Original Research

Plasma NAP-2 levels are associated with critical limb ischemia in peripheral arterial disease patients

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Impact statement

Critical limb ischemia (CLI) is a serious arterial obstruction, resulting in serious reduction of blood flow to the extremities. CLI is a symptomatic disorder and is frequently not diagnosed in time. This results in a high mortality and elevated risk of limb amputation. Serum or plasma biomarkers play important roles in disease prevention, diagnosis, and prognosis. Elevated plasma neutrophil-activating peptide-2 (NAP-2) was found independently associated with CLI, but not with T2DM. Plasma NAP-2 levels might be an early CLI diagnostic biomarker and might provide a novel target for CLI treatment.

Abstract

Our study evaluates the relationship between plasma neutrophil-activating peptide-2 (NAP-2) levels and critical limb ischemia (CLI); 189 subjects were enrolled in this study: 59 subjects with CLI and type 2 diabetes mellitus (T2DM), 45 subjects with CLI and no T2DM, 43 patients with T2DM and no CLI, and 42 without both T2DM and CLI. Subjects with CLI had higher plasma levels of NAP-2 than those of the healthy group (2.04 ± 0.06 ng/mL vs. 1.75 ± 0.09 ng/mL, $P = 0.011$). Subjects with CLI and T2DM had higher plasma levels of NAP-2 than those of the T2DM group (2.08 ± 0.08 ng/mL vs. 1.73 ± 0.10 ng/mL, $P = 0.007$). However, subjects with CLI including CLI and T2DM+CLI had higher plasma levels of NAP-2 than those of the non-CLI group including healthy and T2DM group (2.06 ± 0.05 ng/mL vs. 1.74 ± 0.07 ng/mL, $P < 0.001$). The ankle brachial index (ABI) and total cholesterol (TC) were significantly and negatively correlated with plasma NAP-2 levels (rho $= -0.250$, $P = 0.001$; rho $= -0.162$, $P = 0.026$, respectively). Systolic blood pressure (SBP) positively correlated with plasma NAP-2 levels (rho $= 0.187$, $P = 0.010$). When adjusting for the factors, plasma NAP-2 levels were still significantly correlated with CLI (odds ratio $= 11.543$, 95% confidence intervals: 1.327–100.403, $P = 0.027$). The area under the curve (AUC) of NAP-2+confounders was 0.992 (95% confidence intervals 0.981 to 1.003, $P < 0.001$) and it was higher than those of NAP-2 alone (95% confidence intervals 0.241 to 0.407, $P < 0.001$) and confounders alone (AUC $= 0.990$, 95% confidence intervals -0.013 to 0.018, $P = 0.797$). In conclusion, elevated plasma NAP-2 was independently associated with CLI, but it was not correlated with T2DM. Plasma NAP-2 levels might be an early CLI diagnostic biomarker and might provide a novel target for CLI treatment.

Keywords: Neutrophil-activating peptide-2, peripheral arterial disease, critical limb ischemia, biomarkers

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Introduction

Peripheral arterial disease (PAD) is an asymptomatic and symptomatic manifestation of atherosclerotic vascular disease. Critical limb ischemia (CLI) is a serious arterial obstruction, which is often not diagnosed in time, resulting in serious reduction of blood flow to the extremities with a high mortality and high risk of amputation.[1–3] Early recognition of PAD and taking protective measures in time is likely to reduce the severity of PAD and reduce the CLI incidence rate.[4]

In general, CLI patients and non-CLI patients can be distinguished by color Doppler ultrasound examination or ankle brachial index (ABI).[2,5] However, the accuracy of

detection techniques is not enough, and the ABI might be unreliable due to medial sclerosis; therefore, CLI in patients is difficult to be identified.[6] The accurate diagnosis of early stage vascular diseases is key. Until now, there were no biochemical markers or serum/plasma assays that can predict these vascular diseases.[7,8] Serum or plasma biomarkers play important roles in disease prevention, diagnosis, and prognosis.[8] However, blood tests are an effective and convenient method for identification of disease markers for prevention, diagnosis, and prognosis.[5]

Chemokines produced by endothelial cells, leukocytes, and platelets are involved in atherogenesis.[9,10] Neutrophil-activating peptide-2 (NAP-2) produced by activated platelets binding to its receptors CXCR1 and CXCR2 is involved in atherogenesis.[9,11] In addition, many evidences indicate that NAP-2 plays important roles in inflammatory regulation.[11–13] Although the role of NAP-2 in atherosclerosis including acute coronary syndromes and myocardial infarction has been studied, in addition, diabetes increases the risk of cardiovascular events and peripheral artery disease;[14,15] however, the NAP-2 involved in human CLI has not been fully clarified, particularly amongst T2DM patients.

Based on the previous studies reporting the NAP-2 involvement in atherosclerosis and inflammatory diseases, therefore, we hypothesized that plasma NAP-2 levels are associated with CLI. Therefore, levels of NAP-2 were measured in patients with CLI in this study, and demonstrated the relationship between NAP-2 and clinicopathological parameters of CLI.

Methods

Study population

T2DM patients, T2DM patients without CLI, non-T2DM patients with CLI, and healthy controls were recruited from May 1, 2015, to September 30, 2017. T2DM, non-CLI, and CLI are defined as previously described.[5,16,17] All participants enrolled in the study underwent color Doppler ultrasound examination and ankle-brachial-index (ABI) evaluations. T2DM with CLI and without CLI was matched for gender, age (±5 years), and duration of diabetes (±5 years), CLI with T2DM and without T2DM was matched for gender, age (±5 years), respectively. Exclusion criteria: renal replacement therapy, cancers, any chronic inflammatory disease on therapy. This study was approved by the Central Hospital of Wuhan Ethics Committee, and informed consent obtained from all participants.

Clinical and laboratory data collection

The main characteristics of all participants were collected including gender, age, body height, body weight, blood routine examination, blood pressure, smoking status, HbA1c, lipid levels, and so on. Hypertension, hypercholesterolemia, and coronary heart disease are defined as previously described.[5,16]

NAP-2 determination

Plasma levels were determined by enzyme-linked immunosorbent assays (ELISA) (Raybiotech, USA) according to protocol. The optical density was determined at 450 nm. All samples were measured in duplicate and were stored at −20°C.

Statistical analysis

For group comparisons, we used *t*-test for normally distributed continuous variables, Mann–Whitney *U* tests for non-normally distributed continuous variables, and χ^2 test for categorical values. Logistic regression analysis was performed to determine the independent predictors of CLI. Receiver operating characteristic curve (ROC) was used to evaluate the diagnostic value of NAP-2 to discriminate CLI. Pearson or Spearman' correlations were used to analyze the normally or non-normally distributed continuous variables, respectively. All tests used a $P < 0.05$ for significance. All data were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA).

Results

Clinical data

The main clinical characteristics are showed in Table 1. Among the 189 participants, mean age was 57.2 ± 9.7 years. The proportion of females was 55.56% of the 189 patients. We diagnosed 85 patients as having T2DM+ non-CLI, 59 patients had T2DM+ CLI, and 45 patients had non-T2DM+ CLI. Prevalence of dyslipidemia, hypertension, smoking, and CAD was 59 (31.22%), 66 (34.92%), 50 (26.46%), and 37 (19.58%), respectively. Compared to non-CLI in T2DM group, T2D duration, ABI, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), triglyceride (TG), total cholesterol (TC), apolipoprotein A (Apo A), serum creatinine (Cr), and blood urea nitrogen (BUN) were significant changes in T2DM with CLI ($P < 0.05$). However, fasting plasma glucose (FPG) and HbA1c were significantly different between T2DM+ CLI and non-T2DM+ CLI groups ($P < 0.05$).

Increased plasma levels of NAP-2 correlated with CLI

Subjects with CLI (range: 1.02–2.95 ng/mL) had higher plasma levels of NAP-2 than those of the healthy group (range: 0.89–2.90 ng/mL, 2.04 ± 0.06 ng/mL vs. 1.75 ± 0.09 ng/mL, $P = 0.011$). But compared to the healthy group, it was insignificantly lower in patients with T2DM (range: 0.98–2.89 ng/mL; 1.73 ± 0.10 ng/mL vs. 1.75 ± 0.09 ng/mL, $P = 0.889$). Subjects with CLI and T2DM (range: 1.05–3.52 ng/mL) had higher plasma levels of NAP-2 than those of the T2DM group (2.08 ± 0.08 ng/mL vs. 1.73 ± 0.10 ng/mL, $P = 0.007$). Subjects with CLI in T2DM had higher plasma levels of NAP-2 than those of the CLI group (2.08 ± 0.08 ng/mL vs. 2.04 ± 0.06 ng/mL, $P = 0.705$), but this did not remain significant (Figure 1(a)).

Based on the above results, we suggested that elevated plasma NAP-2 was associated with CLI, but it

Table 1. General characteristics of the study subjects.

Clinical variables	Healthy	T2DM	CLI	CLI+ T2DM
Case, *n*	42	43	45	59
Age, years	56.86 ± 1.04	58.05 ± 1.34	57.67 ± 1.87	56.64 ± 1.28
T2D Duration, years	–	7.24 ± 0.93	–	8.76 ± 0.59
Women, %	22 (52.38)	22 (51.16)	18 (40.00)	22 (37.29)
Hyperlipidemia, %	0 (0)	14 (32.56)	13 (28.89)	18 (30.51)
Hypertension, %	0 (0)	9 (20.93)	18 (40.00)	29 (49.15)
Smoking, %	9 (21.43)	6 (13.95)	16 (35.56)	19 (32.20)
ABI	1.09 ± 0.01	1.12 ± 0.01	0.72 ± 0.02	0.80 ± 0.03
SBP	107.14 ± 0.73	125.67 ± 2.51	131.93 ± 2.90	142.25 ± 3.39
DBP	75.95 ± 1.08	76.74 ± 1.43	79.73 ± 1.54	77.37 ± 1.34
BMI, kg/m^2	24.37 ± 0.46	25.14 ± 0.45	25.11 ± 0.64	25.46 ± 0.32
CAD, %	0 (0)	9 (20.93)	10 (22.22)	12 (20.34)
Statin use, %	0 (0)	8 (18.60)	7 (15.56)	12 (20.34)
Antihypertensive treatment use, %	0 (0)	7 (16.28)	9 (20.00)	10 (16.95)
FPG, mmol/L	4.52 ± 0.13	9.23 ± 0.66	5.31 ± 0.07	10.67 ± 0.48
HbA1c, %	5.02 ± 0.08	8.00 ± 0.29	5.24 ± 0.07	8.05 ± 0.42
HDL-C, mmol/L	1.07 ± 0.05	1.17 ± 0.05	1.12 ± 0.04	0.95 ± 0.06
LDL-C, mmol/L	2.18 ± 0.08	2.87 ± 0.27	2.14 ± 0.12	2.27 ± 0.14
TG, mmol/L	1.24 ± 0.06	1.74 ± 0.17	1.71 ± 0.24	1.44 ± 0.14
TC, mmol/L	4.37 ± 0.17	4.47 ± 0.18	3.94 ± 0.15	3.71 ± 0.20
Apo A, g/L	1.15 ± 0.05	1.27 ± 0.03	1.25 ± 0.03	1.00 ± 0.05
Apo B, g/L	0.86 ± 0.04	0.88 ± 0.04	0.81 ± 0.03	0.78 ± 0.04
Cr, μmol/L	61.11 ± 3.65	65.43 ± 2.78	91.73 ± 4.62	84.52 ± 7.50
BUN, mmol/L	5.23 ± 0.24	4.88 ± 0.21	6.25 ± 0.34	7.23 ± 0.69
UA, mmol/L	278.34 ± 14.14	332.19 ± 16.86	351.08 ± 13.01	323.53 ± 12.93

ABI: Ankle brachial index; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; CAD: coronary artery disease; FPG: fasting plasma glucose; HbA1c: glycosylated hemoglobin; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; TG: triglyceride; TC: total cholesterol; Apo A: Apolipoprotein A; Apo B: Apolipoprotein B; Cr: serum creatinine; BUN: blood urea nitrogen; UA: uric acid.
Note: Data are expressed as mean ± SEM, median (interquartile range) or percentage.

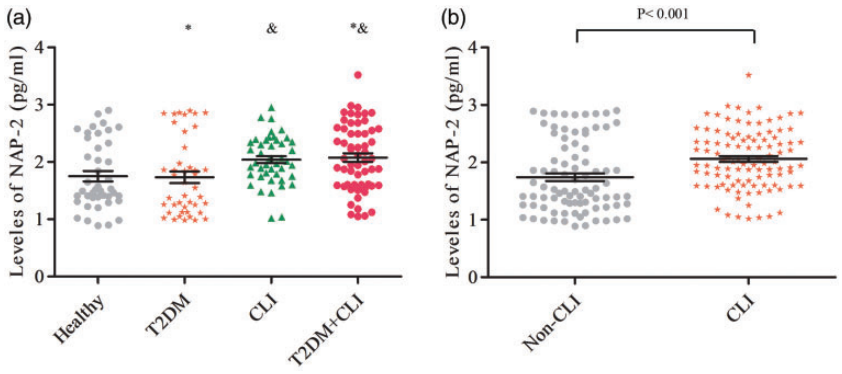

Figure 1. Increased plasma levels of NAP-2 correlated with CLI. Compared to the healthy group, subjects with CLI had higher plasma levels of NAP-2 (2.04 ± 0.06 ng/mL vs. 1.75 ± 0.09 ng/mL, $P = 0.011$). Subjects with CLI and T2DM had higher plasma levels of NAP-2 than those of the T2DM group (2.08 ± 0.08 ng/mL vs. 1.73 ± 0.10 ng/mL, $P = 0.007$). *$P > 0.05$ vs. healthy group, or vs. CLI group. &$P < 0.05$ vs. healthy group, or vs. T2DM group (a). Subjects with CLI including CLI and T2DM+CLI had higher plasma levels of NAP-2 than those of the non-CLI group including healthy and T2DM group (2.06 ± 0.05 ng/mL vs. 1.74 ± 0.07 ng/mL, $P < 0.001$) (b). (A color version of this figure is available in the online journal.)

was not associated with T2DM. Therefore, we divided the healthy group and the T2DM group into one group (non-CLI group), and divided the CLI group and the T2DM+CLI group into one group (CLI group). However, subjects with CLI including CLI and T2DM+CLI (range: 1.02–3.52 ng/mL) had higher plasma levels of NAP-2 than those of the non-CLI group including healthy and T2DM group (range: 0.89–2.90 ng/mL, 2.06 ± 0.05 ng/mL vs. 1.74 ± 0.07 ng/mL, $P < 0.001$) (Figure 1(b)).

Correlation analysis of NAP-2 and CLI risk factors

ABI and TC were significantly and negatively associated with plasma NAP-2 level (rho= −0.250, $P = 0.001$; rho= −0.162, $P = 0.026$, respectively). SBP positively correlates with plasma NAP-2 levels (rho= 0.187, $P = 0.010$), but smoking, LDL-C, Apo A, uric acid (UA) negatively correlate with plasma levels of NAP-2. However, there were positive associations between FPG, Cr, BUN and plasma levels of NAP-2, but they did not remain significant ($P > 0.05$) (Table 2).

The risk factors for CLI in T2DM

The confounders including smoking, ABI, FPG, SBP, LDL-C, TC, UA, Cr, Apo A, and BUN were entered a logistic regression analysis. When adjusting for the above factors, plasma levels of NAP-2 still correlated with CLI (odds ratio =11.543, 95% confidence intervals: 1.327–100.403, $P = 0.027$, Table 3).

Evaluation of the diagnostic values of NAP-2 for CLI

Receiver operating characteristic (ROC) curve showed that the area under curve (AUC) of NAP-2 was 0.668 (95% confidence interval 0.586–0.750, $P < 0.001$) and the optimal cut-off point for NAP-2 was 1.575 ng/mL, the sensitivity is 84.62% (95% confidence intervals 0.762–0.909) and the specificity is 54.12% (95% confidence intervals 0.430–0.650). The AUC of confounders was 0.990 (95% confidence intervals 0.979 to 1.001, $P < 0.001$). Importantly, the AUC of NAP-2+confounders was 0.992 (95% confidence intervals 0.981 to 1.003, $P < 0.001$) and it was higher than those of NAP-2 (95% confidence intervals 0.241 to 0.407, $P < 0.001$) and confounders (95% confidence intervals −0.013 to 0.018, $P = 0.797$), respectively. This indicated that the diagnostic performance of the NAP-2 + confounders panel was superior to either NAP-2 or confounders alone (Figure 2).

Table 2. Spearman Rho correlations between plasma levels of NAP-2 and CLI risk factors.

Variable		NAP-2
Smoking	*rho*	−0.076
	P	0.300
ABI	*rho*	−0.250
	P	*0.001*
FPG	*rho*	0.055
	P	0.454
SBP	*rho*	0.187
	P	*0.010*
LDL-C	*rho*	−0.089
	P	0.221
TC	*rho*	−0.162
	P	*0.026*
Apo A	*rho*	−0.019
	P	0.793
Cr	*rho*	0.079
	P	0.282
BUN	*rho*	0.062
	P	0.395
UA	*rho*	−0.009
	P	0.902

ABI: Ankle brachial index; FPG: fasting plasma glucose; SBP: systolic blood pressure; LDL-C: low density lipoprotein cholesterol; TC: total cholesterol; Apo A: Apolipoprotein A; Cr: serum creatinine; BUN: blood urea nitrogen; UA: uric acid.
Note: CLI group including CLI and T2DM+CLI.
Significant values are marked in italic.

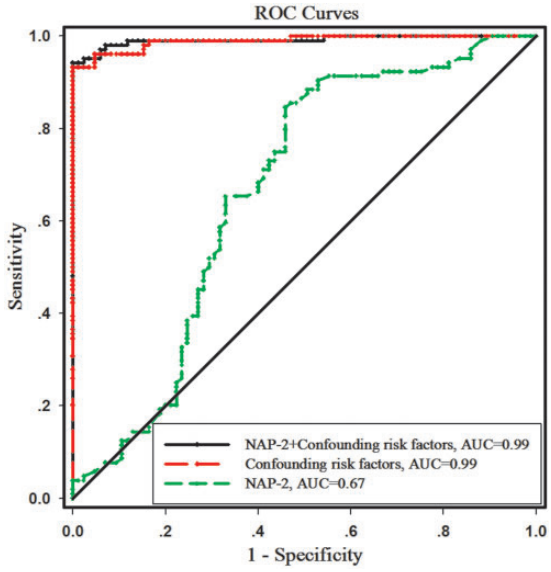

Figure 2. CXCL-6 is a predictive factor for CLI. The area under the curve (AUC) of NAP-2 was 0.668 (95% confidence intervals 0.586–0.750, $P < 0.001$). The optimal cut-off point for NAP-2 was 1.575 ng/mL. The AUC of NAP-2+confounders was 0.992 (95% confidence intervals 0.981 to 1.003, $P < 0.001$), which was higher than that of NAP-2 alone (95% confidence intervals 0.241 to 0.407, $P < 0.001$) and confounders alone (AUC= 0.990, 95% confidence intervals −0.013 to 0.018, $P = 0.797$), respectively. CLI group included CLI and T2DM+CLI. (A color version of this figure is available in the online journal.)

Table 3. Logistic regression analysis for the risk factors of CLI.

	OR	95% CI for OR	*P*	OR[a]	95% CI for OR[a]	*P*[a]
Smoking	2.367	1.187–4.720	*0.014*	/	/	/
ABI	0.000	0.000–0.000	*0.000*	/	/	/
FPG	1.118	1.022–1.223	*0.014*	/	/	/
SBP	1.063	1.041–1.086	*0.000*	/	/	/
LDL-C	0.676	0.480–0.953	*0.025*	/	/	/
TC	0.649	0.497–0.846	*0.001*	/	/	/
Apo A	0.333	0.122–0.905	*0.031*	/	/	/
Cr	1.029	1.015–1.044	*0.000*	/	/	/
BUN	1.397	1.159–1.684	*0.000*	/	/	/
UA	1.003	1.000–1.006	*0.043*	/	/	/
NAP-2	2.662	1.566–4.524	*0.000*	11.543	1.327–100.403	*0.027*

CI: confidence interval; OR: odds ratio; ABI: Ankle brachial index; FPG: fasting plasma glucose; SBP: systolic blood pressure; LDL-C: low density lipoprotein cholesterol; TC: total cholesterol; Apo A: Apolipoprotein A; Cr: serum creatinine; BUN: blood urea nitrogen; UA: uric acid.
[a]Adjusted for smoking, ABI, FPG, SBP, LDL-C, TC, Apo A, Cr, BUN and UA.
Note: CLI group including CLI and T2DM+CLI. Significant values are marked in italic.

Discussion

In this present study, we examined plasma NAP-2 levels and its relationship with CLI. We found that elevated plasma NAP-2 was associated with CLI, but not with T2DM. ABI and TC were significantly and negatively associated with plasma NAP-2 level, and SBP positively correlated with plasma NAP-2 level. When adjusting for the confounders (smoking, ABI, FPG, SBP, LDL-C, TC, UA, Cr, Apo A, and BUN), plasma NAP-2 levels were independently and strongly associated with CLI. Therefore, our findings implicate plasma NAP-2 levels as an early predictor of CLI. Plasma NAP-2 levels might be an early CLI diagnostic biomarker and might provide a novel target for CLI treatment.

In the present study, we found significant differences in smoking, ABI, FPG, SBP, LDL-C, TC, UA, Cr, Apo A, and BUN between CLI (CLI and T2DM+CLI) and non-CLI (healthy and T2DM). However, patients with CLI have lower LDL-C, Apo A, and TC, but have higher plasma levels of Cr, BUN, and UA, which was in accordance with previous findings.[5,16,18] A Spearman Rho correlation analysis showed that ABI and TC were significantly and negatively associated with plasma NAP-2 level, and SBP was shown to positively correlate with plasma NAP-2 levels, this means that with the decrease of ABI and TC, or with the increase of SBP, the levels of NAP-2 increased, and the risk of PAD will increase, which are consistent with previous studies.[5,16,18]

Lower extremity atherosclerosis is one of the main risk factors for diabetic foot ulcer and amputation. Early diagnosis of CLI is important to prevent amputation for the diabetic population.[16] In the current study, plasma levels of NAP-2 were independently associated with CLI, but were not associated with T2DM. Diabetes delayed the healing of foot ulceration,[19,20] and CLI was the main causes for healing of lower limbs ulcers;[21,22] therefore, plasma NAP-2 levels not only may be a biomarker to predict the lower limb ischemia in the non-diabetic CLI population, but they also may be an ideal marker to predict the wound healing in diabetic CLI population.

NAP-2 is encoded by the *CXCL7* gene.[12] NAP-2 is secreted from the α-granules upon platelet activation and plays important roles in regulating inflammation and plaque formation.[13,23] Currently, there is no effective evidence that platelet-derived NAP-2 can promote atherosclerosis. Previous studies reported that NAP-2 is absent in most early carotid atherosclerotic plaques lesions, and foamy macrophages and calcifications were found in coronary and aortic lesions of luminal and neovascular endothelium.[24,25] NAP-2 mainly attracts and activates neutrophil granulocytes, but has no strong chemotactic activities on monocytes and lymphocytes; however, IL-8 and NAP-2 has no effect on arteriogenesis in rabbit.[26] There was little report about the relationship between plasma levels of NAP-2 on atherosclerosis. Only two studies reported that compared with control subjects, patients with stable, unstable, and angina had higher plasma levels of NAP-2,[10,27] and women with high plasma levels of NAP-2 have a higher risk of myocardial infarction.[13] In this study, we found that compared to non-CLI in T2DM, NAP-2 was significantly increased in CLI with non-T2DM patients, especially in CLI with T2DM. However, the pathogenesis of peripheral artery disease differs from that of coronary artery disease. In addition, our study excluded the CAD; thus, the NAP-2 was associated with severe arterial disease, including angina, myocardial infarction, and CLI, especially in T2DM patients.

Previous study reported platelet α-granule secretory proteins including NAP-2 may enhance esterification and oxidized LDL uptake by macrophages. In addition, our studies did not determine the causal relationship between NAP-2 and the pathogenesis of atherosclerosis. However, our data suggest that the release of NAP-2 from locally activated platelets into the vascular wall and probably promotes foam cell formation and atherogenesis.[24,25,28]

Currently, PAD patients, especially the CLI patients, have deficiencies in diagnosis and treatment.[29,30] Primary providers lack professional equipment and trained personnel for ABI measurements. Blood tests are an effective and convenient method for identification of disease markers for prevention, diagnosis, and prognosis. Patients with elevated CLI scores will be referred to a vascular specialist who can provide further assessment and management, and prevent major cardiovascular adverse events.[30,31]

However, there are several limitations for consideration. Firstly, although ABI was $\geq$0.9, this might not rule out the peripheral vascular disease.[32] Secondly, each blood sample was collected only once. Thirdly, although it revealed the close relationship between NAP-2 and CLI in this study, the molecular processes of NAP-2 in CLI require further investigation.

In conclusion, this is the first report that plasma levels of NAP-2 are elevated in patients with CLI and is an independent predictor of patients with CLI. Plasma NAP-2 levels might be an early CLI diagnostic biomarker and might provide a novel target for CLI treatment. Further investigation is needed to exploit the molecular processes of NAP-2 in CLI.

Authors' contributions: XF Wang and Q Liu conceived and designed the experiments. XF Wang and LM Gan performed the experiments. JY Li and Q Liu analyzed the data. XF Wang and Q Liu wrote the paper.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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