

# Expression and Clinical Prognostic Value of Platelet *NLRP3* in Acute Coronary Syndrome

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**Purpose:** Little is known about the relationship between the level of platelet NOD-like receptor protein 3 (*NLRP3*) and the severity of acute coronary syndrome (ACS) or the prognostic value of platelet *NLRP3* for percutaneous coronary intervention (PCI).

**Methods:** Platelets collected from 25 healthy subjects, 23 patients with stable angina pectoris (SAP), and 72 patients with ACS were analyzed by Western blotting and real-time fluorescence quantitative PCR (qPCR). A total of 152 patients with ACS who had undergone PCI were included in this study to evaluate the prognostic value of platelet *NLRP3*.

**Results:** The levels of platelet *NLRP3* in both the healthy and SAP groups were clearly lower than in the ACS group ( $P < 0.001$ ). According to the Pearson correlation analysis, the expression of platelet *NLRP3* was closely related to the mean platelet volume (MPV), left ventricular ejection fraction (LVEF), the Gensini score, and the Global Registry of Acute Coronary Events (GRACE) score (all  $P < 0.001$ ). Multivariate logistic regression analysis identified *NLRP3* as an independent risk factor for adverse cardiovascular events (ACEs) after PCI ( $P = 0.004$ ). The proportion of patients with high *NLRP3* expression (the *NLRP3*-high group) remaining free of adverse events for 3 years was remarkably lower than that in patients with low *NLRP3* expression (the *NLRP3*-low group;  $P = 0.024$ ). The *NLRP3*-high group had a significantly higher proportion of patients with interleukin-1 $\beta$ -expressing (20.4% $\pm$ 6.1%) platelets than the *NLRP3*-low group (10.7% $\pm$ 3.5%,  $P < 0.001$ ). Moreover, the *NLRP3*-high group exhibited higher platelet activity, as indicated by increased PAC-1 binding and CD62P expression, compared with the *NLRP3*-low group ( $P < 0.001$ ).

**Conclusion:** These results indicated that platelet *NLRP3* was a novel potential prognostic factor for patients with ACS that underwent PCI.

**Keywords:** platelet *NLRP3*, acute coronary syndromes, percutaneous coronary intervention, inflammation, prognosis

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## Introduction

Defined as the acute ischemic syndrome of the heart resulting from the rupture of thrombosis or atherosclerotic plaque in the coronary arteries,<sup>1</sup> acute coronary syndrome (ACS) is one of the most serious and potentially fatal diseases. Inflammation contributes to building plaques, regards as determinants of acute coronary.

NOD-like receptor protein 3 (*NLRP3*) inflammasome, an inflammasome with the clear structure and function, consists of *NLRP3*, pro-caspase-1, and apoptosis-associated speck-like protein with a CARD (ASC).<sup>2</sup> *NLRP3* is closely related to the occurrence and development of cardiovascular disease, in which inflammatory bodies are activated by cholesterol crystals in a dose-dependent manner in the

early stages of atherosclerosis.<sup>3</sup> Mice lacking the activation domains, including *ASC* and *caspase-1*, showed reduced inflammation, infarct size, and myocardial fibrosis after ischemic injury.<sup>4</sup>

Wang et al<sup>5</sup> discovered that patients with ACS and acute myocardial infarction (AMI) had increased expression of *NLRP3* and downstream cytokines in monocytes. After activation by external stimuli, *NLRP3* forms a complex with proteins, including *ASC*, *BTK*, and *caspase-1*, and regulates the production of pro-inflammatory factors, such as active interleukin (IL)-1 $\beta$ , thereby controlling platelet activation, aggregation, and thrombosis during ACS.<sup>6</sup> Besides their presence in monocytes, *NLRP3* expression has been recently found in platelets, however, the expression and clinical prognostic value of platelet *NLRP3* in ACS remains unclear.

Patients with ACS participating in the PLATelet inhibition and patient Outcomes trial were at a high risk of residual disease, with 10% of patients experiencing an ischemic endpoint, even after receiving powerful dual antiplatelet therapy.<sup>7</sup> Interestingly, the Canakinumab Anti-inflammatory Thrombosis Outcomes Study trial suggested that the IL-1 $\beta$  monoclonal antibody canakinumab contributed to a 15% reduction in the risk of cardiovascular events in patients with myocardial infarction and C-reactive protein concentration >2 mg/dL based on standard drug therapy.<sup>8</sup> This is the first direct demonstration that anti-inflammatory treatment can reduce cardiovascular events, indicating that inflammation and thrombosis are inseparable in ACS.

Percutaneous coronary intervention (PCI) is an effective treatment for some patients with ACS, although there are still issues surrounding prognosis. There are many factors related to the prognosis after PCI, and the indices studied remain inadequate in terms of sensitivity and specificity. Therefore, there is an urgent need to find ideal indicators to evaluate the prognosis of PCI in patients with ACS. Since platelets are involved in the pathological process of inflammation and thrombosis in ACS, the biomarkers in platelets may be important predictors of the prognosis of ACS.

Platelets can express and release inflammatory factors and chemokines after activation,<sup>9</sup> and inflammatory cells are recruited by platelets-secreted chemokines to vascular lesions,<sup>10</sup> promoting vascular endothelial dysfunction and inducing atherosclerosis. We speculated that platelet *NLRP3* may participate in the platelet hyperactivity in ACS and affect the prognosis of patients with ACS.

Based on this hypothesis, for the first time, we observed the expression of platelet *NLRP3* in patients with ACS. Furthermore, we performed retrospective analysis to examine the relationship between platelet *NLRP3* expression and the clinical endpoints in patients with ACS.

## Materials and Methods

### Participants and Design

In this study, two different patient cohorts were included: one cohort comprised healthy people and patients with coronary heart disease, and the other comprised patients with ACS who underwent PCI. Study subjects were patients hospitalized in Zhongshan Hospital of Fudan University between January 2016 and July 2017. This study was approved by the appropriate Ethics Committee and strictly adhered to the Declaration of Helsinki, and written informed consent was obtained from each patient after a detailed presentation regarding this study.

The first cohort enrolled 25 healthy subjects; 23 patients with stable angina pectoris (SAP); and 72 patients with ACS, comprising 23 patients with unstable angina pectoris (UAP), 26 patients ST-segment elevation myocardial infarction (STEMI), and 23 patients with non-STEMI (NSTEMI). The second cohort comprised 152 patients with ACS who successfully underwent PCI. The inclusion and exclusion criteria defined for both cohorts are shown in [Figure S1](#).<sup>11–13</sup>

### Data Collection and Evaluation

The demographic and clinical characteristics of patients included age (years), body mass index (BMI), sex, smoking status, hypertension, diabetes, blood lipids, low- and high-density lipoprotein cholesterol (LDL-C and HDL-C, respectively), medication taken during this study (administration of  $\beta$  receptor blocker, ACE1, ARB, PPIs, H2RA, statins, and nitrates), platelet parameters, and left ventricular ejection fraction (LVEF).

The blood samples were collected from all patients at the time of enrollment. The venous blood from patients with SAP and ACS was drawn immediately upon hospital admission before medication and angiography. Blood samples were drawn from healthy subjects in the control group on the day of physical examination. For patients in the PCI group, venous blood was drawn immediately upon hospital admission before revascularization (ie, thrombolysis or PCI).

We evaluated the 3-year incidence of cardiovascular events (ACEs) (eg, stroke, AMI, cardiovascular event, and

unplanned coronary revascularization)<sup>14</sup> in 152 patients who underwent PCI.

## Detection of Platelet NLRP3 Expression

Platelet-rich plasma was prepared by centrifuging whole blood samples collected from healthy subjects and patients at  $300 \times g$  for 20 min. Then, the platelet-rich plasma was centrifuged at  $740 \times g$  for 10 min to remove the supernatant and resuspended in Tyrode's buffer (135 mM NaCl, 12 mM NaHCO<sub>3</sub>, 2.9 mM KCl, 0.3 Na<sub>2</sub>HPO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 5 mM D-glucose, 10 mM HEPES, and 1.5% BSA, pH 7.4, 37°C) to obtain a platelet suspension. The concentration was adjusted to  $1 \times 10^5$  cells/ $\mu$ L. Isolated platelets were immediately analyzed by flow cytometry or stored at  $-80^\circ\text{C}$  for further analysis by qPCR and Western blotting.

The manufacturer's protocol (cat. no. 15596–026, Invitrogen) was used to extract the platelet RNA. The total RNA was assessed for purity using an ultraviolet spectrophotometer (Eppendorf, Germany), and then stored at  $-80^\circ\text{C}$  in a refrigerator until use.

In accordance with the PCR amplification kit (cat. no. 206143, Qiagen), primer sequences were matched and amplified using a real-time fluorescence qPCR instrument (Eppendorf, Germany).

Primers were designed and synthesized by a bioengineering company. The following primer sequences were used: *NLRP3* upstream and downstream primers: 5'-A ACGACCCCTTCATTGAC-3' and 5'-GAGGAAGAGGA GGAAAAGGACA-3'; internal reference *GAPDH* upstream and downstream primers: 5'-GAAGGTGAGGTCGGAGTC -3' and 5'-GGAGATGGTGATGGGATT -3'.

The PCR conditions were as follows:  $95^\circ\text{C}$ ,  $95^\circ\text{C}$ ,  $58^\circ\text{C}$ , and  $72^\circ\text{C}$  for 5 min, 20 s, 30s, and 30 s, respectively. Each cycle was repeated 40 times. An analysis was performed for each sample in three parallel duplicate wells. *NLRP3* expression in platelets was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method.<sup>15</sup>

## Western Blotting

Platelet samples were lysed in ice-cold radioimmunoprecipitation assay buffer and vortexed occasionally for 30 min to ensure complete lysis before centrifugation ( $2.1 \times 10^4 \times g$ , 10 min) to remove insoluble debris. Each sample was analyzed by SDS-PAGE and the gel was transferred to a polyvinylidene difluoride membrane, which was incubated with primary antibodies (cat. no. IMG-6668A, 1:1000, NLRP3, from Novus Biologicals or cat. no. 5174S, 1:1000, GAPDH, from Cell Signaling

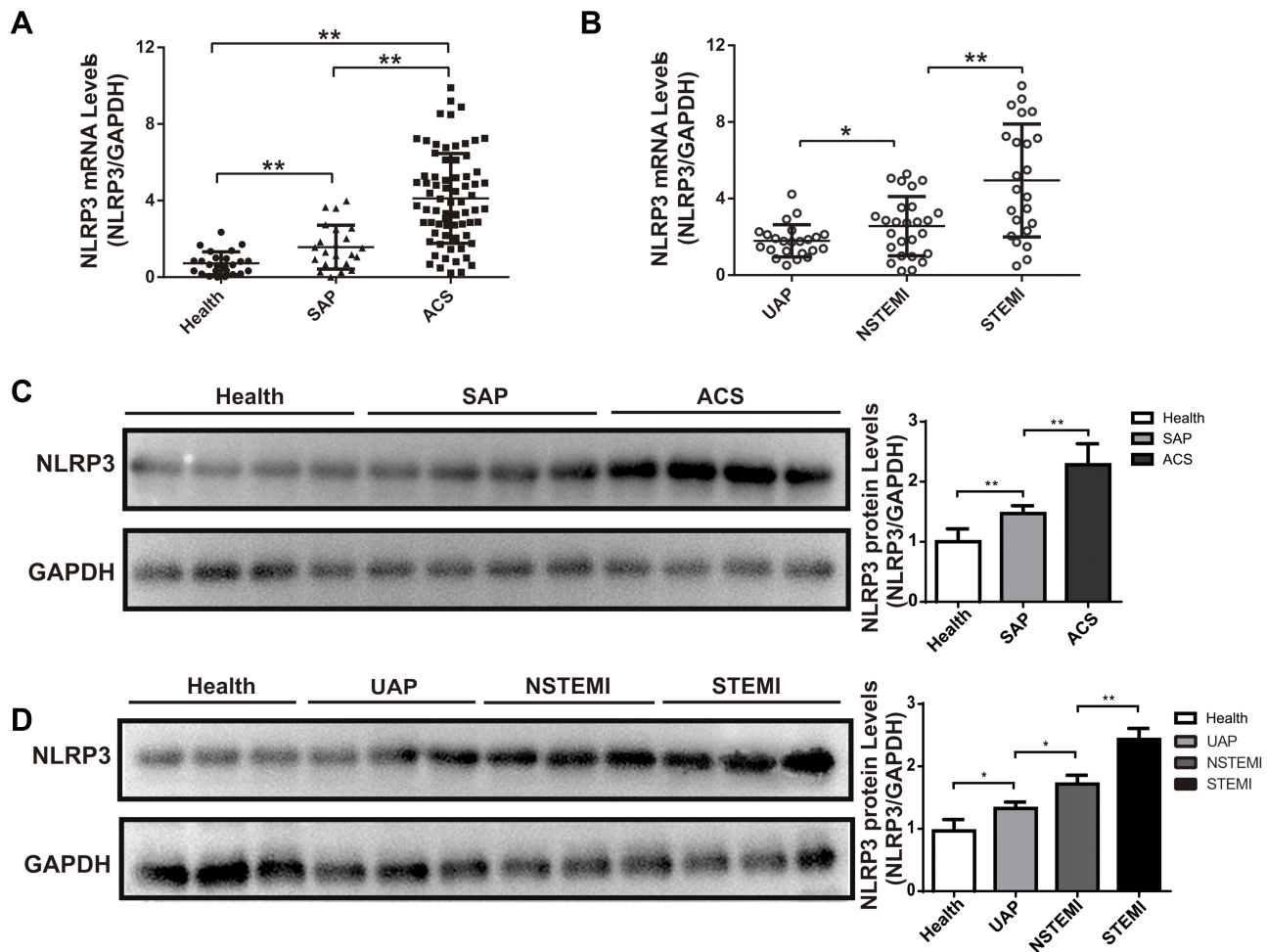
Technology) in 3% BSA in TBS-T at  $4^\circ\text{C}$  for overnight. Anti-rabbit HRP (cat. no. 7074S, 1:5000, from Cell Signaling Technology) was used as a secondary antibody. An ECL Plus kit was used to develop the blot, and bands were quantified by pixel intensity using ImageJ software.

## Flow Cytometry

Platelets were analyzed using a FACS Aria. Approximately 300  $\mu$ L of platelets was fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton for 10 min, and incubated with APC-tagged anti-CD41 (cat. no. MA1-19779, 1:100, Thermo), PE-tagged CD62p antibody (cat. no. 12–0626-82, 1:200, Thermo), FITC-tagged PAC-1 antibody (cat. no. MA5-28564, 1:200, Thermo), or FITC-tagged IL-1 $\beta$  antibody (cat. no. 340515, 1:100, BD) in darkness for 30 min, and then added to PBS (10 mM) for flow cytometric analysis. The activation of platelets can be shown by the percentage of CD62p- or PAC-1 positive platelets. The specific binding of APC-tagged anti-CD41 and characteristic side and forward scattering were used to distinguish platelets. FlowJo version V10 (TreeStar) was used to perform the data analysis.

## Statistical Analysis

SPSS software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Data normality was tested using the one-sample Kolmogorov–Smirnov test. The mean  $\pm$  standard deviation (SD) was used to express normally distributed continuous variables, whereas the median (interquartile range) was used to express abnormally distributed variables. The number (percentage) for frequencies was adopted to express the results. Normally distributed continuous variables were compared using Student's *t*-test, whereas abnormally distributed variables were compared using the Mann–Whitney *U*-test. The Pearson chi-square test was conducted to compare differences in intergroup categorical data. The association between platelet *NLRP3* and clinical parameters was investigated using Pearson's and Spearman's rank correlation coefficients (normally distributed and non-normal data, respectively). Receiver operating characteristic (ROC) curves were used to determine the most meaningful cutoff point. Multivariate logistic regression analysis was performed to identify the independent predictors of ACEs after PCI. The Kaplan–Meier test was conducted to perform survival analysis, and the Log rank test was performed to assess whether the differences in curves were statistically significant. Each test was two-sided and



**Figure 1** Expression of platelet *NLRP3* in health subjects and coronary heart disease patients. **(A)** The relative platelet *NLRP3* mRNA level in healthy subjects (n=25), patients with SAP (n=23), and patients with ACS (n=72). **(B)** The relative platelet *NLRP3* mRNA level in patients with UAP (n=23), NSTEMI (n=26), and STEMI (n=23). **(C)** Representative expression pattern of *NLRP3* protein by Western blotting (Left panel) and relative quantification (Right panel) of *NLRP3* protein in healthy subjects (n=25), patients with SAP (n=23), and patients with ACS (n=72). **(D)** Representative expression pattern of *NLRP3* protein by Western blotting (Left panel) and relative quantification (Right panel) of *NLRP3* protein in healthy subjects (n=25), and patients with UAP (n=23), NSTEMI (n=26), and STEMI (n=23). Data are presented as mean ± SD,  $P < 0.05^*$ ,  $P < 0.01^{**}$ .

**Abbreviations:** ACS, acute coronary syndrome; SAP, stable angina pectoris; UAP, unstable angina pectoris; NSTEMI, non-ST-segment elevation myocardial infarction; STEMI: ST-segment elevation myocardial infarction.

P-values of  $< 0.05$  were considered to indicate statistical significance.

## Results

### *NLRP3* Expression in the Platelets of Patients with ACS

Overall, 25 healthy subjects, 23 patients with SAP, and 72 patients with ACS (23 patients with UAP, 23 patients with STEMI, and 26 patients with NSTEMI) were included in the analysis of platelet *NLRP3* expression. The baseline characteristics of the patients are listed in Table 1. Compared with the healthy and SAP groups, the ACS group demonstrated a significantly higher expression level of platelet *NLRP3*, as detected by qPCR (both

$P < 0.001$ , Figure 1A), and the STEMI group exhibited the highest expression of *NLRP3* among all patients with ACS (Figure 1B). The results were confirmed by Western blotting, which indicated similar differences in *NLRP3* expression in different groups (Figure 1C and D).

### Clinical Significance of Platelet *NLRP3* in Patients with ACS

In total, 152 patients who successfully underwent PCI were examined (average  $66.32 \pm 4.58$  years of age, 57.24% males) to analyze the potential clinical significance of platelet *NLRP3* in patients with ACS.

The focus was on the relationship between platelet parameters and *NLRP3* expression level (detected by qPCR, which



**Table 1** Baseline Characteristics and the Level of Platelet Parameters in the Study Population

Characteristics	Health N=25	SAP N=23	ACS			P1	P2
			UAP N=23	NSTEMI N=26	STEMI N=23		
Age	65.1±4.3	67.0±4.9	66.0±2.1	66.6±4.0	68.4±4.3	0.123	0.071
Male	16(64.0%)	14(60.9%)	11(47.8%)	14(53.8%)	15(65.2%)	0.734	0.797
BMI	22.8±1.7	22.7±2.5	23.0±1.8	21.7±2.0	22.3±2.0	0.551	0.188
Hypertension	10(40.0%)	13(56.5%)	11(47.8%)	14(53.8%)	12(52.2%)	0.485	0.773
Diabetes mellitus	10(40.0%)	8(34.8%)	8(34.8%)	9(34.6%)	9(39.1%)	0.933	0.996
HC	9(36.0%)	8(34.8%)	9(39.1%)	9(34.6%)	8(34.8%)	0.970	0.961
Current smoking	8(32.0%)	9(39.1%)	10(43.5%)	7(26.9%)	9(39.1%)	0.849	0.973
<b>Platelet parameter</b>							
MPV	8.4 (7.8–9.6)	22.7 (20.5–25.0)	21.4 (20.0–23.4)	22.6 (20.7–23.7)	23.2 (21.8–24.8)	0.523	<b>0.044</b>
PLT	247.5±47.6	255.6±59.1	274.3±48.4	247.1±56.5	269.0±64.6	0.485	0.307
PDW	21.4±1.7	21.6±1.5	20.7±0.9	21.14±1.50	20.5±1.7	0.052	0.091
PCT	0.192 (0.168–0.208)	0.183 (0.164–0.211)	0.194 (0.162–0.209)	0.190 (0.155–0.209)	0.182 (0.134–0.206)	0.772	0.665

**Notes:** Values are presented as mean ± SD, as median (interquartile range), or as n (%). A p-value smaller than 0.05, indicated by the bold-italic text. P1: the comparison was made between the healthy, SAP and ACS groups; P2: the comparison was made between the UAP, NSTEMI and STEMI groups.

**Abbreviations:** BMI, body mass index; SAP, stable angina pectoris; ACS, acute coronary syndrome; UAP, unstable angina pectoris; NSTEMI, non-ST-segment elevation myocardial infarction; STEMI, ST-segment elevation myocardial infarction; HC, hypercholesterolemia; PLT, platelet count; MPV, mean platelet volume; PDW, volume distribution width; PCT, the platelet crit.

is a more sensitive and reproducible technique than Western blotting). Mean platelet volume (MPV), an indicator of platelet activity, was positively associated with platelet *NLRP3* expression ( $r=0.599$ ,  $P<0.001$ ) (Figure 2A), whereas platelet count (PLT), platelet volume distribution width (PDW), or platelet crit (PCT) showed no association with platelet *NLRP3* expression ( $P>0.05$ ) (Figure 2B-D). In other clinical parameters, the expression of *NLRP3* in patients with ACS was negatively correlated with LVEF ( $r=-0.288$ ,  $P<0.001$ ) (Figure 2E), and was not related to HDL-C concentration, LDL-C concentration, or WBC count ( $P>0.05$ ) (Figure 2F-H).

The expression of platelet *NLRP3* gradually increased with the severity of the Gensini score, reflecting the severity of coronary lesions and the positive correlation between the expression of platelet *NLRP3* and the Gensini score ( $r=0.353$ ,  $P<0.001$ ) (Figure S2A and S2C). Similar results were observed in the Global Acute Coronary Event (GRACE) score when obtained based on a variety of indicators at the time of admission ( $r=0.561$ ,  $P<0.001$ ) (Figure S2B and S2D).

### ROC Curves for *NLRP3*

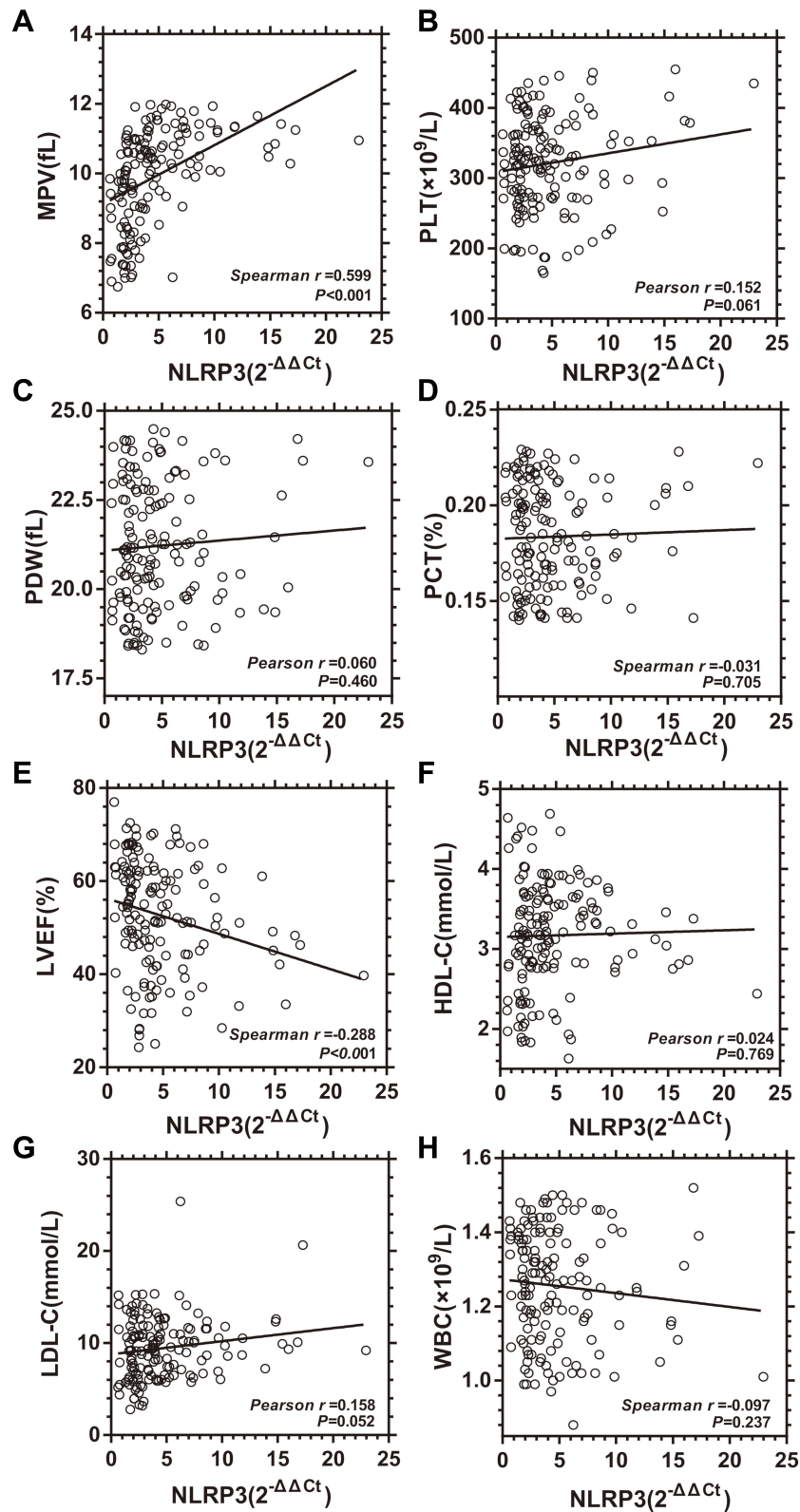
The expression of *NLRP3* ( $2^{-\Delta\Delta Ct}$ ) was used as the detection variable to define ACEs within 36 months as the endpoint, set state variable value as 1, and establish an ROC curve (Figure 3A), which suggested that the best

cutoff value was 3.8850, with a sensitivity and specificity of 75.4% and 70.5%, respectively.

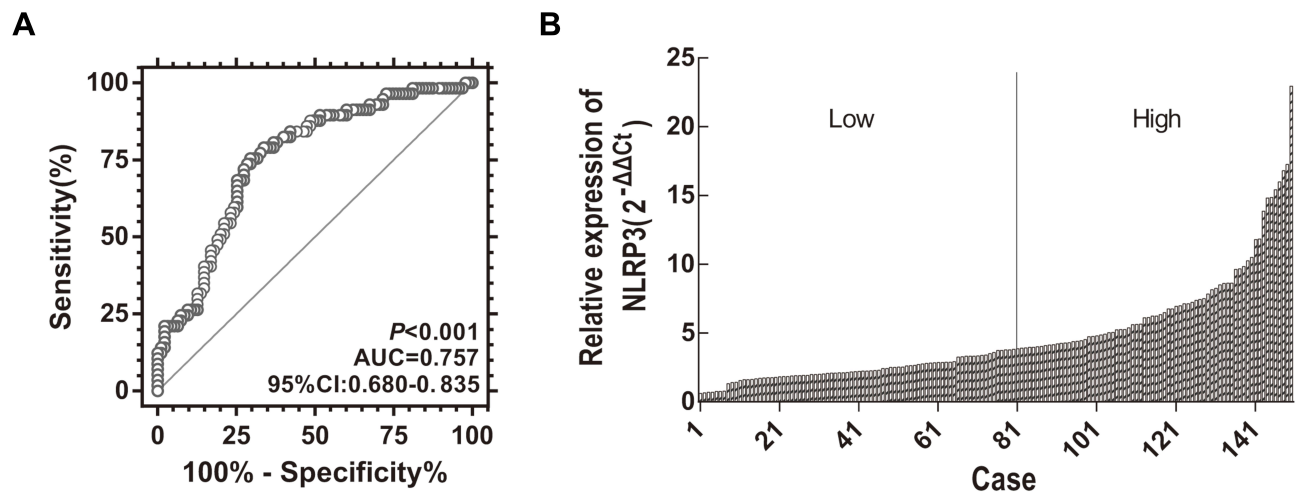
The patients were divided into groups, with low ( $n=81$ ) and high expression ( $n=71$ ) based on platelet *NLRP3* expression (Figure 3B), and the clinical parameters of the two groups were compared. Compared with the group with low *NLRP3* expression, the group with high *NLRP3* expression showed significantly higher STEMI ratio, MPV, hs-CRP, and IL-1 $\beta$ , and significantly lower LVEF (Table 2).

### Prognostic Value of Platelet *NLRP3* in Patients with ACS

Multivariate analyses were performed to further investigate whether platelet *NLRP3* expression was a relatively independent prognostic factor for endpoint events within 3-year after PCI. Patient age (odds ratio [OR]=1.056, 95% confidence interval [CI]: 1.003–1.113,  $P=0.039$ ), hypertension (OR=1.737, 95% CI: 1.046–2.883,  $P=0.033$ ), and platelet *NLRP3* (OR=1.084, 95% CI: 1.035–1.136,  $P=0.001$ ), which showed a significant prognostic value in univariate analyses, were included in the multivariate analyses. The results indicated that platelet *NLRP3* expression (OR=1.071, 95% CI: 1.022–1.123,  $P=0.004$ ) can be considered as a relatively independent factor in prediction of the prognosis of patients that underwent PCI (Table 3).



**Figure 2** Correlation between platelet *NLRP3* expression and clinical parameters. **(A)** Correlation between platelet *NLRP3* mRNA level and mean platelet volume (MPV, n=252). **(B)** Correlation between platelet *NLRP3* mRNA level and platelet count (PLT, n=252). **(C)** Correlation between platelet *NLRP3* expression and platelet volume distribution width (PDW, n=252). **(D)** Correlation between platelet *NLRP3* expression and the plateletcrit (PCT, n=252). **(E)** Correlation between platelet *NLRP3* mRNA level and left ventricular ejection fraction (LVEF, n=252). **(F)** Correlation between platelet *NLRP3* mRNA level and high-density lipoprotein cholesterol (HDL-C, n=252). **(G)** Correlation between platelet *NLRP3* expression and low-density lipoprotein cholesterol (LDL-C, n=252). **(H)** Correlation between platelet *NLRP3* expression and white blood cell count (WBC, n=252).



**Figure 3** ROC curves of platelet *NLRP3* that predicted adverse cardiovascular events in patients who underwent PCI. **(A)** ROC curves with an AUC of 0.757 (95% CI, 0.680–0.835;  $P<0.001$ ) of platelet *NLRP3* that predicted adverse cardiovascular events in patients who underwent PCI. **(B)** Division of relative expression of *NLRP3* in patients with ACS. The boundary value for *NLRP3* is indicated by the black straight line (3.885); Low ( $n=81$ ): The relative expression of *NLRP3*  $\leq 3.885$ ; High ( $n=71$ ): The relative expression of *NLRP3*  $> 3.885$ .

**Abbreviations:** ROC, receiver operating characteristic; AUC, area under the ROC curve; CI, confidence interval.

The Kaplan-Meier curves of groups with low and high platelet *NLRP3* expression were plotted, and Log rank tests were performed. The results indicated that patients with high expression of platelet *NLRP3* had a significantly poorer prognosis after PCI than those with low expression of platelet *NLRP3* ( $P=0.024$ ), as evidenced by the significantly lower proportion of patients remaining free of postoperative events (Figure 4A). Patients in the *NLRP3*-low group had one all-cause mortality, two AMI, and two stroke outcomes, whereas patients in the *NLRP3*-high group had three all-cause mortality, two AMI, five stroke, and three re-PCI /CABG outcomes. Patients in the *NLRP3*-high group ( $20.4\% \pm 6.1\%$ ) showed a significantly higher proportion of IL-1 $\beta$ -expressing platelets ( $P<0.001$ ) than those in the *NLRP3*-low group ( $10.7 \pm 3.5\%$ ) (Figure 4B). Moreover, the *NLRP3*-high group exhibited higher platelet activity, as indicated by integrin  $\alpha$ IIb $\beta$ 3 activation (PAC-1 binding, Figure 4C) and P-selectin exposure (CD62P expression, Figure 4D), compared with the *NLRP3*-low group.

## Discussion

In our study, high expression of *NLRP3* in platelets was found in patients with ACS. Moreover, correlation analysis found that platelet *NLRP3* levels were correlated with the MPV, LVEF, Gensini score, and GRACE score. Multivariate logistic regression analysis showed that platelet *NLRP3* was an independent risk factor for ACEs after PCI in patients with ACS. The Kaplan-Meier Log rank tests indicated that patients with high expression of platelet

*NLRP3* had a significantly poorer prognosis after PCI than those with low expression of platelet *NLRP3*.

Several studies have indicated a close relationship between the *NLRP3* inflammasome and the occurrence and development of cardiovascular diseases.<sup>3,16</sup> Inhibition of *NLRP3* inflammatory bodies in a rat ischemia-reperfusion model reduced the area of myocardial infarction and the level of cardiac troponin I in serum.<sup>17</sup> In ACS, hypoxia and ischemia can increase reactive oxygen species (ROS) production, potassium efflux, and other stressful processes, thereby forming *NLRP3* inflammatory bodies and accelerating atherosclerosis. Liu et al<sup>18</sup> detected up-regulated expression of *NLRP3* and an increase in caspase-1 activity and the production of IL-1 $\beta$  and IL-18 in the ischemic heart of a mouse ischemia-reperfusion model, indicating that the inflammatory response mediated by *NLRP3* inflammatory bodies was of great importance in the development of ACS.

The IL-1 receptor antagonist (IL-1ra) is the endogenous counter-regulator of IL-1. Recently it has been suggested IL-1 blockade could be beneficial in acute coronary syndrome.<sup>19</sup> Over the last three decades, the precise role of AMI-related inflammation response, such as IL-1 $\beta$  production, has been an important topic of pre-clinical animal studies, and clinical research in ACS therapy.<sup>20,21</sup> In the MRC-ILA Heart Study, 182 NSTEMI patients were assigned to anakinra, a recombinant IL-1 receptor antagonist, and the results showed that IL-1 blockade could be sufficient to blunt the acute inflammatory response in

**Table 2** Baseline Demographic and Clinical Characteristics of Low and High NLRP3 Groups

Characteristics	Low NLRP3 (≤3.8850) N=81	High NLRP3 (>3.8850) N=71	P value
Age	66.0±4.6	66.7±4.7	0.307
Male	45 (55.6%)	42 (59.2%)	0.655
BMI	22.7 (20.8–24.0)	22.1 (20.8–23.7)	0.440
Hypertension	42 (51.9%)	35 (49.3%)	0.753
Diabetes mellitus	26 (32.1%)	21 (29.6%)	0.737
Smoking	28 (34.6%)	20 (28.2%)	0.397
Hypercholesterolemia	22 (27.2%)	21 (29.6%)	0.741
Stroke	4 (4.94%)	6 (8.45%)	0.383
<b>The medications during the study, n (%)</b>			
Aspirin	78 (96.3%)	68 (95.8%)	0.545
P2Y12 Inhibitor			
Clopidogrel	77 (95.1%)	62 (87.3%)	0.089
Ticagrelor	3 (3.70%)	4 (5.63%)	0.571
β receptor blocker	42 (51.9%)	36 (50.7%)	0.888
ACEI	40 (49.4%)	37 (52.1%)	0.737
ARB	14 (17.3%)	12 (16.9%)	0.950
PPIs	42 (51.9%)	35 (49.3%)	0.753
H2RA	22 (27.2%)	16 (22.5%)	0.511
Statins	78 (96.3%)	69 (97.2%)	0.760
Nitrates	48 (59.3%)	43 (60.6%)	0.870
<b>ACS</b>			
UAP	61 (75.3%)	46 (64.8%)	0.243
NSTEMI	10 (12.3%)	6 (8.45%)	0.435
STEMI	10 (12.3%)	19 (26.8%)	<b>0.024</b>
<b>Platelet parameters</b>			
PLT (×10 <sup>9</sup> /L)	319.8±60.5	321.8±73.7	0.854
MPV (fL)	9.2±1.3	10.7±0.9	<b>&lt;0.001</b>
PDW (fL)	21.1±1.8	21.4±1.8	0.252
PCT (%)	0.18±0.03	0.18±0.03	0.751
<b>Target lesion</b>			
2-vessel disease	6 (7.4%)	5 (7.0%)	0.950
3-vessel disease	3 (3.7%)	3 (4.2%)	0.588
Multi-vessel disease	10 (12.3%)	9 (12.7%)	0.558
<b>Stent length</b>			
Average number of stents per lesion	1.3±0.3	1.4±0.3	0.230
Average stent length per lesion (mm)	24.9±4.3	25.3±6.0	0.562
Cr (mg/dL)	2.1±2.6	2.2±2.1	0.163
hs-CRP (mg/L)	160.4±6.8	165.4±5.2	<b>0.026</b>
Total cholesterol (mg/dL)	4.7 (4.4–5.3)	4.5 (4.2–5.0)	0.088
IL-1β (pg/mL)	7.3±0.6	7.5±0.6	<b>0.023</b>
LDL-C (mg/dL)	54.5±12.8	58.1±10.4	0.066

(Continued)

**Table 2** (Continued).

Characteristics	Low NLRP3 (≤3.8850) N=81	High NLRP3 (>3.8850) N=71	P value
Blood glucose (mg/dL)	173.8±82.5	165.7±82.9	0.511
LVEF (%)	51.1 (42.8–60.1)	56.0 (45.9–63.1)	<b>0.046</b>

**Note:** Data are presented as mean ± SD or N (%). A p-value smaller than 0.05, indicated by the bold-italic text.

**Abbreviations:** ACS, acute coronary syndrome; BMI, body mass index; Cr, creatinine; hs-CRP, high-sensitivity C-reactive protein; LVEF, left ventricular ejection fraction; LDL-C, low density lipoprotein cholesterol; ACEI, angiotensin converting enzyme I; ARB, angiotensin receptor blocker; PPIs, proton pump inhibitors; H2RA, H2 receptor antagonist; UAP, unstable angina pectoris; NSTEMI: non-ST-segment elevation myocardial infarction; STEMI: ST-segment elevation myocardial infarction.

ACS.<sup>22</sup> Other pre-clinical studies of endogenous or exogenous regulation of IL-1β in ACS models also show the beneficial effects,<sup>8,20,23,24</sup> therefore, IL-1β antagonism could be a promising therapeutic strategy for ACS.

Besides their presence in monocytes, *NLRP3* expression has been recently found in platelets.<sup>25,26</sup> During the activation and thrombosis of platelets, the expression of platelet *NLRP3* is upregulated and the secretion of platelet inflammatory factors, such as IL-1β, increases.<sup>25</sup> Despite the absence of changes in the important platelet surface receptors such as αIIbβ3 integrin, GPIIb and GPVI in *NLRP3*<sup>-/-</sup> mice, *NLRP3*<sup>-/-</sup> mice have a prolonged bleeding time and arterial thrombosis, accompanied by reduced levels of c-Src, Syk, and PLCγ2 phosphorylation. Previous studies have found that engagement of G protein coupled receptors by thrombin can induce the ROS production in platelets, thereby activating platelet *NLRP3*, leading to the assembly of *NLRP3* inflammatory bodies and subsequent caspase-1 activation. Activated caspase-1 transformed immature pre-IL-1β into mature IL-1β, which leads to the phosphorylation of c-Src and Syk and promotes platelet aggregation, spread, and clot shrinkage.<sup>27,28</sup> In our study, the expression of platelet *NLRP3* in patients with ACS was significantly higher than that in healthy individuals. In addition, the STEMI group exhibited the highest *NLRP3* expression, followed by the NSTEMI and UAP groups. These evidences, combined with previous evidence that *NLRP3* is involved in platelet function, indicates an important role of platelet *NLRP3* in the development of ACS.

Platelets are of vital importance for the formation of atherosclerosis, and may promote intracoronary thrombosis if large and super-active.<sup>29</sup> An elevated MPV has been



**Table 3** Univariate and Multivariate Cox Regression Analysis in ACS Patient with PCI

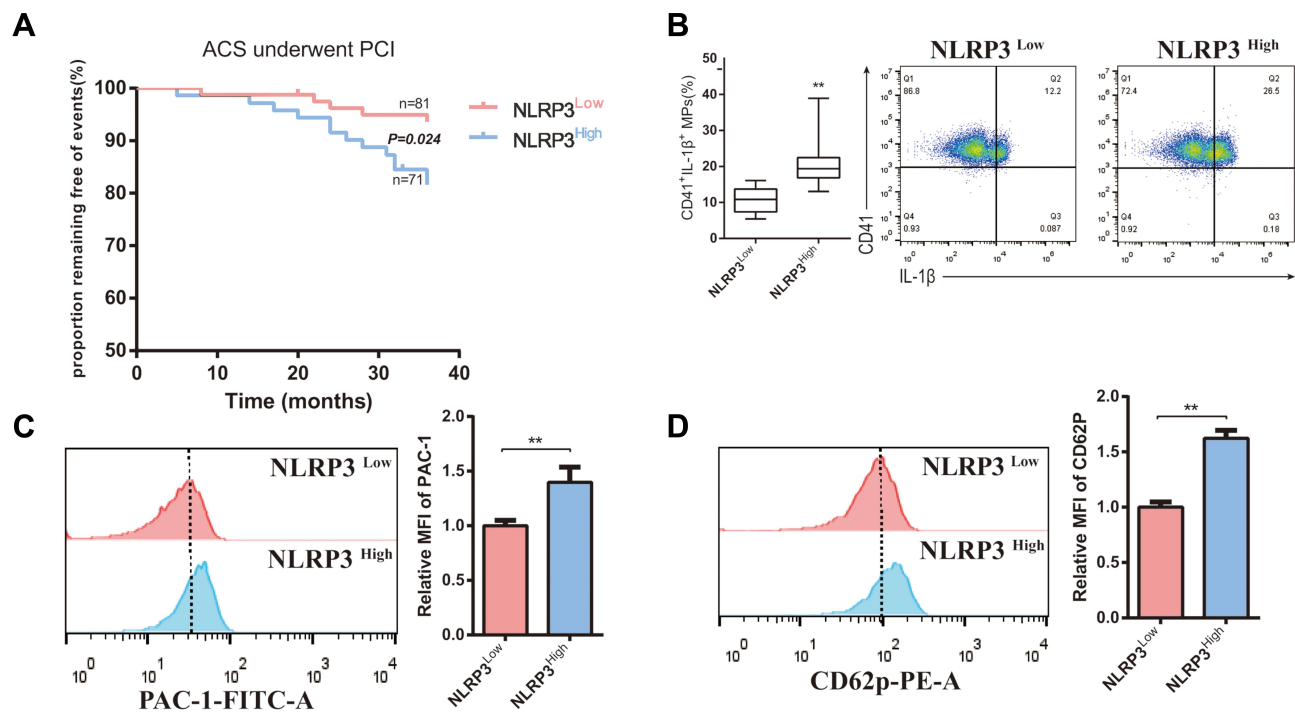
Variable	Single Factor Cox Regression Analysis			Multivariate Cox Regression Analysis		
	OR	95% CI	P value	OR	95% CI	P value
Age	1.056	1.003–1.113	<b>0.039</b>	1.039	0.984–1.098	0.169
Male	1.281	0.769–2.135	0.342			
Hypertension	1.737	1.046–2.883	<b>0.033</b>	1.645	0.988–2.740	0.056
Diabetes	1.257	0.754–2.096	0.380			
Smoke	0.954	0.562–1.619	0.862			
BMI	0.958	0.848–1.082	0.489			
TG	0.929	0.651–1.326	0.685			
TC	0.964	0.173–5.356	0.966			
HDL-C	0.348	0.067–1.822	0.212			
LDL-C	0.993	0.685–1.439	0.971			
Platelet <i>NLRP3</i>	1.084	1.035–1.136	<b>0.001</b>	1.071	1.022–1.123	<b>0.004</b>

**Note:** A p-value smaller than 0.05, indicated by the bold-italic text.

**Abbreviations:** OR, odds ratio; CI, confidence interval; BMI, body mass index; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

demonstrated widely as a prognostic factor for death, myocardial infarction, and composite endpoint events.<sup>30</sup> Therefore, the measurement of MPV at the time of admission is valuable for predicting the degree of arterial openness and the possibility of no-reflow in patients with coronary artery disease (CAD) after PCI.<sup>31,32</sup> Patients with AMI have greater platelet density and higher MPV

values than healthy individuals.<sup>33</sup> With more particles and mitochondria per unit volume, large MPV platelets have a greater ability to produce thromboxane A<sub>2</sub>, and secrete more transforming growth factor- $\beta$ , and more are expressed per unit serosal area of glycoprotein IIb/IIIa.<sup>34</sup> At present, no reports have been published on the expression of platelet *NLRP3* in patients with ACS and its



**Figure 4** Kaplan-Meier curve analysis for estimated proportion of patients remaining free of adverse cardiovascular events. **(A)** The proportion of patients that underwent PCI remaining free of events of in the 3-year follow up was significantly different between the *NLRP3*-low group (n=81) and *NLRP3*-high group (n=71) ( $P=0.024$ ). **(B)** The percentage of  $IL-1\beta$ -expressing platelets was assessed in platelets that were isolated from *NLRP3*-low group (n=81) and *NLRP3*-high group (n=71). **(C)** The expression of PAC-1 in the platelet *NLRP3*-low group (n=81) and *NLRP3*-high group (n=71). **(D)** The expression of CD62p in the platelet *NLRP3*-low group (n=81) and *NLRP3*-high group (n=71). Red line, high *NLRP3* high expression; blue line, low *NLRP3* expression.  $P<0.01$ \*\*.

relationship with the clinical characteristics and prognosis. In our study, the analysis of the platelet parameters in patients with ACS revealed a positive correlation between *NLRP3* expression and MPV ( $r=0.599$ ,  $P<0.001$ ), which was consistent with the involvement of platelet *NLRP3* in platelet activation,<sup>25</sup> suggesting that the upregulation of *NLRP3* may promote platelet activation in ACS.

Patients with ACS may have a poor cardiac function, which manifests as a decreased ejection fraction. The inhibition of *NLRP3* inflammatory bodies/caspase-1/IL-1 $\beta$  can ameliorate cardiac remodeling and attenuate left ventricular systolic dysfunction in mice.<sup>35</sup> Owing to its important role in the measurement of cardiac function, LVEF is an important factor that can be used to predict the prognosis of myocardial infarction.<sup>36</sup> Long-term follow-up showed that LVEF can predict all-cause mortality when echocardiographic measurements were performed for patients after admission as early as possible.<sup>37</sup> We found that platelet *NLRP3* expression levels were negatively correlated with LVEF ( $r=-0.288$ ,  $P<0.001$ ), suggesting that platelet *NLRP3* expression may be associated with the degree of impaired heart function in patients with ACS.

In this study, we analyzed the relationship between the expression of platelet *NLRP3* and Gensini or GRACE risk scores in patients with ACS that underwent PCI for the first time. The Gensini score is a classic criterion used to assess coronary arteries,<sup>38</sup> whereas the GRACE risk score is used to evaluate the severity of CAD. We observed that platelet *NLRP3* expression was positively correlated with the Gensini score ( $r=0.353$ ,  $P<0.001$ ) and GRACE score ( $r=0.561$ ,  $P<0.001$ ), indicating a close relationship between *NLRP3* expression and the severity of CAD.

In the analyses of risk factors for ACEs after PCI in patients with ACS, the Multivariate logistic regression analysis identified platelet *NLRP3* as an independent risk factor ( $P=0.004$ ). The incidence of ACEs was increased ( $P=0.024$ ) in the platelet *NLRP3*-high group after 3 years of follow-up, suggesting that high *NLRP3* expression in platelets was closely related to poor prognosis. The expression of PAC-1 and CD62p was higher in the platelet *NLRP3*-high group, which indicated that high *NLRP3* expression in platelets may be associated with the platelet hyperactivity.

Up to August 30, 2020, coronavirus disease 2019 (COVID-19) has been confirmed in 24854 140 people worldwide, carrying a mortality of approximately 0.15%.<sup>39</sup> COVID-19 has been linked to a number of critical cardiovascular complications, including ACS, and even individuals without a history of cardiovascular disease are at risk of cardiovascular complications.<sup>40</sup> COVID-19-

stimulated innate immune response can triggers activation of the *NLRP3* inflammasome.<sup>41</sup> The relationship between COVID-19 and prognosis of ACS patients is still largely unknown. It is worth exploring to find out the connection between COVID-19 associated ACS and platelet *NLRP3*.

This study, for the first time, detected the expression of platelet *NLRP3* in patients with ACS, and explored the relationship between platelet *NLRP3* expression levels and the clinicopathological characteristics/prognosis of ACS, to provide a novel potential prognostic biomarker and therapeutic target in ACS.

## Limitations

Our study had several potential limitations. First, the sample size of the second cohort was small and subgroup analyses were not carried out. Second, circulating levels of IL-1 $\beta$  at different timeframes from ACS were not monitored, which may further explain the role of IL-1 $\beta$  in ACS. Finally, the patients included in the current analysis were whole spectrum of ACS and the prognostic role of platelet *NLRP3* in patients with NSTEMI and UAP should be verified in future studies.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

## References

1. Yang N, Liu J, Liu J, et al. Performance on management strategies with Class I Recommendation and a level of evidence among hospitalized patients with non-ST-segment elevation acute coronary syndrome in China: findings from the improving care for Cardiovascular Disease in China-Acute Coronary Syndrome (CCC-ACS) project. *Am Heart J*. 2019;212:80–90.

2. Bonaventura A, Vecchié A, Abbate A, Montecucco F. Neutrophil extracellular traps and cardiovascular diseases: an update. *Cells*. 2020;9(1):231. doi:10.3390/cells9010231
3. Duewell P, Kono H, Rayner KJ, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature*. 2010;464(7293):1357–1361. doi:10.1038/nature08938
4. Kawaguchi M, Takahashi M, Hata T, et al. Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. *Circulation*. 2011;123(6):594–604. doi:10.1161/CIRCULATIONAHA.110.982777
5. Wang L, Qu P, Zhao J, Chang Y. NLRP3 and downstream cytokine expression elevated in the monocytes of patients with coronary artery disease. *Arch Med Sci*. 2014;10(4):791–800. doi:10.5114/aoms.2014.44871
6. Deng M, Brickey WJ, Guo H, et al. Platelet activating factor as a novel danger signal for activation of NLRP3 inflammasome. *J Immunol*. 2018;200(1 Supplement):113–115.
7. Berger JS. Oral antiplatelet therapy for secondary prevention of acute coronary syndrome. *Am J Cardiovasc Drugs*. 2018;18(6):457–472. doi:10.1007/s40256-018-0291-2
8. Everett BM, Cornel JH, Lainscak M, et al. Anti-inflammatory therapy with canakinumab for the prevention of hospitalization for heart failure. *Circulation*. 2019;139(10):1289–1299. doi:10.1161/CIRCULATIONAHA.118.038010
9. Schrör K, Huber K. Platelets, inflammation and anti-inflammatory drugs in ACS and CAD. *Thromb Haemost*. 2015;114(3):446–448. doi:10.1160/TH15-08-0632
10. Heger LA, Hortmann M, Albrecht M, et al. Inflammation in acute coronary syndrome: expression of TLR2 mRNA is increased in platelets of patients with ACS. *PLoS One*. 2019;14(10):e0224181. doi:10.1371/journal.pone.0224181
11. Orrem HL, Nilsson PH, Pischke SE, et al. IL-6 Receptor inhibition by tocilizumab attenuated expression of C5a Receptor 1 and 2 in Non-ST-elevation myocardial infarction. *Front Immunol*. 2018;9:2035. doi:10.3389/fimmu.2018.02035
12. Wu H, Qian J, Sun A, Wang Q, Ge J. Association of CYP2C19 genotype with periprocedural myocardial infarction after uneventful stent implantation in Chinese patients receiving clopidogrel pretreatment. *Circ J*. 2012;76(12):2773–2778. doi:10.1253/circj.CJ-12-0635
13. Oz TK, Özbilgin N, Sungur A, et al. Prevalence of metabolic syndrome in young patients with ST-elevation myocardial infarction. *Int J Cardiovasc Acad*. 2018;4(3):53.
14. Lopes RD, de Barros ESPGM, Damiani LP, et al. Major adverse cardiovascular events after 12 months among patients with acute coronary syndrome receiving loading doses of atorvastatin prior to planned PCI. *JAMA*. 2020;323(8):787–789. doi:10.1001/jama.2020.0118
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25(4):402–408. doi:10.1006/meth.2001.1262
16. Baldrihi M, Mallat Z, Li X. NLRP3 inflammasome pathways in atherosclerosis. *Atherosclerosis*. 2017;267:127–138. doi:10.1016/j.atherosclerosis.2017.10.027
17. Marchetti C, Chojnacki J, Toldo S, et al. A novel pharmacologic inhibitor of the NLRP3 inflammasome limits myocardial injury after ischemia-reperfusion in the mouse. *J Cardiovasc Pharmacol*. 2014;63(4):316–322. doi:10.1097/FJC.0000000000000053
18. Liu Y, Lian K, Zhang L, et al. TXNIP mediates NLRP3 inflammasome activation in cardiac microvascular endothelial cells as a novel mechanism in myocardial ischemia/reperfusion injury. *Basic Res Cardiol*. 2014;109(5):415. doi:10.1007/s00395-014-0415-z
19. Szekely Y, Arbel YJ. A review of Interleukin-1 in heart disease: where do we stand today? *Cardiol Ther*. 2018;7(1):25–44.
20. Mahtta D, Sudhakar D, Koneru S, et al. Targeting inflammation after myocardial infarction. *Curr Cardiol Rep*. 2020;22(10):110. doi:10.1007/s11886-020-01358-2
21. Bullenkamp J, Mengoni V, Kaur S, et al. Interleukin-7 and interleukin-15 drive CD4+CD28null T lymphocyte expansion and function in patients with acute coronary syndrome. *Cardiovasc Res*. 2020. doi:10.1093/cvr/cvaa202
22. Morton AC, Rothman AM, Greenwood JP, et al. The effect of interleukin-1 receptor antagonist therapy on markers of inflammation in non-ST elevation acute coronary syndromes: the MRC-ILA Heart Study. *Eur Heart J*. 2015;36(6):377–384. doi:10.1093/eurheartj/ehu272
23. Mann DL. Innate immunity and the failing heart: the cytokine hypothesis revisited. *Circ Res*. 2015;116(7):1254–1268. doi:10.1161/CIRCRESAHA.116.302317
24. Abbate A, Van Tassel BW, Biondi-Zoccai G, et al. Effects of interleukin-1 blockade with anakinra on adverse cardiac remodeling and heart failure after acute myocardial infarction [from the Virginia Commonwealth University-Anakinra Remodeling Trial (2) (VCU-ART2) pilot study]. *Am J Cardiol*. 2013;111(10):1394–1400. doi:10.1016/j.amjcard.2013.01.287
25. Murthy P, Durco F, Miller-Ocuin JL, et al. The NLRP3 inflammasome and bruton's tyrosine kinase in platelets co-regulate platelet activation, aggregation, and in vitro thrombus formation. *Biochem Biophys Res Commun*. 2017;483(1):230–236. doi:10.1016/j.bbrc.2016.12.161
26. Paramel Varghese G, Folkersen L, Strawbridge RJ, et al. NLRP3 inflammasome expression and activation in human atherosclerosis. *J Am Heart Assoc*. 2016;5(5):e003031. doi:10.1161/JAHA.115.003031
27. Qiao J, Wu X, Luo Q, et al. NLRP3 regulates platelet integrin αIIbβ3 outside-in signaling, hemostasis and arterial thrombosis. *Haematologica*. 2018;103(9):1568–1576. doi:10.3324/haematol.2018.191700
28. Haneklaus M, O'Neill LA. NLRP3 at the interface of metabolism and inflammation. *Immunol Rev*. 2015;265(1):53–62. doi:10.1111/imr.12285
29. Ju HY, Kim JK, Hur SM, et al. Could mean platelet volume be a promising biomarker of progression of chronic kidney disease? *Platelets*. 2015;26(2):143–147. doi:10.3109/09537104.2014.890179
30. Koh YY, Kim HH, Choi DH, et al. Relation between the change in mean platelet volume and clopidogrel resistance in patients undergoing percutaneous coronary intervention. *Curr Vasc Pharmacol*. 2015;13(5):687–693. doi:10.2174/1570161112666141017121118
31. Colkesen Y, Coskun I, Muderrisoglu H. The effect of aspirin on mean platelet volume in patients with paroxysmal atrial fibrillation. *Platelets*. 2013;24(4):263–266. doi:10.3109/09537104.2012.682106
32. Haungsaitong R, Udommongkol C, Nidhinandana S, et al. The changes in mean platelet volume after using of antiplatelet drugs in acute ischemic stroke: a randomized controlled trial. *J Med Assoc Thai*. 2015;98(9):852–857.
33. Martin JF, Plumb J, Kilbey RS, Kishk YT. Changes in volume and density of platelets in myocardial infarction. *Br Med J (Clin Res Ed)*. 1983;287(6390):456–459. doi:10.1136/bmj.287.6390.456
34. Murphy AJ, Tall AR. Disordered haematopoiesis and athero-thrombosis. *Eur Heart J*. 2016;37(14):1113–1121.
35. Carbone S, Mauro AG, Prestamburgo A, et al. An Orally Available NLRP3 inflammasome inhibitor prevents western diet-induced cardiac dysfunction in mice. *J Cardiovasc Pharmacol*. 2018;72(6):303–307. doi:10.1097/FJC.0000000000000628
36. Hamada S, Schroeder J, Hoffmann R, et al. Prediction of outcomes in patients with chronic ischemic cardiomyopathy by layer-specific strain echocardiography: a proof of concept. *J Am Soc Echocardiography*. 2016;29(5):412–420.
37. Zghal FM, Boudiche S, Haboubi S, et al. Diagnostic accuracy of strain imaging in predicting myocardial viability after an ST-elevation myocardial infarction. *Medicine*. 2020;99(19):e19528.
38. Nishimura RA, Otto CM, Bonow RO, et al. 2017 AHA/ACC focused update of the 2014 AHA/ACC guideline for the management of patients with valvular heart disease. *Rep Am Coll Cardiol Am Heart Assoc Task Force Clin PractGuidelines*. 2017;70(2):252–289.

39. Coronavirus disease (COVID-19). Weekly epidemiological update. 2020. 8(31).
40. Zheng -Y-Y, Ma Y-T, Zhang J-Y, Xie XJNRC. COVID-19 and the cardiovascular system. *Nat Rev Cardiol.* 2020;17(5):259–260.
41. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *The Lancet.* 2020;395 (10223):497–506.

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