Abstract. It has previously been shown that the number of endothelial progenitor cells (EPCs) is negatively correlated with Syntax score in patients with coronary artery disease (CAD). However, the association between alterations in EPC function and Syntax score is still unknown. The present study evaluated the association between the activity of EPCs as well as endothelial function and Syntax score in patients with CAD and investigated the underlying mechanisms. A total of 60 patients with CAD were enrolled in 3 groups according to Syntax score, and 20 healthy subjects were recruited as the control group. The number and migratory, proliferative and adhesive activities of circulating EPCs were studied. The endothelial function was measured by flow-mediated dilatation (FMD) and the levels of nitric oxide (NO) in plasma or secreted by EPCs were detected. The number and activity of circulating EPCs were lower in patients with a high Syntax score, which was similar to the alteration in FMD. The level of NO in plasma or secreted by EPCs also decreased as Syntax score increased. There was a negative association between FMD or circulating EPCs and Syntax score. A similar association was observed between the levels of NO in plasma or secreted by EPCs and Syntax score. Patients with CAD who had a higher Syntax score exhibited lower EPC numbers or activity and weaker endothelial function, which may be associated with attenuated NO production. These findings provide novel surrogate parameters for evaluation of the severity and complexity of CAD.

Introduction

Coronary artery disease (CAD) is the most common clinical manifestation of ischaemic heart disease and a leading cause of mortality worldwide (1,2). A characteristic feature of CAD is the formation of atherosclerotic plaques, leading to stenosis or obstruction of the coronary artery. Endothelial dysfunction has been implicated as the initiating factor and key event in the pathogenesis of atherosclerosis (3). Therefore, restoration of endothelial activity may prevent the progression of CAD and decrease cardiovascular events.

Circulating endothelial progenitor cells (EPCs) promote neovascularization, repair endothelial injury and improve endothelial function (4,5). Circulating EPCs are impaired in patients with CAD (5) and studies have reported an inverse correlation between the number of circulating EPCs and Syntax score (6,7). Derived from coronary anatomy and lesion characteristics, the Syntax score was devised to grade the anatomical complexity and severity of coronary lesions in patients with CAD (8-11). Moreover, it has been reported as an independent predictor of long-term major adverse cardiac and cerebrovascular events in patients treated with percutaneous coronary intervention (8,12,13). However, the association between the activity of EPCs, endothelial function and Syntax score in patients with CAD is still not clear.

Nitric oxide (NO) serves an important role in the regulation of EPC function and can promote the mobilization, proliferation and migratory activities of circulating EPCs (14-16). Previous studies have shown that the level of NO in plasma or...
secreted by EPCs were correlated to the activity of circulating EPCs and FMD (17,18). Based on the aforementioned studies, it was hypothesized that circulating EPC and endothelial function may be negatively correlated with Syntax score in patients with CAD and may be associated with decreased NO production. Therefore, the present study detected the number and function of circulating EPCs, measured endothelial function and evaluated levels of NO in plasma or culture medium in patients with different Syntax scores. Moreover, the present study detected the correlation between flow-mediated dilatation (FMD) or circulating EPCs and Syntax score.

Materials and methods

Characteristics of patients. A total of 60 patients with CAD were enrolled in 3 groups according to syntax score: Low-(Syntax score <22 points; n=20; 10 male patients and 10 female patients; age, 45-75 years), middle-(Syntax score, 22-32 points; n=20; 10 male patients and 10 female patients; age, 45-75 years) and high-risk (Syntax score ≥32 points; n=20; 10 male patients and 10 female patients; age, 45-75 years) and 20 healthy subjects (10 male patients and 10 female patients; age, 45-75 years) were recruited as a control group. Patients and healthy subjects were recruited between January 2018 and January 2019. In addition, according to the number of lesioned coronary arteries (antero descending branch, circumflex branch and right coronary artery), the patients were divided into single, double and triple lesion vessel groups. The patients with CAD were diagnosed by coronary angiography because of stable angina pectoris admitted to our hospital (Jiangmen Central Hospital). In consideration of the factors that influence the number of EPCs, patients were excluded on the basis of smoking, chronic renal insufficiency (serum creatinine ≥1.4 mmol/l), abnormal liver function, infection, inflammation, diabetes, myocardial infarction, elevated troponin-I or creatine kinase-MB levels and malignant or autoimmune disease. Patients taking antiplatelet, anti-inflammatory, or hypolipidaemic agents were also excluded. Blood samples (50 ml) were taken for routine biochemistry characteristics measurements and EPCs were isolated. Written informed consent was obtained from all patients and healthy subjects and the protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Jiangmen Central Hospital Ethics Committee on Research on Humans.

Isolation of circulating EPCs. Blood samples (35 ml) were diluted with sterile PBS. A total of 15 ml human lymphocyte separation solution (cat. no. HY2015; Tianjin Haoyang Biological Products Technology Co., Ltd.) was added into a 50-ml centrifuge tube (cat. no. 430829; Corning, Inc.). A total of 25 ml diluted peripheral blood was added along the tube wall before centrifugation at 811.7 x g at 4°C for 25 min. A capillary pipette was used to transfer the clouded mononuclear cell layer into another sterile 50-ml centrifuge tube. Then, 5 ml erythrocyte lysate (cat. no. C3702-120 ml; Beyotime Institute of Biotechnology) was added and left to stand for 10 min at 4°C. The mixture was diluted with pre-cooled PBS buffer and mixed before centrifugation at 377.3 x g at 4°C for 10 min. The upper layer was removed using a capillary pipette and discarded. Following the addition of 20 ml pre-cooled PBS buffer to the original centrifuge tube, the mixture was centrifuged again at 377.3 x g at 4°C for 10 min and a capillary pipette was used to remove the upper layer. Then, 10 ml pre-cooled PBS was added to the original centrifuge tube and mixed well. The mixture was transferred to another sterile 50-ml centrifuge tube before centrifugation at 4°C at 377.3 x g for 10 min, and the upper layer was discarded. Isolated circulating EPCs were then cultured in EGM-2 (cat. no. CC-3162; Beijing Bitab Biotech Co., Ltd.) containing 20% high-quality fetal bovine serum at 37°C for 7 days, 100 U/ml penicillin/streptomycin and 50 ng/ml VEGF 50 ng/ml.

Identification and evaluation of circulating EPCs. Circulating EPCs were identified via flow cytometry analysis (FCA) and immunofluorescence microscopy. CD45 (FITC)/CD34+ (PE-Cy7)/KDR+ (APC) (cat. nos. FH045-025, FH034-025 and FH039-025, respectively; all Beijing 4A Biotech Co., Ltd.) were defined as EPCs by FCA. Dil-acLDL+/FITC-lectin+ were defined as EPCs by immunofluorescence microscopy. FCA and immunofluorescence microscopy were performed as previously described (19-26).

Migration and proliferation ability of EPCs. EPC migration was evaluated as previously described (19-22). Proliferation activity was assessed via MTT assay (cat. no. ab211091; Abcam) according to the manufacturer’s instructions and our previous studies (19,20,22,27,28).

Evaluation of EPC adhesive activity. EPCs were digested with Parenzyme (cat. no. CCS001; Tetra-n-Bo Biotechnology Co., Ltd.) at 37°C for 1 min and resuspended in culture medium to a final density of 2x10⁶/ml. A total of 500 µl (2x10⁵/ml per well) cell suspension was inoculated in 24-well plates precoated with fibronectin (cat. no. 5050-1MG; Tetra-n-Bo Biotechnology Co., Ltd.) and incubated at 37°C for 30 min. After being washed twice with PBS, EPCs were fixed with 4% paraformaldehyde for 10 min at 37°C. After two further washes with PBS, adherent EPCs were observed under an inverted immunofluorescence microscope (magnification, x100). Adherent cells were counted in three randomly selected fields of view.

Measurement of plasma NO levels and NO secretion by EPCs. NO levels were measured using the NO levels test kit (cat. no. A012-1-2; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer’s instructions.

FMD and nitroglycerin-mediated dilatation (NMD). FMD was measured as previously described (29,30). For NMD, pressure in an upper-forearm sphygmomanometer cuff was raised to 250 mmHg for 5 min. FMD was calculated as the percentage increase in mean diastolic diameter after reactive hyperaemia at 55-65 sec after deflation to baseline. NMD was calculated as follows: at 15 min after FMD measurement, 400 µg sublingual nitroglycerin was administered and after a further 5 min, the diastolic diameter was remeasured to assess endothelial-independent dilatation.

Western blot analysis. Western blot analysis was performed as previously described (18). Briefly, protein concentrations
were determined using the BCA method. Proteins (50 µg) were separated via 5% SDS-PAGE. Following blocking with 5% skimmed milk powder was used at 4˚C for 12 h, the membranes were incubated at 4˚C for 12 h with the following primary antibodies: β-actin (1:2,000; cat. no. 3700T; Cell Signaling Technology Inc.), endothelial NO synthase (eNOS; 1:1,000; cat. no. 9575; Cell Signaling Technology, Inc.) and phosphorylated (p)-eNOS (1:1,000; cat. no. 9571; Cell Signaling Technology, Inc.). Subsequently, the membranes were incubated with a HRP-conjugated secondary antibody (1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.) at room temperature for 2 h. Protein expression was semi-quantified using ImageJ software (version 1.0; National Institutes of Health) with β-actin as the loading control.

Statistical analysis. Data are presented as the mean ± SD from three independent experiments. Data were analyzed using GraphPad Prism 6.0 software (GraphPad Software, Inc.). Comparisons between multiple groups were analyzed by one-way ANOVA and post hoc Tukey’s test. The persons who analyzed the data were blinded to group assignment. Univariate correlations were calculated using Pearson’s coefficient (r). P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics. Patient characteristics are presented in Table I. Age, BMI, levels of cholesterol, high- and low-density lipoprotein, triglycerides, plasma glucose, and high-sensitivity C-reactive protein were not significantly different between groups.

Levels and activity of circulating EPCs. EPCs were evaluated by fluorescence activated cell sorting analysis (Fig. S1A) and phase-contrast fluorescent microscope labeled by Dil-LDL and FITC-lectin (Fig. S1B).

Number of circulating EPCs is shown in Fig. 1A and B. The levels of circulating EPCs evaluated by FCA (Fig. S2) and immunofluorescence microscopy in patients with CAD were significantly lower compared with the control group. The number of circulating EPCs in middle- and high-risk groups were decreased compared with the low-risk group. The difference between middle- and high-risk groups was also significant. Similarly, the proliferative and adhesive activity of circulating EPCs (Fig. S4) in patients with CAD were significantly impaired compared with control group (both P<0.01; Fig. 1D and E). The migratory activity of circulating EPCs in middle- and high-risk groups was also significantly different between middle- and high-risk groups. Similar results were observed for the proliferative and adhesive function of circulating EPCs (Fig. 1D and E).

FMD and NMD. FMD in patients with CAD decreased compared with the control group (Fig. 2A). The FMD in middle- and high-risk group was lower than in the low-risk group. FMD was also significantly different between the middle- and high-risk groups. NMD (Fig. 2B) showed no significant difference between groups.

Plasma NO levels and secretion by EPCs. Plasma NO levels in patients with CAD were significantly lower than in the Control group (Fig. 2C).
Number and activity of circulating EPCs in groups with different numbers of coronary artery lesions. The number of circulating EPCs evaluated by FCA (Figs. 3A and S5) and immunofluorescence microscopy (Figs. 3B and S1B) in patients with CAD was significantly decreased compared with the Control group. The number of circulating EPCs in multiple (double or triple) vessel groups was decreased compared with the single vessel group. There was also a significant difference in the number of circulating EPCs between the double and triple vessel groups. Similar numbers of migratory, proliferative and adhesive (Fig. 3C-E) circulating EPCs were observed in groups with different number coronary artery lesions.

FMD and NMD in groups with different numbers of coronary artery lesions. FMD in patients with CAD decreased compared with that in the Control group (Fig. 4A). FMD in multiple (double or triple) vessels group was decreased compared with single vessel group. There was also a significant difference in FMD between the double and triple vessels. NMD showed no
significant difference between groups with different numbers of coronary artery lesions (Fig. 4B).

**NO plasma levels and secretion by EPCs in groups with different numbers of coronary artery lesions.** Plasma NO levels in patients with CAD were significantly lower than in the Control group (Fig. 4C). The plasma NO levels in multiple (double or triple) vessel groups were decreased compared with the single vessel group. There was also a significant difference in plasma NO levels between the double and triple vessel groups. Similar results were observed in NO secretion by EPCs in groups with different number of coronary artery lesions (Fig. 4D).

**Western blot results.** eNOS and p-eNOS protein expression levels of circulating EPCs were determined in groups with different Syntax scores and numbers of coronary lesions (Fig. 5A and B). eNOS expression levels were not significantly different, whereas p-eNOS protein expression levels were decreased in the CADs group compared with the Control group. p-eNOS protein expression levels in the middle- and high-risk groups were decreased compared with the low-risk group; there were also significant differences between the high- and middle-risk groups. eNOS expression were not significantly different between groups with different numbers of coronary artery lesions, whereas p-eNOS protein expression levels were decreased in the CAD groups compared with the Control group (Fig. 5B). p-eNOS protein expression levels in the multiple vessel groups were decreased compared with the single vessel group and there was a significant difference between the triple and double vessel groups.

**Correlation between circulating EPC levels and activity and Syntax score.** A negative univariate correlation between the number of circulating EPCs and Syntax score (Fig. 6A and B) was detected. Both were evaluated by FCA (Fig. 6A; R=0.51; P<0.01) and cell culture (Fig. 6B; R=0.55; P<0.01). The numbers of migratory (Fig. 6C; R=0.48), proliferative (Fig. 6D; R=0.54) and adhesive EPCs (Fig. 6E; R=0.58) was significantly inversely correlated with the Syntax score (all P<0.01).

**Correlation between FMD, NMD and Syntax score.** There was a significant inverse correlation between FMD and Syntax score (Fig. 7A; R=0.66; P<0.01). There was no significant correlation between NMD and Syntax score (Fig. 7B; R=0.01; P>0.05).

**Correlation between NO plasma levels or secretion by EPCs and Syntax score.** Both NO plasma levels and secretion by EPCs were negatively correlated with Syntax score (Fig. 7C and D; R=0.58 and R=0.63, respectively; both P<0.01).

**Correlation between circulating EPCs and the number of coronary lesions.** A strong negative univariate correlation was detected between circulating EPCs number and number of coronary lesions, as evaluated by FCA (Fig. 8A; R=0.48; P<0.01) and immunofluorescence microscopy (Fig. 8B; R=0.49; P<0.01). The migratory (Fig. 8C; R=0.75), proliferative (Fig. 8D; R=0.72) and adhesive activity (Fig. 8E; R=0.63) exhibited a significant inverse correlation with the number of coronary lesions (all P<0.01).
Correlation between FMD, NMD and the number of coronary lesions. There was a significant inverse correlation between FMD and the number of coronary lesions (Fig. 9A; R=0.54; P<0.01). By contrast, there was no significant correlation between NMD and the number of coronary lesions (Fig. 9B; R=0.04; P>0.05).

Correlation between NO plasma levels or secretion by EPCs and the number of coronary lesions. Both NO plasma levels and secretion by EPCs were negatively correlated with the number of coronary lesions (Fig. 9C and D; R=0.55 and R=0.71, respectively; both P<0.01).

Discussion

The present study showed that the number and function of circulating EPCs, as well as FMD, were impaired in patients with CAD with a higher Syntax score. Similar alterations were observed in NO levels in plasma or secreted by EPCs into culture medium. In addition, the number and activity of EPCs, FMD, and NO levels in plasma or secreted by EPCs were attenuated in multiple vessel groups compared with the single vessel group. There were significant inverse correlations between the number and activity of circulating EPCs and endothelial function and Syntax score. The NO plasma levels
Figure 4. Endothelial function of EPCs in groups with different numbers of coronary lesions. (A) FMD in patients with coronary artery disease was decreased compared with that in the Control group. FMD in multiple (double or triple) vessel groups was decreased compared with the single vessel group. The differences in FMD were significant between double and triple vessel groups. (B) NMD was not significantly different between groups. NO (C) plasma levels and (D) secretion by endothelial progenitor cells. Data are presented as the mean ± SD. *P<0.05 vs. Control. **P<0.05 vs. single vessel. ¶P<0.05 vs. double vessel. FMD, flow-mediated dilatation; NMD, nitroglycerin-mediated dilation; NO, nitric oxide.

Figure 5. eNOS protein expression levels of circulating EPCs from patients with CAD with different Syntax scores. (A) eNOS protein expression levels were not statistically different between different risk groups. p-eNOS protein expression of circulating EPCs in CAD groups was decreased compared with the Control group. p-eNOS protein expression levels in the middle- and high-risk groups were decreased compared with the low-risk group. The differences were significant between middle-group and high-risk group. eNOS protein expression levels were not statistically different between groups with different numbers of coronary lesions. (B) p-eNOS protein expression in multiple (double or triple) vessel groups were decreased compared with the single vessel group. A significant difference was observed between the double and triple vessel groups. #P<0.05 vs. Control. ¶P<0.05 vs. low-risk or single vessel. ¶P<0.05 vs. middle-risk or double vessel. eNOS, endothelial nitric oxide synthase; EPCs, endothelial progenitor cells; CAD, coronary artery disease; p-, phosphorylated.
Figure 6. Correlation between number and activity of circulating EPCs, endothelial function and Syntax score. (A and B) There was a negative correlation between the number of circulating EPCs and Syntax score. Correlation between numbers of (C) migratory, (D) proliferative and (E) adhesive EPCs and Syntax score. EPCs, endothelial progenitor cells.

Figure 7. Correlations between Syntax score and FMD, NMD, plasma NO or NO secretion by endothelial progenitor cells. (A) Negative correlation between FMD and Syntax score. (B) No correlation between NMD and Syntax score was observed. Correlation between NO (C) plasma levels and (D) secretion by endothelial progenitor cells and Syntax score. FMD, flow-mediated dilatation; NMD, nitroglycerin-mediated dilation; NO, nitric oxide.
and secretion by EPCs were also negatively correlated with Syntax score.

Syntax score is a risk stratification tool for patients with CAD based on the anatomy of the coronary artery, providing a quantifiable objective evaluation of complexity and severity for coronary artery lesions (31-34). The primary cause of CAD is atherosclerosis, which is characterized by defective endothelial function and plaque formation in the inner wall of the artery (35). The present study revealed that FMD decreased in patients with high Syntax score, indicating that endothelial function was impaired and was associated with greater severity and complexity of coronary artery lesions.

A previous study showed that the number and activity of circulating EPCs are decreased in patients with CAD (5), suggesting that attenuated endogenous endothelial repair capacity is involved in coronary artery abnormalities. Studies have reported that the number of circulating EPCs is inversely correlated with Syntax score (6,7), suggesting that the quantitative alteration in circulating EPCs may be a cytobiological parameter to evaluate the severity and complexity of CAD. However, the association between activity of EPCs, as well as endothelial function, and Syntax score is not clear.

Prior studies revealed a correlation between endothelial function and the number and activity of circulating EPCs (17,18). In light of the association between impaired endothelial function and high Syntax score, it was hypothesized that the activity of circulating EPCs is decreased in patients with high Syntax score. Here, the migratory, proliferative and adhesive function of circulating EPCs were shown to be attenuated in patients with a higher Syntax score, indicating that the quantitative and qualitative alteration in circulating EPCs is a surrogate parameter to evaluate the severity and complexity of CAD. These data provide evidence for the potential role of endothelial function and capacity for endogenous repair of endothelial injury in assessment of anatomical abnormalities of the coronary artery. The migration, proliferation and adhesion capability of EPCs, which are characteristic of morphological vascular changes (4,5), were measured and analyzed. FMD is an indicator of vascular functional change (36). Here, the characteristics of vascular morphological and functional changes were assessed to investigate the correlation between functional change and risk levels.

Endothelial function and circulating EPCs were also correlated with the number of lesioned vessels. Compared with single vessel lesions, multiple vessel lesions resulted in
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decreased numbers or activity of circulating EPCs, FMD and NO levels in plasma or secreted by EPCs. This is similar to the correlations with Syntax score, which is primarily based on the anatomy of the coronary artery and includes the number of vessel lesions and severity of lesions.

NO serves an important role in regulating endothelial function and the number and function of circulating EPCs. eNOS is the NOS isoform responsible for the production of NO and key regulators of mobilization and function of EPCs (17,18). In the present study, levels of eNOS in circulating EPCs were preserved in patients with CAD but levels of p-eNOS were decreased compared with the Control group. These results indicate that the decreased phosphorylation of eNOS, but not the alteration of eNOS expression levels, contributed to decreased NO secretion by circulating EPCs in patients with CAD. In addition, phosphorylation of eNOS was lower in patients with high a Syntax score compared with the low Syntax score group. Furthermore, patients with CAD with multiple vessel lesions also exhibited notably decreased eNOS phosphorylation. Therefore, it was inferred that decreased phosphorylation of eNOS may be the mechanism underlying decreased NO in circulating EPCs and FMD in patients with CAD.

In addition, the present study showed a negative correlation between Syntax score and the number and activity of circulating EPCs, as well as endothelial function, further indicating that impaired repair capacity of endothelial injury and subsequent decreased endothelial function is accompanied by serious and complicated coronary artery anatomical abnormalities. NO serves an important role in regulating endothelial function and the number and function of circulating EPCs (37-39). There was an inverse correlation between NO production and Syntax score, suggesting that decreased NO bioavailability may be the mechanism underlying decreased levels of circulating EPCs and endothelial dysfunction in patients with CAD with a high Syntax score.

The present study may have important implications. First, the present data revealed that decreased FMD was associated with high Syntax score, indicating endothelial function may be a clinical indicator of coronary artery anatomical abnormalities. Second, these results showed that decreased number or activity of circulating EPCs was associated with a higher Syntax score, demonstrating that circulating EPCs may serve as an important surrogate biomarker for evaluation of the severity of CAD. Finally, decreased NO biosynthesis may be an important mechanism underlying impaired endothelial repair capacity and dysfunction in patients with a high Syntax score. Therefore, strategies to increase NO production, such as exercise and statin therapy, may be a novel therapeutic approach for endothelial injury in patients with CAD.

To the best of our knowledge, the present study is the first to demonstrate that the number and activity of circulating EPCs, as well as FMD, are negatively correlated with Syntax score, indicating that circulating EPCs and endothelial function may be key surrogate biomarkers for the evaluation of severity and complexity of CAD. Enhancing the number or activity of circulating EPCs and improving endothelial function may be significant therapeutic approaches for serious and complicated CAD.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
BZ and DL performed the experiments, analyzed data and wrote the manuscript. GL and WT performed statistical analysis and discussion and investigated study subjects. GZ and JL designed the study and reviewed the manuscript. BZ and GZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by the Jiangmen Central Hospital Ethics Committee on Research on Humans (approval no. 20170419). Written informed consent was obtained from the participants. Ethics approval and consent to participate confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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