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CYP2C19 Polymorphism is Associated With Amputation Rates in Patients Taking Clopidogrel After Endovascular Intervention for Critical Limb Ischaemia

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WHAT THIS PAPER ADDS

Cardiology studies have shown inter-individual variation of platelet reactivity with clopidogrel treatment. Pharmacogenomics play a major role in investigating antiplatelet effect after clopidogrel use. This study investigated the association between CYP2C19 polymorphisms and the clinical outcomes in critical limb ischaemia (CLI) patients accepting endovascular therapy (EVT) procedures. It was found that genetic profiles significantly influenced amputation free survival and all cause mortality in these patients. Furthermore, the polymorphism number is negatively associated with the clinical outcomes on multivariable analysis. Different antiplatelet therapy other than clopidogrel may be considered in patients with CYP2C19 loss of function allele.

Objective: Clopidogrel is a pro-drug requiring cytochrome P450 (CYP) 2C19 enzyme to be oxidised to its active form. This study evaluated the association between the CYP 2C19 genetic polymorphism and clinical outcomes in patients with critical limb ischaemia (CLI) taking clopidogrel after endovascular therapy (EVT).

Methods: This was a retrospective study. Patients with CLI who had undergone EVT between August 2014 and January 2017 were included. The study subjects were divided into three groups according to the loss of function (LOF) CYP2C19 alleles: (1) extensive metaboliser (EM); (2) intermediate metaboliser (IM); and (3) poor metaboliser (PM). All patients underwent a platelet function test (VerifyNow). Amputation free survival and all cause mortality were estimated using the Kaplan–Meier method. The association between baseline characteristics and clinical outcomes was assessed with the Cox proportional hazard model.

Results: A total of 278 CLI patients (EM: 153, IM: 79, PM: 46) who underwent EVT were included. There were 180/278 (64.7%, EM: 107, IM: 45, PM: 28) patients who completed the 12 month follow up examination. Carriers of at least one CYP2C19 LOF allele (44.9%, 125/278) had diminished pharmacodynamic responses to clopidogrel as measured using VerifyNow (174 $\pm$ 27 platelet reactivity units (PRU), 216 $\pm$ 21 PRU, and 245 $\pm$ 35 PRU for patients with EM, IM, and PM CYP2C19 profiles, respectively; EM vs. IM, $p < .0001$ and EM vs. PM, $p < .0001$). The estimated amputation free 12 month survival rates were EM 82.1%, IM 66.1.0%, and PM 56.6% with significant differences between groups (log-rank test $p = .0006$, p for trend $<.0001$). The estimated all cause 12 month mortality rates were EM 83.7%, IM 72.2%, and PM 71.3% (log rank test $p = .01$, p for trend $p = .007$). The combined group consisting of IM and PM was associated with amputation free survival and all cause mortality based on a univariable analysis (hazard ratio [HR] = 2.23 [1.97–2.46], $p = .011$; HR = 1.43 [1.05–1.85], $p = .043$) and remained significant in a multivariable Cox analysis (HR = 2.65 [2.1–2.9], $p = .009$; HR = 1.39 [1.07–1.74], $p = .037$).

Conclusion: CYP2C19 genetic profiles can significantly influence clinical outcomes (in both amputation free survival and all cause mortality) in CLI patients who are taking only clopidogrel after EVT.

Keywords: Antiplatelet drug resistance, Antiplatelet therapy, Clopidogrel, CYP2C19 polymorphisms, Peripheral artery disease
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INTRODUCTION

Clopidogrel is used as an antiplatelet agent for the treatment of peripheral artery disease (PAD) as advocated by current guidelines.[1,2] Despite its proven efficacy, the inter-individual variability in drug action may significantly influence the therapeutic outcome. Clopidogrel is a pro-drug requiring oxidation into a thiol metabolite within the liver, which occurs mainly via the action of cytochrome P450 enzyme 2C19 (CYP2C19).[3,4] The CYP2C19 gene encodes a member of the cytochrome P450 superfamily of enzymes. Polymorphism within the gene is associated with variable capabilities for metabolizing mephenytoin.[5]

Several genetic polymorphisms exist for CYP2C19 expression, consisting of CYP2C19*1—*3. Thus, individuals homozygous for the *CYP2C19*2* and CYP2C19*3 alleles are considered to be poor metabolisers (PM), whereas subjects with only one *CYP2C19*1* allele are classified as intermediate metabolisers (IM), and those with two *CYP2C19*1* alleles as extensive metabolisers (EM).[6]

Patients with wild type polymorphism (EM) have greater enzyme activity, whereas those with heterozygous (IM) and homozygous (PM) polymorphisms have less activity.[7] Patients taking clopidogrel have different clinical outcomes depending on the type of CYP2C19 polymorphisms, and patients with PM have the worst clinical outcomes.[8,9] Previous studies have focused mainly on CYP2C19 related outcomes of coronary artery disease (CAD) and acute coronary syndrome (ACS), but only a few have concentrated on PAD.[10—13]

The present study aimed to investigate the clinical relevance of CYP2C19 polymorphism in critical limb ischaemia (CLI) patients receiving clopidogrel after endovascular revascularisation. The study examined whether different CYP2C19 polymorphisms led to different clinical outcomes for amputation free survival and mortality in these patients at one year.

MATERIALS AND METHODS

Study design

The study was a retrospective study performed in a single tertiary medical centre in Taipei, Taiwan. The study was conducted with the approval of the local ethics committee and in accordance with the Declaration of Helsinki. All patients gave written informed consent for the procedure.

Patient selection

CLI patients with Rutherford classifications (RC) V and VI were consecutively included in outpatient clinics or admissions from August 2014 to January 2017 if they were adults scheduled to undergo an endovascular revascularisation of the iliac, superficial femoral (SFA), popliteal, anterior tibial, peroneal, or posterior tibial artery and were on clopidogrel treatment prior to the intervention. All CLI patients undergoing EVT were screened monthly for eligibility by the cardiologist from the quality control (QC) section of the catheterisation laboratory. The discharge date of the admission was considered to be the study enrolment date. Exclusion criteria included treatment with aspirin, heparin, oral anticoagulants, and/or P2Y12 inhibitor (other than clopidogrel) treatment at the time of the EVT (Fig. 1). Patients were followed up in clinics at the study hospital at least every three months. According to the reported incidence of amputation (20%) and mortality (20%) during the one year follow up,[14,15] it was assumed that overall event rates (death or amputation) were 20%, 30%, and 40% for

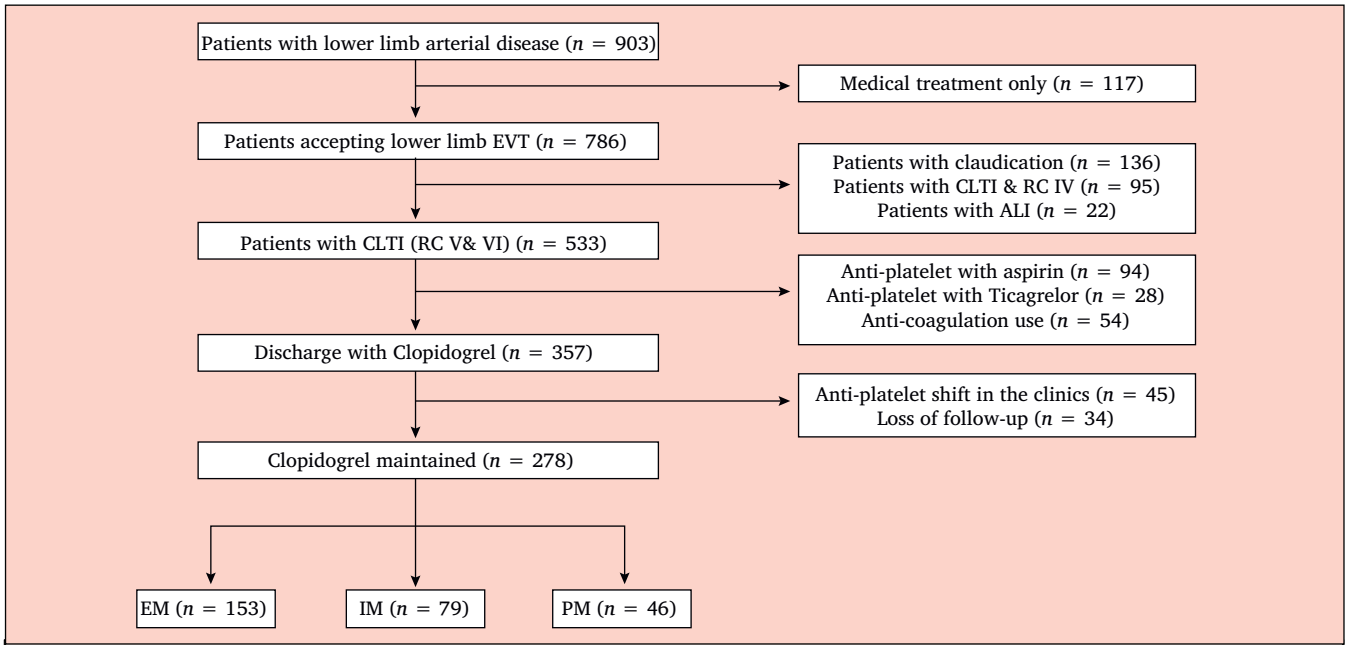

Figure 1. Flow chart of included patients. ALI = acute limb ischaemia; CLTI = chronic limb-threatening ischaemia; EM = extensive clopidogrel metaboliser; EVT = endovascular therapy; IM = intermediate clopidogrel metaboliser; PM = poor clopidogrel metaboliser; RC = Rutherford classification.

EM, IM, and PM, respectively. Given an α level of 0.05, power of 0.8, and HR of 1.5, the estimated sample size was 239. The sample size determination was made by Power and Sample Size Version 08 (NCSS, LLC, Kaysville, UT, USA).

Platelet reactivity measurement

Platelet aggregation assessment was conducted using the VerifyNow P2Y12 assay (Accumetrics, San Diego, CA, USA). VerifyNow P2Y12 (Accumetrics), a whole blood, cartridge based, and point of care turbidimetric method, was performed to test inhibitory effects on platelet aggregation, and the results were reported in platelet reactivity units (PRU).[16,17]

CYP2C19 genotyping

Laboratory testing for CYP2C19 genotyping has been available in at the study hospital since 2014. DNA was isolated from 200 μL peripheral potassium ethylenediaminetetraacetic acid (EDTA) anticoagulated blood. Genotypes were determined with a TaqMan SNP genotyping assay. According to a previous classification, CYP2C19 reduced function alleles were CYP2C19*2 (681G>A; rs4244285) and CYP2C19*3 (636G>A; rs4986893).[18–20] The assay reagent for the single nucleotide polymorphism in the gene was supplied by Applied Biosystem (Foster City, CA, USA). The number of reduced function alleles was calculated. Individuals without reduced function alleles were defined as (*1A/*1A = EM) in the observed alleles, whereas individuals with one and two reduced function alleles were defined as (*1A/*2A or *1A/*3A = IM) and (*2A/*2A, *2A/*3A, or *3A/*3A = PM), respectively.

Outcome measures and variables

The pre-procedural variables considered were age (dichotomised by 80 years), sex, body mass index ([BMI] dichotomised by 18.5 kg/m^2), ambulatory status, RC, infection status, diabetes mellitus, hypertension, dyslipidaemia, regular haemodialysis, C-reactive protein (dichotomised by 3 mg/dL), and serum albumin levels (dichotomised by 3 g/dL) as a nutritional marker. The diseased vessel consisted of a pre-angioplasty artery lesion of > 50% diameter stenosis as measured by digital subtraction angiography (DSA). The anatomical classification of lower extremity arterial disease (LEAD) was evaluated according to the Trans-Atlantic Inter-Society Consensus Document (TASC) II classification. The procedural variables considered in relation to outcome measures in this study were angiosome based EVT, diseased vessel, and TASC D. The outcome measures of this study were major amputation free survival and all cause mortality within one year of the enrolment date derived from reviewing medical charts. This was adjudicated independently by two cardiologists (J. Lee and C. Wu) and two plastic surgeons (N. Cheng and H. Tai). Each patient was followed up for a maximum of one year (365 days), and patient survival information was truncated at one year follow up.

Statistical analysis

Data were analysed using Statistical Package for Social Science (IBM SPSS, version 22, IBM Corp., Armonk, NY, USA). Statistical significance was considered to be a two sided $p < .05$. Non-normally distributed continuous data were displayed as median (interquartile range, IQR) and were compared among the study group (EM vs. IM vs. PM) using Kruskal–Wallis analysis. Normally distributed data were displayed as mean ± SD, and were compared among the study group using one way analysis of variance. Distribution of categorical variables was compared among the study group using the chi-square test. Results of the CYP2C19 test were considered as an ordinal variable: (1) 0 indicated CYP2C19 loss of function allele negative; (2) 1 indicated CYP2C19 loss of function allele; and (3) 2 indicated two CYP2C19 loss of function alleles. There were no missing values in the analysed cohort. Bonferroni's correction was used to adjust for multiple (pairwise) comparison among the study group when the overall test was statistically significant. The Kaplan–Meier estimated survival rates of amputation or mortality were shown along with a log rank test to compare risks among the study group. The study group was treated as an ordinal variable (EM: 0, IM: 1, and PM: 2) in the log rank test. The above mentioned log rank test was further stratified by RC V or VI. To evaluate the independent effect of gene polymorphism (treated as an ordinal variable) on risks of amputation or mortality, a multivariable Cox proportional hazard model was conducted with adjustment of 15 pre-specified clinical characteristics (listed in Tables 3 and 4). The assumption of proportional hazard was tested by Schoenfeld partial residuals, in which the study group was the only explanatory ordinal variable. Sensitivity analyses were conducted using multivariable logistic regression models. Finally, another sensitivity analysis was conducted in which only patients with complete follow ups (whose outcomes occurred within one year or whose follow up duration was 365 days) were retained.

RESULTS

Patient demographics and clinical features

Fig. 1 includes all patients assessed for eligibility and the detailed selection process. Between August 2014 and January 2017, a total of 903 patients with LEAD signed inform consent forms and were screened for enrolment in National Taiwan University Hospital, Taipei, Taiwan. Of these, 117 patients who received medical therapy only were excluded, and an additional 253 patients were excluded who experienced claudication (136/253), RC IV (95/253), and acute limb ischaemia (22/253). Among the remaining 533 patients, only 357 patients were taking clopidogrel after EVT, whereas patients taking aspirin (94/176), ticagrelor (28/176), and anticoagulants (54/176) were excluded. Patients were also excluded with antiplatelet changes (45/79) or loss of follow up within 14 days (34/79) in the outpatient clinics. Finally, a total of 278 patients with CLI (RC V and VI)

Table 1. Baseline characteristics of 278 patients undergoing endovascular treatment of chronic limb-threatening ischaemia, stratified by CYP2C19 genotype and metabolism capacity for clopidogrel

	Total (n = 278)	Extensive metaboliser (n = 153)	Intermediate metaboliser (n = 79)	Poor metaboliser (n = 46)	p
Age − years	72 ± 13	73 ± 12	71 ± 11	72 ± 13	.47
Male gender	183 (65.8)	99 (64.7)	55 (69.6)	29 (63.0)	.68
Body mass index − kg/m^2	23 ± 4	23 ± 5	24 ± 3	23 ± 4	.22
Non-ambulatory status	93 (33.4)	55 (35.9)	24 (30.3)	14 (30.4)	.62
Genotyping result					
*1/*1	153 (55.0)	153 (55.0)	0	0	
*1/*2	71 (25.5)	0	71 (25.5)	0	
*1/*3	8 (2.9)	0	8 (2.9)	0	
*2/*2	42 (15.1)	0	0	42 (15.1)	
*2/*3	0	0	0	0	
*3/*3	4 (1.4)	0	0	4 (1.4)	
Risk factors					
Current smoking	78 (28.4)	46 (30.0)	21 (26.6.8)	12 (26.1)	.79
Diabetes mellitus	215 (76.9)	117 (76.4)	60 (75.9)	37 (80.4)	.82
Glycated haemoglobin A1C	7.1 ± 1.5	7.2 ± 1.6	7.1 ± 1.7	7.3 ± 1.3	.79
Hypertension	219 (78.7)	124 (81.0)	62 (78.5)	33 (71.7)	.39
Dyslipidaemia	200 (71.9)	113 (73.8)	53 (67.1)	34 (73.9)	.52
Dialysis	114 (41.0)	63 (41.2)	30 (37.9)	21 (45.6)	.70
Kidney transplantation	17 (4.2)	8 (5.2)	5 (6.3)	4 (8.6)	.68
Atrial fibrillation	55 (19.8)	29 (18.9)	18 (22.8)	8 (17.3)	.71
Coronary artery disease	224 (80.5)	123 (80.4)	62 (78.5)	39 (84.7)	.74
Heart failure	57 (20.5)	28 (18.3)	17 (21.5)	12 (26.1)	.69
Left ventricular ejection fraction − %	55.2 ± 16.8	58.3 ± 14.7	57.5 ± 15.1	59.6 ± 15.2	.75
Stroke	39 (14.0)	19 (12.4)	12 (15.2)	8 (17.4)	.65
Albumin − g/dL	3.4 ± 0.5	3.4 ± 0.5	3.4 ± 0.6	3.5 ± 0.6	.52
C-reactive protein − mg/dL	4.7 ± 3.8	5.4 ± 4.4	4.4 ± 3.9	4.8 ± 3.7	.21
Medication					
Oral hypoglycaemic agent only	162 (64.3)	98 (64.1)	53 (67.1)	29 (63.0)	.86
With insulin	34 (12.2)	19 (12.4)	7 (8.8)	8 (17.3)	.96
Antibiotics	82 (29.4)	43 (28.1)	27 (34.1)	12 (26.1)	.54
Cilostazol	249 (89.5)	138 (90.1)	68 (86.1)	43 (93.5)	.39
Statin	231 (83.1)	131 (85.6)	63 (79.7)	37 (80.4)	.45
Proton pump inhibitor	24 (8.6)	12 (7.8)	7 (8.8)	5 (10.9)	.81

Data are presented as n (%) or mean ± standard deviation, as appropriate.

after EVT were included. Patient demographics and comorbidities were stratified by LOF allele status in Table 1. The mean follow up was 245 days (SD = 128 days). There were 105/278 (37.8%) patients who completed follow up without amputation, 75/278 (27%) patients underwent amputation within 365 days, and 98/27 (35.3%) patients were lost to follow up within 365 days. In this study cohort, CYP2C19 genotyping revealed 153 non-carriers of reduced function CYP2C19 alleles (55.0%), and 125 carriers (45.0%), including 79 subjects with one reduced function allele (28.4%), and 46 subjects with two reduced function alleles (16.5%). The genotype distribution did not deviate from the Hardy—Weinberg equilibrium and was consistent with previous reports. The baseline demographics and clinical features were not significantly different from each group classified according to CYP2C19 profiles. All patients treated with clopidogrel alone for 12 months were obtained by reviewing medical charts from outpatient clinics and admission records.

Presentation, lesion severity, and technical details

The mean ankle brachial index (ABI) was 0.41 ± 0.22 before patients underwent EVT. RC V was observed in 69% (192/278) of limbs, and RC VI in 86 limbs (30.9%). The TASC D classification of femoropopliteal lesion was 36.3% (103/278). The diseased vessel was located in iliac arteries in 23.1% (64/278), in SFA in 67.7% (188/278), anterior tibial artery in 56.1% (156/278), posterior tibial artery in 38.8% (108/278), and peroneal artery in 24.8% (69/278) of patients. The diseased below ankle vessel was in the dorsalis pedis artery in 20.8% (58/278) and in the plantar artery in 15.1% (42/278). There were 64/278 (23.0%) stents deployed in the iliac arteries, 102/278 (36.7%) stents deployed in the SFA, and no stents deployed in the below knee (BTK) arteries. Angiosome guided EVT with direct revascularisation was 81.6% (227/278). Baseline lesion characteristics and severity were not significantly different among the three groups (Table 2).

Table 2. Lesion characteristics and clinical severity of limb ischaemia in 278 patients undergoing endovascuar treatment of chronic limb-threatening ischaemia, stratified by CYP2C19 genotype and metabolism capacity for clopidogrel

	Total (n = 278)	Extensive metaboliser (EM) (n = 153)	Intermediate metaboliser (IM) (n = 79)	Poor metaboliser (PM) (n = 46)	p
Ankle brachial index (ABI) before angioplasty	0.41 ± 0.22	0.43 ± 0.24	0.49 ± 0.28	0.41 ± 0.31	.16
Rutherford classification of clinical severity					
Stage V	192 (69.0)	104 (67.9)	57 (72.1)	31 (67.3)	.78
Stage VI	86 (30.9)	49 (32.0)	22 (27.8)	15 (32.6)	.78
TASC D (femoro-popliteal lesion)	103 (36.3)	54 (35.2)	30 (37.9)	14 (30.4)	.69
Diseased vessel distribution					
Iliac artery	64 (23.0)	33 (21.6)	20 (25.3)	11 (23.9)	.44
Superficial femoral artery (SFA)	188 (67.7)	98 (64.0)	58 (73.4)	32 (69.6)	.34
Anterior tibial artery	156 (56.1)	83 (54.2)	46 (58.2)	27 (58.6)	.78
Posterior tibial artery	108 (38.8)	63 (41.1)	31 (39.2)	14 (30.4)	.42
Peroneal artery	69 (24.8)	34 (22.2)	22 (27.8)	12 (26.1)	.61
Dorsalis pedis artery	58 (20.8)	33 (21.5)	14 (17.7)	11 (23.9)	.67
Plantar artery	42 (15.1)	23 (15.0)	13 (16.4)	6 (13.0)	.88
Stent deployment					
Iliac artery	64 (23.0)	33 (21.6)	20 (25.3)	11 (23.9)	.44
SFA	102 (36.7)	55 (35.9)	31 (39.2)	16 (34.7)	.84
Revascularisation strategy					
Direct revascularisation	227 (81.6)	125 (81.6)	65 (82.2)	36 (78.2)	.84
Completeness of follow up[a]	180 (64.7)	107 (69.9)	45 (60.0)	28 (60.1)	.89

Data are presented as n (%) or mean ± standard deviation, as appropriate.; TASC D = Trans-Atlantic Inter-Society Consensus Document.
[a] Follow up was considered complete if ≥ 365 days or if death or amputation occurred within 365 days of inclusion into the study.

Table 3. Association between baseline and treatment characteristics and risk of major amputation at 1 year in 278 patients undergoing endovascular treatment of chronic limb-threatening limb ischaemia

	Major amputation at 1 year (bivariable)		Major amputation at 1 year (multivariable)	
	HR (95% CI)	p	HR (95% CI)	p
Age > 80 years	1.54 (0.91–1.78)	.072	1.41 (0.82–1.86)	.15
Male gender	1.26 (0.74–1.56)	.45	1.15 (0.71–1.56)	.59
Body mass index < 18.5 kg/m^2	1.65 (0.85–1.88)	.32	1.46 (0.81–1.96)	.43
Non-ambulatory status	1.68 (1.13–1.92)	.036	1.71 (1.18–1.91)	.031
Diabetes mellitus	1.24 (0.85–1.46)	.22	1.37 (0.65–1.78)	.35
Hypertension	1.13 (0.61–1.52)	.56	1.19 (0.68–1.46)	.51
Dyslipidaemia	1.57 (0.54–2.67)	.66	1.65 (0.61–2.28)	.48
Haemodialysis	1.87 (1.31–2.59)	.032	1.67 (1.43–1.92)	.022
TASC D (femoro-popliteal lesion)	1.78 (1.24–2.76)	.021	1.69 (1.21–2.67)	.026
Rutherford classification VI (*versus* V)	2.14 (1.47–2.43)	.015	2.42 (1.68–3.12)	.012
Albumin < 3 g/dL	1.57 (1.03–2.21)	.047	1.42 (0.86–2.91	.082
C reactive protein > 3 mg/dL	1.87 (1.56–2.27)	.024	2.16 (1.85–2.47)	.015
Gene polymorphism number	2.23 (1.97–2.46)	.011	2.65 (2.1–2.9)	.009
Insulin	1.78 (1.56–2.12)	.041	1.96 (1.67–2.11)	.034
Proton pump inhibitor	1.52 (0.72–2.37)	.26	1.41 (0.61–2.46)	.39
Direct revascularisation	0.78 (0.49–0.94)	.023	0.73 (0.52–0.91)	.012

TASC D = Trans-Atlantic Inter-Society Consensus Document; HR = hazard ratio; CI = confidence interval.

Genotype and platelet function

VerifyNow was used in this study to measure platelet function after antiplatelet treatment, and PRU indicated the platelet reactivity unit. Carriers of at least one CYP2C19 LOF allele had diminished pharmacodynamic responses to clopidogrel. The mean PRU was 196.3 ± 36.4, while EM patients had 174.6 ± 27.1, IM patients had 216.4 ± 21.3, and PM patients had 245.7 ± 35.6. The antiplatelet effect after clopidogrel use was negatively associated with CYP2C19 polymorphism numbers with a dose dependent effect (EM vs. IM, $p < .0001$, EM vs. PM, $p < .0001$). There was a gradient effect (EM vs. IM: −42.06 ± 3.48 [95% CI: −48.82 to −35.18] PRU; EM vs. PM: −71.02 ± 4.89 [95% CI: −80.57 to −61.43] PRU); patients without the CYP2C19 LOF

Table 4. Association between baseline and treatment characteristics and risk of death (any cause) at 1 year in 278 patients undergoing endovascular treatment of chronic limb-threatening limb ischaemia

	Death at 1 year (bivariable)		Death at 1 year (multivariable)	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age > 80 years	1.43 (1.13–1.72)	.035	1.51 (1.19–1.88)	.029
Male gender	1.14 (0.85–1.42)	.57	1.17 (0.82–1.56)	.62
Body mass index < 18.5 kg/m^2	1.38 (0.81–1.89)	.37	1.42 (0.85–1.93)	.45
Non-ambulatory status	1.78 (1.05–2.48)	.048	1.69 (0.94–2.61)	.057
Diabetes mellitus	1.16 (0.66–1.82)	.58	1.26 (0.69–1.91)	.64
Hypertension	1.21 (0.87–1.59)	.44	1.19 (0.91–1.48)	.37
Dyslipidaemia	1.05 (0.76–1.53)	.68	1.12 (0.80–1.49)	.53
Haemodialysis	2.41 (1.83–2.92)	.014	2.32 (1.95–2.78)	.011
TASC D (femoro-popliteal lesion)	1.56 (0.88–1.87)	.48	1.65 (0.86–2.06)	.56
Rutherford classification VI (*versus* V)	1.33 (0.73–1.83)	.42	1.41 (0.71–1.94)	.56
Albumin < 3 g/dL	1.73 (1.12–2.34)	.044	1.61 (0.92–2.46)	.052
C-reactive protein > 3 mg/dL	1.65 (0.77–2.12)	.56	1.53 (0.79–2.04)	.51
Gene polymorphism number	1.43 (1.05–1.85)	.043	1.39 (1.07–1.74)	.037
Insulin	1.28 (0.76–1.72)	.38	1.23 (0.64–1.99)	.47
Proton pump inhibitor	1.22 (0.62–1.88)	.49	1.38 (0.69–2.19)	.58
Direct revascularisation	1.13 (0.69–1.67)	.47	1.18 (0.71–1.54)	.43

TASC D = Trans-Atlantic Inter-Society Consensus Document; HR = hazard ratio; CI = confidence interval.

allele had the greatest platelet inhibition, whereas CYP2C19 LOF allele carriers had lower platelet inhibition (Fig. 2).

Prognosis of critically ischaemic limbs after EVT

The number of amputation events for CLI patients was 28/153 (18.3%) in EM, 24/79 (30.4%) in IM, and 20/46 (43.5%) in PM at one year after EVT, respectively (log rank test $p = .0006$, p for trend $< .0001$, Fig. 3A) The number of death at one year was 25/153 (16.3%) in EM, 18/79 (22.8%) in IM, and 14/46 (30.4%) in PM (log rank test $p = .01$, p for trend $< .007$, Fig. 3B). In addition, the results of the sensitivity analysis including only patients with one year follow up information showed a similar trend to that of the primary analysis (Figs. S1 and S2).

Predictors of amputation and all cause mortality after EVT

As shown in Table 3, the univariable analysis revealed a significant association with amputation free survival for several variables: (1) age > 80 years; (2) non-ambulatory status; (3) haemodialysis; (4) TASC D; (5) RC VI; (6) albumin < 3 g/dL, (7) CRP > 3 mg/dL; (8) gene polymorphism number; (9) insulin use; and (10) indirect revascularisation. Multivariable Cox analysis indicated that non-ambulatory status, haemodialysis, TASC D, RC VI, CRP > 3 mg/dL, gene polymorphism number, insulin use, and indirect revascularisation remained significant. On the other hand, age > 80 years, non-ambulatory status, haemodialysis, albumin < 3 g/dL, and gene polymorphism number were significantly associated with all cause mortality based on the univariable analysis. However, only age > 80 years, haemodialysis, and gene polymorphism number remained significant in the multivariable Cox analysis (Table 4). Testing of Schoenfeld partial residuals revealed insignificant correlations (amputation: number of events = 75, $r = -.18$, $p = .116$; mortality: number of events = 57, $r = -.20$, $p = .132$), which indicated that the assumption of proportional hazard was not violated (data not shown). Furthermore, the results of the sensitivity analysis including only patients with one year follow up showed that gene polymorphism number was significantly associated with risks of either amputation or death (Tables S1 and S2).

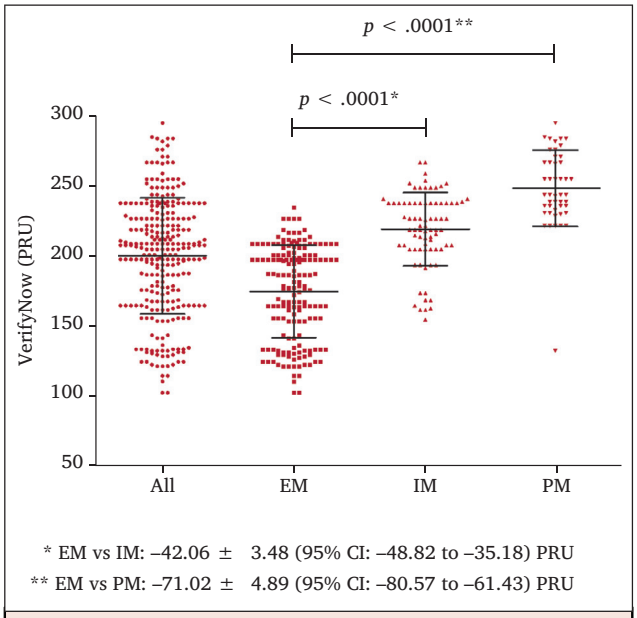

Figure 2. CYP2C19 gene polymorphism and platelet function test measured by VerifyNow. The mean level of platelet reactivity units (PRU) was 196.3 ± 36.4, while extensive metaboliser (EM) patients were 174.6 ± 27.1, intermediate metaboliser (IM) patients were 216.4 ± 21.3, and poor metaboliser (PM) patients were 245.7 ± 35.6 (EM vs. IM, $p < .0001$, EM vs. PM, $p < .0001$). The mean difference and 95% CI between EM and IM was (−42.06 ± 3.48) PRU and (−48.82 to −35.18) PRU. The mean difference and 95% CI between EM and PM was −71.02 ± 4.89 PRU and −80.57 to −61.43) PRU, respectively.

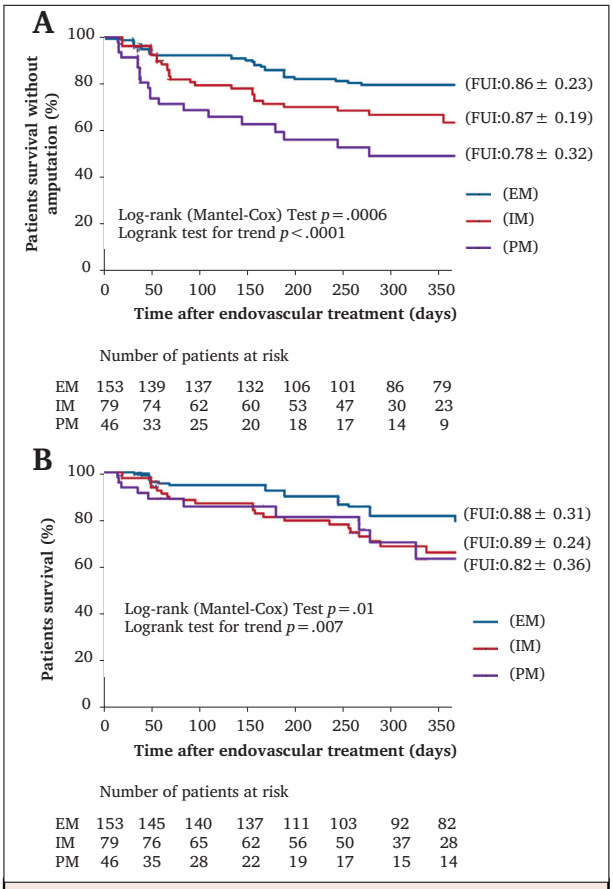

Figure 3. Cumulative Kaplan Meier estimates of amputation free survival (A) and overall survival (B), stratified by metabolism phenotypes for clopidogrel (i.e. CYP2C19 genotype). (A) The estimated freedom from major amputation in surviving patients treated by endovascular revascularisation of chronic limb-threatening ischaemia at 1 year follow up was 82.1% in extensive metabolisers (EM), 66.1% in intermediate metabolisers (IM, and 56.6% in poor metabolisers (PM). (B) The estimated overall 1 year survival in the same groups was 83.7%, 72.2% and 71.3%, respectively. FUI = follow up index.

DISCUSSION

The present study clearly shows that CYP2C19 polymorphism has a strong association with clinical outcomes, particularly amputation free survival in patients with CLI having endovascular therapy and clopidogrel. This study considered only patients undergoing EVT who took clopidogrel, as opposed to any other antiplatelet agents or anticoagulants (as detailed in Fig. 1). This is important when discussing the relationship between pharmacogenomic effects for clopidogrel use as it eliminates the confounding effect of antiplatelet activity from other medications. Guo et al. reported that patients carrying CYP2C19 LOF alleles had a reduced response to clopidogrel therapy,[10] leading to a greater risk of in-stent restenosis after endovascular treatment of lower extremity peripheral arterial disease. CLI implies an increased risk of cardiovascular morbidity and mortality, and optimal antiplatelet treatment is definitely needed.[21–24] However, there were only five CLI patients (19.8%) among the 50 included study subjects, which offered little information about this particular group. Díaz-Villamarín et al. had genotyped CYP2C19 and ABCB1 polymorphisms in 72 peripheral artery disease patients following percutaneous transluminal angioplasty.[11] Patients presented with claudication to different extents, but none had CLI. Neither study described above could eliminate aspirin's antiplatelet effects, an important confounding factor for antithrombotic therapy.

The traditional light transmission aggregometry (LTA) method with adenosine diphosphate as an agonist is the gold standard for assessing clopidogrel's antiplatelet effects. However, LTA, which is not available in most hospitals, is time consuming, technically demanding, and poorly reproducible. This limits its wider applicability.[3,25] VerifyNow is now well accepted as a useful tool for monitoring platelet function because of its quick and reproducible properties.[16,17] In the study cohort, platelet function evaluated by VerifyNow was significantly different in each group divided by CYP2C19 polymorphisms. The antiplatelet effect was negatively associated with the number of CYP2C19 LOF alleles, which was compatible with previous studies performed in coronary artery disease (CAD) patients.[3,8,9] Few studies have addressed the issue of platelet function tests in LEAD, and none of them reported that platelet function after clopidogrel use was associated with the clinical outcomes.[26,27] In the present study, it was found that platelet function test variability was significant in univariable but not multivariable Cox analysis. By contrast, the effects of CYP2C19 polymorphisms remained prominent even in the multivariable Cox analysis, which may have diminished the effects of the platelet function test. The fact that platelet function testing was done at only one time point may have reduced the test's significance.[27–29] The optimal and typical measuring point for a platelet function test is under investigation, whether for LEAD or CAD. In previous studies, the results of platelet function varied significantly with the measurement time point.[27–29]

The significant prognostic factors for poor outcome in this study, whether amputation free survival or mortality, are similar to those of previously reported studies.[30–32] There are discrepancies regarding the EVT strategy for CLI, and the present study cohort supported the idea of a directed revascularisation strategy with angiosome based EVT. Iida et al. reported that angiosome based EVT improved the clinical outcomes, but Kawarada et al. reported that physiology based EVT was even more important.[33–36] The proper EVT strategy for CLI remains under debate, and current guidelines suggest direct in line flow to the foot.[1,2] The present authors always perform EVT for CLI using direct in line flow, and most tests are angiosome guided. On the other hand, although many prognostic factors are the same for both amputation free survival and all cause mortality, this procedural variable is only statistically significant in predicting amputation free survival. This suggests that amputation is not absolutely linked to all cause mortality. Other variables such as age and haemodialysis acts systemically instead of locally. Indeed, PAD patient

mortality is high and complex as described in previous studies and includes patients with sepsis, myocardial infarction, and cancer.[37,38] Thorough and comprehensive treatment rather than only wound care is needed for CLI patients. Therefore, introduction of genetic profiles into the treatment plan to optimise antiplatelet use may be warranted.

Restenosis after EVT with stent deployment is a major limitation for favourable outcomes and is influenced by a number of factors such as vascular inflammation, and platelet activation and aggregation.[39] However, stent deployment is necessary if the vessel is dissected or recoils, or to maintain patency. While primary stenting in iliac artery stenosis is recommended by current guidelines, SFA stent implantation should be carefully evaluated from lesion to lesion.[1] The primary patency rate after stent deployment was shown to be greatest for lesions in the common iliac artery decreasing distally.[40] There is high rate of re-occlusion and target lesion stenosis post-stent deployment, and this rate is associated with higher amputation rates in CLI patients.[1] The ideal antiplatelet regimen and appropriate duration of treatment has not been well validated in clinical trials. The MIRROR study showed that treatment with antiplatelet agents reduced target lesion revascularisation, improved the patency of treated lesions and decreased the need for revascularisation.[41] Intensive antiplatelet treatment after infra-inguinal stent implantation is advocated by the European Society for Vascular Surgery guidelines.[1] Therefore, optimised antithrombolytic treatment may be far more important in patients undergoing EVT with stent deployment, especially in patients with IM and PM genotyping.

This study showed that CLI patients taking clopidogrel after EVT had diminished antiplatelet effects and a poor outcome if their genotyping was IM or PM. However, screening all of the LEAD patients by genotyping before the procedure may not be applicable. Genetic testing for CYP2C19 polymorphisms is now readily available, but it costs $300—$400 for each allele.[42] The results of a screening test may lend themselves to non-ambiguous interpretation for clinicians and patients. In the case of pharmacogenetic testing for clopidogrel, previous reports have shown that there remains uncertainty as to the genotype at risk in PCI patients.[43] This uncertainty partially stems from the observation that CYP2C19 LOF allele carriers (such as individuals carrying either one or two loss of function alleles) are quite common in the population, but have only modestly increased risk. Poor metabolisers (such as individuals carrying two LOF alleles) have a higher risk but are uncommon, at least in Caucasian populations, in which they are found in an estimated 2% of individuals.[44] However, these alleles may warrant more attention in Asian populations because the prevalence rate of PM is up to 20%, such as in the present patient cohort.[45] On the other hand, the choice of antithrombolytic treatment for LEAD patients is limited, unlike that for ACS patients who can take prasugrel or ticagrelor as alternatives. More importantly, there is no definitive proof that intervening on the basis of genotype would improve outcome, even in ACS patients. Nevertheless, there remains a need for larger and longer clinical trials for CLI patients to have optimised antithrombolytic treatments based on genotyping because the rate of amputation and mortality rate is so high.

Limitations

This study has several noteworthy limitations. First of all, it was a single centre cohort study with limited patient numbers. However, it is the largest cohort addressing the issue of genetic impact on EVT outcome so far. Second, the existence of numerous confounding factors in the present cohort study may have influenced the results obtained. Attempts were made to use a multivariable Cox analysis model to diminish the possible confounding effect of other clinical factors. Third, the methods and timing of platelet function testing are controversial. The methods mentioned in most clinical trials were followed with the VerifyNow platelet function instrument and “on treatment” timing, although these parameters may not have been sufficient to fully evaluate the response to antiplatelet therapy. Finally, external validity from the patient cohort analysis may not be strong. During patient enrolment, patients were excluded if taking other antiplatelets or anticoagulants. Also excluded were patients with RC I-IV, and those with antiplatelet changes during the follow up period, which are often encountered in general clinical practice. On the other hand, the present study may show a high degree of internal validity in this highly selective group.

CONCLUSION

This study found that CYP2C19 genetic profiles significantly influenced the clinical outcomes (both amputation free survival and all cause mortality) in patients with CLI taking only clopidogrel after EVT. Treatment for CLI should be tailored to each patient. Patients with CYP2C19 LOF alleles have a trend towards poor outcomes; thus, genotypic assessment may aid in identification of high risk individuals before treatment. A multicentre study with a longer follow up is needed to confirm these results and to provide guidelines for clinical practice.

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CONFLICT OF INTEREST

None.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejvs.2019.02.011.

REFERENCES

1 Aboyans V, Ricco JB, Bartelink MEL, Bjorck M, Brodmann M, Cohnert T, et al. Editor's choice - 2017 ESC guidelines on the diagnosis and treatment of peripheral arterial diseases, in collaboration with the european society for vascular Surgery (ESVS). *Eur J Vasc Endovasc Surg* 2018;**55**:305–68.

2 Gerhard-Herman MD, Gornik HL, Barrett C, Barshes NR, Corriere MA, Drachman DE, et al. 2016 AHA/ACC guideline on the management of patients with lower extremity peripheral artery disease: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *J Am Coll Cardiol* 2017;**69**:e71–126.

3 Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Alfonso F, Macaya C, Bass TA, et al. Variability in individual responsiveness to clopidogrel: clinical implications, management, and future perspectives. *J Am Coll Cardiol* 2007;**49**:1505–16.

4 Bates ER, Lau WC, Angiolillo DJ. Clopidogrel-drug interactions. *J Am Coll Cardiol* 2011;**57**:1251–63.

5 Fleming I. The pharmacology of the cytochrome P450 epoxygenase/soluble epoxide hydrolase axis in the vasculature and cardiovascular disease. *Pharmacol Rev* 2014;**66**:1106–40.

6 Li-Wan-Po A, Girard T, Farndon P, Cooley C, Lithgow J. Pharmacogenetics of CYP2C19: functional and clinical implications of a new variant CYP2C19*17. *Br J Clin Pharmacol* 2010;**69**:222–30.

7 Wu CK, Tsai CT, Chang YC, Luo JL, Wang YC, Hwang JJ, et al. Genetic polymorphisms of the angiotensin II type 1 receptor gene and diastolic heart failure. *J Hypertens* 2009;**27**:502–7.

8 Bauer T, Bouman HJ, van Werkum JW, Ford NF, ten Berg JM, Taubert D. Impact of CYP2C19 variant genotypes on clinical efficacy of antiplatelet treatment with clopidogrel: systematic review and meta-analysis. *BMJ* 2011;**343**:d4588.

9 Liang ZY, Han YL, Zhang XL, Li Y, Yan CH, Kang J. The impact of gene polymorphism and high on-treatment platelet reactivity on clinical follow-up: outcomes in patients with acute coronary syndrome after drug-eluting stent implantation. *EuroIntervention* 2013;**9**:316–27.

10 Guo B, Tan Q, Guo D, Shi Z, Zhang C, Guo W. Patients carrying CYP2C19 loss of function alleles have a reduced response to clopidogrel therapy and a greater risk of in-stent restenosis after endovascular treatment of lower extremity peripheral arterial disease. *J Vasc Surg* 2014;**60**:993–1001.

11 Díaz-Villamarín X, Dávila-Fajardo CL, Martínez-González LJ, Carmona-Sáez P, Sánchez-Ramos J, Álvarez Cubero MJ, et al. Genetic polymorphisms influence on the response to clopidogrel in peripheral artery disease patients following percutaneous transluminal angioplasty. *Pharmacogenomics* 2016;**17**:1327–38.

12 González A, Moniche F, Cayuela A, García-Lozano JR, Torrecillas F, Escudero-Martínez I, et al. Effect of CYP2C19 polymorphisms on the platelet response to clopidogrel and influence on the effect of high versus standard dose clopidogrel in carotid artery stenting. *Eur J Vasc Endovasc Surg* 2016;**51**:175–86.

13 Kinlay S. Outcomes for clinical studies assessing drug and revascularization therapies for claudication and critical limb ischemia in peripheral artery disease. *Circulation* 2013;**127**:1241–50.

14 Rooke TW, Hirsch AT, Misra S, Sidawy AN, Beckman JA, Findeiss LK, et al. 2011 ACCF/AHA focused update of the guideline for the management of patients with peripheral artery disease (updating the 2005 guideline): a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol* 2011;**58**:2020–45.

15 Dormandy JA, Rutherford RB. Management of peripheral arterial disease (PAD). TASC Working Group. TransAtlantic Inter-society Consensus (TASC). *J Vasc Surg* 2000;**31**(1 Pt 2):S1–296.

16 Harrison P, Frelinger 3rd AL, Furman MI, Michelson AD. Measuring antiplatelet drug effects in the laboratory. *Thromb Res* 2007;**120**:323–36.

17 De Morais SM, Wilkinson GR, Blaisdell J, Meyer UA, Nakamura K, Goldstein JA. Identification of a new genetic defect responsible for the polymorphism of (S)-mephenytoin metabolism in Japanese. *Mol Pharmacol* 1994;**46**:594–8.

18 Jeong YH, Bliden KP, Antonino MJ, Park KS, Tantry US, Gurbel PA. Usefulness of the VerifyNow P2Y12 assay to evaluate the antiplatelet effects of ticagrelor and clopidogrel therapies. *Am Heart J* 2012;**164**:35–42.

19 Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT, et al. Cytochrome p-450 polymorphisms and response to clopidogrel. *N Engl J Med* 2009;**360**:354–62.

20 Wu Cho-Kai, Su Mao-Yuan, Lee Jen-Kuang, Chiang Fu-Tien, Hwang Juey-Jen, Lin Jiunn-Lee, et al. Galectin-3 level and the severity of cardiac diastolic dysfunction using cellular and animal models and clinical indices. *Sci Rep* 2015;**5**:17007.

21 Wu CK, Lee JK, Chiang FT, Yang CH, Huang SW, Hwang JJ, et al. Plasma levels of tumor necrosis factor-α and interleukin-6 are associated with diastolic heart failure through downregulation of sarcoplasmic reticulum Ca2+ ATPase. *Crit Care Med* 2011;**39**:984–92.

22 Wu CK, Lin JW, Caffrey JL, Chang MH, Hwang JJ, Lin YS. Cystatin C and long-term mortality among subjects with normal creatinine-based estimated glomerular filtration rates: NHANES III (Third National Health and Nutrition Examination Survey). *J Am Coll Cardiol* 2010;**56**:1930–6.

23 Lin HH, Lee JK, Yang CY, Lien YC, Huang JW, Wu CK. Accumulation of epicardial fat rather than visceral fat is an independent risk factor for left ventricular diastolic dysfunction in patients undergoing peritoneal dialysis. *Cardiovasc Diabetol* 2013;**12**:127.

24 Wu CK, Tsai HY, Su MM, Wu YF, Hwang JJ, Lin JL, et al. Evolutional change in epicardial fat and its correlation with myocardial diffuse fibrosis in heart failure patients. *J Clin Lipidol* 2017;**11**:1421–31. https://doi.org/10.1016/j.jacl.2017.08.018.

25 Geisler T, Langer H, Wydymus M, Göhring K, Zürn C, Bigalke B, et al. Low response to clopidogrel is associated with cardiovascular outcome after coronary stent implantation. *Eur Heart J* 2006;**27**:2420–5.

26 van der Loo B, Braun J, Koppensteiner R. On-treatment function testing of platelets and long-term outcome of patients with peripheral arterial disease undergoing transluminal angioplasty. *Eur J Vasc Endovasc Surg* 2011;**42**:809–16.

27 Leunissen TC, Peeters Weem SM, Urbanus RT, den Ruijter HM, Moll FL, Asselbergs FW, et al. High on-treatment platelet reactivity in peripheral arterial disease: a pilot study to find the optimal test and cut off values. *Eur J Vasc Endovasc Surg* 2016;**52**:198–204.

28 Gurbel PA, Becker RC, Mann KG, Steinhubl SR, Michelson AD. Platelet function monitoring in patients with coronary artery disease. *J Am Coll Cardiol* 2007;**50**:1822–34.

29 Gorog DA, Fuster V. Platelet function tests in clinical cardiology: unfulfilled expectations. *J Am Coll Cardiol* 2013;**61**:2115–29.

30 Shiraki T, Iida O, Takahara M, Soga Y, Yamauchi Y, Hirano K, et al. Predictors of delayed wound healing after endovascular therapy of isolated infrapopliteal lesions underlying critical limb ischemia in patients with high prevalence of diabetes mellitus and hemodialysis. *Eur J Vasc Endovasc Surg* 2015;**49**:565–73.

31 Teraa M, Conte MS, Moll FL, Verhaar MC. Critical limb ischemia: current trends and future directions. *J Am Heart Assoc* 2016;**5**. pii: e002938.

32 Vogel TR, Dombrovskiy VY, Carson JL, Graham AM. In-hospital and 30-day outcomes after tibioperoneal interventions in the US

Medicare population with critical limb ischemia. *J Vasc Surg* 2011;**54**:109–15.
33 Iida O, Soga Y, Hirano K, Kawasaki D, Suzuki K, Miyashita Y, et al. Long-term results of direct and indirect endovascular revascularization based on the angiosome concept in patients with critical limb ischemia presenting with isolated below-the-knee lesions. *J Vasc Surg* 2012;**55**:363–370.e5.
34 Iida O, Nanto S, Uematsu M, Ikeoka K, Okamoto S, Dohi T, et al. Importance of the angiosome concept for endovascular therapy in patients with critical limb ischemia. *Catheter Cardiovasc Interv* 2010;**75**:830–6.
35 Kawarada O, Fujihara M, Higashimori A, Yokoi Y, Honda Y, Fitzgerald PJ. Predictors of adverse clinical outcomes after successful infrapopliteal intervention. *Catheter Cardiovasc Interv* 2012;**80**:861–71.
36 Kawarada O, Yokoi Y, Higashimori A, Waratani N, Fujihara M, Kume T, et al. Assessment of macro- and microcirculation in contemporary critical limb ischemia. *Catheter Cardiovasc Interv* 2011;**78**:1051–8.
37 Mueller T, Hinterreiter F, Luft C, Poelz W, Haltmayer M, Dieplinger B. Mortality rates and mortality predictors in patients with symptomatic peripheral artery disease stratified according to age and diabetes. *J Vasc Surg* 2014;**59**:1291–9.
38 Caro J, Migliaccio-Walle K, Ishak KJ, Proskorovsky I. The morbidity and mortality following a diagnosis of peripheral arterial disease: long-term follow-up of a large database. *BMC Cardiovasc Disord* 2005;**5**:14.
39 Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG, et al. Inter-society consensus for the management of peripheral arterial disease (TASC II). *Eur J Vasc Endovasc Surg* 2007;**33**(Suppl 1):S1–75.
40 Bradbury AW, Adam DJ, Bell J, Forbes JF, Fowkes FG, Gillespie I, et al. Multicentre randomised controlled trial of the clinical and cost-effectiveness of a bypass-surgery-first versus a balloon-angioplasty-first revascularisation strategy for severe limb ischaemia due to infrainguinal disease. The Bypass versus Angioplasty in Severe Ischaemia of the Leg (BASIL) trial. *Health Technol Assess* 2010;**14**:1–210.
41 Tepe G, Bantleon R, Brechtel K, Schmehl J, Zeller T, Claussen CD, et al. Management of peripheral arterial interventions with mono or dual antiplatelet therapy – the MIRROR study: a randomised and double-blinded clinical trial. *Eur Radiol* 2012;**22**:1998–2006.
42 Hresko A, Haga SB. Insurance coverage policies for personalized medicine. *J Pers Med* 2012;**2**:201–16.
43 Paré G, Mehta SR, Yusuf S, Anand SS, Connolly SJ, Hirsh J, et al. Effects of CYP2C19 genotype on outcomes of clopidogrel treatment. *N Engl J Med* 2010;**363**:1704–14.
44 Mega JL, Simon T, Collet JP, Anderson JL, Antman EM, Bliden K, et al. Reduced-function CYP2C19 genotype and risk of adverse clinical outcomes among patients treated with clopidogrel predominantly for PCI: a meta-analysis. *JAMA* 2010;**304**:1821–30.
45 Holmes Jr DR, Dehmer GJ, Kaul S, Leifer D, O'Gara PT, Stein CM. ACCF/AHA clopidogrel clinical alert: approaches to the FDA "boxed warning": a report of the American College of Cardiology Foundation Task Force on clinical expert consensus documents and the American Heart Association endorsed by the Society for Cardiovascular Angiography and Interventions and the Society of Thoracic Surgeons. *J Am Coll Cardiol* 2010;**56**:321–41.