



Reduced Vitamin D Receptor on Circulating Endothelial Progenitor Cells:

A New Risk Factor of Coronary Artery Diseases

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Aim: Endothelial progenitor cells (EPCs) are shown to participate in the pathological processes of atherosclerosis. While Vitamin D and its receptor axis might exert some effects on EPCs' function. But their exact relationship with clinical patients is still elusive, which inspired us to explore the potential association of vitamin D receptor (VDR) expression on circulating EPCs and serum vitamin D levels among patients with coronary artery disease (CAD).

Methods: Two hundred patients with CAD after their admission to hospital and one hundred healthy controls were enrolled. Medical history data were retrieved and fresh blood samples were collected for flow cytometry analysis. VDR expressions on EPCs were evaluated according to the standardized protocol. Logistic regression analysis was used to investigate the potential risk factor of CAD.

Results: CAD patients were found to have lower $\log_{10}^{\text{VDR-MFIs}}$ than those of control group, especially for patients with diabetes ($p < 0.001$). $\log_{10}^{\text{VDR-MFIs}}$ were inversely correlated with glycated hemoglobin ($R = -0.472$, $p < 0.001$), and while EPCs challenged with high glucose had lower VDR expression. Multivariate logistic regression analysis revealed that lower $\log_{10}^{\text{VDR-MFIs}}$ were independently associated with the risk of CAD ($OR = 0.055$, $p = 0.008$).

Conclusion: A significant decrease of VDR expression on circulating EPCs was observed among CAD patients, particularly among those also with diabetes. VDR expression on EPCs was independently negatively correlated with HbA_{1c} and high glucose decreased EPCs' VDR expression. Low levels of VDR expression on circulating EPCs might serve as a potential risk factor of CAD.

Key words: Coronary artery disease, Endothelial progenitor cells, Vitamin D receptor, Diabetes

Abbreviations; CAD: coronary artery disease, VDR: vitamin D receptor, HbA_{1c}: glycated hemoglobin, SA: stable angina, UA: unstable angina, eNOS: endothelial nitric oxide synthase, MFI: mean fluorescence intensity, LDL-C: low-density lipoprotein cholesterol, HDL: High-density lipoprotein cholesterol, Hs-CRP: High-sensitive C-reactive protein, AGEs: advanced glycation end products, RAGE: receptor for advanced glycation end products, FGF23: fibroblast growth factor 23

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Introduction

Atherosclerosis is featured by imbalance of endothelium injury and repair, which often leads to some severe clinical complications such as myocardial infarct, stroke, and so on¹⁾. Data from recent studies

uncovered the key role of endothelial progenitor cells (EPCs) during such pathological process by mediating the activation of endogenous endothelial repair². After sensing the damage to endothelial layer of arteries and following tissue ischemia, EPCs are mobilized from the bone marrow or adjacent arteries, homing to the affected areas and mediating tissue recovery³. Abnormal levels and dysfunction of circulating EPCs were shown to be associated with severity of coronary artery disease (CAD)⁴⁻⁷. EPCs have been defined by several cell surface markers, such as CD34, CD133, and kinase insert domain receptor (KDR). According to the standardized International Society of Hematology and Graft Engineering (ISHAGE) protocol described before, combination of CD45^{dim}, CD34⁺, and KDR⁺ could be used to define human circulating EPC population⁸.

Recently several researches reported the increased proportion of EPCs with the osteoblastic marker osteocalcin (OCN) among patients with atherosclerosis⁹⁻¹¹, which implied that such specific markers on EPCs would be used as potential biomarkers of endothelium regeneration and prognosis of atherosclerosis. In addition to being involved with the maintenance of mineral homeostasis *in vivo*, Vitamin D receptor (VDR) was also suggested to modulate endothelial function in response to inflammation, thrombosis, and vasodilation^{12, 13}. Data from a cross-sectional study about dialysis patients showed that VDR expressions on circulating EPCs decreased among dialysis patients compared with those of control group and significantly increased after calcitriol or paricalcitol therapy^{14, 15}, which implied that change of VDR expression on circulating EPCs might be used to evaluate patient's therapy response.

Since patients with end-stage renal disease had been confirmed with high risk of atherosclerotic cardiovascular disease (ASCVD), but no data about the correlation between VDR expressed on circulating EPCs and ASCVD was available. Our study aimed to evaluate the expression of VDR on circulating EPCs and serum vitamin D among patients with coronary artery disease (CAD).

Methods

Ethics Statement

Protocol of this study was approved by the ethics committee of Shanghai Changzheng Hospital, which was conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consents were given to all study participants.

Enrolled Participants Selection

100 healthy volunteers and 200 patients with diagnosed CAD were enrolled in the study. Diagnosis of CAD was based on typical chest pain, ischemic proofs of electrocardiogram, and angiographically proven coronary artery stenosis (>70%)¹⁶. Unstable angina (UA) was defined as angina pain at rest lasting for at least 10 min within 24 h before enrollment or more severe and prolonged angina pectoris or angina precipitated by less exertion than in the past and electrocardiographic (ECG) changes compatible with the clinical diagnosis of UA (new ST-segment depression more than 0.1 mV and T wave inversion \geq 0.1 mV in at least two adjacent leads)^{17, 18}. Others were diagnosed as stable angina (SA). Control group included healthy volunteers from physical examination centers without previous history or current symptoms or signs of ischemic heart disease. All CAD patients underwent coronary angiography. Stenosis severity was evaluated based on Syntax scores by two experienced cardiologists blinded to the experimental data, and patients were divided into two groups (low Syntax score \leq 22, high Syntax score >22)¹⁹.

Data about enrollers' risk factor of CAD, such as age, gender, smoking habits, diabetes mellitus (DM), and hypertension were also collected. Hypertension was diagnosed based on repeated blood pressure measurements \geq 140/90 mmHg (at least two times in different environments) or the current medication of antihypertensive drugs. Diagnose of DM was based on a fasting serum glucose level \geq 6.99 mmol/L on multiple occasions and/or oral glucose tolerance test result \geq 11.01 mmol/L and/or random plasma glucose level \geq 11.01 mmol/L and/or glycated hemoglobin (HbA_{1c}) level \geq 47.54 mmol/mol, and/or the use of insulin or oral antidiabetic agents²⁰.

Exclusion criteria for all groups were: 1) age >80 years or age <50 years, 2) patients with myocardial infarction, 3) evidence of any systemic inflammatory or infectious diseases, organ failure, malignancies, immunologic or hematologic diseases, 4) current treatment with non-steroidal anti-inflammatory drugs other than aspirin, 5) evidence of chronic renal insufficiency (evaluated glomerular filtration rate less than 90 ml/min \times 1.73m²) and medical history of exogenous vitamin D supplements.

Laboratory Tests

Laboratory data were acquired from venous blood samples obtained after a 12 h overnight fast prior to coronary angiography. Serum levels of lipids, 25(OH) vitamin D, C-reactive protein (CRP), and HbA_{1c} were examined with the commercial kits. IFCC-HbA_{1c} was transformed according to the

Table 1. Primers for quantitative real-time PCR

Genes	Primer sequence	Product size (bp)	Annealing temperature (°C)
VDR	S: 5'-GGTACAGCGTAAGAGGGAGATGA-3' AS: 5'-TAGGTGGGGTTCATAGGTCTTGTG-3'	144	61
GAPDH	S: 5'-TATGACTCTACCCACGGCAAGT-3' AS: 5'-ATACTCAGCACCAGCATCACC-3'	138	60

IFCC-aligned standards for reporting HbA_{1c} using the following formula: HbA_{1c} (%) = HbA_{1c} (mmol/mol)/10.929 + 2.15²¹.

Flow Cytometry Quantification of VDR Expression on Circulating EPCs

EPCs were quantified using the following protocol: fresh blood collected into EDTA collection tubes were transported on wet ice and preceded within 2 to 3 h. FcR-blocking antibody was added and incubated for 10 min at room temperature. All staining procedures were performed on ice. 100 µl whole blood was incubated with 2 µl of CD45 (FITC; Beckton Dickinson), 1 µl of CD34 (PerCP; Abcam), 1 µl of KDR (PE; Beckton Dickinson), and 1 µl of VDR (Abcam) for 30 min. After a gentle wash, the sample was incubated with 1 µl of rat anti-mouse antibody (APC; Beckton Dickinson). Before flow cytometry analysis, the samples were lysed and washed to remove the erythrocytes. Circulating EPCs were sequential gating according to our previous study protocol⁷ derived from the ISHAGE strategy⁸ using FlowJo, and expression of VDR on circulating EPCs was shown as geometric mean fluorescence intensity (VDR-MFIs). For the level of VDR-MFIs was too high and too discrete to count, log₁₀^{VDR-MFIs} were used for statistics.

Preparation of Human EPCs

EPCs were prepared as described previously²². Briefly, blood obtained from healthy volunteers was diluted 1:2 in phosphate-buffered saline layered over Histopaque 1077 (Amersham Biosciences, Piscataway, NJ, USA) and centrifuged for 30 min at 400 g at room temperature. Peripheral blood mononuclear cells were incubated in M-199 (GIBCO, Los Angeles, CA, USA) medium supplemented with recombinant human vascular endothelial growth factor (30 ng/mL, from Pepro Tech, London) in six-well tissue culture plates (10⁷ per well) at 37°C². The medium was replaced every 3 days, and cells not adhering to the bottom were washed away. On day 7, the cells were changed to serum-free medium for another 24 h²³ and then treated with different culture media: one containing normal glucose (5.5 mmol/L) as negative control, one containing normal glucose and mannitol

(16.5 mmol/L) as osmotic pressure control, and the last one with high glucose (22 mmol/L).

VDR mRNA Expression

Total RNA was extracted from the sample by the TRIzol method strictly following the manufacturer's protocol (Takara, Dalian, China). Concentrations and purities of isolated RNA were measured at 260 nm using Nanodrop and with A260/280 ratio respectively. The first-strand cDNA used for quantitative real-time PCR was synthesized from 1 µg of RNA with Prime-Script RT Master Mix (Takara, Dalian, China). Expression levels of VDR gene were quantified with Bio-Rad real-time PCR system (Bio-rad, USA) and SYBR green PCR reaction mix (Bio-Rad, USA). Primers of VDR and GAPDH (synthesized by Takara Biological Technology Co. Ltd, Dalian, China) are listed in **Table 1**. The program was as follows: 95°C for 3 min, 45 cycles of 95°C for 15 s, and annealing temperature for 45 s. Melting curve analysis and agarose gel electrophoresis were performed to confirm the specificity of the PCR products obtained using each primer pair. Relative expression levels of genes were analyzed using the 2^{-ΔΔCt} method by normalizing with GAPDH housekeeping gene expression and presented as fold change relative to cells cultured in normal glucose.

Statistical Analysis

Continuous variables were tested for normal distribution with the Kolmogorov–Smirnov test. Comparisons between the two groups were analyzed by *t* test (two-sided) for normally distributed variables or ANOVA for those with more than two subgroups. Data were expressed as mean ± SD. Not normally distributed continuous variables were compared by the Mann–Whitney *U* test. Categorical variables were presented as frequency and percentage, which were compared using Pearson chi-square test. Multivariate linear regression analysis and nonparametric bivariate correlation (Spearman rank correlation coefficient) were used to correlate log₁₀^{VDR-MFIs} with cardiovascular risk factors. Statistical significance was assumed if a null hypothesis could be rejected at *p* ≤ 0.05. All statistical analysis was performed with SPSS 17.

Table 2. Clinical characteristics of study population based on with or without DM

	Control (n=100)	CAD only (n=120)	CAD with DM (n=80)	P-value
Male (%)	62 (62)	88 (73.33)	58 (72.50)	0.149
Age, years	58.31 ± 8.52	60.39 ± 9.57	59.13 ± 10.81	0.270
Hypertension, n (%)	39 (39)	56 (46.67)	44 (55)	0.101
History of smoking, n (%)	40 (40)	54 (45.45)	45 (56.25)	0.088
Current Medication				
Aspirin, n (%)	4 (4)	116 (96.67)	78 (97.5)	<0.001
Clopidogrel, n (%)	0 (0)	109 (90.83)	73 (91.25)	<0.001
β-blocker, n (%)	14 (14)	92 (76.67)	63 (78.75)	<0.001
ACEI/ARBs, n (%)	21 (21)	57 (47.5)	58 (72.5)	<0.001
Statin, n (%)	0 (0)	110 (91.67)	76 (95)	<0.001
Insulin/Oral antidiabetic drugs, n (%)	2 (2)	0 (0)	75 (93.75)	<0.001
Laboratory testing				
Fasting glucose, mmol/L	5.34 ± 0.57	5.27 ± 0.69	9.05 ± 1.79	<0.001*, #
Total cholesterol, mmol/L	4.43 ± 1.02	4.28 ± 1.15	4.39 ± 1.24	0.597
LDL-C, mmol/L	2.41 ± 0.77	2.30 ± 1.06	2.55 ± 0.92	0.181
HDL-C, mmol/L	1.07 ± 0.31	1.06 ± 0.38	0.96 ± 0.45	0.107
Triglycerides, mmol/L	1.92 ± 1.43	1.95 ± 1.27	2.08 ± 1.02	0.916
25(OH) vitamin D, ng/ml	33.05 ± 7.28	32.70 ± 6.72	30.79 ± 7.13	0.074
hs-CRP, mg/L	6.34 ± 7.25	5.62 ± 4.98	6.50 ± 7.51	0.581
HbA _{1c} , mmol/mol	38.72 ± 4.29	39.35 ± 4.50	61.63 ± 9.91	<0.001*, #
Syntax Score	/	12.26 ± 9.43	16.57 ± 8.82	0.001

Continuous variables were presented as mean ± S.D. for normal distribution. One-way ANOVA was used to compare the difference among groups and post-hoc (Tukey) test was used for multiple comparisons.

* $P < 0.001$, CAD with diabetes vs control, # $P < 0.001$, CAD with diabetes vs CAD only

Categorical variables were presented as frequency and percentage, which were compared using Chi-square test.

Results

Characteristics of enrolled 300 participants are summarized in **Tables 1** and **2**.

Notably, patients with CAD and DM had higher levels of HbA_{1c} and fasting glucose than those of the other two groups ($p < 0.001$). No statistical differences of ages, serum lipids, and CRP were seen among the three groups. Comparing with those with CAD only, no significant difference of Syntax Score with patients with CAD and DM was revealed. As summarized in **Table 2**, patients with UA had higher levels of HbA_{1c} than those of control ($p < 0.001$), but no statistical difference was observed between the groups of UA and SA. Patients with UA had higher levels of fasting glucose than those of the other two groups ($p < 0.001$).

As **Fig. 1** showed, the numbers of EPCs were significantly higher in healthy controls compared with those of patients with CAD, no matter with or without DM ($p = 0.001$). Comparison of $\log_{10}^{\text{VDR-MFIs}}$ on circulating EPCs among the three groups, lower $\log_{10}^{\text{VDR-MFIs}}$ was found within the patients with CAD only ($p = 0.001$) and those with CAD and DM ($p <$

0.001), and the latter had even lower $\log_{10}^{\text{VDR-MFIs}}$ ($p = 0.005$). In addition to $\log_{10}^{\text{VDR-MFIs}}$, no significant differences of serum 25(OH) vitamin D levels were seen among the three groups ($p = 0.074$, **Fig. 1C**). $\log_{10}^{\text{VDR-MFIs}}$ among the control group were significantly higher than those among UA groups ($p < 0.001$), but not higher than those within SA group ($p = 0.065$), and no difference was shown between the two CAD subgroups ($p = 0.127$, **Fig. 2A**). While no significant differences of serum 25(OH) vitamin D levels were shown among the three groups ($p = 0.063$, **Fig. 2C**).

Patients with CAD were divided into two groups based on Syntax Score as shown in **Table 3**. More patients with high Syntax Score had DM ($p < 0.001$) and hypertension ($p < 0.001$) than those with low Syntax Score, and those with high Syntax Score had higher levels of fasting glucose ($p < 0.001$), LDL ($p < 0.001$), and HbA_{1c} ($p < 0.001$). But no differences of $\log_{10}^{\text{VDR-MFIs}}$ ($p = 0.179$), EPCs numbers ($p = 0.230$), and serum 25(OH) vitamin D levels ($p = 0.068$) were observed between these two groups.

Table 4 shows the potential correlations between $\log_{10}^{\text{VDR-MFIs}}$ on circulation EPCs and other clinical

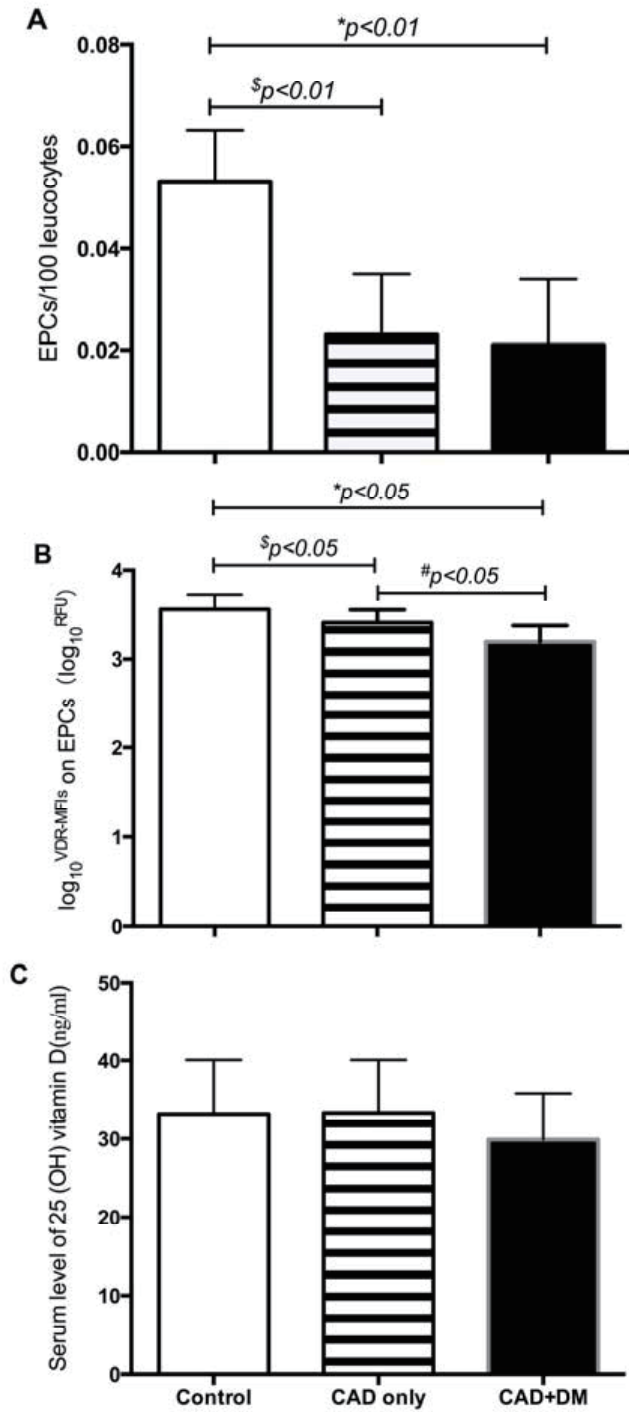


Fig. 1. Differences of VDR-MFIs on circulating EPCs and serum 25(OH) vitamin D levels among three groups

A. EPC levels were significantly higher among healthy controls compared with those of patients with CAD, no matter with or without DM, but no difference was observed between the two CAD groups.

B. VDR-MFIs on circulating EPCs were significantly lower in CAD patients compared with those in control groups, no matter with DM or not, and patients with DM had lower Log₁₀^{VDR-MFIs} than patients with CAD only.

C. No difference was observed on serum 25(OH) vitamin D levels among these groups.

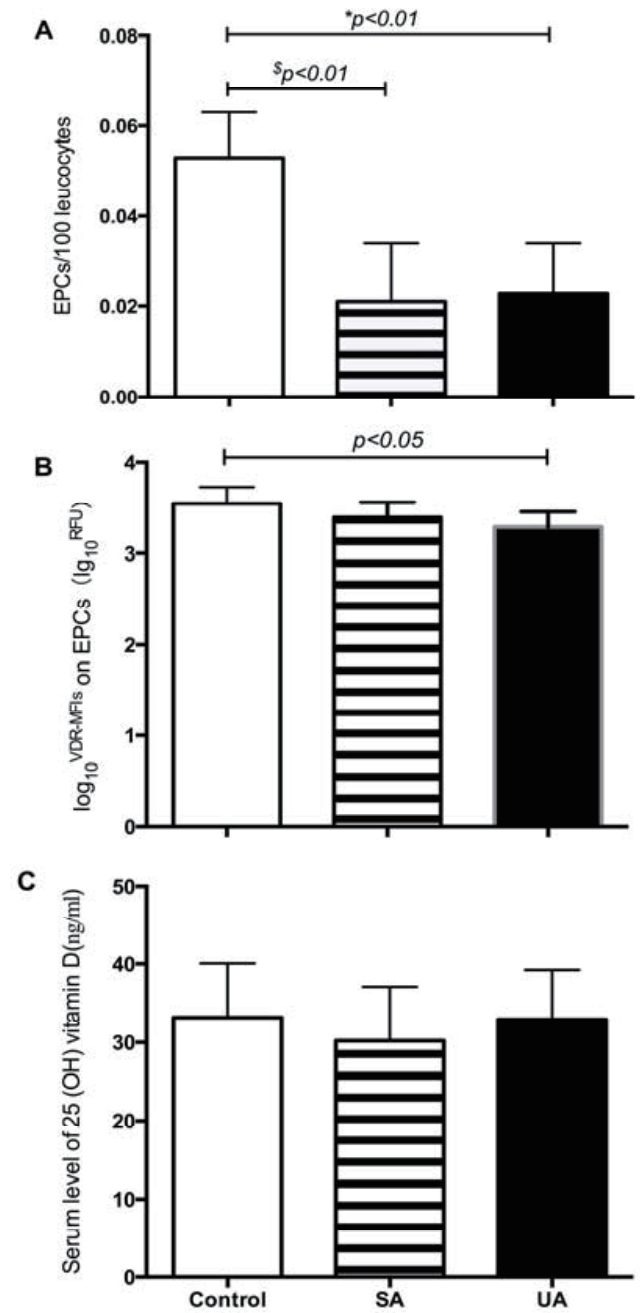


Fig. 2. Differences of log₁₀^{VDR-MFIs} on circulating EPCs and serum 25(OH) vitamin D levels among three groups

A. EPC levels were significantly higher among healthy controls compared with those of patients with CAD, no matter stable or not, but no difference was observed between the two CAD groups.

B. Log₁₀^{VDR-MFIs} on circulating EPCs were significantly lower in CAD patients with UA compared with those in control groups, but no difference was observed between SA and control groups, and also no difference between the two CAD groups.

C. No difference was observed on serum 25(OH) vitamin D levels among these groups.

Table 3. Clinical characteristics of study population based on severity of angina

	Control (n=100)	SA (n=73)	UA (n=127)	P-value
Male (%)	62 (62)	51 (69.86)	95 (74.80)	0.115
Age, yeas	58.31 ± 8.52	60.58 ± 9.32	61.03 ± 10.42	0.089
Hypertension, n (%)	39 (39)	33 (45.21)	67 (52.76)	0.116
DM, n (%)	2 (2)	17 (23.29)	63 (49.61)	<0.001
History of smoking, n (%)	40 (40)	34 (46.58)	65 (51.18)	0.187
Current Medication				
Aspirin, n (%)	4 (4)	70 (95.89)	124 (97.64)	<0.001
Clopidogrel, n (%)	0 (0)	65 (89.04)	117 (92.13)	<0.001
β-blocker, n (%)	14 (14)	63 (86.30)	92 (72.44)	<0.001
ACEI/ARBs, n (%)	21 (21)	36 (49.36)	79 (62.20)	<0.001
Statin, n (%)	0 (0)	64 (87.67)	122 (96.06)	<0.001
Insulin/Oral antidiabetic drugs, n (%)	2 (2)	15 (20.55)	60 (47.24)	<0.001
Laboratory testing				
Fasting glucose, mmol/L	5.34 ± 0.57	7.13 ± 1.57	8.08 ± 0.91	<0.001*, #, &
Total cholesterol, mmol/L	4.43 ± 1.02	4.46 ± 0.95	4.58 ± 1.23	0.556
LDL-C, mmol/L	2.41 ± 0.77	2.43 ± 0.78	2.50 ± 0.97	0.713
HDL-C, mmol/L	1.07 ± 0.31	1.05 ± 0.40	0.96 ± 0.42	0.072
Triglycerides, mmol/L	1.92 ± 1.43	2.07 ± 1.35	1.96 ± 1.14	0.744
25(OH) vitamin D, ng/ml	33.05 ± 7.28	30.68 ± 7.25	32.67 ± 6.37	0.063
hs-CRP, mg/L	6.34 ± 7.25	4.98 ± 4.90	5.43 ± 9.77	0.509
HbA _{1c} , mmol/mol	38.72 ± 4.29	44.15 ± 12.51	50.05 ± 13.82	<0.001*, #, &
Syntax Score	/	12.71 ± 0.25	15.37 ± 8.77	0.127

Continuous variables were presented as mean ± S.D. for normal distribution. One-way ANOVA was used to compare the difference among groups and post-hoc (Tukey) test was used for multiple comparisons.

*P<0.001, UA vs control; #P<0.001, SA vs control; &P<0.01, UA vs SA

Categorical variables were presented as frequency and percentage, which were compared using Chi-square test.

Table 4. Clinical characteristics based on Syntax Score grouping

	Low Syntax Score (≤ 22, n=134)	High Syntax Score (> 22, n=66)	P-value
Male (%)	96 (71.64)	50 (75.76)	0.538
Age, yeas	60.54 ± 9.13	61.02 ± 9.74	0.528
Hypertension, n (%)	54 (40.30)	46 (69.70)	<0.001
DM, n (%)	32 (23.88)	48 (72.73)	<0.001
History of smoking, n (%)	70 (57.46)	29 (43.94)	0.270
Current Medication			
Aspirin, n (%)	130 (97.01)	64 (96.97)	0.986
Clopidogrel, n (%)	120 (89.55)	62 (93.94)	0.308
β-blocker, n (%)	109 (81.34)	46 (69.70)	0.064
ACEI/ARBs, n (%)	73 (54.48)	42 (63.64)	0.218
Statin, n (%)	123 (91.79)	63 (95.45)	0.340
Insulin/Oral antidiabetic drugs, n (%)	30 (22.39)	45 (68.18)	<0.001
Laboratory testing			
Fasting glucose, mmol/L	5.62 ± 1.20	6.99 ± 1.35	<0.001
Total cholesterol, mmol/L	4.24 ± 1.20	4.46 ± 1.31	0.238
LDL-C, mmol/L	2.38 ± 0.86	2.89 ± 0.98	<0.001
HDL-C, mmol/L	1.02 ± 0.45	0.95 ± 0.29	0.251
Triglycerides, mmol/L	1.99 ± 1.17	1.82 ± 1.15	0.332
25(OH) vitamin D, ng/ml	32.05 ± 7.35	30.21 ± 5.02	0.068
hs-CRP, mg/L	5.81 ± 11.52	3.45 ± 2.63	0.102
HbA _{1c} , mmol/mol	44.86 ± 12.35	55.25 ± 10.93	<0.001
Log ₁₀ ^{VDR-MFIs} , (lgRFU)	3.25 ± 0.25	3.20 ± 0.24	0.179

Continuous variables were presented as mean ± S.D. for normal distribution. Student *t* test was used to compare the difference between groups.

Categorical variables were presented as frequency and percentage, which were compared using Chi-square test.

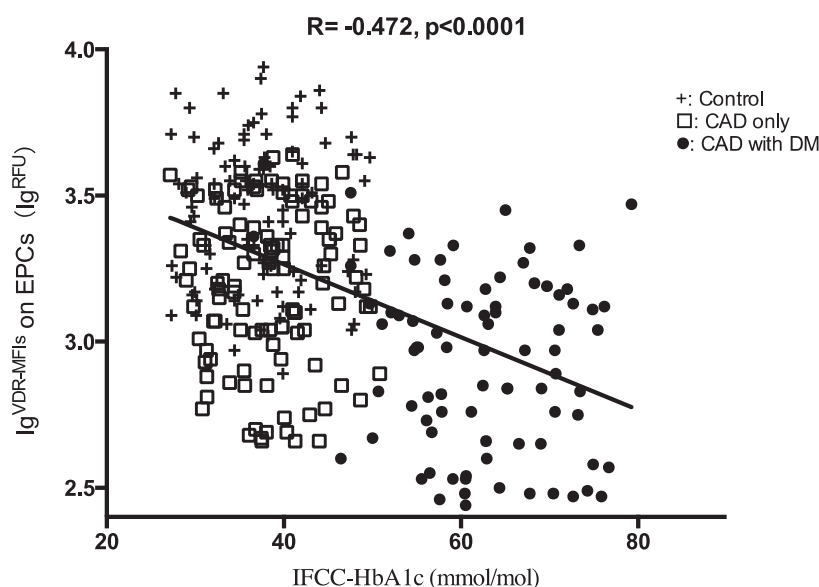


Fig. 3. Correlation between $\log_{10}^{\text{VDR-MFIs}}$ on circulating EPCs and IFCC-HbA_{1c} among all participants

$\log_{10}^{\text{VDR-MFIs}}$ were shown to be negatively correlated with HbA_{1c} ($R = -0.472$, $p < 0.001$). IFCC-HbA_{1c} was transformed according to the IFCC-aligned standards for reporting HbA_{1c} using the following formula: HbA_{1c} (%) = HbA_{1c} (mmol/mol) / 10.929 + 2.15.

variables. A negative correlation between $\log_{10}^{\text{VDR-MFIs}}$ and HbA_{1c} was uncovered ($R = -0.472$, $p < 0.001$, **Fig. 3**). Except for HbA_{1c}, no correlations between VDR-MFIs and other factors such as age, lipids, CRP, serum 25(OH) vitamin D levels, and Syntax Score were observed.

Univariate and Multivariate Logistic Regression Analysis

Among those included parameters, such as dyslipidemia, DM, age, genders, and *et al.*, univariate logistic regression revealed that the following four variables were significantly associated with severity of CAD: DM [OR = 13.56, 95% CI (2.61–70.45), $p = 0.002$], HbA_{1c} [OR = 4.37, 95% CI (1.76–10.85), $p = 0.003$], EPCs number [OR = 0.039, 95% CI (0.012–0.13), $p = 0.001$], and $\log_{10}^{\text{VDR-MFIs}}$ [OR = 0.025, 95% CI (0.003–0.21), $p = 0.001$]. Other variables including age, sex, serum lipid, and 25(OH) vitamin D levels, were not statistically significant in our analysis (**Table 3**). Based on the above, lower $\log_{10}^{\text{VDR-MFIs}}$ of circulating EPCs were significantly associated with the severity of CAD after adjusting for those covariates [OR = 0.055, 95% CI (0.006–0.508), $p = 0.008$] using multivariate logistic regression analysis. HbA_{1c} and DM were not found to be significantly associated in the multivariate logistic regression analysis (**Table 5**). More importantly, as shown in **Table 6**,

Table 5. Correlations between $\log_{10}^{\text{VDR-MFIs}}$ on circulating EPCs and clinical features of all subjects involved

Variable	R-value	P-value
Age	0.091	0.402
Fasting glucose	0.207	0.099
Total cholesterol	0.168	0.215
LDL-C	0.173	0.198
HDL-C	0.158	0.251
Triglycerides	-0.122	0.302
25(OH) vitamin D	0.095	0.430
Hs-CRP	0.053	0.701
Syntax Score	0.103	0.274
HbA _{1c}	-0.472	< 0.001

the results of the multivariate logistic regression analysis of variables predicting a high Syntax Score suggested that only the hypertension was independent predictor of the severity of CAD (OR = 11.82, 95% CI (3.07–45.51), $p = 0.005$), after adjusting for DM, dyslipidemia, fasting glucose, and HbA_{1c}.

In order to explore the effects of high glucose on EPCs VDR expression, we analyzed the change of VDR mRNA expression by human EPCs with different culture medium. Compared with normal glucose, expressions of VDR mRNA were reduced by approximately 61% at 24 h and 82% at 48 h (**Fig. 4**) after

Table 6. Univariate and multivariate logistic regression analysis to determine independent risk factors of CAD

Variables	Univariate		P-value	Multivariate		P-value
	OR	95%CI		OR	95%CI	
Age	1.04	0.97-1.12	0.097	/	/	/
Sex	1.99	0.77-5.14	0.115	/	/	/
Smoking history	1.72	0.87-3.40	0.269	/	/	/
Hypertension	2.27	0.91-5.66	0.082	/	/	/
Fasting glucose	1.13	0.75-1.70	0.195	/	/	/
Total cholesterol	0.65	0.39-1.08	0.113	/	/	/
LDL-C	0.67	0.41-1.09	0.153	/	/	/
HDL-C	0.83	0.37-1.86	0.528	/	/	/
Triglycerides	1.05	0.86-1.28	0.317	/	/	/
25(OH) vitamin D	0.94	0.81-1.09	0.206	/	/	/
Hs-CRP	0.99	0.96-1.02	0.751	/	/	/
DM	13.56	2.61-70.45	0.002	0.61	0.06-7.44	0.586
HbA _{1c}	4.37	1.76-10.85	0.003	1.07	0.92-1.24	0.335
EPCs number	0.039	0.012-0.13	0.001	0.45	0.097-2.09	0.229
Log ₁₀ ^{VDR-MFIs}	0.025	0.003-0.21	0.001	0.055	0.006-0.508	0.008

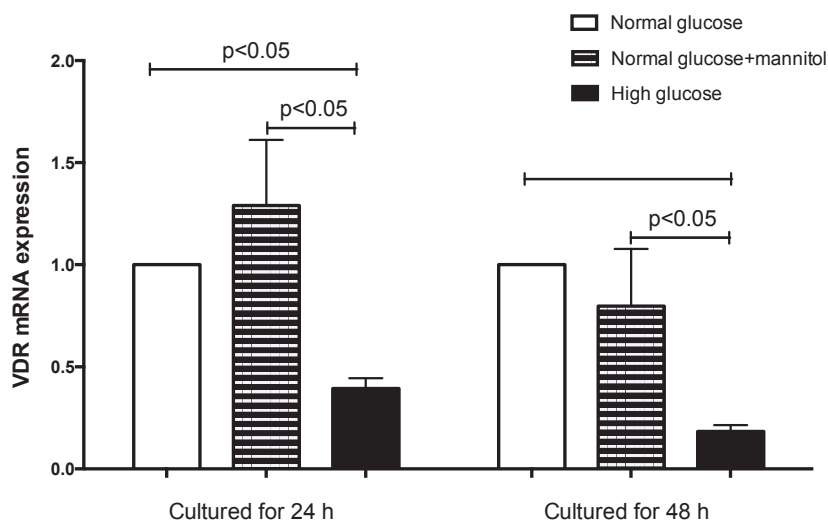


Fig. 4. Changes of VDR mRNA expression in EPCs cultured in different medium

Compared with normal glucose cultured EPCs, expression of VDR mRNA reduced by approximately 61% at 24 h and 82% at 48 h (Fig. 4) in high glucose ($p < 0.05$). No reduced levels of VDR mRNA in mannitol cultured EPCs were observed compared with those in normal glucose group.

changing to high glucose medium ($p < 0.05$), whereas no reduced levels were found with medium containing mannitol, which implied such effects were not caused by osmotic pressure change of high glucose.

Discussion

To our knowledge, this might be the first study to evaluate the expression of VDR in circulating EPCs

among CAD patients. Our observations indicated that: 1) decreased VDR expression on EPCs might be associated with CAD, and 2) persistent high serum glucose state might lower VDR expression on EPCs, which would accelerate the pathological process of atherosclerosis.

Previous studies demonstrated that among patients with diabetes circulating EPCs were shown to be lower and abnormal compared with those in non-

Table 7. Univariate and multivariate logistic regression analysis to identify independent predictors of a high Syntax Score (>22)

Variables	Univariate		P-value	Multivariate		P-value
	OR	95%CI		OR	95%CI	
Age	1.03	0.99-1.07	0.125	/	/	/
Sex	1.48	0.81-2.63	0.263	/	/	/
Smoking history	2.09	0.88-4.96	0.104	/	/	/
Hypertension	5.53	2.36-12.96	0.013	11.82	3.07-45.51	0.005
Fasting glucose	2.31	1.38-3.87	0.002	1.70	0.53-5.45	0.426
Total cholesterol	1.73	0.92-3.25	0.092	/	/	/
LDL-C	3.11	2.07-4.67	0.006	2.81	0.95-8.31	0.06
HDL-C	0.35	0.09-1.36	0.249	/	/	/
Triglycerides	0.88	0.54-1.43	0.481	/	/	/
25(OH) vitamin D	0.92	0.84-1.01	0.091	/	/	/
Hs-CRP	0.93	0.79-1.09	0.314	/	/	/
DM	8.47	4.32-16.61	0.001	3.55	0.05-252.05	0.539
HbA _{1c}	1.37	1.07-1.75	0.008	0.93	0.82-1.05	0.358
EPCs number	0.28	0.053-1.48	0.130	/	/	/
Log ₁₀ ^{VDR-MFIs}	0.12	0.01-1.44	0.118	/	/	/

diabetics, which could be improved by tight glycemic control²⁴. While patients with HbA_{1c} in the prediabetic range were shown to have a significant increase of EPCs co-expressing osteoblastic marker osteocalcin²⁵. However, behind mechanisms is still unknown. In the condition of diabetes, the formation and accumulation of advanced glycation end products (AGEs) is accelerated, which is considered to accelerate the development of vascular atherosclerosis²⁶⁻²⁸. AGEs would impair the migration, adhesion, and secretion potential of EPCs, and promote EPCs apoptosis²⁹. *In-vitro* studies demonstrated that AGEs could induce endothelial cells dysfunction by decreasing expression of endothelial nitric oxide synthase (eNOS)^{22, 30}. High glucose was shown to promote EPCs dysfunction and apoptosis through inhibition of eNOS-AMP-activated protein kinase pathway³¹. All the above might contribute to endothelial dysfunction and abnormal wound healing in patients with diabetes, which would eventually lead to atherosclerosis progression.

Vitamin D and VDR had been thoroughly investigated for their potentially beneficial cardiovascular effects. Data from our studies implied that the expression of VDR on EPCs was independently associated with CAD. Similarly, among hemodialysis patients, VDR-MFIs of EPCs in patients was lower than those of control, and those receiving calcitriol therapy had higher VDR-MFIs of EPCs^{14, 15}, and while an inverse relationship between vitamin D levels and circulating EPCs levels were uncovered among patients with diabetes³². Furthermore, intravenous transfusion of EPCs overexpressing VDR could inhibit atherosclerosis in apoE^{-/-} mice by elevating eNOS expression

within arteries and serum concentration of NO³³. Vitamin D could improve the angiogenic properties of EPCs³⁴, and an *in-vitro* study showed that vitamin D might modulate NO metabolism and blunt the pathological effects of AGEs against EPCs³⁰. Above all, Vitamin D and VDR might play a protective role in atherosclerotic disease by enhancing the function of EPCs and attenuating the impairment of AGEs.

While as our research demonstrated, serum levels of 25(OH) vitamin D among CAD patients were not significantly lower than those of control. A research involving 1568 community-dwelling elderly participants and following-up 4.4 years also suggested that 25(OH) vitamin D could not be used to predict the onset of peripheral artery disease³⁵. In fact, the 1,25(OH) vitamin D, but not the 25(OH) vitamin D, is the active form of vitamin D transforming from 25(OH) vitamin D in kidney by 1 α -hydroxylase³⁶. Compared with 25(OH) vitamin D, detection serum 1,25(OH) vitamin D is a high demanding task for its low level and instability, so the serum level of 25(OH) vitamin D is often considered as the indicator of vitamin D status³⁷. No such data are available about the association between serum level of 1,25(OH) vitamin D and atherosclerosis in human until now. Recent study showed that swine administered with vitamin D deficient diet were found with accelerated CAD progression, which implied that lower serum level of 1,25(OH) vitamin D, but not 25(OH) vitamin D would be the risk factor of atherosclerosis³⁸.

Data from our research showed that log₁₀^{VDR-MFIs} were inversely associated with HbA_{1c}, and decreased expression of VDR of EPCs challenged with high glu-

cose. Similar data has shown that a significant decrease of VDR expression in pancreas, skeletal muscle, and adipose tissue of experimentally-induced type 2 diabetes rats compared with those of the control³⁹). A research using osteoblast-like MG-63 cells demonstrated that high glucose would inhibit VDR expression of in such cells and subsequently impair the ability to synthesize osteocalcin, which might explain the potential mechanism of diabetic osteopenia⁴⁰). Therefore, the diabetic-like environment may impair the EPCs by decreasing VDR expression, which might be partly reversed by vitamin D.

This study had several potential limitations. Firstly, the markers we used for gating EPCs only included KDR, CD34, and CD45, which might contribute to selection bias. Secondly, only the expression of VDR on EPCs was reported without such cells' function. Association of VDR on EPCs, function of EPCs, and the prognosis of CAD patients would need to be studied in the future.

In conclusion, we demonstrated a significant decrease of EPCs VDR expression among CAD patients, particularly among those with elevated HbA_{1c}. VDR expression on EPCs would be independently correlated with HbA_{1c}, and high glucose might lead to lower VDR expression in EPCs. EPCs with low VDR expression could play a role in the pathogenesis of CAD and might serve as a potential biomarker for evaluating the severity of CAD.

Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

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