin (B2M), and 25-OH vitamin D. Measurements were performed using the immunoenzymatic method with chemiluminescence values determined using Immulite 1000 equipment (Siemens, Erlange, Germany).[7] For each marker, the cutoff value was defined according to the guidelines of the kit.

Urinary Gene Expression of BTP

The urinary gene expression of BTP was determined by plasma RNA isolation (initial amount, 1 μg). Synthesis of the cDNA was performed using SSIII first-strand quantitative polymerase chain reaction (qPCR) Supermix (Invitrogen, cat no. 11752050; Carlsbad, CA, USA).

The BTP urinary gene expression was evaluated by quantitative real-time reverse transcriptase-PCR. Specific primers for each selected gene were designed using Input Primer3 Blast program version 0.4.0.

Glyceraldehyde-3-phosphate dehydrogenase was used as a reference gene to normalize the relative expression of the target gene expression values. The initial standardization of quantitative real-time reverse transcriptase-PCR amplifications occurred in a thermocycler: Applied Biosystems 7500 real-time PCR

system (Applied Biosystems, Dusseldorf, Germany). The final volume was 15 μl, which contained 1× SYBR Green mix (QuantiTect SYBR Green PCR kit, QIAGEN Cat. No. 204 054), 10 pmol of each specific primer, and 2 μl of cDNA initially diluted 10×. The initial cyclic parameters consisted of a hot start step at 95 °C for 15 seconds and 60 °C for the primer sequence.

Statistical Analyses

The ability of the glomerular and tubular function markers to discriminate between different levels of eGFR impairment was assessed by receiver operating characteristic (ROC) analysis. Renal dysfunction was defined as an eGFR equal to or less than 60 ml/min per 1.73 m^2.

Multivariate analysis via logistic regression was used to identify the covariates associated with the occurrence of renal dysfunction. Initially, univariate binary logistic regression analyses were conducted to test the association between each covariate and the variable binary response. Then, the covariates with $P < 0.05$ in the univariate regression analyses were considered in a multiple logistic regression model. The final model was obtained following a manual backward stepwise elimination process wherein models were examined for indications of an influence attributable to the removed nonsignificant variable.

When the assumption was not fulfilled, the quantitative covariates in the logistic regression were dichotomized for use by utilizing the optimal cutoff point obtained from the ROC analysis. The presence of multicollinearity was assessed by estimating the variance inflation factors. The calibration and discriminatory ability of the final multiple logistic regression model were evaluated by the Hosmer-Lemeshow test and ROC analysis, respectively.

Linear correlation analysis and comparison of the quantitative variables between 2 groups were conducted using Spearman's correlation and the Mann-Whitney test. Data normality was assessed using both visual inspection of histograms and the Shapiro-Wilk normality test. Quantitative variables were described as medians and percentiles, and correlation coefficients were presented as 95% confidence intervals.

All statistical analyses were performed using R software (R Foundation, Austria). Statistical significance was evaluated at the 95% confidence level ($P < 0.05$).

RESULTS

Patients at the outpatient diabetic kidney setting of ABC Medical School, Sao Paulo, Brazil, were included sequentially during the years 2013–2015. They were evaluated after giving their informed consent, resulting in the inclusion of 100 patients with T2D. Ten patients had kidney disease with a cause other than diabetes and were excluded, resulting in 90 patients in this sample.

Table 1 shows the demographics of the sample. Among the patients, the HbA1c was 6.21% for the group with better glycemic control and 9.2% for the group with worse glycemic control. Furthermore, the average eGFR for both groups was greater than 60 ml/min per 1.73 m^2, specifically 83.8 ml/min per 1.73 m^2 for the group with HbA1c less than 7% and 76.7 ml/min per 1.73 m^2 for the group with worse glycemic control.

ROC Curves for the Various Biomarkers in Patients With T2D

The performance of each tested marker was analyzed by considering the change in renal function endpoint, which is defined by the eGFR calculated by the simplified formula Modification of Diet in Renal Disease and the value of serum creatinine, which are shown in Table 2.

The area under the curve (AUC) of serum CysC was 0.635. The ACR showed the best performance in determining the presence of renal dysfunction in

Table 1. Demographics and baseline characteristics of the whole study population

Variable	Group 1 (HbA1c < 7) n = 30	Group 2 (HbA1c ≥ 7) n = 60
Male (%)	40.00	53.30
Age (yr)	61.50 (9.80)	61.20 (9.68)
Ethnicity (%)		
Caucasian	76.60	80.00
Black	33.40	20.00
Hypertension (%)	90.00	85.00
BMI	32.00 (4.80)	29.60 (4.98)
Urea (mg/dl)	41.00 (20.90)	48.40 (28.40)
Creatinine (mg/dl)	1.07 (0.41)	1.22 (0.64)
eGFR (ml/min per 1.73 m^2)	83.80 (35.80)	76.70 (36.12)
sCys C (ng/ml)	3.42 (1.44)	3.94 (1.55)
uCys C (ng/ml)	0.50 (0.10–0.50)	0.50 (0.07–0.50)
sNGAL (ng/ml)	0.80 (0.37–1.07)	0.80 (0.40–1.08)
uNGAL (ng/ml)	0.01 (0.01–0.37)	0.01 (0.01–0.37)
uBTP	0.10 (0.10–3.13)	0.10 (0.10–3.04)
ACR (mg/g)	36.10 (9.82–103.60)	38.30 (10.10–104.00)
HbA1c (%)	6.21 (2.02)	9.20 (4.24)
TNF-α (pg/ml)	9.82 (4.83)	9.09 (4.84)
IL6 (pg/ml)	2.00 (2.00–3.08)	2.00 (2.00–3.08)
CRP (mg/l)	5.15 (1.93–11.70)	5.15 (1.82–11.02)
Homocisteine (μmol/l)	11.58 (6.00)	12.30 (5.86)
B2M (ng/ml)	2365.00 (1786.50–3028.50)	2439.00 (1850.25–3049.00)
Vitamin D (ng/ml)	17.46 (8.11–22.16)	17.55 (8.11–21.71)

Data presented as mean ± SD and median with interquartile ranges.
ACR, albumin-to-creatinine ratio; B2M, beta 2 microglobulin; BMI, body mass index; BTP, beta-trace protein; CRP, ultrasensitive C-reactive protein; CysC, cystatin C; eGFR, estimated glomerular filtration rate; HbA1c: glycated hemoglobin; IL6, interleukin 6; NGAL, neutrophil gelatinase-associated lipocalin.

Table 2. Receiver operating characteristics curve values for the biomarkers tested in patients with type 2 diabetes

Variable	Sensitivity	Specificity	AUC (95% CI)
uNGAL	0.15	0.96	0.526 (0.42–0.64)
sNGAL	0.75	0.53	0.621 (0.51–0.71)
sCysC	0.37	0.88	0.635 (0.52–0.73)
uCysC	0.70	0.46	0.569 (0.44–0.69)
ACR	0.70	0.62	0.660 (0.53–0.78)
uBTP	0.85	0.35	0.567 (0.46–0.67)

ACR, albumin-to-creatinine ratio; AUC, area under the curve; BTP, beta-trace protein; CI, confidence interval; CysC, cystatin C; HbA1c, glycated hemoglobin; NGAL, neutrophil gelatinase-associated lipocalin.

patients with T2D, with an AUC value of 0.660. The values of the other markers were not considered to be significant.

In this study, the urinary gene expression of BTP had an AUC of 0.567.

Interaction Among the Biomarkers and Outcomes Tested

Table 3 shows the results of Spearman's correlation test using creatinine values, GFR, and the urinary gene expression of BTP.

Serum CysC and ACR had significant *P* values with respect to the worst outcome of renal function in patients with T2D.

Urinary NGAL showed a positive correlation with the urinary gene expression of BTP ($P = 0.02$). We did not find a positive correlation between urinary BTP and renal outcome but did find a tendency for a positive correlation between urinary BTP and eGFR ($P = 0.06$).

The results of the univariate and multivariate regression models are shown in Table 4, with the variables as dichotomous outcomes for the presence of abnormal kidney function. Markers that showed a positive association in both models are also indicated in Table 4.

In the univariate model, a positive association was observed between the values of serum CysC ($P = 0.01$), serum NGAL ($P < 0.001$), increased age, and male gender.

Table 3. Correlation of *P* values of urinary gene expression of BTP and eGFR with the biomarkers tested in patients with type 2 diabetes

	Variables		
Biomarkers	Creatinine (mg/dl) *P* value	eGFR (ml/min per 1.73 m^2) *P* value	uBTP *P* value
sNGAL (ng/ml)	0.140	0.370	0.310
uNGAL (ng/ml)	0.960	0.740	0.020
sCysC (ng/ml)	<0.001	<0.001	0.010
uCysC (ng/ml)	0.680	0.740	0.800
ACR (mg/g)	0.020	0.030	0.250
uBTP	0.090	0.060	–
HbA1c (%)	0.980	0.700	0.280

ACR, albumin-to-creatinine ratio; BTP, beta-trace protein; CysC, cystatin C; eGFR, estimated glomerular filtration rate; HbA1c: glycated hemoglobin; NGAL, neutrophil gelatinase-associated lipocalin; Spearman's correlation test, $P < 0.05$.

Table 4. Variables associated with worse kidney function in patients with type 2 diabetes. Univariate and multivariate regression models

	Univariate			Multivariate		
Variables	OR	95% CI	*P*	OR	95% CI	*P*
Age	1.05	1.00–1.10	0.030	1.06	0.99–1.14	0.080
Male	9.80	3.69–25.98	<0.001	14.85	3.34–65.93	<0.001
sNGAL	3.39	1.36–8.42	<0.001	3.53	0.80–15.46	0.090
sCysC	1.48	1.08–2.04	0.010	1.48	0.84–2.60	0.170
B2M	4.01	1.49–10.77	0.005	1.80	0.37–8.55	0.450
IL6	2.70	1.06–6.85	0.030	1.93	0.40–9.24	0.400
Homocysteine	1.15	1.04–1.26	0.004	1.07	0.93–1.23	0.320
Vitamin D	0.91	0.87–0.96	0.002	0.88	0.81–0.96	0.007

B2M: beta 2 microglobulin; BMI, body mass index; CI, confidence interval; CysC, cystatin C; HbA1c, glycated hemoglobin; IL6, interleukin-6; NGAL, neutrophil gelatinase-associated lipocalin; OR, odds ratio.

All inflammatory markers in the univariate model showed positive associations with the outcome of abnormal kidney function (eGFR < 60 ml/min per 1.73 m^2).

The multivariate model showed a positive association between the inflammatory markers. Serum 25-OH vitamin D levels showed a negative association with worse renal function, with an odds ratio of 0.91 and a *P* value of 0.002 in the univariate model and an odds ratio of 0.81 with $P = 0.007$ in the multivariate model. Male gender also showed a strong association with worse renal function in both models.

The multivariate logistic regression model built with a backward selection of variables (Table 5) confirmed the association of male gender, serum CysC, and age with the outcome of worse kidney function. However, vitamin D was associated with better kidney function.

DISCUSSION

Patients with diabetes can develop macro- and microvascular complications. The generation of glucose degradation products and the endothelial damage triggered by hyperglycemia contribute to a permanent state of inflammation and encourage the persistence of oxidative stress. Thus, the possibility of jointly evaluating previously reported inflammatory markers—interleukin-6, tumor necrosis factor-α, C-reactive protein, homocysteine, and vitamin D—makes the results of this study even more interesting.

Urinary Markers

BTP is widely found in body tissues and fluids, and its elevation has been studied as a marker of reduced GFR.[8] However, BTP presents great variability in study outcomes due to the differences in measurement methods and the variations between individuals.[9]

Table 5. Multivariate logistic regression model built with backward selection of variables with $P < 0.05$

Variables	OR	95% CI	P
Age (yr)	1.07	1.00–1.14	0.03
sCysC (ng/ml)	2.04	1.23–3.40	0.005
Vitamin D (ng/ml)	0.89	0.83–0.96	0.003
Male	19.95	4.96–80.22	<0.001

CI, confidence interval; CysC: cystatin C; OR, odds ratio.

Selvin *et al.*[9] evaluated the coefficient of variability of various markers of the GFR, such as CysC, B2M, and BTP. Among all of the evaluated markers, BTP showed the highest intraindividual variability by mass analysis.[9] The RNA extraction and gene amplification process was the most sensitive method, but the amount of free RNA was small. This problem resulted in an isolation issue, thereby requiring fast and optimal processing of the urinary samples.[10]

Compared with nondiabetic individuals, diabetic individuals had an association between BTP and renal dysfunction, with an AUC value of 0.567.

This result differs from previous studies, such as that by Uehara *et al.*,[11] who found an AUC value of 0.84 when measuring urinary BTP by reaction to latex.

There is a direct association between the urinary values of NGAL and worse progression of renal function in T2D without the presence of albuminuria, as shown by Wu *et al.*[12] Thus, the presence of a larger amount of this protein before creatinine elevation is an early marker of tubular dysfunction. The AUC value of urinary NGAL in this experiment was 0.526, which was not significant; however, the increase in urinary NGAL value and the urinary gene expression of BTP were positively correlated, according to Spearman's test.

Matys *et al.*[13] found an AUC value of 0.59 for urinary NGAL in diabetic patients with stable coronary disease. The authors concluded that in addition to serum and urinary NGAL, CysC was also not higher than the eGFR in these patients.[12,13]

When the other urinary markers were evaluated, NGAL and CysC did not have the same result as the ACR but had AUC values of 0.526 and 0.569, respectively.

An elevated ACR is clinically relevant because it indicates a loss in glomerular selectivity and/or reduced tubular reabsorption. In an Indian cohort of Pima ethnicity, those with microalbuminuria had a 2.1 times higher risk of progression to terminal kidney disease and were 9.3 times more likely to develop diabetes than those with normoalbuminuria.[14]

In this sample, the average ACR was 86.6 mg/g in the group with HbA1c less than 7% and was 116.5 mg/g in the group with HbA1c exceeding 7%. Moreover, there was no division into groups based on ACR, unlike the approach used by most of our peers in their respective analyses. To assess the ACR and its interaction with the other markers, we categorized patients who showed no reduction in GFR by their glucose levels.

The observed AUC of ACR was 0.660, which was the best value among the evaluated urinary markers. This value was positively correlated with creatinine and the eGFR as expected, and the correlation was a linear relationship, as indicated by univariate linear regression.

Serum Biomarkers

HbA1c was tested for its ability to predict renal injury in accordance with the combined outcome of creatinine and eGFR, which was estimated using the Modification of Diet in Renal Disease formula. The AUC value was found to be unsuitable, having a value of 0.568. However, this study used a cross-sectional analysis, and the best performance of HbA1c lies in the long-term monitoring of diabetes.

During a 4-year period, Lee *et al.*[15] analyzed the effects of glycemic control as determined by HbA1c values in GFR in T2D. The subjects were divided according to HbA1c control; intermediate control was considered to be approximately 7% to 9%, and the worst control was considered to be above 9%. Lee *et al.* noted that a higher baseline HbA1c was associated with a greater decline in GFR over the studied period. The most significant outcome was shown in patients with poor glycemic control and an ACR higher than 300 mg/g.[15]

CysC and NGAL were positively correlated with worse eGFR, with AUC values of 0.635 and 0.621, respectively; in this analysis, these values are only lower than the value for ACR. In addition, the authors showed a positive relationship with the combined endpoint of renal injury assessment, with the univariate logistic regression model showing $P = 0.01$ and <0.001 for CysC and NGAL, respectively.

CysC showed a positive relationship with renal injury outcome when there was a choice of variables in the adjusted multivariate logistic regression model with $P = 0.005$. There were also linear relationships with the continuous values of creatinine and eGFR.

Assal *et al.*[16] assessed the pattern of CysC and urinary NGAL, among other markers, in diabetic subjects with different levels of eGFR. Subjects were stratified according to their baseline ACR, and the CysC values were higher among those with macroalbuminuria and increased creatinine values. Nevertheless, the AUC value in this experiment was 0.727.[16]

NGAL indicates the elevation of early known problems in secondary acute kidney injury models—such as

ischemia, cardiopulmonary bypass, and vasoconstriction—as shown in vascular examinations. NGAL has a good accuracy in predicting early kidney damage in these situations, but its use in chronic renal injury models is still under debate.[17,18]

In a prospective analysis, Chou *et al*.[19] followed a cohort of diabetic patients stratified according to GFR and albuminuria. They noted that those with greater reduction in GFR during the follow-up period, and therefore, a greater variation in GFR value, had a higher serum NGAL value, and NGAL had a positive correlation with eGFR.[19]

Inflammatory Biomarkers

In our sample, serum homocysteine was related to kidney dysfunction in the univariate analysis and linear model built using the continuous values of creatinine and eGFR. These findings are consistent with studies showing deterioration of GFR and elevated levels of homocysteine in both diabetic and nondiabetic patients.[20,21]

However, low vitamin D levels were associated with worse eGFR and a greater creatinine value in all of the analyses. Zoppini *et al*.[21] conducted a retrospective analysis of the serum values of 715 patients with T2D and found vitamin D deficiency in 36.6% of these diabetic patients. Nevertheless, these patients had more microvascular complications than those with higher vitamin D levels.[21]

The other inflammatory markers, C-reactive protein, interleukin-6, tumor necrosis factor-α, and B2M, did not show the same behavior as homocysteine and vitamin D. B2M had a positive association in the univariate logistic regression model using the outcome of kidney damage.

This study has some limitations. First, this is a cross-sectional analysis. In a longitudinal follow-up, perhaps the effect of HbA1c variation in kidney function could be better evaluated. Another important point to consider is the variability in urine sample analysis, particularly with regard to the extraction of RNA and its amplification in the process of gene expression analysis of urinary BTP. However, our results confirm that it is possible to assess markers that compare gene expression in samples of urinary sediment and to understand the correlation with other markers. In addition, ACR was found to be a reliable marker in diabetic patients with poor glycemic control, in the absence of detected renal dysfunction.

In conclusion, NGAL and serum CysC were positively associated with the presence of renal dysfunction and had better performance in terms of ROC analysis than that of the other markers evaluated in patients with T2D without kidney dysfunction. NGAL had an AUC of 0.621, with a sensitivity of 75% and a specificity of 53.06%. CysC had an AUC of 0.635, with a sensitivity of 37.5% and a specificity of 88%. The urinary concentrations of these markers did not show the same performance.

Homocysteine and vitamin D were associated with the presence of renal dysfunction in all the models tested. Furthermore, B2M and interleukin-6 showed a positive correlation with renal dysfunction in the multivariate logistic regression model.

DISCLOSURE

All the authors declared no competing interests.

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