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Prevalence of high on-treatment (aspirin and clopidogrel) platelet reactivity in patients with critical limb ischemia☆

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ABSTRACT

Objectives: The goal of this study is to establish the prevalence of high on-treatment platelet reactivity to aspirin (HPRA) and clopidogrel (HPRC) in patients with critical limb ischemia (CLI).
Background: CLI is associated with an increased risk of death and cardiovascular events. Unlike other patient populations with atherosclerotic cardiovascular disease, previous studies failed to demonstrate a benefit of antiplatelet therapy in patients with CLI.
Methods: From June 2014 to November 2016, we performed platelet reactivity studies for P2Y12 and thromboxane A2 (TXA2) inhibition in 100 CLI patients receiving daily treatment with aspirin and clopidogrel. P2Y12 inhibition was measured by two assays: vasodilator-stimulated phosphoprotein (VASP) and VerifyNow P2Y12 assays. HPRC was defined as VerifyNow P2Y12 reactive units (PRU) >208 and VASP-platelet reactivity index (VASP-PRI) >50%. TXA2 inhibition was measured with the VerifyNow aspirin test and HPRA was defined as aspirin reaction units (ARU) >550.
Results: Mean age was 67 ± 11 years, 50% were male, 80% had diabetes mellitus, and 26% had chronic renal insufficiency. Thirty-three percent of patients had a PRU >208 and 46% a VASP-PRI >50%. HPRC was present in 26% of patients based on the criteria of both a PRU >208 and VASP-PRI >50%. HPRA was present in 25% of patients. The overall prevalence of HPR to ASA or clopidogrel was 35% and HPR to both drugs was present in 8% of patients. Clinical characteristics were similar between groups.
Conclusions: HPR to aspirin or clopidogrel is highly prevalent in patients with CLI. Nearly one in ten patients with CLI is a hyporesponder to both aspirin and clopidogrel.

1. Introduction

Critical limb ischemia (CLI) is defined as limb pain that occurs at rest or impending limb loss caused by severe compromise of blood flow to the affected extremity. CLI is a major cause of death and disability, secondary to myocardial infarction, stroke, and amputation. The mortality in patients with CLI approaches 22% and 50% at one and five years, respectively [1–3]. High on-treatment platelet reactivity (HPR) in patients treated with aspirin and clopidogrel (previously referred to as "resistance") is associated with an increased risk of recurrent cardiovascular events after percutaneous coronary interventions and acute coronary syndromes [4,5]. The prevalence of HPR in patients with CLI treated with ASA and/or clopidogrel is not known.

2. Methods

This prospective clinical study investigated the prevalence of HPR in 100 CLI patients at the Keck Hospital of the University of Southern California (USC) and Los Angeles County Medical Center. The study

Abbreviations: ADP, adenosine diphosphate; ASA, aspirin; CLI, critical limb ischemia; DAPT, dual antiplatelet therapy; DES, drug-eluting stent; DM, diabetes mellitus; HPR, high on-treatment platelet reactivity; HPRA, high on-treatment platelet reactivity to aspirin; HPRC, high on-treatment platelet reactivity to clopidogrel; PAD, peripheral arterial disease; APRA, appropriate platelet reactivity on aspirin; APRC, adequate platelet reactivity on clopidogrel; ARU, aspirin reaction units; BMI, body mass index; CRI, chronic renal insufficiency; HbA1C, glycosylated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MmHg, millimeters of mercury; PARADISE, Preventing Amputations Using Drug-Eluting Stents (trial); PREVENT III, Project or Ex-Vivo Vein graft Engineering via Transfection III; PRI, P2Y12 reactivity index; PRU, P2Y12 reactive units; SAS, statistical analysis software; TXA2, thromboxane A2; USC, University of Southern California; VASP, vasodilator-stimulated phosphoprotein; VASP-PRI, vasodilator-stimulated phosphoprotein-platelet reactivity index; WBC, white blood count.

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was approved by the USC institutional review board and all participants gave written, informed consent.

Platelet inhibition to ASA was evaluated with the VerifyNow ASA test. Clopidogrel platelet inhibition was evaluated with two different tests: the vasodilator-stimulated phosphoprotein (VASP) and the VerifyNow P2Y12 assays. All platelet function analyses were performed after a minimum of one week of uninterrupted dual antiplatelet therapy (DAPT) with ASA and clopidogrel. The HPR on ASA treatment (HPRA) group was defined as having ASA reaction units (ARU) >550 by the VerifyNow ASA assay. The HPR on clopidogrel (HPRC) group was defined as having P2Y12 reaction units (PRU) >208 by the VerifyNow assay and VASP-platelet reactivity index (VASP-PRI) >50% by the VASP assay. All other patients were assigned to the adequate platelet reactivity on therapy (APR) group. The prevalence of HPR was calculated for ASA and/or clopidogrel. Compliance to aspirin and clopidogrel was monitored for one week prior to blood drawing with phone calls and confirmed on the day of blood sample drawing by pill count and review of the patient's medication record. For every 10 subjects, a control blood sample was obtained from a healthy volunteer to confirm validity of the assays.

3. Study endpoint

The aim of this study is to establish the prevalence of HPR in CLI patients treated with ASA and clopidogrel, and to evaluate the variability of response to antiplatelet therapy in CLI.

4. Study population

4.1. Inclusion criteria

Patients were included who had a diagnosis of CLI and who received uninterrupted treatment with ASA and/or clopidogrel for ≥1 week prior to platelet inhibition testing.

4.2. Exclusion criteria

Patients were excluded if there was chronic use of nonsteroidal anti-inflammatory drugs, use of an oral anticoagulant (warfarin), glycoprotein IIb/IIIa inhibitors, fibrinolytic drugs, or thrombocytopenia (platelet count $<100 \times 10^3/\mu l$) within 30 days before testing. Patients with any documented history of a hypercoagulable state or history of medication non-compliance were also excluded.

5. Experimental design

5.1. Blood sample collection

Venous blood samples (12 mL) for platelet function analyses were collected once from an antecubital vein, six hours after daily dosing. The first 4 mL of blood were discarded to avoid spontaneous platelet activation. Samples were processed by blinded laboratory personnel. Platelet function assays included flow cytometric analysis of the status of phosphorylation of the VASP and the VerifyNow P2Y12 and ASA systems.

5.2. VerifyNow® assay

A venous blood sample was collected in a tube containing 3.2% sodium citrate. The VerifyNow ASA assay and VerifyNow P2Y12 assay (Accumetrics®, SanDiego, CA, USA) were used to test the effects of ASA and clopidogrel treatments, respectively. VerifyNow platelet assays were processed at the USC Clinical Trials Research Unit. VerifyNow is a validated measure of adenosine diphosphate (ADP) and thromboxane A2-induced platelet aggregation, and thus evaluates the antiplatelet effect of P2Y12 receptor inhibitors (clopidogrel) and ASA, respectively [6–8]. With this assay, platelet reactivity is expressed in P2Y12 reaction units (PRU) and aspirin reaction units (ARU). Based on current recommendations, HPR on ASA is defined as ARU >550 and HPR on clopidogrel as PRU >208 [6].

5.3. Vasodilator-stimulated phosphoprotein (VASP) assay

The VASP assay was used to determine the P2Y12 reactivity index (PRI) as per standard protocol. In brief, VASP phosphorylation (VASP-P) was measured by quantitative flow cytometry using commercially available, labeled monoclonal antibodies (Biocytex Inc., Marseille, France) at the USC Flow Cytometry Core Laboratory (Eli & Edythe Broad CIRM Center for Regenerative Medicine and Stem Cell Research Center, Los Angeles, CA, USA). PRI was calculated after measurements as previously described by Aleil et al. [9]. A reduced PRI is indicative of greater inhibition of the $P2Y_{12}$ signaling pathway. Based on current recommendations, HPR on clopidogrel is defined as VASP-platelet reactivity index (VASP-PRI) >50% [6].

6. Data analysis

Data were analyzed using SAS statistical software. The prevalence of HPR to clopidogrel and to ASA was estimated by the proportion of patients with HPR. Patients were divided into groups based on platelet reactivity study results. Groups based on clopidogrel platelet reactivity results included: high on-treatment platelet reactivity on clopidogrel (HPRC, defined as patients with PRU >208 and VASP-PRI >50%) and adequate platelet reactivity on clopidogrel (APRC, defined as patients not in the HPRC group). Groups based on ASA platelet reactivity results included high on-treatment platelet reactivity on aspirin (HPRA, defined as patients with ARU >550) and adequate platelet reactivity on aspirin (APRA, defined as patients with ARU <550). Demographic and laboratory variables were reported descriptively using Chi-Square test or Fisher's exact test and Student's *t*-test.

7. Results

Mean age was 67 ± 11 years, 50% were male, 80% had diabetes mellitus, and 26% had chronic renal insufficiency. DAPT duration prior to testing was 4.0 ± 19.1 months for the overall cohort and was similar in both groups. Vascular-related history included amputation in 43% of patients, lower extremity bypass in 39%, and 81% had lower extremity angioplasty (Table 1). Thirty-three percent of patients had a PRU >208 and 46% a VASP-PRI >50%. HPRC was found in 26% of patients based on the criteria of both a PRU >208 and VASP-PRI >50%. HPRA was present in 25% of patients and APRA in 75%. The overall prevalence of HPR to ASA or clopidogrel was 35% and HPR to both drugs was present in 8% of patients (Fig. 1). Clinical characteristics were similar between groups (Tables 2 and 3).

8. Discussion

Symptomatic peripheral arterial occlusive disease is caused by inadequate blood flow causing oxygen supply and demand mismatch. CLI occurs as a result of chronic lack of blood supply, setting off a cascade of events that leads to pain at rest, tissue loss, chronic limb ulcerations, and inadequate healing [1]. Peripheral arterial disease (PAD) affects approximately 8.5 million Americans and CLI represents 1–3% of the total number of patients with PAD [1]. There are approximately 500–1000 new cases of CLI per million people (150,000–300,000 per year) in United States [10]. The estimated number of deaths in CLI patients is 30,000 to 60,000 during the first year, and 75,000 to 150,000 during the first five years after diagnosis. Most deaths are related to cardiovascular events (sudden cardiac death, myocardial infarction, and stroke). Patients with CLI have a greater risk of cardiovascular ischemic events than those with PAD alone, which may be related to higher rates of

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