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Utility of the ACD-GENE-CLI Score in Asian Patients with Critical Limb Ischemia Undergoing Endovascular Interventions

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Aims: Critical limb ischemia (CLI) is an emerging public health threat and lacks a reliable score for predicting the outcomes. The Age, Body Mass Index, Chronic Kidney Disease, Diabetes, and Genotyping (ABCD-GENE) risk score helps identify patients with coronary artery disease who have cytochrome P450 2C19 (*CYP2C19*) polymorphism-related drug resistance and are at risk for cardiovascular adverse events. However, its application to CLI remains unknown. In this study, we aim to validate a modified ACD-GENE-CLI score to improve the prediction of major adverse limb events (MALEs) in patients with CLI receiving clopidogrel.

Methods: Patients with CLI receiving clopidogrel post-endovascular intervention were enrolled prospectively in two medical centers. Amputation and revascularization as MALEs were regarded as the outcomes.

Results: A total of 473 patients were recruited, with a mean follow-up duration of 25 months. Except for obesity, old age, diabetes, chronic kidney disease (CKD), and *CYP2C19* polymorphisms were significantly associated with MALEs. Using bootstrap regression analysis, we established a modified risk score (ACD-GENE-CLI) that included old age (≥ 65 years), diabetes, CKD, and *CYP2C19* polymorphisms. At a cutoff value of 8, the ACD-GENE-CLI score was superior to the *CYP2C19* deficiency only, and the conventional ABCD-GENE score in predicting MALEs (area under the curve: 0.69 vs. 0.59 vs. 0.67, $p=0.01$). The diagnostic ability of the ACD-GENE-CLI score was consistent in the external validation. Also, Kaplan–Meier curves showed that in *CYP2C19* deficiency, the ABCD-GENE and ACD-GENE-CLI scores could all differentiate patients with CLI who are free from MALEs.

Conclusions: The modified ACD-GENE-CLI score could differentiate patients with CLI receiving clopidogrel who are at risk of MALEs. Further studies are required to generalize the utility of the score.

Key words: ACD-GENE-CLI score, Critical limb ischemia, Clopidogrel, *CYP2C19* polymorphism

Abbreviations: ACEI/ARB: angiotensin converting enzyme inhibitors/angiotensin Ⅱ receptor blockers; BMI: body mass index; CAD: coronary artery disease; CKD: chronic kidney disease; *CYP2C19*: cytochrome P450 2C19; DM: diabetes mellitus; eGFR: estimated glomerular filtration rate; HPR: high on-treatment platelet reactivity; LDL: low-density lipoprotein; LOF: loss of function; MACEs: major adverse cardiovascular events; MALEs: major adverse limb events; CLI: critical limb ischemia

Introduction

Critical limb ischemia (CLI) is a clinical phenomenon of ischemic pain at rest or in poorly healing ulcers or gangrene, caused by peripheral artery disease (PAD)[1-4]. With an estimated incidence of 500–1000 million per year, it mainly occurs among elders with comorbidities[2]. To note, CLI contributes to the rate of limb loss and amputation from 10% to 40% every year and has become an emerging public health threat[1, 5]. CLI management has evolved from open surgery to an endovascular approach, and the refinements in endovascular intervention have improved its outcomes[6]. To maintain the patency of target vessels after endovascular intervention, the use of antiplatelet drugs to suppress platelet reactivity is crucial[7-9]. Clopidogrel is frequently prescribed in Asians and is associated with a lower risk of gastrointestinal bleeding compared with aspirin or other P2Y12 inhibitors[8, 10-12]. However, loss-of-function (LOF) alleles of the cytochrome P450 2C19 (*CYP2C19*) enzyme are associated with a poor response to clopidogrel and result in high platelet reactivity[8, 13]. Compared with non-carriers, *CYP2C19* LOF allele carriers, especially in Asian populations, were found to have a greater risk of adverse cardiovascular events while receiving clopidogrel for coronary artery disease (CAD)[8, 13]. In contrast to the relatively low prevalence (2%–5%) of *CYP2C19* poor metabolizers in Caucasians and Africans, its prevalence in Asians can be up to 15%[13, 14]. To note, Lee *et al.* reported that *CYP2C19* genetic profiles significantly differentiated clinical outcomes in patients with CLI receiving clopidogrel after endovascular interventions[15]. Thus, whether genetic testing is worthwhile for decision-making regarding drug therapy remains debatable[16, 17].

The Age, Body Mass Index, Chronic Kidney Disease, Diabetes, and Genotyping (ABCD-GENE) risk score has been developed by Angiolillo *et al.* to assess the high on-treatment platelet reactivity (HPR) status and the risk of major adverse cardiovascular events (MACEs) in patients with CAD receiving clopidogrel[18]. The score incorporates four clinical parameters: old age (>75 years), body mass index (BMI) >30 kg/m^2, chronic kidney disease [CKD, determined as an estimated glomerular filtration rate (eGFR) of <60 ml/min according to the Cockcroft–Gault formula], and diabetes mellitus (DM); and the genetic parameters of *CYP2C19* LOF alleles[18]. Previous studies have shown that a cutoff score of ≥ 10 was sensitive in identifying patients with HPR status and was correlated with a higher risk of MACEs[18]. Two independent CAD cohorts in France, the Popular (Do Platelet Function Assays Predict Clinical Outcomes in Clopidogrel-Pretreated Patients Undergoing Elective PCI) and the FAST-MI (Registry of Acute ST-Elevation and Non–ST-Elevation Myocardial Infarction) registries, also validated this score[19, 20]. However, the frequencies of *CYP2C19* LOF and obesity vary between Asian and Western populations[8, 13, 21]. Besides, whether this score can also be applied in the risk assessment of patients with CLI remains unknown.

Thus, in the present study, we assessed the ABCD-GENE score's ability to predict the development of major adverse limb events (MALEs) in Asian patients treated with clopidogrel after endovascular interventions. Based on the clinical and genetic parameters associated with MALEs, we optimized the score and developed a modified score with a better prediction for the risk of MALEs in the Asian population.

Material and Methods

Patients and The Study Design

We prospectively and consecutively collected clinical data and blood samples from patients with CLI who underwent endovascular interventions at two medical centers from January 2018 to December 2021. Patients with CLI who presented with rest pain, ischemic ulceration, or gangrene were classified according to the Fontaine III or IV classification[2, 3]. Patients should be above 20 years old and have been receiving clopidogrel for more than one year after the intervention, which included either balloon angioplasty or stenting. Patients with end-stage renal disease, a predicted lifespan of less than one year, scheduled amputation surgeries or bypass surgery, and receiving no interventions were excluded. The derivation cohort (CMMC cohort) were patients who were recruited from Chi Mei Medical Center of Tainan City, and those assigned to the validation cohort (KMUH cohort) were patients treated at Kaohsiung Medical University Hospital of Kaohsiung. The flow chart of the study design is displayed in **Fig. 1**. Demographic data, including age, BMI,

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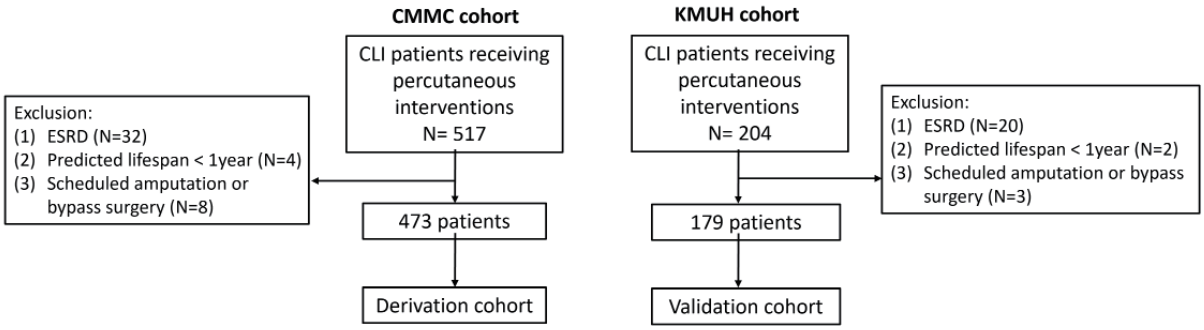

Fig. 1. The study design flow chart

underlying diseases, biochemistry tests, Fontaine classification, locations or main lesions, types of endovascular interventions, and drug prescriptions were collected. CKD was defined as an eGFR of <60 ml/min/1.73 [2, 22]. DM was defined as a glycated hemoglobin of $>6.5\%$[23]. As our primary outcome, MALE endpoints were defined as amputation or revascularization. The study was conducted in strict accordance with the Declaration of Helsinki on Biomedical Research involving Human Subjects and was approved by the local ethics committee (Institutional Review Board approval no. 10705-003). The enrolled patients provided written informed consent for their participation.

Measurement of *CYP2C19* Polymorphism

The blood samples were collected during endovascular interventions. Genomic DNA was isolated from the patient's blood samples, as previously described[8, 18]. The TaqMan single nucleotide polymorphism genotyping assay (Applied Biosystems, Foster City, CA, USA) was used to measure the LOF alleles of *CYP2C19**2 (681G>A; rs4244285) and *CYP2C19**3 (636G>A; rs4986893) using an Applied Biosystems 7500 Real-Time Polymerase Chain Reaction System. Patients were classified into three genotype groups accordingly: normal metabolizers (*1/*1, no LOF allele), intermediate metabolizers (*1/*2 or *1/*3, one LOF allele), and poor metabolizers (*2/*2, *2/*3, or *3/*3, two LOF alleles).

ABCD-GENE Risk Score Modification

As previously described[18], the ABCD-GENE risk score was calculated using one genetic and four clinical parameters as follows: age >75 years (4 points), obesity (BMI >30 kg/m^2) (4 points), CKD (3 points), DM (3 points), one *CYP2C19* LOF allele (6 points), and two LOF alleles (24 points). A cutoff score of ≥ 10 was applied to predict HPR to clopidogrel.

Statistical Analyses

Categorical variables are presented numerically and as percentages and compared using the chi-square test of heterogeneity or Fisher's exact test. Continuous variables are presented as means with standard deviation, or median with interquartile range according to the data distribution. Comparison tests were estimated using Student's *t*-test or Mann–Whitney *U* test. Statistical tests were 2-sided, and a p value of <0.05 indicated statistical significance. Odds ratios (ORs) and 95% confidence intervals (CIs) were assessed using logistic regression. On the basis of the derivation cohort (CMMC cohort), univariate and multivariate regression analyses were performed to assess the association between key factors and MALE outcomes. Factors with a statistical significance were integrated for further score modification. Based on the selected independent risk factors, we applied a multivariate logistic regression model with 1,000 bootstrap samples to modify the score and designated it as the "ACD-GENE-CLI score." To compare the two scores and *CYP2C19* deficiency only (one allele or two alleles), receiver operating characteristic (ROC) curve analyses were performed based on the presence of MALEs to assess the diagnostic ability with the area under the curve (AUC) corresponding to the maximum average sensitivity and specificity. Furthermore, to evaluate the ability of the new model in correctly reclassifying and categorizing different risks, net reclassification index (NRI) was also applied[24]. The validation cohort (KMUH cohort) was included for external validation to ensure the generalizability of the ACD-GENE-CLI score. Subsequently, Kaplan–Meier curves were utilized to understand MALEs-free survival based on the cutoff values of the ACD-GENE-CLI scores. The best cutoff value was used for the sensitivity and specificity analysis. In addition, by separately considering the outcomes of amputation and revascularization, subgroup analyses were performed using the ACD-

GENE-CLI score as either a continuous or dichotomized variable. Model 1 included all patients enrolled in the CMMC cohort. In Model 2, as a sensitivity test, we excluded patients who reached the endpoint within the first month of enrollment. Further, we performed another sensitivity test focusing on patients with or without antiplatelet agent combination therapies after endovascular interventions. Combination therapy was defined as clopidogrel plus aspirin or cilostazol for more than three months. In a sensitivity test, patients in the CMMC cohort were divided into those who received clopidogrel only and those who received a combination therapy. Statistical Package for the Social Sciences software (Version 22.0; IBM, Armonk, NY, USA) was used for all statistical analyses.

Results

Baseline Characteristics of The Derivation (CMMC) Cohort

During the study period, 473 and 179 patients were recruited into the CMMC and KMUH cohorts, respectively. All patients received clopidogrel during the follow-up period, while the mean follow-up duration was 25 months after endovascular interventions. In the CMMC cohort **(Table 1)**, the mean age was 66.9±13.4, with 153 (32.3%) patients who are >75 years old. Obesity was found in 13.5% of patients in this cohort. Hypertension was the most common comorbidity, and more than half of the patients have DM (53.3%) and about one-third have CKD (32.7%). In terms of endovascular interventions, half of the patients had stenotic lesions above the knee, at the level of the iliac, common femoral, and superficial femoral arteries. Around 40% of the studied population were classified at Fontaine stage Ⅳ while the rest are at Fontaine stage Ⅲ. Around 40% received stenting while the others received balloon angioplasty. Regarding the *CYP2C19* allele, 47.1% of the patients had at least one LOF allele, while 11 (2.3%) patients had two LOF alleles.

In the cohort, 29.4% (139/473) of the patients developed MALEs, with 80 patients receiving revascularization and 59 patients having an amputation. In comparison with those who were free from MALEs, the average age of patients who developed MALEs was greater (69.1±12.8 vs. 66.2±12.6 years, p=0.02; **Table 1**). In addition, patients with CLI who developed MALEs presented with more underlying DM (66.2% vs. 47.9%, p=0.01) and CKD (74.8% vs. 61.4%, p<0.01). Conversely, obesity, hypertension, CAD, smoking, and concomitant use of other cardiovascular drugs, including aspirin, cilostazol, anticoagulants, angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, and statins, were not significantly different between the two groups. Concerning genetic polymorphisms, the *CYP2C19* LOF allele was significantly associated with developing MALEs. The majority (66.2%) of patients who developed MALEs had at least one LOF allele, compared with 39.2% of those free from MALEs (p< 0.01). The distribution of the ABCD-GENE scores are presented in the bar charts in **Fig. 2A**. As the scores increased, more patients developed MALEs. When the score exceeded 10, more than half of the patients reached the endpoint.

Prediction of The Development of MALEs Using The ABCD-GENE Score

Logistic regression analysis revealed that CKD (OR: 1.88, 95% CI: 1.21–2.93, p=0.005), DM (OR: 2.14, 95% CI: 1.42–3.2, p=0.001), and the *CYP2C19* LOF allele (one allele, OR: 2.35, 95% CI: 1.57–3.52, p=0.001; two alleles, OR: 11.49, 95% CI: 2.4–53.9, p=0.002) were significantly associated with MALE outcomes **(Table 2)**. Notably, old age at the cutoff value of 65 years (OR: 2.08, 95% CI: 1.35–3.19, p=0.001) instead of 75 years (OR: 1.44, 95% CI: 0.95–2.19, p=0.08) specifically correlated with MALEs. Obesity, which was included in the original ABCD-GENE scores, failed to show a significant association with MALEs. Similarly, in the multivariable analysis, >65 years of age (OR: 1.94, 95% CI: 1.19–3.17, p=0.008), CKD (OR: 1.25, 95% CI: 1.03–2.1, p=0.040), DM (OR: 1.17, 95% CI: 1.01–1.73, p=0.020), and the *CYP2C19* LOF allele (one allele, OR: 2.74, 95% CI: 1.78–4.21, p=0.001; two alleles, OR:20.42, 95% CI:4.21–90.8, p=0.001) were still significantly associated with the development of MALEs.

Establishment of The ACD-GENE-CLI Score and Comparison with The ABCD-GENE Score

Given the differences between MALE-associated risk parameters in the present CMMC cohort compared with the ABCD-GENE scores, we used the bootstrap regression analysis to establish a modified risk score, designated as ACD-GENE-CLI. As shown in the analysis in **Table 3**, each of the variables was assigned an integer weighted score of 1.5 proportional to the OR for the development of MALEs. The integer points assigned included age >65 years (+3 points), CKD (+3 points), DM (+3 points), and *CYP2C19* polymorphism (one LOF allele +5 points; two LOF alleles +10 points). Compared with the distribution of the ABCD-GENE scores in case

Table 1. Baseline characteristics in patients with critical limb ischemia (CLI) with and without major adverse limb events (MALEs) in the CMMC cohort (N=473)

Variable	All population (N=473)	MALE (-) (N=334)	MALE (+) (N=139)	*P* value*
Age (y/o)	66.9 ± 13.4	66.2 ± 12.6	69.1 ± 12.8	0.02
Age >75 y/o, *n* (%)	153 (32.3)	100 (29.9)	53 (38.1)	0.05
Age >65 y/o, *n* (%)	285 (60.2)	185 (55.3)	100 (71.9)	0.001
Men, *n* (%)	304 (64.2)	222 (65.9)	82 (59)	0.14
BMI (kg/m^2)	25.1 ± 4.7	25.1 ± 4.5	24.5 ± 4.7	0.23
BMI >25 kg/m^2, *n* (%)	208 (43.9)	152 (45.5)	56 (40.3)	0.36
BMI >30 kg/m^2, *n* (%)	64 (13.5)	47 (14.1)	17 (12.2)	0.35
Medical history				
Hypertension, *n* (%)	333 (70.4)	242 (72.5)	91 (65.4)	0.08
DM, *n* (%)	252 (53.3)	160 (47.9)	92 (66.2)	0.01
Hyperlipidemia, *n* (%)	286 (60.5)	199 (59.5)	87 (62.5)	0.08
CAD, *n* (%)	129 (27.3)	90 (26.9)	39 (28)	0.8
Previous stroke, *n* (%)	36 (7.6)	27 (8.1)	9 (6.5)	0.69
Heart failure, *n* (%)	29 (6.2)	17 (5.1)	12 (8.6)	0.06
Atrial fibrillation, *n* (%)	61 (12.9)	48 (14.3)	13 (9.3)	0.17
Cancer, *n* (%)	34 (7.2)	28 (8.3)	6 (4.3)	0.18
Smoking, *n* (%)	320 (67.6)	230 (68.8)	90 (64.7)	0.07
Lab data				
eGFR (ml/min/1.73 ^{2})	53.5 ± 40.3	58.6 ± 41.7	42.6 ± 35.7	0.007
CKD (eGFR<60), *n* (%)	169 (35.7)	205 (61.4)	104 (74.8)	0.006
Fasting glucose (mg/dl)	173.6 ± 79.9	166.7 ± 75.1	184.6 ± 86.6	0.07
Cholesterol (mg/dl)	153.4 ± 42.6	153.5 ± 36.7	155.5 ± 52.5	0.78
LDL (mg/dl)	91.5 ± 41.8	91.6 ± 36.4	93.5 ± 50.2	0.80
Triglyceride (mg/dl)	165.1 ± 209.9	175.7 ± 229.8	126.1 ± 43.3	0.16
Endovascular angiography and interventions				
Location (above knee), *n* (%)	259 (54.7)	176 (52.7)	83 (59.7)	0.18
Stenting, *n* (%)	192 (40.6)	130 (38.9)	62 (44.6)	0.26
Fontaine stage Ⅳ	186 (39.3)	123 (36.8)	63 (45.3)	0.09
Cardiovascular drugs				
Aspirin, *n* (%)	80 (16.9)	54 (16.2)	26 (18.7)	0.66
Cilostazol, *n* (%)	45 (9.5)	26 (7.8)	19 (13.6)	0.56
ACEIs/ARBs, *n* (%)	228 (48.2)	167 (50)	61 (43.8)	0.26
Statins, *n* (%)	257 (54.3)	188 (56.3)	69 (49.6)	0.22
*CYP2C19**2 alleles				
None, *n* (%)	250 (52.9)	203 (60.8)	47 (33.8)	0.001
One allele LOF, *n* (%)	212 (44.8)	129 (38.6)	83 (59.7)	0.001
Two alleles LOF, *n* (%)	11 (2.3)	2 (0.6)	9 (6.5)	0.001

**P* value represents for the comparison between MALE(-) and MALE (+)
DM= diabetes mellitus; CAD= coronary arterial disease; eGFR= estimated glomerular filtration rate; CKD= chronic kidney disease; LDL= low-density lipoprotein; ACEI/ARB= angiotensin converting enzyme inhibitors/angiotensin Ⅱ receptor blockers; LOF=loss of function

numbers and the percentage of MALEs in patients with CLI **(Fig. 2A)**, the distribution of the ACD-GENE-CLI scores showed a more positive correlation between the score and the percentage of MALEs **(Fig. 2B)**.

A logistic regression analysis was performed to examine the efficacy of *CYP2C19* deficiency (one allele or two alleles) and the scores in predicting MALEs. Only *CYP2C19* deficiency was positively associated with MALEs (OR: 1.07, 95% CI: 1.03–1.17, p=0.001). Likewise, both scores also correlated with the outcomes: ABCD-GENE score, OR: 1.13, 95% CI: 1.08–1.17, p=0.001; ACD-GENE-CLI score, OR: 1.2, 95% CI: 1.13–1.26, p=0.001 **(Table 4)**. Even though using the previously validated cutoff value of 10 in the ABCD-GENE score could

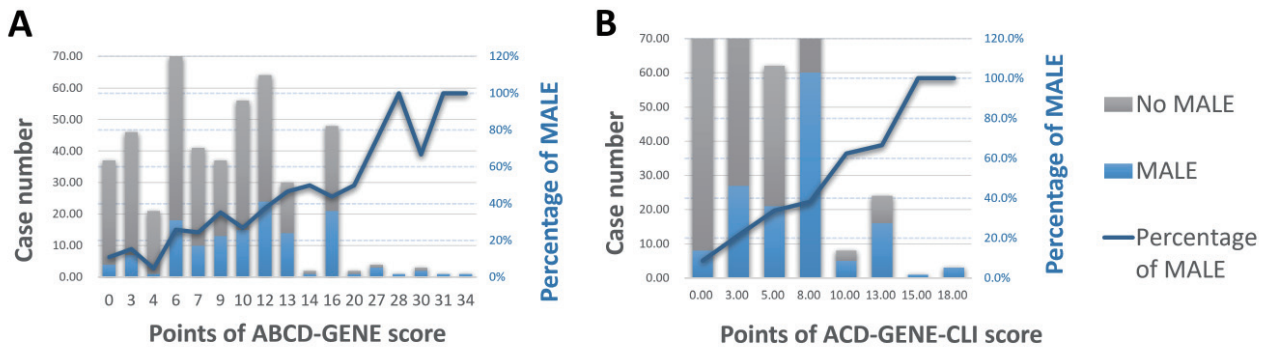

Fig. 2. Distribution of the (A) ABCD-GENE and (B) ACD-GENE-CLI scores in case numbers and percentage of MALEs in patients with CLI (CMMC cohort)

Table 2. The univariate and multivariable logistic regression analyses of clinical and genetic risk parameters in predicting MALEs in CMMC cohort

Parameters	Univariate analysis			Multivariable analysis		
	Odds ratio	95% CI	*p* value	Odds ratio	95% CI	*p* value
Age >75 y/o	1.44	0.95-2.19	0.08			
Age >65 y/o	2.08	1.35-3.19	0.001	1.94	1.19-3.17	0.008
Obesity (BMI>30)	0.85	0.4-1.5	0.59			
CKD	1.88	1.21-2.93	0.005	1.25	1.03-2.1	0.040
Diabetes	2.14	1.42-3.2	0.001	1.17	1.01-1.73	0.020
One allele	2.35	1.57-3.52	0.001	2.74	1.78-4.21	0.001
Two alleles	11.49	2.4-53.9	0.002	20.42	4.21-90.8	0.001

Abbreviations as listed in Table 1

Table 3. The Bootstrap Regression derived points in ACD-GENE-CLI score

Parameters	Bootstrap Regression			Integer points assigned
	Odds ratio	95% CI	*P* value	
Clinical factors				
Old age (>65 y/o)	2.04	1.10-3.55	0.022	+3
CKD (eGFR<60)	1.93	1.19-3.12	0.006	+3
Diabetes	1.89	1.12-3.03	0.016	+3
Genetic factors				
One allele	3.69	1.57-4.50	<0.001	+5
Two alleles	6.89	5.29-19.26	<0.001	+10

Abbreviations as listed in Table 1

differentiate patients at risk for MALEs (OR: 2.44, 95% CI: 1.62–3.68, p=0.001), it was shown that an ACD-GENE score of ≥ 9 was more sensitive in correlating with the risks of MALEs in our study (OR: 2.77, 95% CI: 1.81–4.25, p=0.001). Further, using a cutoff value of 8 (the median value in the ACD-GENE-CLI score), patients who had the highest risk of MALEs scored ≥ 8 (OR: 3.92, 95% CI: 2.52–6.08, p=0.001). In the ROC curve analyses shown in **Fig. 3A**, compared with the *CYP2C19* deficiency and ABCD-GENE score, the ACD-GENE-CLI score was more significantly associated with MALEs (AUC 0.69 vs. 0.59 vs. 0.67, p=0.01). If we merely compared the ACD-GENE-CLI score with the conventional

Table 4. The logistic regression analyses of *CYP2C19* deficiency, ABCD-GENE and ACD-GENE-CLI scores associated with the development of MALEs

Parameters	Logistic regression analysis		
	Odds ratio	95% CI	*p* value
CYP2C19 deficiency	1.07	1.03-1.17	0.001
ABCD-GENE	1.13	1.08-1.17	0.001
ABCD-GENE ≥ 8	2.77	1.81-4.25	0.001
ABCD-GENE ≥ 9	2.79	1.82-4.27	0.001
ABCD-GENE ≥ 10	2.44	1.62-3.68	0.001
ACD-GENE-PAD	1.2	1.13-1.26	0.001
ACD-GENE-CLI ≥ 7	3.56	2.26-5.61	0.001
ACD-GENE-CLI ≥ 8	3.92	2.52-6.08	0.001
ACD-GENE-CLI ≥ 9	2.79	1.84-4.23	0.001

Abbreviations as listed in Table 1

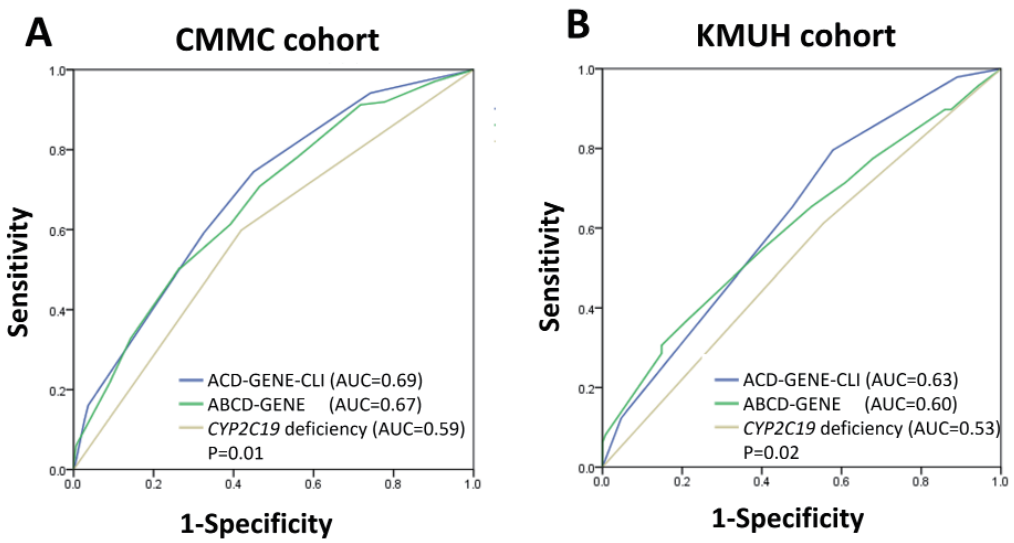

Fig. 3. ROC curves of *CYP2C19* deficiency (one alle or two alleles), the ABCD-GENE score, and the ACD-GENE-CLI score in predicting MALEs in the (A) CMMC cohort and (B) KMUH cohort

ABCD-GENE score, the difference was not significant ($p=0.08$). However, as we reclassified the population according to those with MALEs ($n=139$) and those without MALEs ($n=334$), NRI was calculated as 16.5% and significantly differentiated the risks of MALEs in comparison with the ACD-GENE-CLI and ABCD-GENE scores ($p<0.001$). Further, using ACD-GENE-CLI score ≥ 8, the sensitivity and specificity are above 60%, with a less positive predictive value specificity (45.8%, 95% CI: 38.7%–53.2%) but a better negative predictive value (82.1%, 95% CI: 77.1%–86.4%) **(Supplemental Table 3)**. Nevertheless, the accuracy statistics of the score was superior to those using only *CYP2C19* deficiency or the ABCD-GENE score. Further, as shown in **Fig. 4**, *CYP2C19* deficiency and the ABCD-GENE (using a cutoff value of 9) and ACD-GENE-CLI (using a cutoff value of 8) scores could all sensitively differentiate patients with CLI free from MALEs in the Kaplan–Meier plotter.

External Validation of the ACD-GENE-CLI Score

To further examine whether the ACD-GENE-CLI score is applicable to other populations with CLI, we included an external cohort with 179 patients from KMUH. Compared with the CMMC cohort, the patients in the KMUH cohort were relatively older (65.9 ± 13.4 vs. 69.1 ± 11.3 years, $p=0.06$) and predominantly female **(Table 1 and Supplemental Table 1)**. More patients in this cohort have DM (53.3% vs. 67%, $p=0.001$) and low eGFR (58.5 ± 40.3 vs. 36.8 ± 36.8 ml/min/1.73m^2, $p=0.001$).

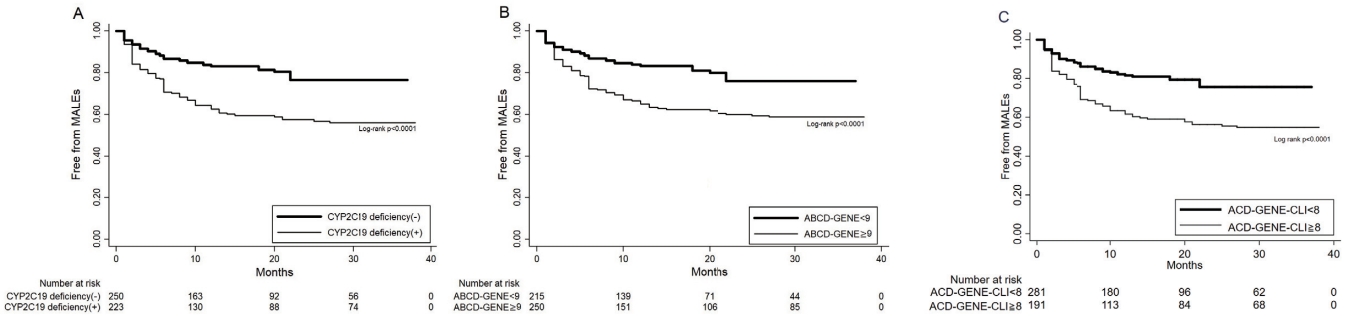

Fig. 4. The Kaplan–Meier plotter for (A) *CYP2C19* deficiency (one alle or two alleles), (B) ABCD-GENE score (using a cutoff value of 9), and (C) ACD-GENE-CLI score (using a cutoff value of 8) associated with CLI patients free from MALEs in the CMMC cohort

However, fewer patients in the KMUH cohort received angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, or statins. In terms of the *CYP2C19* LOF allele, more patients in the KMUH cohort presented with at least one LOF allele than those in the CMMC cohort (54.2% vs. 47.1%). Among the 179 patients, 27.3% developed MALEs during follow-up. Similarly, patients with MALEs had a higher ratio of underlying DM, CKD, and the *CYP2C19* LOF allele **(Supplemental Table 1)**. In this cohort, obesity also failed to differentiate between patients with or without the development of MALEs.

As a result, the patients from the KMUH cohort had higher ABCD-GENE and ACD-GENE-CLI scores **(Supplemental Table 2)**. Notably, despite certain differences in characteristics between the two cohorts, the ACD-GENE-CLI score was superior in predicting MALEs compared with *CYP2C19* deficiency and the ABCD-GENE score (AUC 0.63 vs. 0.53 vs. 0.60, $p=0.02$) **(Fig. 3B)**.

Subgroup Analysis and Sensitivity Assessment

Subgroup analyses were performed to separately assess the outcomes of amputation and revascularization **(Fig. 5)**. In Model 1, whether the ACD-GENE-CLI scores were set as a continuous or dichotomized variable (<8 or ≥ 8), patients who had higher scores had a significantly higher hazard ratio (HR) for amputation **(Fig. 5A, B)**. Regarding the outcome of revascularization, trends of increasing risks in patients with higher scores were evident but were not statistically significant. As a sensitivity analysis, in Model 2, we excluded patients who reached the endpoint within the first month of enrollment to avoid the influence of management during the initial period after endovascular interventions. Similarly, we observed that the higher the ACD-GENE-CLI scores, the higher the risk of amputation, revascularization, and composite endpoints of MALEs in the studied patients **(Fig. 5C, D)**.

A Sensitivity Test Focusing on Patients with or without Combination Therapies of Antiplatelet Agents

For the second sensitivity test, given that clopidogrel has been prescribed alone or in a combination with other antiplatelet agents such as aspirin after the endovascular intervention, we further investigated whether the ACD-GENE-CLI score is applicable in patients who received different types of therapies. We divided the patients according to those who are receiving clopidogrel only ($n=368$) and those receiving a combination therapy (clopidogrel plus aspirin or cilostazol, $n=105$). Noteworthily, in both sets, an ACD-GENE-CLI score of ≥ 8 was still specifically associated with MALEs (clopidogrel only: OR: 2.60, 95% CI: 1.60–4.21, $p=0.001$, combination therapy: OR: 3.56, 95% CI: 1.41–9.01, $p=0.007$) **(Supplemental Table 4)**.

Discussion

In conjunction with clinical parameters and *CYP2C19* polymorphism, the ABCD-GENE score was developed as a simple tool to identify patients with CAD who are at increased risk for adverse ischemic events[21]. Nevertheless, whether it could also be applied in patients with CLI remains unknown. In this Asian cohort, we found that although old age, diabetes, CKD, and *CYP2C19* polymorphisms, which were included in the ABCD-GENE score, were also positively associated with MALEs, obesity was not. Instead, we developed ACD-GENE-CLI, a modified score that included old age (≥ 65 years), diabetes, CKD, and *CYP2C19* polymorphisms, to predict the risk of MALEs in patients with CLI receiving clopidogrel. A cutoff value of ≥ 8 was adopted, and it presented with good accuracy in both the derivation

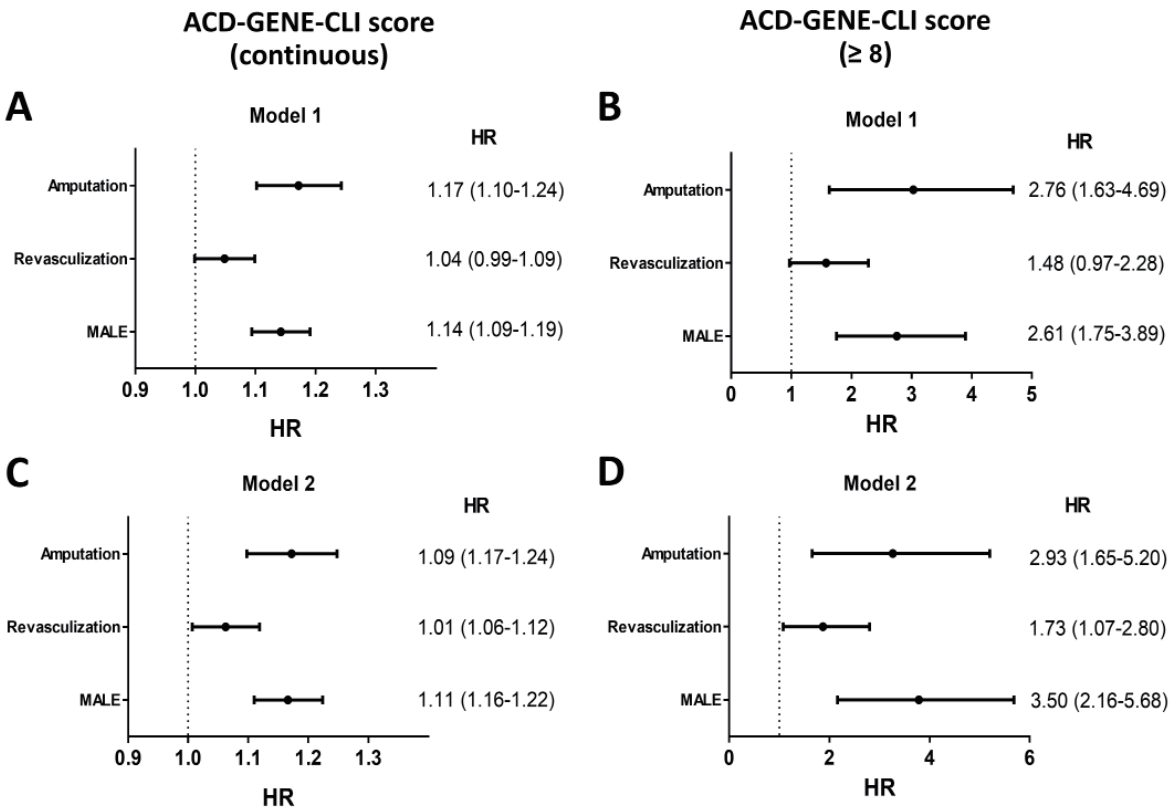

Fig. 5. Forest plots of hazard ratios (HRs) are shown according to different outcomes, including amputation, revascularization, and the composite endpoints of both outcomes

Model 1 includes the ACD-GENE-CLI score plus clinical variables. In Model 2, we excluded patients who reached the endpoint in the first month to avoid the immediate influence of management after interventions. Model 2 was also shown in a sensitivity analysis including only the population of patients free from endpoints in the first month. In the left panel, the ACD-GENE-CLI score is regarded as a continuous variable, while in the right panel, the ACD-GENE-CLI score is regarded as a dichotomous variable (≥ 8).

and validation cohorts. Our findings also support that combining clinical risks and genetic testing appears helpful in stratifying the risk for MALEs in patients with CLI receiving clopidogrel **(Fig. 6)**.

This is the first study applying a modified score to predict MALEs in patients with CLI based on the ABCD-GENE score. Although several studies have attempted to validate its efficiency in different cohorts, the score was applied primarily in patients with CAD. In The Tailored Antiplatelet Initiation to Lessen Outcomes Due to Decreased Clopidogrel Response after Percutaneous Coronary Intervention (TAILOR-PCI) trial, Capodanno *et al.* reported that among 3,883 patients with CAD treated with clopidogrel, MACEs at 12 months were significantly increased in patients with high ABCD-GENE scores[25]. In another multi-site and real-world investigation focusing on patients who underwent percutaneous coronary intervention, the risk for MACEs was also higher among patients with ABCD-GENE scores greater than 10 [26]. In terms of the high prevalence of *CYP2C19* LOF alleles in Asia, a Japanese cohort pooled from four prospective studies found that, even with a high proportion (60%) of HPR on clopidogrel, the ABCD-GENE score has a significant and moderate diagnostic ability in predicting HPR status in the population[21]. In addition to its feasibility in CAD, in The Clopidogrel With Aspirin in Acute Minor Stroke or Transient Ischemic Attack (CHANCE) trial focusing on patients with acute non-disabling cerebrovascular events, Dai *et al.* also found that the efficacy of clopidogrel–aspirin therapy decreased in patients with higher ABCD-GENE scores[27]. Nevertheless, in a Chinese cohort including patients with acute coronary syndrome, Wu *et al.* established a novel GeneFA score that only included the *CYP2C19* genotype, fibrinogen, and age[28]. The authors found that, compared with the ABCD-GENE score, the GeneFA score presented a better predictive value for HPR in these patients[28].

Noteworthily, our study found that obesity failed to differentiate the risk of MALEs in patients with

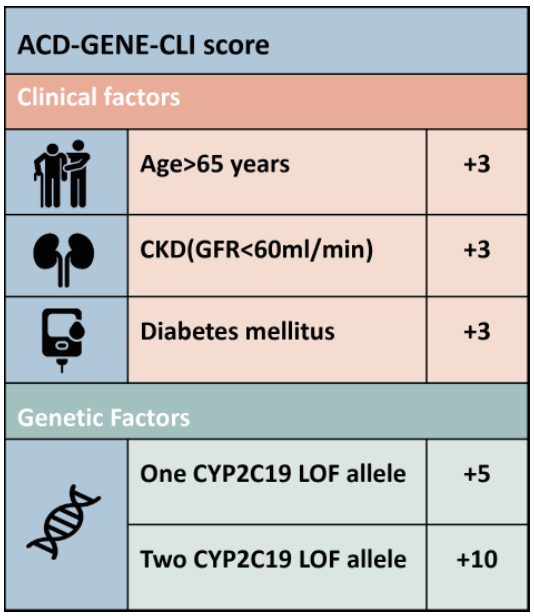

Fig. 6. Illustration of the ACD-GENE-CLI score, combining clinical risks and genetic testing, to stratify the risk for MALEs in patients with CLI receiving clopidogrel

CLI, and the simplified ACD-GENE-CLI score precisely reflects the outcomes. Different from western countries, patients having ischemic events in Asia usually presented with a paradoxical phenomenon of BMI. FOCUS registry (ClinicalTrials.gov Identifier: NCT 00868829), a large-scale, prospective study consisting of 5,084 patients, indicated an inverse association between BMI and long-term prognosis in Asian patients with CAD[29]. Likewise, in a retrospective cohort study in South Korea, it was observed that compared with normal weight, obesity was more likely to reduce mortality risk in patients with ischemic stroke, especially the elderly[30]. In another Asian cohort, Chen *et al.* highlighted a U-shaped association between BMI and death from overall cardiovascular diseases[31]. Therefore, BMI may not be an ideal parameter to reflect the risks of cardiovascular morbidity in patients with ischemic events such as CLI.

Regarding the impact of genetic polymorphisms, unlike monogenic vascular syndromes, CLI usually results from hundreds of genes interacting with the environment[9, 32]. Despite the current immature state of genetic screening to assess the outcomes of CLI, scientists still attempt to investigate the applicability of genetic testing in risk stratification and prognostication[9, 32]. In a retrospective study, Lee *et al.* reported that the *CYP2C19* polymorphism is associated with higher amputation rates among patients with CLI treated with clopidogrel[15]. Similarly, Gou *et al.* described that *CYP2C19* LOF allele carriers have a greater risk of in-stent restenosis after endovascular treatment for CLI[33]. In a systematic review focusing on *CYP2C19* polymorphisms in patients with CLI, Osnabrugge *et al.* reported an association between *CYP2C19* LOF alleles and reduced clopidogrel function[34]. The authors suggested that *CYP2C19* testing is necessary for patients with CLI receiving clopidogrel to improve the prediction of clinical outcomes[34]. However, in the double-blind, multicenter, randomized EUCLID trial, patients with symptomatic CLI were randomly assigned to receive ticagrelor (90 mg twice daily) or clopidogrel (75 mg twice daily)[35]. Although 59% of clopidogrel users harbored a *CYP2C19* polymorphism, the risk of major adverse cardiac or bleeding events was similar to that of non-carriers[35]. Based on the collective evidence, a de-escalation-guided strategy merely accounting for the *CYP2C19* LOF alleles may not be adequate to predict the MALE outcomes in patients with CLI. The newly modified ACD-GENE-CLI score integrates both clinical and genetic parameters to provide specific weight of the individual variables. Further research, including a cost-effectiveness study, is necessary to investigate the feasibility and applicability of the test for *CYP2C19* polymorphism in clinical situations.

Limitations

Despite the good accuracy of the newly established score, this study still had some limitations.

First, given that only Asians were included, the prevalence of *CYP2C19* LOF alleles was higher than that reported in other studies. The findings highlight the complexity of multiple comorbidities and genetic polymorphisms in Asian patients with CLI. Another limitation is the lack of HPR measurements. Although tailored antiplatelet therapy based on HPR has been previously discussed, the application of platelet reactivity testing in CLI remains inconclusive[36, 37]. In contrast to the dynamic changes in platelet reactivity, CYP2C19 LOF alleles could guide decision-making regarding antiplatelet agents and improve clinical outcomes. Second, the newly modified score is based on the ABCD-GENE score. Since many unidentified confounding factors might interfere with the outcome in patients with either CAD or CLI, the sensitivity and specificity of the scoring system might not be adequate to identify all patients with risks. Also, if we compared the predictive power for MALEs between the ACD-GENE-CLI score with the conventional ABCD-GENE score, the results in the ROC curves showed that the difference was not statistically significant (p=0.08). However, using NRI to reclassify the populations according to the MALE outcomes , the ACD-GENE-CLI score significantly differentiated the risks compared with the ABCD-GENE score. It implied that further verification is required to test whether the ACD-GENE-CLI score could replace the ABCD-GENE score. Third, different from CAD, CLI includes a wide variety of clinical presentation and morphology, which impact the outcomes, especially MALEs. Lastly, a sample size of 473 in the derivation cohort might be too small to make the conclusion. Further studies with larger sample sizes are needed to improve the generalizability and enhance the robustness of applying prediction scores in clinical situations.

Conclusions

By studying the genetic polymorphism and clinical outcomes of patients with CLI receiving clopidogrel, we established a modified risk score, ACD-GENE-CLI that included old age (>65 years), DM, CKD, and the *CYP2C19* LOF allele and compared it with the ABCD-GENE score. In the external validation cohort, the ACD-GENE-CLI score also demonstrated the ability to differentiate patients at risk for MALEs. Further studies are required to validate the feasibility of the score and to generalize its application in patients with CLI.

Conflicts of Interest

None.

Funding

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Author Contributions

Study design: WTC, PSH, LWS, CTL, HST, ZCC, PCH, and CSH; Data collection: WTC, PSH, PCH, and CSH; Data analysis: WTC, CTL, HST, YCL, JYS, and CSH; Writing: WTC, PSH, LWS, CTL, HST, ZCC, PCH, and CSH; Final approval: WTC, PSH, LWS, CTL, HST, ZCC, PCH, and CSH; Agreement to be accountable for this work: WTC, PSH, LWS, CTL, HST, ZCC, PCH, and CSH

Data Availability Statement

The original data is available upon reasonable request to the corresponding author

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Supplemental Table 1. Baseline characteristics in patients with critical limb ischemia (CLI) with and without major adverse limb events (MALE) in validation cohort of KMUH cohort (N = 179)

Variable	All population (N = 179)	MALE (-) (N = 130)	MALE (+) (N = 49)	*P* value
Age (y/o)	69.1 ± 11.3	68.5 ± 11.1	70.2 ± 11.9	0.51
Age > 75 y/o, *n* (%)	64 (35.7)	42 (32.3)	22 (44.9)	0.16
Age > 65 y/o, *n* (%)	122 (68.2)	85 (65.3)	37 (75.5)	0.05
Men, *n* (%)	84 (41.3)	51 (39.2)	23 (46.9)	0.39
BMI (kg/M^2)	24.5 ± 4.3	24.6 ± 4.3	24.3 ± 4.5	0.58
BMI > 25, *n* (%)	68 (37.9)	52 (40)	16 (32.6)	0.39
BMI > 30, *n* (%)	17 (9.5)	13 (10)	4 (8.1)	0.47
Medical history				
Hypertension, *n* (%)	138 (77.1)	103 (79.2)	35 (71.4)	0.32
Diabetes, *n* (%)	120 (67)	82 (63.1)	38 (77.5)	0.04
Hyperlipidemia, *n* (%)	12 (6.7)	12 (9.2)	0 (0)	0.69
CAD, *n* (%)	15 (8.4)	11 (8.4)	4 (8.1)	0.37
Previous stroke, *n* (%)	2 (1.1)	2 (1.5)	0 (0)	0.67
HF, *n* (%)	3 (1.6)	1 (0.7)	2 (4)	0.17
Atrial fibrillation, *n* (%)	27 (15)	23 (17.7)	4 (8.2)	0.16
Cancer, *n* (%)	15 (8.4)	12 (9.2)	3 (6.1)	0.76
Smoking, *n* (%)	32 (17.8)	93 (71.5)	32 (65.3)	0.06
Lab data				
eGFR(ml/min/1.73^2)	36.8 ± 36.8	38.6 ± 38.5	33.2 ± 22.3	0.04
CKD (eGFR < 60) , *n* (%)	140 (78.2)	100 (76.9)	40 (81.6)	0.54
Fasting glucose (mg/dl)	175.1 ± 81.2	158.3 ± 67.9	129.7 ± 49.1	0.4
Cholesterol (mg/dl)	159.3 ± 28.7	136.6 ± 56.7	189 ± 25.4	0.48
LDL (mg/dl)	96.9 ± 24.9	93.1 ± 24.2	124 ± 7.1	0.17
TG (mg/dl)	142.8 ± 56.2	154 ± 27.3	186.3 ± 21.5	0.24
Cardiovascular drugs				
Aspirin, *n* (%)	59 (32.9)	49 (37.6)	10 (20.4)	0.54
Cilostazol, *n* (%)	59 (32.9)	46 (35.3)	13 (26.5)	0.11
ACEI/ARB, *n* (%)	67 (37.4)	48 (36.9)	19 (38.7)	0.86
Statins, *n* (%)	78 (43.5)	55 (42.3)	23 (46.9)	0.61
*CYP2C19**2 allele				
None, *n* (%)	82 (45.8)	66 (50.7)	16 (32.6)	0.001
One allele, *n* (%)	94 (52.5)	64 (50)	30 (61.2)	0.04
Two alleles, *n* (%)	3 (1.7)	0 (0)	3 (6.1)	0.02

Abbreviation as listed in Table 1

Supplemental Table 2. The comparison of risk scores and outcomes in patients with critical limb ischemia (CLI) in the derivate (CMMC) and the validation (KMUH) cohorts

Variable	CMMC (N=473)	KMUH (N=179)	*P* value
Risk scores			
ABCD-GENE (IQR)	9 (6, 12)	10 (6, 13)	
ABCD-GENE ≥ 9, n (%)	250 (52.8)	113 (63.1)	0.02
ABCD-GENE-CLI (IQR)	8 (6, 11)	9 (6, 14)	
ABCD-GENE-CLI ≥ 8, *n* (%)	248 (52.4)	124 (69.2)	0.001
Outcomes			
MALEs, *n* (%)	139 (29.3)	49 (27.3)	0.69
Time to events (IQR)	8 (2, 15)	6 (2, 25)	
Amputation, *n* (%)	59 (12.4)	17 (9.5)	0.3
Time to events (IQR)	8 (2, 15)	6 (2, 18)	
Revasculization, *n* (%)	80 (16.9)	32 (17.8)	0.73
Time to events (IQR)	6 (2, 8)	8 (6, 25)	

Abbreviation as listed in Table 1

Supplemental Table 3. Statistics of *CYP2C19* deficiency, ABCD-GENE (using a cut-off value of 9) and ACD-GENE-CLI score (using a cut-off value of 8) in CMMC cohort

	CYP2C19 deficiency (+)		ABCD-GENE (≥ 9)		ACD-GENE-CLI(≥ 8)	
	Value	95% CI	Value	95% CI	Value	95% CI
Sensitivity	59.7%	51.1% to 67.9%	66.2%	57.7% to 73.9%	64.0%	55.5% to 71.9%
Specificity	58.1%	52.6% to 63.4%	52.4%	46.9% to 57.9%	68.6%	63.3% to 73.5%
Positive Likelihood Ratio	1.42	1.18 to 1.72	1.39	1.18 to 1.64	2.04	1.66 to 2.49
Negative Likelihood Ratio	0.69	0.56 to 0.87	0.65	0.50 to 0.83	0.52	0.42 to 0.66
Positive predictive value	37.2%	30.9% to 43.9%	36.7%	30.7% to 42.9%	45.8%	38.7% to 53.2%
Negative predictive value	77.6%	71.9% to 82.6%	78.8%	72.9% to 84.0%	82.1%	77.1% to 86.4%
Accuracy	58.6%	53.9% to 63.0%	56.5%	51.9% to 60.9%	67.2%	62.8% to 71.5%

Supplemental Table 4. The logistic regression analyses of ABCD-GENE-CLI scores associated with the development of MALEs in patients with Clopidogrel use only (N=368) or Clopidogrel combination use (N=105)

Parameters	Logistic regression analysis		
	Odds ratio	95% CI	*P* value
Clopidogrel use only			
ABCD-GENE-CLI ≥ 8	2.60	1.60-4.21	0.001
Clopidogrel combination use			
ABCD-GENE-CLI ≥ 8	3.56	1.41-9.01	0.007