Effect of cilostazol on plasma levels of proprotein convertase subtilisin/kexin type 9

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ABSTRACT

The protein complex proprotein convertase subtilisin/kexin type 9 (PCSK9) serves as an important target for the prevention and treatment of atherosclerosis and lipid homeostasis. This study investigated the effect of cilostazol on plasma PCSK9 concentrations. We performed a post hoc analysis of two prospective, double-blind, randomized controlled trials including 115 patients of whom 61 received cilostazol 200 mg/day and 54 received placebo for 12 weeks. Linear regression analysis was performed to determine the associations between several parameters and changes in PCSK9 levels. Use of cilostazol, but not placebo, significantly increased plasma PCSK9 concentrations, high-density lipoprotein cholesterol levels, and number of circulating endothelial progenitor cells (EPCs), and decreased triglyceride levels with a trend toward an increase in total cholesterol (TC) levels. A reduction in hemoglobin A1C and an increase in plasma vascular endothelial growth factor and adiponectin levels with cilostazol treatment were also found. Changes in the number of circulating EPCs were positively correlated and the TC concentrations were inversely correlated with changes in the PCSK9 levels. After adjusting for changes in levels of TC and numbers of circulating EPCs and history of metabolic syndrome, use of cilostazol remained independently associated with changes in plasma PCSK9 levels. In conclusion, cilostazol treatment was significantly and independently associated with an increase in plasma PCSK9 levels in patients with peripheral artery disease or at a high risk of cardiovascular disease regardless of background statin use and caused an improvement in some metabolic disorders and levels of vasculo-angiogenic biomarkers.

INTRODUCTION

Proprotein convertase subtilisin/kexin type 9 (PCSK9), a newly recognized protein, plays an important role in cholesterol homeostasis and affects plasma lipoprotein levels by enhancing the degradation of hepatic low-density lipoprotein receptors and subsequently reducing expression of these receptors on hepatocytes, thereby leading to increased plasma levels of total cholesterol (TC) and low-density lipoprotein cholesterol.
(LDL-C) [1]. From a genetic point of view, individuals with certain sequence variations in the PCSK9 gene have not only lower plasma LCL-C levels but also a lower incidence of coronary artery disease (CAD) [2]. Plasma PCSK9 levels are not only correlated with atherogenic lipoprotein levels but also with many other cardiovascular risk factors, such as fasting plasma glucose, age, and blood pressure [3, 4]. In addition to traditional risk factors, PCSK9 is also associated with non-traditional risk factors involving inflammatory and oxidative processes [5–9]. Moreover, plasma PCSK9 levels have been reported to be associated with the severity of CAD [10], and the presence of peripheral artery disease (PAD), especially those with extensive, severe, and complicated PAD [11]. We found that circulating endothelial progenitor cell (EPC) dysfunction, in particular, the number of apoptotic circulating endothelial cells, and some vasculo-angiogenic and oxidative biomarkers were significantly correlated with PCSK9 levels [11]. Taken together, PCSK9 protein is an important target for the prevention and treatment of atherosclerosis.

Some studies have revealed that plasma PCSK9 levels were affected by some drugs, such as an increase in PCSK9 levels with the use of statins [12], ezetimibe [13], and fibrates [14], and a decrease with nicotinic acid treatment [15]. Recently, some studies have indicated a relationship between PCSK9 and the metabolism of high-density lipoprotein cholesterol (HDL-C) [16] and triglyceride [17].

Cilostazol, a phosphodiesterase 3 inhibitor, is licensed for treatment of patients with PAD and intermittent claudication owing to its antiplatelet and vasodilatory effects [18–20]. Recently, we and other researchers found that this compound has beneficial effects on metabolic parameters, angiogenesis, numbers and functions of circulating human early EPCs in vitro [18, 21–24], and in clinical settings [19, 20]. In particular, cilostazol treatment is beneficial for a reduction in triglyceride levels and an increase in HDL-C levels in patients with PAD [19, 25] or at high risk of cardiovascular disease (CVD) [20]. However, no study has investigated the effect of cilostazol on plasma PCSK9 levels.

In our study, we included participants from two double-blind, randomized, placebo-controlled trials [19, 20] to evaluate the effect of cilostazol treatment on plasma PCSK9 levels and the potential mechanisms of action in patients with PAD or at high risk of CVD.

RESULTS

The participant flow of the two cohorts is demonstrated in Figure 1. The mean patient age was 65.6 ± 9.3 years, and 66.1% of the patients were male. The background characteristics and parameters between the cilostazol treatment and placebo groups were similar and well matched (Table 1). All participants could tolerate the treatment protocol and completed the entire study.

Comparisons of changes in the background parameters, number of circulating EPCs and apoptotic endothelial cells, in vitro EPC functions, and serum/plasma biomarker levels, after treatment between the groups are demonstrated in Table 2 and Figure 2. Cilostazol treatment significantly increased plasma PCSK9 levels (18.7 ± 8.9% vs. -6.5 ± 5.1%, \( P = 0.02 \)) and improved triglyceride and HDL-C levels (-13.0 ± 4.0% vs. 16.5 ± 5.8%, \( P < 0.001; 10.1 ± 2.2\% \text{ vs.} 2.6 ± 3.3\%, P = 0.05 \), respectively). Despite no statistically significant changes in the TC and LDL-C levels between the groups, cilostazol treatment led to a marginal increase in TC levels. Cilostazol had a beneficial effect on glucose homeostasis, as shown by a significant improvement in hemoglobin A1C levels and a trend toward a decrease in fasting insulin levels and homeostasis model assessment index. Use of cilostazol, but not placebo, significantly increased the circulating EPC count [91.2 (25.9, 469.2)% vs. -28.1 (-76.0, 95.0)%], and reduced migration capacity of EPCs, without any effects on the apoptotic endothelial cell count or other EPC functions. Cilostazol treatment tended to enhance EPC 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) absorbance, a marker of cell viability (\( P = 0.06 \)). Vascular endothelial growth factor (VEGF)-A165 and adiponectin concentrations significantly increased in the cilostazol treatment group [41.8 (16.1, 91.0)% vs. -6.6 (-39.9, 15.5)%], \( P < 0.001; 10.5 (-21.1, 50.9)% \text{ vs.} -18.8 (-35.5, 5.0)% , \( P = 0.002 \), respectively], whereas other serum/plasma biomarker levels did not change significantly. Moreover, heart rate and platelet counts significantly increased with cilostazol treatment.

Table 3 demonstrates that changes in PCSK9 plasma levels were similar with respect to the background characteristics or medications, except for history of metabolic syndrome. Patients with a history of metabolic syndrome had smaller changes in PCSK9 concentrations than other patients. Of note, changes in PCSK9 levels was similar in patients with or without statin treatment during the 3-month study period (3.9 ± 7.2% vs. 9.7 ± 8.1%, \( P = 0.60 \)).

Changes in the number of circulating EPCs was positively correlated, and the TC concentration was inversely correlated, with changes in PCSK9 levels (\( r = 0.21, P = 0.04; r = -0.22, P = 0.03 \), respectively) (Table 4). After adjusting for changes in TC levels, numbers of circulating EPCs, and history of metabolic syndrome, use of cilostazol remained independently associated with changes in the plasma PCSK9 levels \( [\beta = 0.20 (95\% \text{ confidence interval} = 0.02-0.48), P = 0.04] \) (Table 5).
DISCUSSION

By evaluating two prospective, randomized, double-blinded, placebo-controlled trials in the current post hoc analysis, we found, for the first time, that cilostazol treatment was significantly and independently associated with an increase in plasma PCSK9 levels in patients with PAD or at a high risk of CVD regardless of background statin use. Although cilostazol treatment exerted a beneficial effect on some metabolic disorders and vasculo-angiogenic biomarkers, the increase in PCSK9 concentrations with this drug were probably mediated through other mechanisms. To the best of our knowledge, these findings have never been reported.

To date, no clinical trial has investigated the effects of cilostazol on plasma PCSK9 levels. Statins [12] and fibrates [14] significantly increase PCSK9 concentrations via upregulation of sterol regulatory element-binding protein-2 (SREBP-2). Fibrates may also function via an adiponectin-inducing effect [14]. In contrast, the mechanisms for ezetimibe and niacin are not well known [13, 15]. The ability of niacin to decrease PCSK9 concentrations might be attributed to a reduction in triglyceride synthesis and very low-density lipoprotein particle secretion from the liver [26]. The mechanism underlying the effect of cilostazol on PCSK9 concentrations is poorly understood and could only be speculated. Interestingly, the expression of PCSK9 can be regulated not only by SREBP-2 but also by peroxisome proliferator-activated receptor-γ (PPARγ) [27, 28]. Furthermore, activation of the adiponectin receptor can upregulate PCSK9 expression through activation of PPARγ and the adenosine monophosphate-activated protein kinase signaling pathway [28]. Our previous study revealed that cilostazol could serve as an activator of adenosine monophosphate-activated protein kinase signaling molecules [21] and adiponectin receptors (unpublished data), and our current study demonstrates that cilostazol treatment can increase plasma adiponectin concentrations. In addition, Sanada et al. found that induction of angiogenesis by cilostazol was through activation of PPARγ [29].

According to the current study, a history of metabolic syndrome and changes in TC levels and EPCs count are independently associated with changes in plasma PCSK9 concentrations. The mechanism responsible for this finding is not well known and probably very complex. It might be due to mixed effects

Figure 1: Participant flow diagram. AE, adverse event. SAE, serious adverse event.
Table 1: Baseline characteristics and parameters between patients in the cilostazol treatment and placebo groups

| Variables                        | Cilostazol group (n = 61) | Placebo group (n = 54) | P value |
|----------------------------------|---------------------------|------------------------|---------|
| Age, years                       | 65.5 ± 1.3                | 65.6 ± 1.2             | 0.95    |
| Male gender                      | 38 (62.3)                 | 38 (70.4)              | 0.36    |
| Underlying disease               |                           |                        |         |
| PAD                              | 24 (39.3)                 | 20 (37.0)              | 0.80    |
| CAD                              | 16 (26.2)                 | 14 (25.9)              | 0.97    |
| Old myocardial infarction        | 10 (16.4)                 | 3 (5.6)                | 0.07    |
| Old cerebrovascular accident     | 5 (8.2)                   | 3 (5.6)                | 0.72    |
| Diabetes mellitus                | 30 (49.2)                 | 23 (42.6)              | 0.48    |
| Hypertension                     | 48 (78.7)                 | 40 (74.1)              | 0.56    |
| Hyperlipidemia                   | 47 (77.0)                 | 39 (72.2)              | 0.55    |
| Metabolic syndrome               | 37 (60.7)                 | 33 (61.1)              | 0.96    |
| Tobacco smoking                  | 10 (16.4)                 | 13 (24.1)              | 0.30    |
| Peptic ulcer disease             | 2 (3.3)                   | 3 (5.6)                | 0.66    |
| Chronic kidney disease           | 6 (9.8)                   | 7 (13.0)               | 0.60    |
| Aspirin use                      | 37 (60.7)                 | 23 (42.6)              | 0.06    |
| Clopidogrel use                  | 3 (4.9)                   | 5 (9.3)                | 0.47    |
| ACEI use                         | 10 (16.4)                 | 10 (18.5)              | 0.76    |
| ARB use                          | 29 (47.5)                 | 22 (40.7)              | 0.46    |
| CCB use                          | 37 (60.7)                 | 25 (46.3)              | 0.12    |
| Statin use                       | 33 (54.1)                 | 23 (42.6)              | 0.22    |
| Thiazolidinedione use            | 5 (8.2)                   | 7 (13.0)               | 0.40    |
| Fasting plasma glucose, mg/dL    | 126.3 ± 8.2               | 112.5 ± 3.9            | 0.13    |
| Hemoglobin A1C, %                | 6.7 ± 0.2                 | 6.5 ± 0.1              | 0.32    |
| Fasting insulin, mU/L            | 9.7 (6.7, 15.6)           | 9.7 (5.5, 17.7)        | 0.86    |
| HOMA index                       | 2.6 (1.5, 5.3)            | 2.4 (1.4, 5.0)         | 0.80    |
| Body weight, kg                  | 72.0 ± 1.5                | 73.0 ± 2.1             | 0.68    |
| Waist circumference, cm          | 97.0 ± 1.2                | 96.9 ± 1.6             | 0.93    |
| Body mass index, kg/m²           | 28.1 ± 0.5                | 27.8 ± 0.7             | 0.74    |
| Blood pressure, mmHg             |                           |                        |         |
| Systolic                         | 136.6 ± 2.4               | 130.9 ± 2.1            | 0.07    |
| Diastolic                        | 80.5 ± 2.0                | 76.5 ± 1.5             | 0.11    |
| Heart rate, beats/min            | 78.1 ± 1.7                | 77.4 ± 1.9             | 0.79    |
| White blood cell count, 10³/μL   | 6550.8 ± 213.3            | 6557.4 ± 266.3         | 0.98    |
| Hemoglobin, g/dL                 | 13.8 ± 0.2                | 14.1 ± 0.5             | 0.57    |

(Continued)
### Table 2: Changes in parameters between patients in the cilostazol treatment and placebo groups

| Variables                              | Cilostazol group (n = 61) | Placebo group (n = 54) | P value |
|----------------------------------------|---------------------------|------------------------|---------|
| Fasting plasma glucose, %              | 1.1 ± 2.8                 | 5.4 ± 2.9              | 0.29    |
| Hemoglobin A1C, %                      | -1.8 ± 1.3                | 2.6 ± 1.4              | 0.03    |
| Fasting insulin, %                     | 7.4 ± 7.1                 | 36.4 ± 15.0            | 0.09    |
| HOMA index, %                          | 12.0 ± 8.2                | 50.0 ± 19.6            | 0.08    |
| Body weight, %                         | 0.4 ± 3.3                 | 0.7 ± 2.1              | 0.94    |
| Waist circumference, %                 | -2.0 ± 0.6c               | -2.1 ± 0.7c            | 0.95    |
| Body mass index, %                     | 28.5 ± 29.2               | 14.9 ± 13.4            | 0.68    |

ACEI, angiotensin-converting enzyme inhibitor. ARB, angiotensin receptor blocker. CCB, calcium channel blocker. HOMA, homeostasis model assessment. PBMC, peripheral blood mononuclear cell. SDF-1α, stromal cell-derived factor-1α. TM, thrombomodulin.

Numerical values are expressed as mean ± standard deviation or median (interquartile range), as appropriate, and categorical variables are expressed as number (%).
of concomitant medications and disease, and inverse cause-and-effect relationships. A reduction in TC can lead to an increase in circulating EPC count [30] and might be, on the other hand, associated with increased PCSK9 concentrations due to less PCSK9 use up in certain situations [31]. Furthermore, PCSK9 could probably increase the circulating EPC count via the activation of PPARγ [28] and thereafter VEGF [11, 32], an enhancer of EPC mobilization from bone marrow [33].

The effect of cilostazol on TC and LDL-C is controversial. In the current study, cilostazol treatment had marginally increased TC levels, possibly due to the stimulating effect of cilostazol on PCSK9. Currently, the prognostic role of PCSK9 concentrations in cardiovascular outcomes remains a subject of debate.

| Changes in parameters | Cilostazol group (n = 61) | Placebo group (n = 54) | P value |
|-----------------------|---------------------------|------------------------|---------|
| Blood pressure, %     |                           |                        |         |
| Systolic              | -0.9 ± 1.6                | 0.8 ± 1.2              | 0.41    |
| Diastolic             | 14.5 ± 14.5               | 0.6 ± 1.6              | 0.35    |
| Heart rate, %         | 9.9 ± 2.0                 | -0.3 ± 1.9             | < 0.001 |
| White blood cell count, % | -3.2 ± 3.0          | 7.2 ± 6.5              | 0.14    |
| Hemoglobin, %         | -1.3 ± 0.8                | -3.4 ± 2.1             | 0.35    |
| Platelet count, %     | 5.3 ± 1.6                 | 0.1 ± 1.4              | 0.02    |
| TC, %                 | 4.0 ± 2.7                 | -2.6 ± 2.7             | 0.08    |
| Triglyceride, %       | -13.0 ± 4.0               | 16.5 ± 5.8             | < 0.001 |
| HDL-C, %              | 10.1 ± 2.2                | 2.6 ± 3.3              | 0.05    |
| LDL-C, %              | 5.4 ± 4.8                 | 0.3 ± 3.1              | 0.37    |
| Colony-forming units, % | 60.2 ± 25.9          | 93.3 ± 27.9            | 0.39    |
| KDR+CD34+ cells, %    | 91.2 (25.9, 469.2)       | -28.1 (-76.0, 95.0)    | < 0.001 |
| CD146+annexin V+ cells, % | -33.3 (-76.0, 75.0) | 0 (-48.1, 100.0)      | 0.13    |
| BrDU incorporation, % | 26.3 ± 10.6               | 15.1 ± 10.7            | 0.46    |
| XTT absorbance, %     | 32.6 ± 14.3               | 3.5 ± 5.7              | 0.06    |
| Nucleosome fragmentation, % | 5.5 (-41.8, 49.9) | 6.1 (-26.0, 83.4)     | 0.47    |
| Migrated cells, %     | -14.7 (-41.8, 25.3)      | 10.6 (-17.5, 34.0)     | 0.01    |
| Soluble TM, %         | 4.6 ± 4.5                 | 14.9 ± 8.3             | 0.27    |
| VEGF-A165, %          | 41.8 (16.1, 91.0)         | -6.6 (-39.9, 15.5)     | < 0.001 |
| SDF-1α, %             | 18.1 (-28.9, 65.6)        | 3.8 (-24.0, 57.5)      | 0.58    |
| Adiponectin, %        | 10.5 (-21.1, 50.9)        | -18.8 (-35.5, 5.0)     | 0.002   |
| PCSK9, %              | 18.7 ± 8.9                | -6.5 ± 5.1             | 0.02    |

Abbreviations as shown in Table 1.

aChanges in parameters = (post-treatment value – baseline value) / baseline value × 100%. Values are expressed as mean ± standard deviation, number (%), or median (interquartile range), as appropriate.

bP < 0.05 vs. baseline. cP < 0.01 vs. baseline.
Figure 2: Effects of cilostazol on proprotein convertase subtilisin/kexin type 9 (PCSK9) concentrations, lipid parameters, and circulating number and functions of endothelial progenitor cells (EPCs). Our data revealed the effects of cilostazol treatment in (A) PCSK9 concentrations, (B) total cholesterol (TC) levels, (C) triglyceride levels, (D) high-density lipoprotein cholesterol (HDL-C) levels, (E) circulating number of EPCs, and (F) migrated EPCs. Values are expressed as mean ± standard deviation or median (interquartile range), as appropriate.
Table 3: The association of baseline characteristics and changes in plasma levels of PCSK9 in the entire cohort

|                         | Changes in PCSK9 levels (%) |       |       |       |
|-------------------------|-----------------------------|-------|-------|-------|
|                         | In patients with history of | In patients without history of |       |       |
| Male gender             | 8.5 ± 7.2                   | 3.7 ± 7.7 | 0.68  |       |
| Underlying disease      |                             |       |       |       |
| Diabetes mellitus       | 0.6 ± 7.1                   | 12.3 ± 8.0 | 0.29  |       |
| Hypertension            | 7.0 ± 6.4                   | 6.3 ± 10.1 | 0.96  |       |
| Hyperlipidemia          | 9.2 ± 6.5                   | -0.1 ± 9.5 | 0.46  |       |
| Metabolic syndrome      | -1.7 ± 6.1                  | 20.2 ± 9.9 | 0.05  |       |
| Tobacco smoking         | -2.3 ± 11.9                 | 9.2 ± 6.1 | 0.40  |       |
| PAD                     | -2.2 ± 8.0                  | 12.5 ± 7.2 | 0.19  |       |
| CAD                     | -4.8 ± 9.6                  | 11.0 ± 6.5 | 0.20  |       |
| Myocardial infarction   | 4.1 ± 16.6                  | 7.2 ± 5.8 | 0.86  |       |
| Cerebrovascular accident| 19.4 ± 29.9                 | 5.9 ± 5.4 | 0.53  |       |
| Chronic kidney disease  | -5.2 ± 11.9                 | 8.4 ± 5.9 | 0.43  |       |
| Aspirin use             | 9.8 ± 7.1                   | 3.7 ± 8.3 | 0.58  |       |
| Clopidogrel use         | -0.9 ± 14.3                 | 7.4 ± 5.7 | 0.70  |       |
| RASI use                | 12.5 ± 7.4                  | -1.9 ± 7.8 | 0.20  |       |
| CCB use                 | 1.7 ± 6.0                   | 12.9 ± 9.5 | 0.32  |       |
| Diuretic use            | -6.1 ± 7.2                  | 11.0 ± 6.7 | 0.18  |       |
| Statin use              | 3.9 ± 7.2                   | 9.7 ± 8.1 | 0.60  |       |

Abbreviations as shown in Table 1. RASI, renin-angiotensin system inhibitor.

*aChanges in parameters = (post-treatment value – baseline value) / baseline value × 100%. Values are expressed as mean ± standard deviation.

Table 4: Correlation between changes in PCSK9 levels and significant changes of parameters in the entire cohort

|                         | Changes in PCSK9 levels | r     | P value |
|-------------------------|-------------------------|-------|---------|
| Changes in TC levels    | -0.22                   | 0.03  |         |
| Changes in KDR·CD34+ cells | 0.21                   | 0.04  |         |

Abbreviations as shown in Table 1.

Table 5: Independent predictors of changes in plasma levels of PCSK9 by multivariable linear regression analysis in the entire cohort

|                         | Changes in PCSK9 levels | Beta (95% CI) | P value |
|-------------------------|-------------------------|---------------|---------|
| Cilostazol use          | 0.20 (0.02, 0.48)       | 0.04          |         |
| History of metabolic syndrome | -0.19 (-0.47, -0.01) | 0.04          |         |
| Changes in TC levels    | -0.27 (-1.43, -0.26)   | 0.01          |         |
| Changes in KDR·CD34+ cells | 0.19 (0, 0.02)       | 0.05          |         |

Abbreviations as shown in Table 1.
effect of additional PCSK9 inhibition during cilostazol treatment warrants further investigation.

MATERIALS AND METHODS

Patient population

This post hoc analysis was performed using two previous prospective, double-blind, randomized, placebo-controlled trials, which consecutively enrolled eligible patients with PAD ($n = 44$; started in January 2012 and completed in September 2013; cohort 1: ClinicalTrials.gov identifier: NCT01952756) or at high risk of CVD without pre-existing atherosclerotic disease ($n = 71$; started in January 2013 and completed in August 2014; cohort 2: ClinicalTrials.gov identifier: NCT02194686). The results of both studies have been published [19, 20]. The detailed inclusion and exclusion criteria were described previously [19, 20]. Briefly, patients who had mild-to-moderate PAD identified by ankle-brachial indices ($< 0.9$) but without obvious symptoms of intermittent claudication or critical limb ischemia were enrolled in cohort 1, whereas patients who had multiple cardiovascular risk factors but not pre-existing atherosclerotic diseases such as PAD or CAD were included in cohort 2. The current study without necessity of obtaining informed consent was conducted according to the Declaration of Helsinki and under the approval of the Ethics Committee of National Cheng Kung University Hospital (IRB number: A-ER-104-345).

Randomization and allocation to treatment

During a 1-week run-in period, 124 eligible patients were screened (44 in cohort 1 and 80 in cohort 2). Eight patients were excluded prior to randomization (7 consent withdrawal; 1 screening failure due to active malignancy). During the 12-week study period, the eligible patients were randomly assigned to receive either 200 mg cilostazol ($n = 61$) or placebo ($n = 55$) daily (Figure 1) via the unrestricted randomization method with sealed envelopes for allocation concealment. The patients, care providers, and those who administered the interventions or performed assays were all blinded after assignment to interventions. One patient in the placebo group withdrew informed consent after treatment.

Measurement of serum or plasma biomarkers

The baseline blood samples were obtained from the peripheral veins in all participants before treatment and the final samples were obtained using the same procedure one day immediately after the end of the study period. The blood samples were sent for isolation, cell culture, and assay of human EPCs, and prepared and stored for use in an enzyme-linked immunosorbent assay, as described previously [11, 19, 20, 37, 38]. Plasma or serum concentrations of biomarkers were measured using commercial kits (R&D Systems Inc., Minneapolis, MN, USA; and Mercodia AB, Uppsala, Sweden).

Isolation and culture of EPCs, and determination of numbers of circulating EPCs and apoptotic endothelial cells

Isolation of early EPCs was performed by Ficoll density gradient centrifugation according to standard protocols, and colony formation by EPCs was identified and quantified, as described previously [11, 19–21]. EPCs were defined as CD45−CD34+ kinase insert domain receptor (KDR)+ cells, and circulating endothelial cells were defined as CD45−CD146− annexin V− cells [11, 20]. All fluorescence-labeled antibodies were purchased from Becton Dickinson (Arlington, VA, USA).

Measurement of EPC functions in vitro

As previously described [11, 19–21], EPC migration was measured using modified Boyden chambers, proliferation and viability were assessed by bromodeoxyuridine (BrdU) and XTT assays, and EPC apoptosis was determined using a terminal 2′-deoxyuridine 5′-triphosphate nick end labeling assay kit (Roche, Basel, Switzerland).

Primary measurement

As reported previously, the primary endpoint of both studies was the number of circulating EPCs, and the secondary endpoint was the viability of EPCs [19, 20]. The sample size estimation and power calculation were performed accordingly and described previously [20]. However, the current post hoc analysis aimed to detect the effect of cilostazol treatment on plasma PCSK9 levels as the primary measurement.

Statistical analysis

Each analysis was performed in a per-protocol manner. Distributions of numerical variables in both groups were expressed as mean ± standard deviation, and skewed data were reported as median (interquartile range). Chi-square or Fisher’s exact test were performed for comparing categorical variables between groups, whereas the Mann-Whitney U test or unpaired Student’s $t$-test were performed for comparing numerical variables, as appropriate. Differences between baseline and post-treatment values were analyzed by the Wilcoxon signed-rank test or paired Student’s $t$-test, as appropriate. The Pearson correlation was used to assess the relationship between the changes in metabolic factor levels in the
plasma or serum, vasculoangiogenic biomarkers, EPC parameters, and changes in plasma PCSK9 levels after treatment over the entire cohort. The independent correlation of cilostazol use with the changes in plasma PCSK9 levels was adjusted for all uni-variables (a $P$ value < 0.1) with a multivariable linear regression model by a stepwise regression method. A $P < 0.05$ (2-sided) was considered to indicate statistical significance. All statistical analyses were performed using SPSS for Windows (Version 13.0, SPSS Inc., Chicago, IL, USA).

Author contributions

ICC, WKT and THC conceived and designed the study; ICC, WKT, SYT, PYL and THC collected data; THC performed statistical analysis; ICC, WKT, YHL, PYL and THC reviewed studies and interpreted the data; ICC, WKT and THC wrote the manuscript; SYT prepared figures; ICC, WKT and THC obtained financial support for this study. All authors critically reviewed the manuscript and contributed to manuscript revision.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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