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RESEARCH ARTICLE

Plasma soluble C-type lectin-like receptor-2 is associated with the risk of coronary artery disease

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Abstract **Accumulating evidence suggests that C-type lectin-like receptor-2 (CLEC-2) plays an important role in atherothrombosis. In this case-control study, we investigated the association between CLEC-2 and incidence of coronary artery disease (CAD). A total of 216 patients, including 14 cases of stable angina pectoris (SAP, non-ACS) and 202 cases of acute coronary syndrome (ACS), and 89 non-CAD control subjects were enrolled. Plasma levels of soluble CLEC-2 (sCLEC-2) were measured using the enzyme-linked immunosorbent assay (ELISA). Compared with the control group (65.69 (55.36–143.22) pg/mL), the plasma levels of sCLEC-2 were significantly increased in patients with CAD (133.67 (88.76–220.09) pg/mL) and ACS (134.16 (88.88–225.81) pg/mL). The multivariate adjusted odds ratios (95% confidence interval) of CAD reached 2.01 (1.52–2.66) ($P_{trend} < 0.001$) for each 1-quartile increase in sCLEC-2. Restricted cubic splines showed a positive dose-response association between sCLEC2 and CAD incidence ($P_{linearity} < 0.001$). The addition of sCLEC-2 to conventional risk factors improved the C statistic (0.821 vs. 0.761, $P = 0.004$) and reclassification ability (net reclassification improvement: 57.45%, $P < 0.001$; integrated discrimination improvement: 8.27%, $P < 0.001$) for CAD. In conclusion, high plasma sCLEC-2 is independently associated with CAD risk, and the prognostic value of sCLEC-2 may be evaluated in future prospective studies.**

Keywords soluble C-type lectin-like receptor-2; coronary artery disease; risk factor

Introduction

Coronary artery disease (CAD), the most prevalent cardiovascular disease worldwide, causes more deaths than from malignancies annually [1]. Moreover, epidemiological studies have revealed the increasing incidence of CAD in most countries [2,3]. Mechanistically, the causes of CAD have been attributed to endothelial inflammation, lipid deposition, leukocyte infiltration, and platelet activation [4]. Platelet activation is a crucial switch of thrombotic events that follow atherosclerotic plaque rupture. Additionally, cytokines from activated platelets may promote leukocyte activation and transmigration into the subendothelium, which then promotes atherosclerosis progression. The important role of platelets in atherosclerosis has rated cytokines as essential contributors to CAD [5]. For decades, signaling molecules, which are responsible for platelet activation under proatherogenic conditions, have been investigated and proposed to be potential diagnostic markers and therapeutic targets for CAD [6–8].

Colonna *et al.* identified C-type lectin-like receptor-2 (CLEC-2) as a cell membrane receptor 17 years ago [9]. Emerging studies have revealed the structure, function, and disease relevance of CLEC-2 since its discovery [10]. Two major ligands of CLEC-2, including the snake venom called rhodocytin and an endogenous sialic acid-like glycoprotein podoplanin, are available. Their ligation

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with CLEC-2 activates downstream signals in platelets through Src/Syk/SLP-76 pathway, which interacts with the glycoprotein VI pathway [11,12]. CLEC-2 is an important receptor for platelet activation and thrombosis. Mice with platelet conditional deletion of CLEC-2 are protected from arterial thrombus formation [13]. Moreover, CLEC-2 is an activation receptor in leukocytes that mediates inflammatory and immune responses [14]. The proteomic analysis of rhodocytin-activated platelets indicates the cardiovascular disease- and inflammation-related pathways underlying CLEC-2 signaling [15]. Furthermore, patients with rheumatoid arthritis, which is a chronic inflammatory disease, show increased plasma level of CLEC-2 from platelet-borne microparticles [16]. CAD progression involves leukocyte activation and accumulation, which may increase plaque vulnerability and contribute to thrombotic events [17]. CLEC-2 may play an important role in CAD by regulating leukocyte and platelet activation.

The increasing burden of CAD calls for the early identification of high-risk patients using simple and cost-effective methods. The combination of electrocardiogram (ECG) and cardiac enzymes can be used to screen most probands. However, few patients without substantial myocardial damage may be underestimated [18]. A recent large-scale clinical trial showed that coronary artery contrast-enhanced CT is more effective than traditional non-invasive methods in ruling-in and ruling-out CAD patients. However, its application may be blunted by high economical costs and technical requirements [19]. Coronary angiography (CAG), as the golden diagnostic criteria for CAD, may not be a preferred initial screening tool in outpatient or emergency conditions [20]. In this regard, novel biomarkers that reflect early ischemic events, especially platelet activation, are warranted to identify high-risk CAD patients [21]. One candidate molecule set may exhibit platelet-borne factors that can reflect platelet reactivity during early acute coronary events [22]. This study aims to examine the association of plasma soluble CLEC-2 (sCLEC-2) with CAD.

Materials and methods

Inclusion criteria

A total of 216 CAD patients hospitalized in the First and Second Affiliated Hospitals of Soochow University and the First Affiliated Hospital of China Medical University from December 2014 to May 2016 were consecutively enrolled in this study. CAD diagnosis was confirmed by characteristic syndromes and signs, ECG findings, cardiac enzyme, troponin, echocardiography, and CAG results in accordance with the latest AHA/ACC guideline for CAD diagnosis [23–25]. Among all the recruited patients, 158 were male and 58 were female, with an average age of 61.3 ± 11.0 years. CAD patients were divided into acute coronary syndrome (ACS) group (202 cases) and non-ACS group (14 cases). A total of 89 age-matched subjects without a previous history of atherosclerotic disease undergoing routine health examinations, including 52 males and 37 females, were enrolled as the control group (non-CAD). Elevated cardiac enzymes or abnormal ECG results obtained from subjects with CAD symptoms were excluded from the control group. The study protocols, which conformed to the *Declaration of Helsinki*, were approved by the ethics committee from the First and the Second Affiliated Hospitals of Soochow University and by the ethics committee from the First Hospital of China Medical University [26]. Informed consent was obtained from each participant.

Exclusion criteria

CAD patients who underwent intravenous thrombolytic therapy or complicated with severe congestive heart failure, angina due to coronary myocardial bridge, coronary vasospasm, idiopathic cardiomyopathy, acute infection, and acute exacerbation of chronic diseases, malignancy, severe hepatic or renal dysfunction, autoimmune disease, and connective tissue disease were excluded.

Clinical information

Upon admission, basal information, including age, gender, and post-medical history, were recorded for each patient. Physical exam and ECG were performed within 10 min after admission. Venous blood was drawn through venipuncture of veins in the forearm. Blood biochemistry tests, including hepatic and renal functions, serum electrolytes, cardiac enzymes, and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) were issued. For ACS patients, global registry of acute coronary events (GRACE) scores, which are based on age, gender, systolic blood pressure, creatinine, Killip class, cardiac arrest, increased cardiac index, and changed ST segment in ECG, were calculated for risk stratification (< 85, low risk; 85–133, medium risk; > 133, high risk) [27]. Modification of Diet in Renal Disease (MDRD) Study Equation was used to calculate estimated glomerular filtration rate (eGFR).

Assessment of sCLEC-2 plasma levels

Five milliliters of venous blood collected from each patient upon admission, or control subjects were anticoagulated with sodium citrate, and centrifuged at 3000 rpm for 15 min at room temperature. Supernatants were carefully collected, and 250 μL aliquots were transferred into

Eppendorf tubes. Samples were immediately stored at − 80 °C for further measurement. Each tube was used only for a single test to avoid freezing–thawing cycles. The plasma levels of sCLEC-2 were assessed using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Xinle, Shanghai, China).

CAG

All patients with CAD underwent emergency or elective CAG after hospitalization. Patients were divided into single, double, and triple branch lesion groups based on the number of affected coronary branches revealed by CAG. Subgroups were assigned according to the Gensini scores that were calculated according to the location and degree of coronary artery stenosis (< 20, low risk; 20–40, medium risk; > 40, high risk) [28].

Statistical analysis

Continuous variables with normal and skewed distribution were presented as mean with standard deviation (SD) and medians with interquartile ranges, respectively. Mean and medians were compared using the unpaired Student's *t*-test and Mann Whitney test. The percentages for categorical variables were compared using χ^2 test. SCLEC-2 levels among different affected coronary vessel, Gensini score, and GRACE score subgroups were compared using the one-way analysis of variance (ANOVA) test. Multivariate logistic regression, which was adjusted for sex, age, smoking, hypertension, diabetes mellitus (DM), and hyperlipidemia, was used to calculate the odds ratio (OR) and 95% confidence interval (CI) of CAD for increasing sCLEC-2 levels. A forest plot was used to visualize ORs and corresponding 95% CIs of sCLEC-2 and other variables for predicting CAD risk. Restricted cubic splines were plotted to explore the association between increasing sCLEC-2 and CAD with four knots positioned at the 5th, 35th, 65th, and 95th percentiles of the in-study sCLEC-2 level. Receiver operating characteristic (ROC) curve (e.g., area under curve (AUC)) and an upgraded model with sCLEC-2 were constructed for a conventional model without sCLEC-2 to determine whether the addition of sCLEC-2 improved CAD risk prediction, and the C statistic from both models were compared using the likelihood χ^2 test reported by Delong *et al*. Integrated discrimination improvement (IDI) and net reclassification improvement (NRI) statistics evaluated the improvement of discriminatory ability by sCLEC-2. Net benefits were estimated by the decision curve analysis. A 2-tailed P value < 0.05 was considered statistically significant. Statistical analyses were performed by using SPSS (version 22.0, IBM, Armonk, New York) and R (Version 3.4.3, R Foundation for Statistical Computing, Vienna, Austria).

Results

Baseline characteristics

Table 1 displays the clinical information of the enrolled subjects. The proportion of males in CAD patients was higher than that in non-CAD subjects. Subjects with CAD were more likely to experience hypertension and DM. A high proportion of smokers was observed in CAD patients. Subjects with CAD exhibited higher serum N-terminal B type natriuretic peptide (NT-proBNP) levels ($P < 0.001$) but lower eGFR than non-CAD subjects ($P < 0.001$) (Table 1).

Comparison of plasma sCLEC-2 levels among different populations

We measured the sCLEC-2 plasma levels and healthy controls in patients with CAD to determine the association between sCLEC-2 and CA. As expected, the result showed that patients with CAD had significantly increased plasma

Table 1 Baseline characteristics of enrolled patients with CAD and control subjects

Variables	non-CAD (n = 89)	CAD (n = 216)	P value
Age (year)	59.88±9.69	61.90±11.45	0.144
Male gender (n (%))	52 (58.4)	158 (73.1)	0.012
Smoking (n (%))	15 (16.9)	87 (40.3)	<0.001
Hypertension (n (%))	24 (27.0)	123 (56.9)	<0.001
DM (n (%))	6 (6.7)	55 (25.5)	<0.001
Hyperlipidemia (n (%))	15 (16.9)	39 (18.1)	0.803
NT-ProBNP (mmol/L)	60.00 (36.00–88.50)	161.90 (37.25–1123.50)	<0.001
eGFR (mL/(min·1.73m^2))	116.00 (99.00–132.00)	71.00 (58.25–81.75)	<0.001

NT-proBNP, N-terminal prohormone of brain natriuretic peptide; eGFR, estimated glomerular filtration rate.

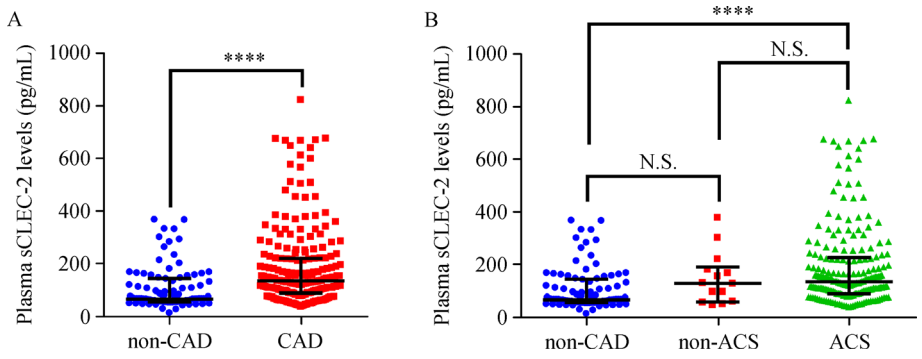

Fig. 1 Plasma levels of sCLEC-2 were measured in CAD patients and control subjects. (A) Plasma sCLEC-2 was higher in CAD patients compared with control subjects. (B) No significant difference in plasma sCLEC-2 was detected between ACS and non-ACS patients. ****$P < 0.0001$; N.S., not significant.

sCLEC-2 levels than non-CAD patients (133.67 (88.76–220.09) pg/mL versus 65.69 (55.36–143.22) pg/mL, $P < 0.0001$) (Fig. 1A).

We assessed the sCLEC-2 plasma levels in ACS patients, non-ACS CAD patients, and non-CAD controls to determine whether plasma sCLEC-2 level can reflect the severity of CAD. As expected, ACS patients demonstrated higher levels of sCLEC-2 compared with the non-ACS CAD and non-CAD groups. However, the difference between ACS and non-ACS CAD patients did not reach statistical significance ($P > 0.05$) (Fig. 1B).

Next, we compared plasma sCLEC-2 levels from CAD patients with single ($n = 80$), double ($n = 65$), and triple ($n = 71$) coronary branch lesions. No significant difference was found among these three groups (single-branch 147.19 (105.93–217.28) pg/mL, double-branch 132.79 (86.36–228.13) pg/mL, triple-branch 127.18 (75.19–254.05) pg/mL, $P > 0.05$) (Fig. 2A).

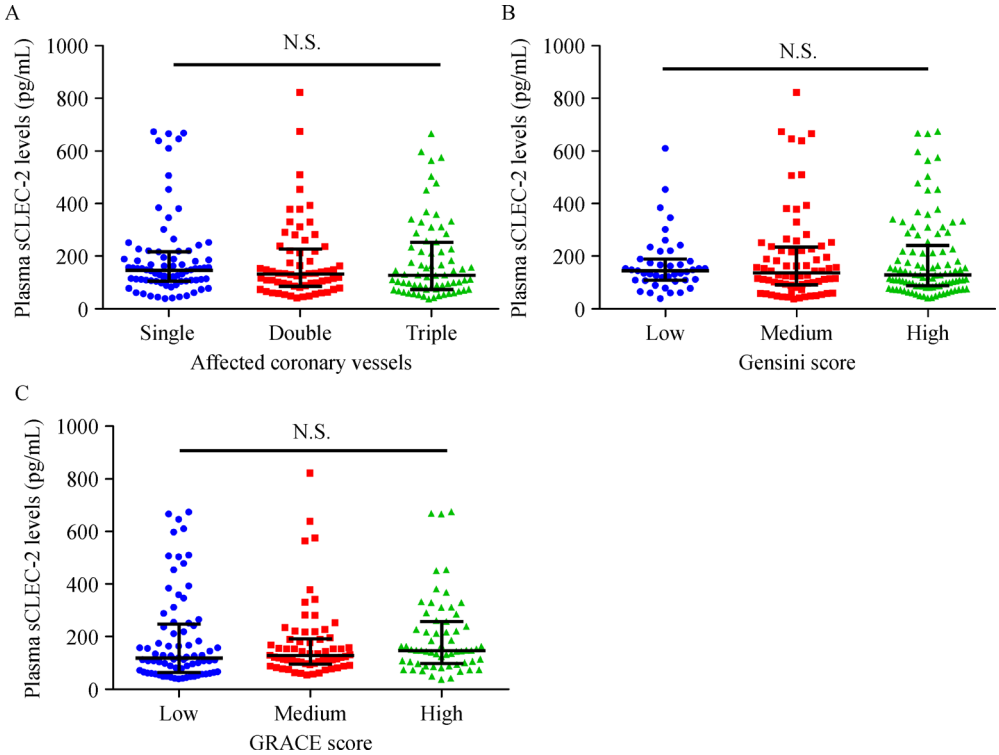

Fig. 2 Subgroup analyses of plasma sCLEC-2 levels in CAD patients. Plasma sCLEC-2 levels were compared among patient subgroups with different (A) affected coronary branches, (B) Gensini score classes, and (C) GRACE score classes. N.S., not significant.

Patients with CAD were stratified according to quantified coronary lesions by Gensini score to determine whether plasma sCLEC-2 can reflect the severity of coronary lesions. Accordingly, 39, 73, and 104 patients were included in the low-, medium-, and high-risk groups, respectively. No significant difference was found in plasma sCLEC-2 levels among these groups (low-risk 147.19 (107.10–180.02) pg/mL, medium-risk 130.04 (79.19–239.58) pg/mL, high-risk 127.22 (87.12–223.71) pg/mL, $P > 0.05$) (Fig. 2B).

In CAD, platelet activation is linked to increased plaque vulnerability and high-risk coronary lesions [16]. We compared plasma sCLEC-2 levels among ACS patients from different GRACE risk subgroups to elucidate the role of sCLEC-2 in CAD risk stratification. Plasma sCLEC-2 levels were comparable among low-risk ($n = 59$), medium-risk ($n = 81$), and high-risk ($n = 62$) ACS patients (low risk 113.43 (65.52–236.01) pg/mL, medium risk 130.04 (97.31–217.07) pg/mL, high risk 147.19 (99.04–266.40) pg/mL, $P > 0.05$) (Fig. 2C).

Association between plasma sCLEC-2 and CAD incidence

Compared with the lowest quartile of sCLEC-2, multivariate analysis adjusted for conventional risk factors showed an elevating risk of CAD associated with the increase in sCLEC-2 quartiles (Quartile 2: OR 4.83, 95% CI 2.21 – 10.53; Quartile 3: OR 5.84, 95% CI 2.60 – 13.11; Quartile 4: OR 7.72, 95% CI 3.26 – 18.28; $P_{trend} < 0.001$). Furthermore, the odds of CAD by each 1-SD increase of sCLEC-2 was 2.55 (95% CI 1.58–4.13, $P < 0.001$) (Table 2). Fig.3A shows the forest plot of the multivariate model with sCLEC-2 quartile for CAD risk (OR 2.01, 95%CI 1.52 – 2.66, $P < 0.001$). Restricted cubic spline analysis with multivariate adjustment is positively associated with the increase in sCLEC-2 and risk of CAD ($P_{linearity} < 0.001$) (Fig. 3B).

Improvement of C statistic and reclassification ability by adding sCLEC-2 to conventional risk factors

We constructed ROC and evaluated the C-index for multivariate logistic models before and after adding sCLEC-2 to determine whether sCLEC-2 improved CAD prediction beyond conventional risk factors. As expected, incorporating sCLEC-2 improved the C-index for distinguishing CAD cases from 0.761 to 0.821 ($P = 0.004$). Next, we evaluated that improvement of the discriminatory ability by sCLEC-2 by using the NRI and IDI statistics. Consistently, the addition of sCLEC-2 exhibited satisfactory performance to reclassify CAD cases or controls into their corresponding categories (Continuous NRI = 57.45%, 95% CI 34.15%–80.75%, $P < 0.001$; Categorical NRI = 20.35%, 95% CI 11.22%–29.49%, $P < 0.001$). This improvement was confirmed by the improved performance in identifying CAD events in cases rather than in controls (IDI = 8.27%, 95% CI 4.73%–11.82%; $P < 0.001$) (Table 3).

Finally, we used decision curve analysis to compare the predictive ability for CAD between models with and without sCLEC-2. Fig. 4 shows that the model that contains sCLEC-2 exhibits higher net benefit from intermediate through high threshold probabilities than the basic model. For example, at a threshold of 75% CAD risk, sensitivity (0.66 vs. 0.62) and specificity (0.82 vs. 0.78) were increased after sCLEC-2 was added to the basic model.

Discussion

Platelet-borne proteins that reflect their activation are proposed as potential diagnostic markers for CAD, considering the roles of platelets in CAD [29–31]. CLEC-2, a membrane receptor that mediates platelet activation, is emerging as a molecular target for diagnosing and treating thrombotic disease [16,32]. However, the clinical significance of CLEC-2 in CAD remains unclear. In this multicenter, cross-sectional study, we demonstrated that the plasma level of sCLEC-2 is significantly increased in CAD, especially in the setting of ACS. Moreover, a high sCLEC-2 level is an independent risk factor for CAD. Hence, sCLEC-2 may be used as an early biomarker for identifying high-risk CAD probands.

Human CLEC-2 is a non-classical C-type lectin receptor encoded by *CLEC1B*. The cytoplasmic tail of CLEC-2 binds to spleen tyrosine kinase (Syk), thereby forming dimers that are predisposed to platelet activation, aggregation, and thrombus formation [33]. In various

Table 2 OR and 95% CI of CAD risk associated with CLEC-2

	Q1	Q2	Q3	Q4	*P* for trend	Per SD increase
No. of CAD cases (%)	78 (25.6)	75 (24.6)	75 (24.6)	77 (25.2)		
Odds of CAD						
Unadjusted OR (CI)	Reference	4.90 (2.43–9.92)	5.75 (2.79–11.86)	8.63 (3.95–18.84)	0.009	2.76 (1.71–4.45)
Adjusted OR (CI)[a]	Reference	4.83 (2.21–10.53)	5.84 (2.60–13.11)	7.72 (3.26–18.28)	<0.001	2.55 (1.58–4.13)

[a] Adjusted for sex, age, smoking, hypertension, DM, and hyperlipidemia.

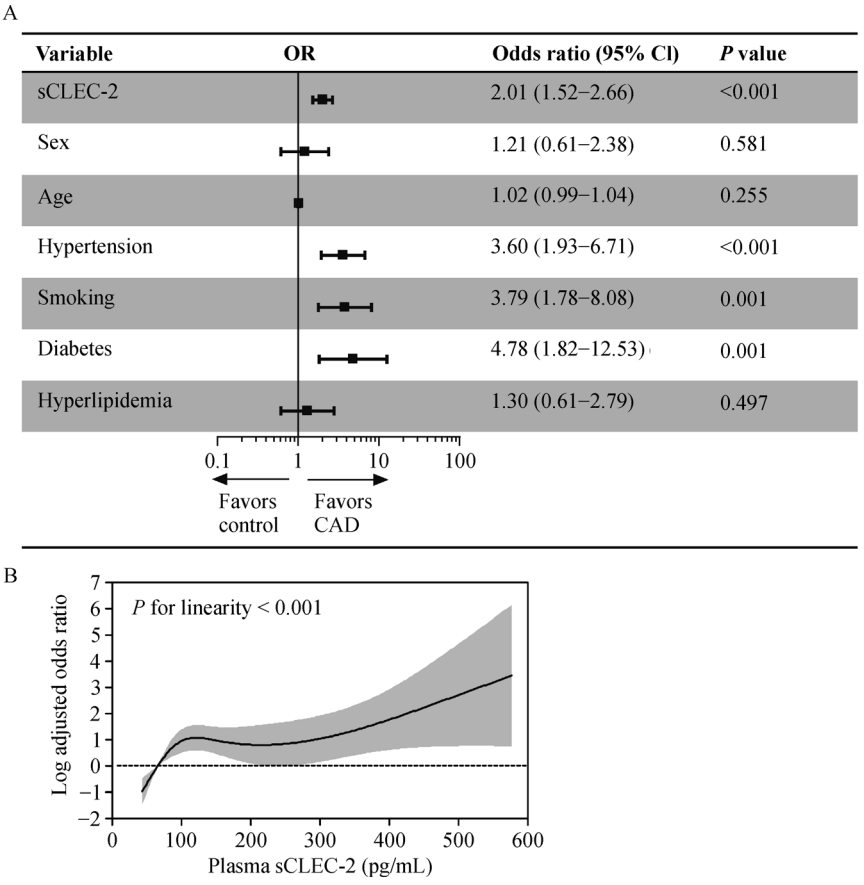

Fig. 3 Association between plasma sCLEC-2 and CAD. (A) Forest plot of the multivariate logistic model for the association of sCLEC-2 with CAD. (B) The positive association between plasma sCLEC-2 and CAD was displayed in a restricted cubic spline.

Table 3 Reclassification and discrimination statistics for CAD incidence by plasma CLEC-2

	C statistics		NRI continuous		NRI categorical[a]		IDI	
	Estimate (95% CI)	*P* value	Estimate (95% CI)	*P* value	Estimate (95% CI)	*P* value	Estimate (95% CI)	*P* value
Basic model	0.761 (0.709–0.808)		Reference		Reference		Reference	
Basic model + CLEC-2 quartile	0.821 (0.774–0.863)	0.004	57.45 (34.15–80.75)	<0.001	20.35 (11.22–29.49)	<0.001	8.27 (4.73–11.82)	<0.001

Basic model included sex, age, smoking, hypertension, DM, and hyperlipidemia status. CI indicates confidence interval; NRI, net reclassification index; and IDI, integrated discrimination index. [a] Risk categories: < 10%, 10%−30%, > 30%.

inflammatory and thrombotic disorders, endogenous podoplanin associates with CLEC-2, activates downstream signals, and triggers thrombus formation [34–38]. Additionally, S100A13 secreted by vascular smooth muscle cells in atherosclerotic lesions activates CLEC-2 [39]. In accordance with the atherothrombotic nature of CAD, we showed increased plasma sCLEC-2 in these patients and found the highest sCLEC-2 level in those with ACS. These findings suggest that CLEC-2 may reflect platelet activation and plaque vulnerability in CAD.

In the current study, elevated sCLEC-2 in plasma may originate from three major sources. First, CLEC-2 can shed from platelet surface by proteolytic mechanism upon activation. Second, activated platelets may release microparticles that carry CLEC-2 on their membranes [32]. A third reservoir may be neutrophils, which also express functional CLEC-2 [40]. In patients with rheumatoid arthritis, an inflammatory disorder characterized by increased microparticle production, Ozaki *et al.* reported the dominant increase in plasma sCLEC-2 in

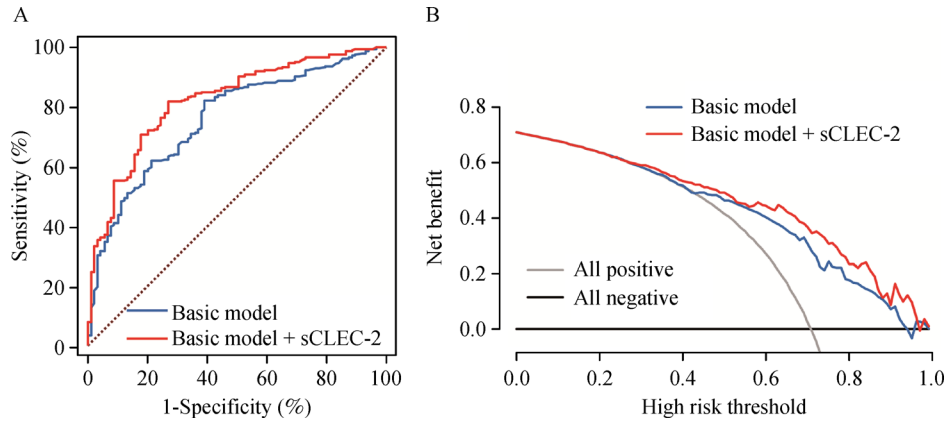

Fig. 4 Improvement distinguishing capacity for CAD by plasma sCLEC-2. (A) Addition of plasma sCLEC-2 to conventional risk factors improved the AUC of the receiver operating curve (ROC) for CAD. (B) Decision curve analyses for the odds of CAD by adding sCLEC-2 to a basic model. Basic model includes sex, age, smoking, hypertension, DM, and hyperlipidemia status.

microparticles [41]. Additionally, plasma sCLEC-2 is increased in those with DM, a common cause of platelet activation [32]. Furthermore, CLEC-2 regulates phagocytosis, an essential step of macrophage infiltration and foam cell formation in atherogenesis [40]. Regarding these aspects, we proposed that sCLEC-2, as an important regulator of atherothrombosis, might reflect the extent of CAD. Gensini and GRACE scores were calculated to obtain a comprehensive estimation of the severity of CAD. However, we did not find a significant correlation between sCLEC-2 level and coronary atherosclerotic burden (determined by Gensini score) or major adverse cardiac events (MACE) risk (determined by GRACE score) in ACS. One explanation for this may be the complicated sCLEC-2 pools *in vivo*. To further explore the value of CLEC-2 in CAD stratification, future studies are required to differentiate the origin of CLEC-2 by measuring platelet-, microparticle-, and leukocyte-borne sCLEC2. Given similar levels of sCLEC-2 between healthy volunteers and patients with stable CAD, activated platelets and platelet-derived microparticles may play a role in the increase of plasma sCLEC-2. In this study, we focused on the association between plasma sCLEC-2 and CAD and did not measure functional parameters of platelets and leukocytes. Therefore, further verification is required to elaborate the cell origin of increased plasma sCLEC-2, especially when a small size of the non-ACS population in the present study may underscore the role of leukocyte-derived sCLEC-2. Whether plasma sCLEC-2 can be a practical marker to predict the severity and prognosis of CAD remains to be determined.

For decades, platelet-borne factors have been investigated due to their potential use in diagnosing CAD. One surrogating example is the membrane protein CD40 ligand (CD40L) [42]. A soluble form of CD40L is generated by proteolytic cleavage from platelet surface upon activation [43]. In CAD, an increased CD40L level has been associated with exacerbated coronary lesion and higher risk of developing MACE [44–46]. Similar to CLEC-2, CD40L mediates platelet activation, leukocyte accumulation, and inflammatory response [47,48]. However, only the ratios of platelet surface to plasma or serum CD40L expression show sufficient diagnostic efficacy in CAD [49]. The use of CD40L as an initial diagnostic marker for CAD may be hampered by an advanced technical requirement of flow cytometry and relatively slow output. Another potential marker include PF4 and β-thromboglobulin. However, their plasma concentrations may be affected by commonly used medications, including propranolol and nitrates [50,51]. Apolipoprotein B has shown prognostic value for CAD, but its plasma level may vary upon statin intake [52]. Circulating microRNAs are emerging as novel biomarkers for CAD, and their application requires additional procedures, including RNA isolation [53]. By contrast, ordinary blood collection and clinical laboratory tests are sufficient for analyzing sCLEC-2 levels by conventional ELISA method, thereby facilitating its use in outpatient and emergency departments [32].

Regarding limitations of the current study, the association of sCLEC-2 with CAD should be interpreted cautiously. First, we only conducted a cross-sectional case-control study, which may not show the prognostic value of sCLEC-2 in CAD. Second, the sample size was small, which may compromise our current findings. A relatively small proportion of patients with non-ACS CAD may make the drawing of a significant conclusion from the link of sCLEC-2 in this population difficult. We may not rule out subclinical atherosclerosis in the control group in the current study because not all control participants underwent CAG.

In conclusion, we showed that plasma sCLEC-2 is

increased in patients with CAD and ACS. The elevated level of plasma sCLEC-2 is an independent risk factor for CAD and may be presented as a useful biomarker for identifying high-risk ACS patients.

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Compliance with ethics guidelines

Min Fei, Li Xiang, Xichen Chai, Jingchun Jin, Tao You, Yiming Zhao, Changgeng Ruan, Yiwen Hao, and Li Zhu declare that they have no conflict of interest. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the *Helsinki Declaration* of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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