Brain-Derived Neurotrophic Factor, a New Predictor of Coronary Artery Calcification

Hong Jin, PhD[1], Jing-jing Ji, PhD[1], Yi Zhu, PhD[1] ⓘ,
Xiao-dong Wang, PhD[2], Yi-ping Li, PhD[1], Qiu-yin Shi, MD[1],
and Yi-fei Chen, MD[3]

Abstract

Brain-derived neurotrophic factor (BDNF) plays a functional role in vascular endothelium homeostasis and the alleviation of atherosclerosis. Matrix gla protein (MGP) and Nε-(1-carboxymethyl)-l-lysine (CML) are both confirmed to be VC predictors. This study investigated the association between BDNF, MGP, CML and coronary artery calcification (CAC). Plasma BDNF, MGP, and CML levels were measured in 274 patients who underwent computed tomography to determine the CAC score (Agatston score). It was found that patients with CAC exhibited lower BDNF and MGP and higher CML levels than those without CAC. Plasma BDNF levels in patients with diabetes or hypertension were lower compared with the control groups. In logistic regression analysis, age, hypertension, BDNF, and MGP were independent predictors of CAC. Plasma BDNF and MGP levels were both correlated with the Agatston score even after adjustment for age, total cholesterol level, triglycerides, low-density lipoprotein level, creatinine clearance rate, and the presence of hypertension and diabetes mellitus. In 167 patients with CAC, circulating BDNF level was inversely associated with CML level and positively related to MGP level. In the receiver operating characteristic analysis for CAC, the areas under the curves for BDNF, MGP, and CML were 0.757, 0.777 and 0.653, respectively. In summary, plasma BDNF levels are associated with the Agatston score, and BDNF further predicts the occurrence of CAC.

Background

Coronary artery calcification (CAC), including arterial media calcification and intima calcification, is a pathophysiological phenomenon related to advanced atherosclerosis, which disrupts the elasticity and function of the vessel wall and is further linked with plaque instability, adverse cardiovascular events, and patient mortality. Early computed tomography (CT) scanning and coronary angiography (CAG) can accurately assess the severity of CAC.[1] However, considering the cost and invasiveness of CT angiography (CTA) and CAG, the identification of biomarkers of CAC could assist in the management and treatment of vascular lesions.

Extensive research has shown that endothelial-mesenchymal transition,[2] imbalances in vascular smooth muscle cell (VSMC) osteoblastic phenotype transition,[3] advanced glycation end products (AGEs) formation,[4] and stimulated oxidative stress and apoptosis[5] can all contribute to vascular calcification (VC). Matrix gla protein (MGP) and Nε-(1-carboxymethyl)-l-

lysine (CML) are both confirmed to be VC predictors. Brain-derived neurotrophic factor (BDNF) is one of the members of the neurotrophin family and mainly functions biologically through the receptor tyrosine kinase receptor.[6] BDNF improves the process of embryonic stem cell differentiation to endothelial cells (ECs) and upregulates nitric oxide synthase expression, regulating the endothelial oxidative stress level, and

[1] Department of Cardiology, Zhongda Hospital, School of Medicine, Southeast University, Nanjing, People's Republic of China
[2] Department of Cardiology, Affiliated Hospital of Jiangsu University, Zhenjiang, People's Republic of China
[3] Department of Cardiology, Xishan Hospital, Wuxi, People's Republic of China

Corresponding Author:
Hong Jin, Department of Cardiology, Zhongda Hospital, School of Medicine, Southeast University, Nanjing 210000, People's Republic of China.
Email: jinhong5985@163.com

reducing endothelial damage.[7] Our previous research demonstrated that BDNF could alleviate high glucose-induced EC damage through Bcl-2/adenovirus E1B-19 kDa-interacting protein 3 (BNIP3)-mediated mitophagy.[8] In clinical studies, we found that plasma BDNF levels are positively correlated with the levels of vascular protective factor von Willebrand factor (vWF) and can be used as an independent predictor of stable coronary artery disease (CAD).[9] Although there is no research on the relationship between BDNF and VC, we speculated that BDNF, as a protective factor of vascular endothelial secretion, decreases endothelial oxidative stress and renders osteoprogenitor cell transition under hemodynamic disturbance or diabetes; furthermore, via paracrine signaling, it acts on VSMCs, stabilizing the VSMC contraction phenotype, reducing VSMC apoptosis, and alleviating VC to a certain extent.

Based on the above, the aims of the present study were to specifically establish the associations between BDNF and CAC in subjects who underwent coronary CT scanning.

Patients and Methods

Ethics Approval and Consent to Participate

This study was performed in compliance with the Declaration of Helsinki, and it was approved by the Committee of Clinical Investigation of Southeast University School of Medicine and Jiangsu University School of Medicine (ZDSYLL066-P01). Informed consent was obtained from all individual participants included in the study. The enrolled patients provided written informed consent, including questions about their general conditions and atherogenic risk factors.

Clinical Study Design

This study was registered in the China Clinical Trial Registration Center (ChiCTR1800020259).

A total of 300 consecutive patients with angina pectoris or suspected CAD, including outpatients and inpatients, were included in this cross-sectional study. All subjects who underwent coronary CTA using 320-row-detector dynamic volume CT at Zhongda Hospital Affiliated with Southeast University and Affiliated Hospital of Jiangsu University between November 2018 and May 2020 were recruited. Patients with coronary stenosis detected by coronary CTA were followed by CAG examination. Patients with > 50% stenosis of the primary coronary artery or its major branches were diagnosed with CAD.

Coronary CTA images were analyzed by 1 of 3 experienced radiologists, all of whom were blinded to the laboratory and clinical details of the participants at the time of analysis. The quantitative CAC score was calculated using dedicated software and was expressed as an Agatston score.[10] The presence of CAC was defined as an Agatston score of > 0.

The exclusion criteria included the following: (1) malignant tumor (n = 2), (2) pregnancy, (3) severe liver dysfunction, (4) severe hematological disorders, (5) a history of coronary artery bypass grafting, (6) allergy to the contrast agent, and (7) hemolysis of collected blood samples or poorly saved samples (n = 24). After the exclusion criteria were checked, a total of 274 patients were finally enrolled.

Dyslipidemia was diagnosed when the fasting plasma total cholesterol (TC) was > 5.2 mmol/L, triglycerides (TG) were > 1.7 mmol/L or low-density lipoprotein cholesterol (LDL-C) was > 2.6 mmol/L according to the Chinese Guidelines for the Prevention and Treatment of Dyslipidemia.[11] A fasting lactate dehydrogenase (LDH) > 228 U/L and a creatinine clearance rate (Ccr) < 70 ml/min were considered harmful to the cardiovascular system. Diabetes was diagnosed according to data obtained from medical records. Hypertension was diagnosed when systolic blood pressure (SBP) was at least 140 mmHg and/or diastolic blood pressure was at least 90 mmHg or if the patient was taking antihypertensive drugs.

Laboratory Procedure

Blood samples were collected in tubes containing EDTA (pH 7.5) and immediately centrifuged at 3000 rpm for 20 min at 4°C. Plasma samples were stored at −80°C for analysis. The samples to be tested will be evaluated within 3 months after acquisition. Plasma BDNF, CML and MGP levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (CUSABIO, China). The measurements were performed strictly according to the manufacturer's instructions. Other biochemical parameters were analyzed using standard laboratory methods in a typical hospital laboratory.

Cell Culture

Human VSMCs (HVSMCs) were obtained from ATCC cell bank (PCS-100-012, Shanghai, China). HVSMCs were incubated in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 10% fetal bovine serum and antibiotics at 37°C with 5% CO_2. HVSMC calcification was induced with calcified medium (CM) containing β-glycerophosphate (β-GP, Sigma-Aldrich, St. Louis, MO, USA) according to previous protocols. Briefly, HVSMCs were cultured with 10 mM β-GP for 7 days, the medium was renewed twice a week.

Measurement of the Calcium Content

Calcium content of HVSMCs were solubilized with RIPA lysis buffer (Beyotime Biotechnology, Jiangsu, China) and measured using the Calcium Assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) and normalized to the total protein content with a Bicinchoninic Acid (BCA) Protein Assay kit (KeyGEN Biotechnology, Jiangsu, China).

Alizarin Red S Staining

HVSMCs were fixed in 4% paraformaldehyde for 30 min, and then underwent a series of treatments according to previous protocols.[12] For Alizarin red S staining, the calcium phosphate salts were visualized as a red staining.

Table 1. Clinical Characteristics of Subjects Based on the Agatston Score (n = 274).

Variables	0 (n = 107)	1-100 (n = 58)	101-400 (n = 60)	>400 (n = 49)	p value
Age, years	60 ± 12	66 ± 11	68 ± 10	73 ± 9	< 0.001
Gender, male, n (%)	51 (47.7)	35 (60.3)	36 (60.0)	28 (57.1)	0.298
BMI (kg/m2)	24.9 ± 3.7	25.4 ± 3.4	25.0 ± 3.6	24.7 ± 4	0.770
Currently smoking, n (%)	23 (21.5)	19 (32.8)	14 (23.3)	16 (32.7)	0.282
SBP (mmHg)	132 ± 19	139 ± 18	140 ± 19	141 ± 18	0.006
DBP (mmHg)	83 (70-86)	80 (71-89)	78 (69-83)	79 (70-89)	0.624
LDH (U/L)	188 (163-205)	184 (163-203)	187 (157-205)	187 (155-207)	0.985
TG (mmol/L)	1.8 (0.9-2.0)	1.8 (1.0-2.3)	1.6 (0.9-1.7)	1.5 (0.9-1.7)	0.096
TC (mmol/L)	4.5 ± 1	4.8 ± 1.1	4.4 ± 1.0	4.3 ± 1.1	0.055
LDL-C (mmol/L)	2.3 ± 0.9	2.6 ± 1.0	2.2 ± 0.8	2.0 ± 1.0	0.023
HDL-C (mmol/L)	1.5 (1.1-1.7)	1.6 (1.1-1.6)	1.7 (1.1-2.1)	1.7 (1.2-2.1)	0.271
FBG (mmol/L)	5.7 (4.9-5.7)	6.3 (5.0-7.0)	6.3 (4.9-6.7)	6.1 (4.8-6.9)	0.027
*HbA1c (%)	5.9 (5.5-6.1)	6.6 (5.8-7.1)	6.4 (5.7-6.6)	6.5 (5.7-7.1)	0.001
Ccr (ml/min)	100 (80-111)	92 (76-110)	91 (67-106)	73 (57-81)	< 0.001
CAD, n (%)	9 (8.4)	17 (29.3)	33 (55.0)	35 (71.4)	< 0.001
#Number of stenosed coronary vessel, n	0.1 (0-0)	0.7 (0-1)	1.1 (0-2)	2 (1-3)	< 0.001
Diabetes mellitus, n (%)	10 (9.3)	12 (20.7)	16 (26.7)	15 (30.6)	0.002
Hypertension, n (%)	48 (44.9)	40 (69.0)	51 (85.0)	42 (85.7)	< 0.001
Cerebral infarction, n (%)	9 (8.4)	4 (6.9)	2 (3.3)	3 (6.1)	0.632
Remote infarct, n (%)	2 (1.9)	0 (0.0)	1 (0.2)	0 (0)	0.581
Aortic valve calcification, n (%)	13 (12.1)	11 (19.0)	13 (21.7)	18 (36.7)	0.008
LVEF (%)	68 ± 7	69 ± 5	67 ± 8	67 ± 10	0.572
BDNF (ng/mL)	1.77 ± 0.32	1.59 ± 0.38	1.44 ± 0.22	1.33 ± 0.31	< 0.001
CML (pg/mL)	215.5 (128.5-297.5)	251.8 (179.6-314.4)	273.8 (188.7-358.1)	304.2 (221.9-388.4)	< 0.001
MGP (pg/mL)	184.7 ± 39.9	159.5 ± 36.1	146.6 ± 35.6	123.7 ± 32.9	< 0.001

Values are shown as the means ± SD, median (interquartile range) or percentage. BMI body mass index, SBP systolic blood pressure, DBP diastolic blood pressure, LDH lactate dehydrogenase, TG triglycerides, TC total cholesterol, LDL-C low-density lipoprotein cholesterol, HDL-C high-density lipoprotein cholesterol, FBG fasting blood glucose, Ccr creatinine clearance rate, CAD coronary artery disease, LVEF left ventricular ejection factor, BDNF brain-derived neurotrophic factor, CML Nε-(1-carboxymethyl)-l-lysine, MGP matrix Gla protein.
*Data from 168 subjects.
#Left main coronary artery, left anterior descending branch, left circumflex branch, right coronary artery and main branches were included in the statistics.

Statistical Analysis

All data were performed a normal distribution test, and are expressed as the mean ± standard deviation for approximately normally distributed data and as the median (interquartile range) for skewed continuous variables. Skewed continuous variables were ln transformed for analysis. Comparisons of continuous variables between 2 groups were performed using Student's t-tests. One-way ANOVA with post hoc comparisons by Tukey's multiple comparisons test was used to compare the results for multiple group comparisons. The chi-square test was used for comparisons of categorical variables between groups. Pearson's correlation method was used for correlation analysis. Associations between key variables and the Agatston score were analyzed using multiple linear regression analysis. Univariable and multiple logistic regression analyses were performed to identify significant parameters for the presence of CAC. Receiver operating characteristic (ROC) analysis was performed to determine whether the explored variables were predictive of CAC. Comparisons of areas under the ROC curves (AUCs) were performed as recommended by DeLong et al.[13] All tests were 2-sided. P values < 0.05 were considered significant. Statistical analyses were performed using SPSS software 22.0 (SPSS Inc., Chicago, IL, USA).

Results

Baseline Clinical Characteristics

Table 1 shows the clinical and biochemical characteristics of the enrolled patients. A total of 274 patients were stratified into 4 groups based on their Agatston scores (0; 1-100; 101-400; > 400) according to ACCF/AHA 2007 clinical expert consensus.[14] Significant differences in clinical and metabolic parameters were observed among the groups. Larger Agatston scores were significantly associated with older age ($p < 0.001$). Hypertension ($p < 0.001$), diabetes ($p = 0.002$), and aortic valve calcification ($p = 0.008$) were more prevalent in the higher Agatston score groups. As the Agatston score increased, the proportion of CAD ($p < 0.001$), the number of coronary artery lesions ($p < 0.001$), LDL-C level ($p = 0.023$), HbA1c level ($p = 0.001$), fasting blood glucose (FBG) level ($p = 0.027$), and plasma CML level ($p < 0.001$) also increased, while Ccr level ($p < 0.001$) and plasma BDNF ($p < 0.001$) and MGP ($p < 0.001$) level decreased. There were no differences between the groups in sex, body mass index (BMI), current smoking status, diastolic blood pressure, TG, TC, high-density lipoprotein cholesterol (HDL-C), LDH, left ventricular
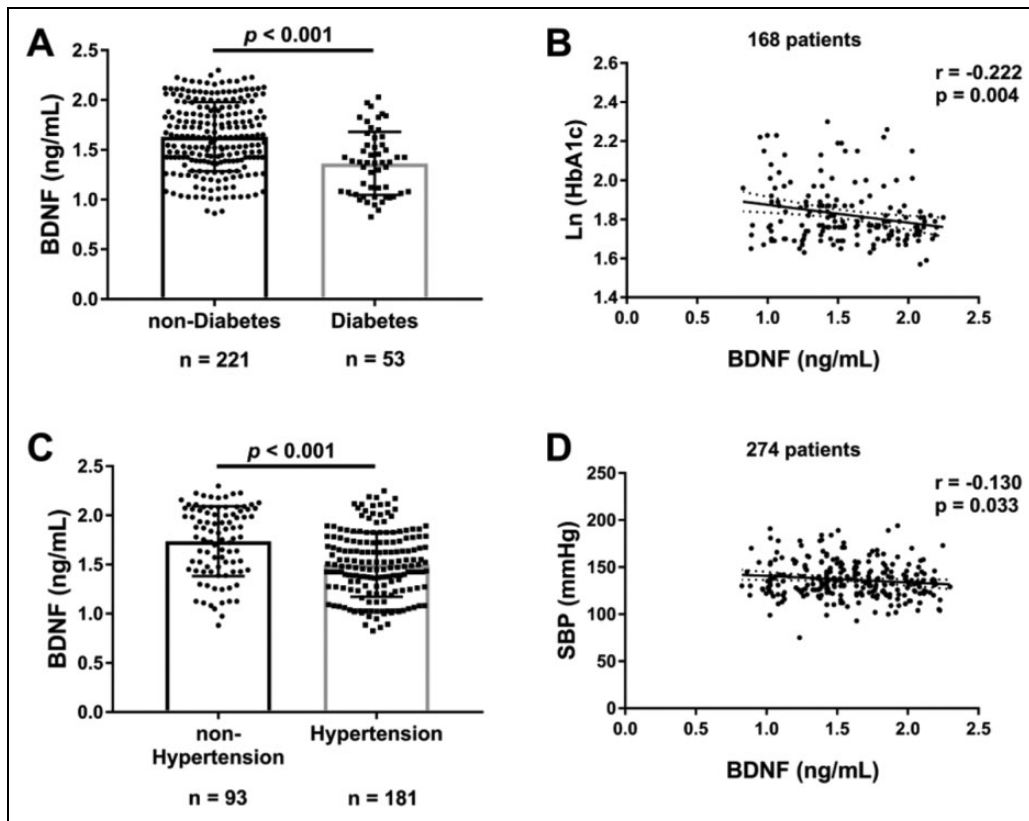
Figure 1. Basic expression of BDNF in patients with diabetes or hypertension. A, Plasma BDNF levels in 221 non-diabetic patients and 53 diabetic patients. B, Bivariable correlation analysis between BDNF and HbA1c (ln transformed) in 168 patients. C, Plasma BDNF levels in 93 non-hypertensive patients and 181 hypertensive patients. D, Bivariable correlation analysis between BDNF and SBP in 274 patients.

ejection factor (LVEF), or history of cerebral infarction and remote infarct.

Plasma BDNF Levels Were Decreased in Patients With Diabetes or Hypertension

Since the accumulation of toxic metabolites in diabetes and the hemodynamic disturbances in hypertension could both damage vascular endothelium and disturb the normal secretion of BDNF.[15,16] As seen in Figure 1A and 1C, plasma BDNF levels were markedly decreased in 274 patients with diabetes ($p < 0.001$) or hypertension ($p < 0.001$) compared with the control groups. For the BDNF and HbA1c correlation, it was demonstrated that BDNF and HbA1c (ln transformed) were statistically related in 168 patients (Figure 1B, r = −0.222, $p = 0.004$). In addition, SBP and BDNF also presented a negative association in 274 patients (Figure 1D, r = −0.13, $p = 0.033$).

Logistic Regression Analysis of Risk Factors for Predicting CAC

Univariable logistic analysis revealed that age, hypertension, diabetes mellitus, Ccr, CML, MGP, and BDNF were significantly associated with the presence of CAC (Agatston score > 0) (Table 2). These parameters were then entered into a

multivariable logistic regression analysis, and age (HR = 1.078; 95% CI, 1.040-1.117; $p < 0.001$), hypertension (HR = 2.838; 95% CI, 1.444-5.576; $p = 0.002$), MGP (HR = 6.379; 95% CI, 3.266-12.460; $p < 0.001$) and BDNF (HR = 5.447; 95% CI, 2.744-10.810; $p < 0.001$) were independent predictors of the occurrence of CAC (Table 2).

As seen in Supplementary Table 1, univariable logistic analysis revealed that age, Ccr, and MGP were significantly associated with the presence of severe CAC (Agatston score > 400) in patients with CAC. These parameters were then entered into a multivariable logistic regression analysis, and age (HR = 1.058; 95% CI, 1.012-1.107; p = 0.014), and MGP (HR = 3.501; 95% CI, 3.266-12.460; p = 0.004) were independent predictors of the occurrence of the Agatston score > 400.

Multiple Linear Regression Analysis of Cardiovascular Risk Factors for the Agatston Score

We next investigated the correlation between cardiovascular risk factors and the Agatston score in patients with CAC. Agatston score was strongly associated with age (r = 0.256, $p = 0.001$), the presence of diabetes mellitus (r = 0.209, $p = 0.007$), hypertension (r = 0.218, $p = 0.005$), TG (r = −0.167, $p = 0.033$), TC (r = −0.163, $p = 0.038$), LDL-C (r = −0.216, $p = 0.006$), Ccr (r = −0.233,

Table 2. Logistic Regression Analysis of Cardiovascular Risk Factors for Predicting Patients With CAC (n = 274).

	Univariate analysis			Multivariate analysis		
	HR	95% CI	p value	HR	95% CI	p value
Age (per year)	1.069	1.043-1.095	< 0.001	1.078	1.040-1.117	< 0.001
Male sex	1.599	0.980-2.607	0.060			
Currently smoking	1.517	0.859-2.679	0.151			
Hypertension	4.808	2.814-8.216	< 0.001	2.838	1.444-5.576	0.002
Diabetes mellitus	3.391	1.622-7.092	0.001	1.635	0.654-4.087	0.293
BMI > 23 (kg/m^2)	1.107	0.632-1.940	0.722			
TG > 1.7 (mmol/L)	1.158	0.686-1.953	0.583			
TC > 5.2 (mmol/L)	1.331	0.767-2.312	0.310			
LDL-C > 2.6 (mmol/L)	1.278	0.767-2.129	0.346			
HDL-C < 1.02 (mmol/L)	1.056	0.562-1.984	0.866			
LDH > 228 (U/L)	1.208	0.553-2.638	0.636			
Ccr < 70 (ml/min)	2.806	1.561-5.043	0.001	1.271	0.543-2.978	0.581
CML > median (pg/mL)	2.042	1.246-3.348	0.005	1.001	0.522-1.919	0.997
MGP < median (pg/mL)	5.718	3.329-9.821	< 0.001	6.379	3.266-12.460	< 0.001
BDNF < median (ng/mL)	4.923	2.894-8.376	< 0.001	5.447	2.744-10.810	< 0.001

BMI body mass index, TG triglycerides, TC total cholesterol, LDL-C low-density lipoprotein cholesterol, HDL-C high-density lipoprotein cholesterol, LDH lactate dehydrogenase, Ccr creatinine clearance rate, CML Nε-(1-carboxymethyl)-l-lysine, MGP matrix Gla protein, BDNF brain-derived neurotrophic factor.

$p = 0.004$), MGP (r = −0.378, $p < 0.001$), CML (r = 0.157, $p = 0.043$) and BDNF (r = −0.393, $p < 0.001$) (Table 3). Multiple linear regression analysis revealed that age, MGP level, and BDNF level were independently associated with the Agatston score in patients with CAC (Table 4).

Sensitivity and Specificity of BDNF in Predicting CAC

Bivariable correlation analysis demonstrated that plasma BDNF levels are correlated with MGP levels (Figure 2A, r = 0.176, $p = 0.023$) or CML levels (ln transformed, Figure 2B, r = −0.235, $p = 0.002$) in 167 patients with CAC, then we analyzed the ROC curve of the 3 parameters. ROC analysis for the detection of CAC revealed AUCs of 0.757 (95% CI: 0.699 to 0.816) for BDNF, 0.777 (95% CI: 0.719 to 0.835) for MGP, and 0.653 (95% CI: 0.587 to 0.719) for CML levels (Figure 2C and D), with significant differences between the AUCs of BDNF and CML ($p = 0.019$). We further analyzed the combined diagnostic value of BDNF+MGP and BDNF+CML for CAC. The AUCs for BDNF+MGP and BDNF+CML were 0.813 (95% CI: 0.761 to 0.865) and 0.769 (95% CI: 0.712 to 0.825), respectively. A significant difference was found between the AUC of BDNF and the AUC of BDNF+MGP ($p = 0.015$) (Figure 2C). In addition, BDNF+CML demonstrated a greater AUC than that of CML alone ($p < 0.001$) (Figure 2D). These results indicate that plasma BDNF levels predict the occurrence of CAC, and the diagnostic value of BDNF for CAC is further improved when combined with MGP.

BDNF Inhibited Calcium Deposition in VSMC

To further verify the role of BDNF in arterial calcification, and considering that VSMC is the key driving cell of arterial intimal calcification and media calcification,[3] we selected HVSMC for the construction of an *in vitro* VC model. 100 ng/mL human

Table 3. Correlations Between Cardiovascular Risk Factors and Agatston Score in Patients With CAC (n = 167).

Variable	r	p value
Age, years	0.256	0.001
Male sex	0.013	0.865
Currently smoking	−0.034	0.658
Hypertension	0.218	0.005
Diabetes mellitus	0.209	0.007
BMI	−0.119	0.142
TG	−0.167	0.033
TC	−0.163	0.038
LDL cholesterol	−0.216	0.006
HDL cholesterol	0.080	0.315
LDH	0.003	0.968
Ccr	−0.233	0.004
MGP	−0.378	< 0.001
CML	0.157	0.043
BDNF	−0.393	< 0.001

BMI body mass index, TG triglycerides, TC total cholesterol, LDL-C low-density lipoprotein cholesterol, HDL-C high-density lipoprotein cholesterol, LDH lactate dehydrogenase, Ccr creatinine clearance rate, MGP matrix Gla protein, CML Nε-(1-carboxymethyl)-l-lysine, BDNF brain-derived neurotrophic factor.

recombinant BDNF (rBDNF) was chosen for intervention according to the previous research.[8] As seen in Figure 3A, rBDNF treatment reversed calcium deposition in calcified HVSMCs, and Alizarin red S staining also showed that calcium nodule formation in HVSMCs were inhibited after rBDNF exposure (Figure 3B).

Discussion

The current study demonstrated the following main points: (1) plasma BDNF and MGP levels are both associated with the

Table 4. Multiple Linear Regression Analysis of Cardiovascular Risk Factor for Agatston Score in Patients With CAC (n = 167).

	Unstandardized coefficient	Standardized coefficient	p value
Age, years	0.039	0.243	0.015
Hypertension	0.553	0.135	0.060
Diabetes mellitus	0.445	0.121	0.098
TG	−0.280	−0.099	0.196
TC	0.012	0.008	0.926
LDL cholesterol	−0.123	−0.070	0.396
Ccr	−0.246	−0.057	0.540
MGP	−0.015	−0.332	< 0.001
CML	−0.042	−0.014	0.849
BDNF	−1.530	−0.305	< 0.001

TG triglycerides, TC total cholesterol, LDL-C low-density lipoprotein cholesterol, Ccr creatinine clearance rate, MGP matrix Gla protein, CML Nε-(1-carboxymethyl)-l-lysine, BDNF brain-derived neurotrophic factor.

Agatston score, and BDNF levels could both predict the presence of CAC; (2) plasma BDNF levels are associated with MGP and CML levels in CAC patients; (3) plasma BDNF levels predict the occurrence of CAC, and the diagnostic value of BDNF for CAC is further improved when combined with MGP; (4) BDNF could inhibit calcium deposition in calcified HVSMCs.

VC and adverse cardiovascular events are strongly correlated.[17] CAC is a predictor of adverse cardiac events in asymptomatic patients.[18] The Agatston score, a non-traditional risk factor for cardiovascular disease, is also beneficial for clinical evaluations.[19] VC often manifests as calcifications in atherosclerotic plaques and media and can be understood to a certain extent as an extension of atherosclerosis.[20] The research on traditional biomarkers of atherosclerosis and VC is well accepted. In this study, we found that increased age and hypertension are independent predictors of CAC, and multiple linear regression analysis revealed that age is independently associated with the Agatston score. Diabetes and chronic kidney disease (CKD) are recognized as the main causes of VC. AGEs accumulation in the diabetic state can accelerate the osteoblastic phenotype transition of VSMCs, and the high phosphate levels induced by CKD *in vivo* are the basis of the ectopic deposition of calcium salts.[21] However, in our study, diabetes and Ccr were not statistically significant in either logistic regression analysis or multiple linear regression analysis. We speculated that there are 4 main reasons for this: (1) of the 274 enrolled patients, the proportion of diabetic patients was relatively small; (2) the vast majority of diabetic patients received standardized diabetes treatment, which alleviated diabetic vascular complications; (3) among the 167 CAC patients included in this study, more than half of the patients had a mild degree of CAC (Agatston score < 100),[14] which reduced the predictive sensitivity of the score; (4) the proportion of patients with CKD was also low, and most patients with CKD were in stage II. This finding indicates that in future researches, we should adjust the inclusion criteria while expanding the sample size, so that the risk factors are more hierarchical.

Low BDNF levels have been reported to be detected in atherosclerotic coronary vascular tissue, whereas it is higher at baseline, suggesting that BDNF expression is suppressed during vascular injury.[22] However, research on the relationship between BDNF and VC, especially CAC, is currently lacking. Our study is the first, preliminary exploration of the relationship between BDNF and CAC. We found that plasma BDNF levels decreased as the Agatston score increased. Plasma BDNF levels were closely related to Agatston and can be used as independent predictors of CAC. In addition, we analyzed the predictive value of plasma BDNF for severe CAC (Agatston score > 400) in CAC patients. Plasma BDNF was not statistically significant. Two reasons may be existed: (1) the number of sample cases is small; (2) Patients with severe CAC have severe basic diseases, which may interfere with the levels of BDNF in the circulation. Plasma BDNF levels were also lower in patients in diabetes or hypertension, which was consistent with previous researches.[15,16] However, we must also point out that BDNF exists as a secretory factor in the vascular endothelium, and the expression of BDNF in the circulation is affected by many factors, such as hemodynamics, hypothalamic-pituitary-adrenal axis, and cognitive dysfunction,[23-25] so the specificity of the expression of markers in peripheral blood is low. MGP, a VC inhibitor, was selected as a positive control. In multivariable logistic regression analysis and multiple linear regression analysis models, BDNF and MGP were both significantly correlated with CAC even after adjustment for several cardiovascular risk factors. In the bivariable correlation analysis, we found that plasma BDNF levels were significantly correlated with MGP levels. These results indicate that in VC patients, BDNF may also be a protective factor to delay the progression of VC. Therefore, we continued to explore the effects of BDNF on HVSMC calcification *in vitro*. As we expected, BDNF can effectively inhibit calcium deposition in HVSMCs. Previous views propose that VSMC calcification is mainly manifested in arterial media calcification. However, in recent years, studies have confirmed that the osteogenic differentiation of VSMCs and calcium deposition caused by vesicle release also play a key role in arterial intimal calcification.[3] Therefore, the degree of VSMC calcification can correspond to the Agatston score to a certain extent. The main targets of MGP are BMPs, and studies have confirmed that in striatal neurons, BDNF can also regulate BMPs,[26] so the two may have common targets in intracellular signaling pathways.

Among the 274 enrolled patients, multiple linear regression indicated that there was no statistical correlation between plasma CML level and Agatston score, and multiple logistic regression found that plasma CML level could not significantly predict the presence of CAC; however, as shown in Table 1, elevated CML levels were found in patients with CAC. At the same time, in CAC patients with or without diabetes, plasma BDNF levels were negatively correlated with CML levels. We propose the following reasons for these observations: (1) the diagnostic value of CML for diabetic VC has been confirmed, and the small proportion of diabetic patients in this study affected the diagnostic specificity of CML to a certain extent;
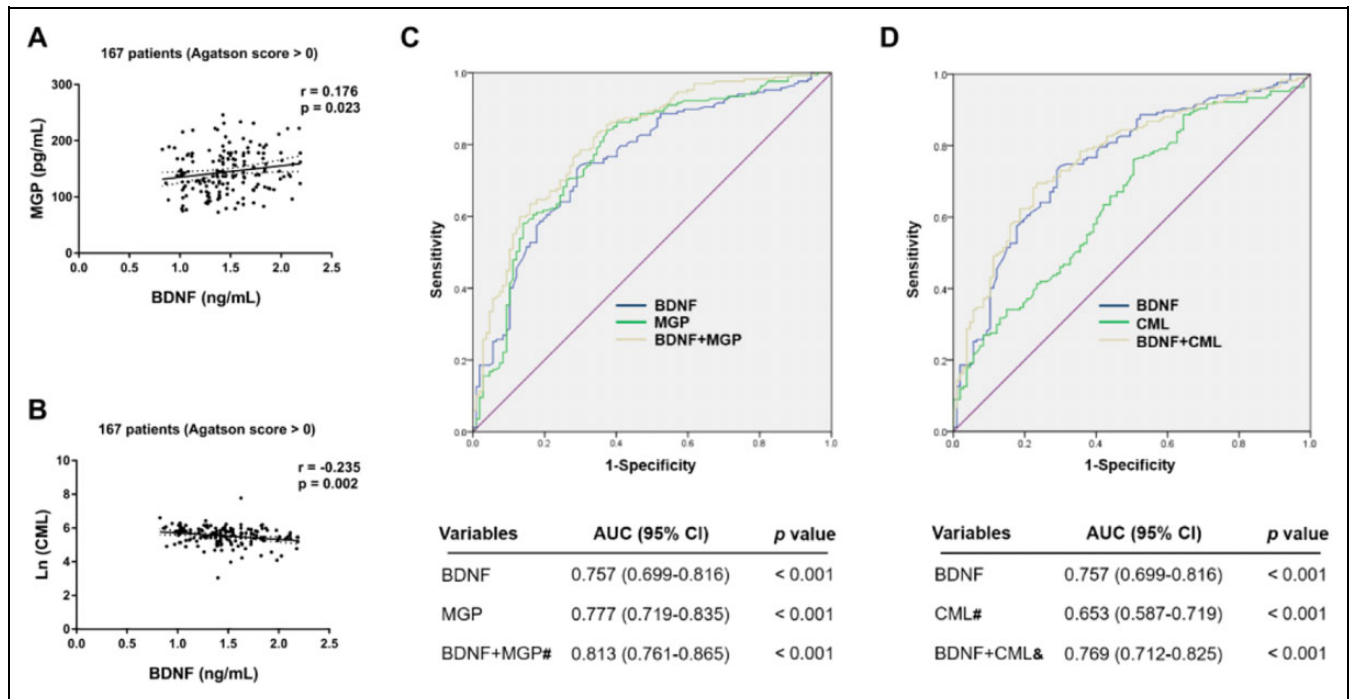
Figure 2. ROC curves for BDNF as a marker to predict the presence of CAC. Bivariable correlation analysis between BDNF and MGP (A) or CML (ln transformed) (B) in 167 patients with CAC. (C) The AUCs of BDNF, MGP, and BDNF+MGP for predicting the occurrence of CAC were 0.757 (95% CI: 0.699 to 0.816), 0.777 (95% CI: 0.719 to 0.835), and 0.813 (95% CI: 0.761 to 0.865), respectively. (D) The AUCs of CML and BDNF+CML for predicting the presence of CAC were 0.653 (95% CI: 0.587 to 0.719) and 0.769 (95% CI: 0.712 to 0.825), respectively. **#** $p < 0.05$ compared with BDNF. **&** $p < 0.05$ compared with CML.
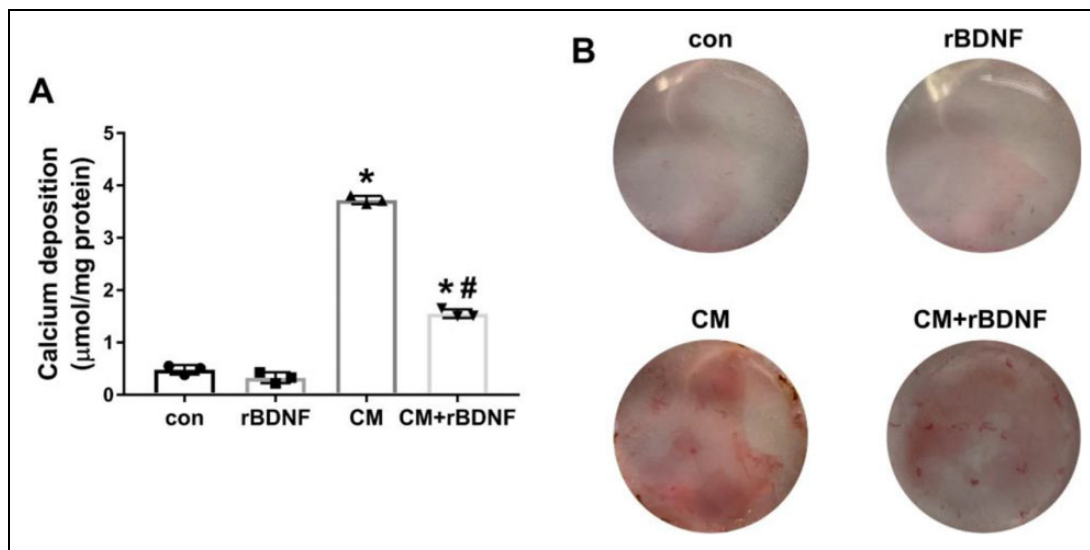

Figure 3. BDNF alleviated calcium deposition in HVSMCs. A, HVSMCs were treated with or without CM or rBDNF (100 ng/mL) for 7 days, and calcium deposition was evaluated. * $p < 0.05$ compared with the control groups. # $p < 0.05$ compared with the CM groups. B, HVSMCs were cultured with or without CM or rBDNF (100 ng/mL) for 21 days, and calcium nodules were stained with Alizarin red S.

(2) AGEs are derived from a combination of excessive sugar and protein, which can be acquired from 2 sources: the first is excessive sugar and protein synthesis, and the second is the ingestion of AGEs present in food through eating. Although there were fewer patients diagnosed with diabetes in this study, Asian people have been adopting western diets in recent decades, and therefore the AGEs ingested in these diets cannot be ignored[27]; (3) as also shown in Table 1, HbA1c and FBG levels were significantly greater in CAC patients than in non-CAC patients. Although the proportion of diabetic patients

increased, this cannot fully explain the changes in HbA1c and FBG, and therefore some CAC patients may be pre-diabetic; (4) CKD patients with calcium and phosphorus metabolism imbalance are usually accompanied by the calcification of small and medium arteries,[28] and deficits in the body fluid metabolic capacity aggravates the accumulation of CML to a certain extent; (5) the secretion of BDNF is dysfunctional in the presence of high glucose and AGEs[8,29]; moreover; in the calcification microenvironment, the basic expression of endothelium-derived cytokines is disturbed.[30] In ROC analysis, we found that the AUC of BDNF+CML was markedly greater than the AUC of CML, suggesting that CML combined with BDNF is more suitable for the diagnosis of CAC in a mixed population of diabetes and non-diabetes patients. Therefore, we concluded that in CAC patients, CML levels are not entirely dependent on the diagnosis and progression of diabetes, but in a calcified microenvironment, CML and BDNF are significantly related, and the combined diagnostic capabilities of the 2 markers have clinical significance.

Our study also has some limitations: (1) the study population was relatively small; (2) circulating BDNF levels are influenced by several factors, not all of which were analyzed; (3) this study was a cross-sectional study and lacks follow-up data; and (4) measurements of these markers at multiple time points for each subject might be useful to confirm their pathophysiological role.

Conclusions

Plasma BDNF levels were associated with the Agatston score, and could predict the presence of CAC. Plasma BDNF levels were associated with MGP and CML levels in CAC patients. In addition, plasma BDNF levels predicted the occurrence of CAC, and the combined diagnostic capabilities of BDNF+MGP or BDNF+CML have clinical diagnostic value. Moreover, calcium deposition in VSMCs could be reversed after BDNF treatment. Further studies should determine the specific mechanisms underlying the relationship between BDNF and VC.

Authors' Note

Hong Jin and Jing-jing Ji contributed equally. HJ was involved in the experimental design and writing of the manuscript. JJJ performed biochemistry detection and data analysis. YZ, XDW, YPL, QYS, and YFC contributed to the blood samples and clinical characteristics collection.

Declaration of Conflicting Interests

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ORCID iD

Yi Zhu https://orcid.org/0000-0003-0845-0164

Supplemental Material

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