

## Effect of Intensive Atorvastatin Therapy on Periprocedural PTEN Expression in CD4+T Lymphocytes of Patients with Unstable Angina Undergoing Percutaneous Coronary Intervention

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### Abstract

**Objectives:** To investigate the effects of intensive atorvastatin therapy on PTEN expression by CD4+ T lymphocytes in patients with unstable angina (UA) that received PCI.

**Methods:** All patients with UA were randomly divided into the pretreatment with an intensive atorvastatin (ATV) group (80mg 12h before PCI, with a further 20mg every day after PCI, n = 56) or a conventional (control) group (only 20 mg/day, n = 56). Circulating CD4+ T cells were subsequently obtained prior to PCI and 18–24 h after successful PCI, using a magnetic cell sorting system. Plasma cTnI, CK-MB, hsCRP, IL-10 and TNF- $\alpha$  levels were measured just prior to the PCI and 18–24 h after PCI. PTEN mRNA and protein were determined by Real-time PCR and western blots, respectively.

**Results and Discussion:** PTEN mRNA and protein were dramatically decreased in ATV group ( $p < 0.05$ ). In contrast, TNF- $\alpha$  and hsCRP significantly increased following PCI in two groups, with the ATV group being higher than control group ( $p < 0.05$ ). IL-10 also markedly increased following PCI for the two groups. However, higher values were associated with the ATV group ( $p < 0.05$ ). Compared to the control group, the incidence of elevated cTnI levels post-PCI was lower in the ATV group ( $p < 0.05$ ); however, no difference could be found between the two groups regarding the incidence of elevated CK-MB post-PCI ( $p > 0.05$ ).

**Conclusion:** Intensive atorvastatin treatment reduced the post-PCI myocardial inflammatory response in patients with UA, possibly by enhancing PTEN expression in CD4+ T lymphocytes.

**Keywords:** Unstable angina; Atorvastatin; CD4+ T lymphocytes; PTEN; TNF- $\alpha$ ; IL-10.

## Introduction

PMI is a common complication associated with PCI, and has been reported for approximately 15% to 20% of patients undergoing PCI [1]. Most patients with PMI remain asymptomatic and no changes in cardiac function. However, PMI has been associated with an increased risk of short-term and long-term mortality following PCI. Once PMI occurs, there is no effective treatment to improve it. Therefore, the prevention of PMI might be clinically important. One such therapy involves hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins) which can be administered in a loading dose prior to PCI. In the Atorvastatin for Reduction of Myocardial Damage during Angioplasty (ARMYDA) trial demonstrated that intensive atorvastatin could reduce in PMI of patients undergoing PCI, and it is hypothesized that the favorable clinical prognosis associated with intensive statin therapy results from an anti-inflammatory effect mediated by these agents [2 - 4].

Plaque rupture can occur following PCI, and may induce both a local and systemic inflammatory response that is accompanied by the release of inflammatory factors for leukocyte and platelet activation (e.g., TNF- $\alpha$ , IL-6) [5 - 7]. Phosphatase and tension homolog deleted on chromosome ten (PTEN) has been identified as a tumor-suppressor gene and has been associated with maintaining the balance between cell survival and cell death, and which has been shown to inhibit an inflammatory response. Gunzl et al. reported that PTEN-deficient immunocytes increase inflammatory cytokine production, and reduce anti-inflammatory cytokine IL-10 production [8]. In addition, our preliminary experiment identified a significant decrease in PTEN expression by CD4<sup>+</sup> T lymphocytes in patients undergoing PCI compared with controls. Based on these findings, it is hypothesized that PTEN plays a role in the inflammatory response following PCI while atorvastatin could up-regulate the expression of PTEN in CD4<sup>+</sup>T lymphocytes, reduce inflammatory cytokine production and result in the reduction of PMI.

It remains unclear whether statin agents can inhibit the unfavorable inflammatory responses associated with coronary intervention procedures, and thereby decrease the incidence of PMI. Therefore, the goal of this study was to investigate the impact of intensive statin therapy on periprocedural PTEN expression by CD4<sup>+</sup> T lymphocytes in patients with UA in order to assess the potential protective mechanism(s) mediated by statin agents.

## Subjects and Methods

### Study Population and Protocol

This was a random, prospective, double-blind, placebo-controlled clinical trial. A total of 128 patients fulfilling the inclusion criteria from September 2013 to August 2014 were initially evaluated. Nine patients were excluded because of current or earlier treatment with statins, five because of low ejection fraction, and two for renal failure. A total of 112 patients fulfilling the inclusion criteria were included in the study. Eligible patients (n=112) were randomly divided into the pretreatment with an intensive atorvastatin (ATV) group (80mg 12h before PCI, with a further 20mg every day after PCI, n = 56) or a conventional (control) group (20 mg/day, n = 56). After coronary angiography, 5 patients were excluded from the study (two were treated medically and three with bypass surgery). Thus, 107 patients were enrolled and they were the study population. According to our standard protocol, all patients without contraindications were pretreated with aspirin (100mg/d) and with Clopidogrel (300mg) at least 6h before the procedure.

Unstable angina was diagnosed according to the guideline for diagnosis and treatment of patients with unstable angina [9]. Successful PCI was defined according to the American Heart Association/American College of Cardiology Task Force recommendations [10]. The criteria used were as follows: no in-hospital complication; improvement in luminal narrowing to 30% of a contiguous normal vessel, and complete resolution of clinical manifestation of ischemia after PCI.

Exclusion criteria included: 1) previous or current treatment with statins, 2) allergy or contraindication to statins, 3) the presence of severe infectious diseases or tumors, 4) severe liver or renal dysfunction, 5) unstable angina with high-risk features warranting an emergency coronary angiography, 6) acute stroke, 7) a left ventricular ejection fraction < 30%, or 8) previous MI or previous revascularization (PCI or CABG). This study was approved by Ethics Committee, and all patients provided informed consent before participation.

### Interventional Procedure

PCI was performed using a standard technique, through the radial artery route. Routine care was taken before and after the procedure for all patients, including pretreatment with a loading dose of clopidogrel (300 mg initial oral bolus) at least 6h before the procedure, followed by 75 mg/day at least for 12 months, in addition to lifelong aspirin medication (100mg/day). No additional heparin or glycoprotein IIb/IIIa inhibitors were needed during or after the procedure. The radial artery sheath was removed immediately after the end of the procedure.

## Angiographic Analysis

Classification of coronary artery morphology based on the report of the American Heart Association/American College of Cardiology Task Force was used [10]. Coronary angiograms were reviewed by independent observers blinded to the results of biochemical assays. Intimal major or minor dissection, thrombus, abrupt closures in a previously patent vessel, no-reflow, spasm and side-branch occlusion were assessed. The degree of perfusion was evaluated according to TIMI criteria [11]. No-reflow phenomenon was defined as TIMI flow grade 0, 1, or 2 without a mechanical obstruction on angiograms after PCI. Left ventricular function was assessed by angiography in all patients.

## Blood Sampling and Analyzing

Peripheral venous blood (20 ml) was collected from each patient 18–24 h before and after PCI, respectively, and blood serum was stored at  $-20^{\circ}\text{C}$ . Cardiac troponin I (cTnI), creatine kinase MB(CK-MB) and highly sensitive C-reactive protein (hs-CRP) levels of plasma were tested before and 18 - 24 h after PCI. Serum concentrations of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-10 (IL-10) were detected by Enzyme-Linked Immunosorbent Assay, ELISA, according to the manufacturer's protocol (eBioscience, San Diego, Calif., USA).

## Cell Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Hypaque as an established protocol [12]. Isolated PBMCs were washed twice and resuspended in 1640 culture medium (Hyclone, USA). CD4<sup>+</sup> T lymphocytes were isolated from prepared PBMCs using an immunomagnetic sorting technique (Dyna, NOR). Subsequently, cells were stained with 0.4% trypan blue to assay cell viability. Overall purity of isolated CD4<sup>+</sup>T cells was above 95%, as confirmed by fluorescence activated cell sorter (FACS) analysis.

## Real-time PCR Assay for Quantification of PTEN mRNA

RNA was extracted from isolated PBMCs using a kit (KeyGEN, China). The nanodrop method was used to detect RNA concentrations, and RNA degradation was detected using agarose gel electrophoresis. cDNA were synthesized using a cDNA reverse transcription kit (Fermentas, Lithuania). Amplification of PTEN mRNA was performed using a real-time PCR assay. The reaction mixture contained PTEN-primer (Sangon Biotech, China):

5'-CTATTCCCAGTCAGAGGCGCTAT-3', 5'-TGAAGT-TGTCTTCCCGTCGTGT-3', and a GAPDH-control (Sangon Biotech, China):5'-GA-

GTCAACGGATTTGGTCGT-3',5'  
GACAAGCTTCCCGTTCTCAG-3'.

For telomerase mediated extension of the PTEN primer, reactions were incubated at  $23^{\circ}\text{C}$  for 30 min. The mixture was then heated to  $94^{\circ}\text{C}$  for 5 min to inactivate telomerase activity, then was subjected to 35 cycles of  $94^{\circ}\text{C}$  for 30 s,  $50^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 90 s. PCR reaction products were separated using 10% polyacrylamide gel electrophoresis and were detected by SYBR green (Gene Inc.) staining. Fluorescent intensity was detected using a spectrophotometer. PTEN mRNA was quantified using a PCR ELISA kit according to the manufacturer's instructions (KeyGEN, China).

## Western Blotting for PTEN Protein Expression

Briefly, cells were centrifuged and supernatants were removed. Cells were lysed with  $1\times$  SDS sample buffer, and protein concentration was detected using the BCA method. Proteins were separated by SDS-PAGE, transferred to PVDF membranes for 40 min, then blocked in 5% non-fat dry milk in TBS for 1 h. Membranes were incubated with primary antibodies raised against PTEN (Abcam, USA) and GAPDH (Jackson ImmunoResearch, USA) overnight at  $4^{\circ}\text{C}$ . Membranes were then washed 5x with 0.05% Tween-20/TBS at RT. Membranes were reblocked in 10% non-fat dry milk in 0.05% Tween-20/TBS for 10 min before being incubated with IRDye® secondary antibodies (LI-COR, USA) for 2 h at RT. After washing with 0.05% Tween-20/TBS, immunoblots were imaged and bands were quantified by densitometry using Odyssey Infrared Imaging System software (LI-COR Biosciences, USA). GAPDH was used as an internal control.

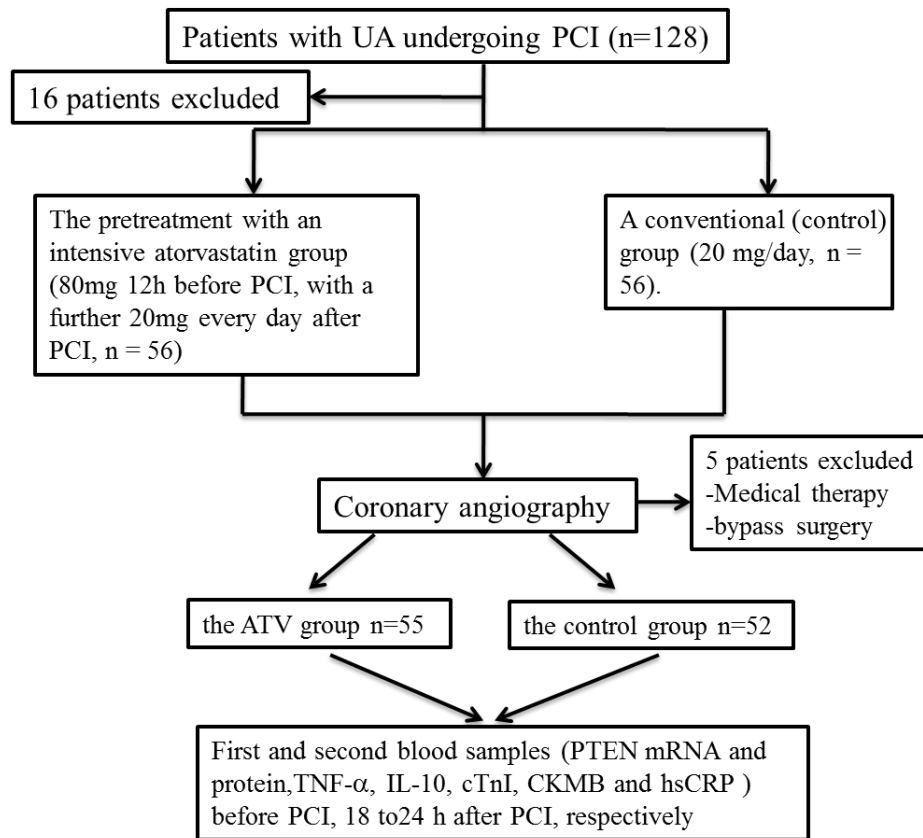
## Statistical Analysis

Statistical analyses were performed with SPSS version 17.0 software. Data are expressed as mean  $\pm$  SD or percentages for categorical variables. To compare parametric continuous variables, the independent Student t-test was used. For categorical variables, the  $\chi^2$  test was used.  $P < 0.05$  was considered to be statistically significant.

## Results

The studied population was composed of 55 patients in the ATV group, 52 patients in the control group. A CONSORT (Consolidated Standards of Reporting Trials) flow diagram is shown in Figure 1. Clinical and procedural variables in the ATV and ATV+TMZ groups are shown in Tables 1, 2 and 3 respectively. The two groups were similar with regard to age, sex, cardiovascular risk factors, mean time to angiography, and medical therapy at the time of intervention. Coronary anatomy, lesion type, procedural characteristics, use of drug-eluting stents, diameter and length of implanted stents were similar.

**Figure 1:** A CONSORT (Consolidated Standards of Reporting Trials) flow diagram



UA: unstable angina; Cardiac troponin I cTnI: cardiac troponin; CK-MB: creatine kinase MB; hs-CRP: highly sensitive C-reactive protein; PTEN: phosphatase and tension homolog deleted on chromosome ten; TNF- $\alpha$ : tumor necrosis factor- $\alpha$  and IL-10: interleukin-10

**Table 1:** Clinical characteristics of the study population

Variables	ATV (n=55)	Control (n=52)	P value
Mean (SD) age (years)	63(12)	61(13)	0.935
Sex, male, n (%)	35(64)	31(60)	0.891
Renal insufficiency, n (%)	21(38)	18(35)	0.702
Angina class,*n (%)			
1~2	34(62)	33(63)	0.861
3~4	21(38)	19(37)	0.861
Risk factors, n (%)			
Smoking (past or current)	36(65)	33(63)	0.830
Hypercholesterolemia	28(51)	23(44)	0.489
Hypertension	38(69)	32(62)	0.412
Diabetes mellitus	21(38)	15(29)	0.307
Family history	18(33)	10(19)	0.112
Mean $\pm$ SD BMI (kg/m <sup>2</sup> )	26.4 $\pm$ 3.5	25.8 $\pm$ 3.8	0.894
Medication, n (%)			
ACEI or ARB	40(73)	38(73)	0.968
$\beta$ Blockers	35(64)	30(58)	0.529
Calcium antagonist	28(51)	24(46)	0.623
Aspirin	55(100)	52(100)	1.000
Clopidogrel	55(100)	52(100)	1.000
Insulin	13(24)	8(15)	0.283
Nitrates	28(51)	30(58)	0.482

ACEI: angiotensin-converting enzyme inhibitors; ARB: angiotensin receptor blocker; BMI: body mass index; \*Classification of unstable angina according to Braunwald.

**Table 2:** Angiographic characteristics of the study population

Variable, n (%)	ATV(n=55)	Control (n=52)	P Value
Lesion class*			
A+B1	40(73)	41(79)	0.461
B2+C	15(27)	11(21)	0.461
Artery involved			
Left main artery	8(15)	5(10)	0.435
Left anterior descending artery	26(47)	29(56)	0.379
Left circumflex artery	13(24)	14(27)	0.696
Right coronary artery	15(27)	13(25)	0.789
Diagonal	4(7)	3(6)	1.000
Saphenous venous graft	0	0	1.000
Bifurcation lesion	9(16)	8(13)	0.890
Mean±SD LVEF (%)	58.3±9.5	60.3±8.6	0.823

\*According to the American Heart Association/American College of Cardiology classification.

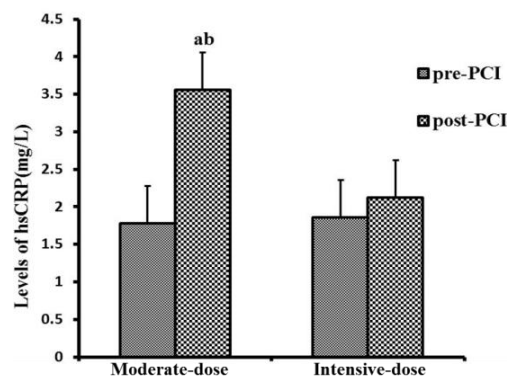
**Table 3:** Procedural characteristics and complications of the study population

Parameters	ATV (n=55)	Control (n=52)	P Value
drug eluting stents ,n (%)	55(100)	52(100)	1.000
Stent length (mm)	24.2±2.9	23.8±2.2	0.832
Stent diameter (mm)	2.92±0.46	3.01±0.52	0.786
Total inflation time (s)	27±5	26±6	0.762
Inflation maximal pressure (atm)	15.3±2.6	15.2±3.3	0.925
TIMI flow grade≤2, n (%)	0	0	1.000
TIMI flow grade = 3, n (%)	55(100)	52(100)	1.000
ST change during inflation, n (%)	10(18)	8(15)	0.699
Procedural complication, n (%)			
Side-branch occlusion	5(9)	4(8)	1.000
Coronary dissection	4(7)	3(5)	1.000
No reflow	0	0	1.000
Coronary spasm	7(13)	5(10)	0.586
Coronary embolization	2(4)	1(2)	1.000

TIMI: Thrombolysis in Myocardial Infarction Trial.

Serum levels of CK-MB and cTnI did not statistically differ between the two groups prior to PCI and both were in the normal range. Compared to the control group, the incidence of elevated cTnI levels 18–24 h post-PCI was lower in the ATV group (38.4% and 63.6%, respectively,  $p=0.000$ ); and, difference could be found between the two

groups regarding the incidence of elevated CK-MB 18–24 h post-PCI (25.5% and 32.8%, respectively,  $p=0.000$ ). Moreover, serum levels of hs-CRP significantly increased following PCI in both the ATV and control groups, with the latter being higher than the former ( $p < 0.05$ ; Figure 2).

**Figure 2:** Changes of the serum levels of hs-CRP

The a indicates a P-value  $< 0.05$  compared with the intensive-dose (post-PCI) group. The b indicates a P-value  $< 0.05$  compared with the moderate-dose (pre-PCI) group.

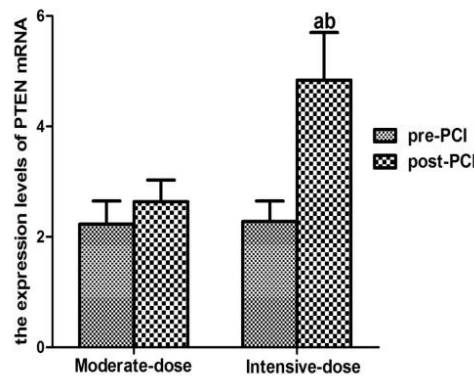
Prior to PCI, no significant differences were observed in the mRNA ( $2.28 \pm 0.37$  vs  $2.23 \pm 0.42$ ) and protein levels of PTEN ( $0.243 \pm 0.032$  vs  $0.215 \pm 0.054$ ) detected for the ATV and control groups ( $P > 0.05$ ). However, for the intensive-dose atorvastatin group, both mRNA ( $4.84 \pm 0.86$ ) and protein ( $0.625 \pm 0.043$ ) levels of PTEN were found to markedly increase between 18-24h after PCI compared with the levels detected prior to PCI ( $P < 0.05$ ). In contrast, there was no statistical difference between the mRNA ( $2.64 \pm 0.39$ ) and protein ( $0.285 \pm 0.046$ ) levels of PTEN detected prior to and 18-24h after PCI for the control group ( $P > 0.05$ ), as summarized in Figures 1, 2.

Serum levels of TNF- $\alpha$  and IL-10 did not statistically differ between the two groups prior to PCI ( $P < 0.05$ , Figures 3, 4). For example, levels of TNF- $\alpha$  for the ATV group were  $7.74 \pm 1.23$  pg/ml versus  $7.62 \pm 1.38$  pg/ml for the control

group ( $P > 0.05$ ). Similarly, levels of IL-10 were  $7.26 \pm 0.94$  pg/ml and  $7.42 \pm 1.12$  pg/ml, respectively ( $P > 0.05$ ). 18-24 h after PCI, patients in the ATV group exhibited a slight increase in serum levels of TNF- $\alpha$  ( $P > 0.05$ ). However, patients in the control group exhibited a significant increase in serum levels of TNF- $\alpha$  ( $P < 0.05$ ). Furthermore, for the ATV group, the level of TNF- $\alpha$  was lower and IL-10 was higher than the control group (TNF- $\alpha$ :  $8.19 \pm 1.56$  pg/ml vs.  $14.13 \pm 2.42$  pg/ml; IL-10:  $12.26 \pm 1.34$  pg/ml vs.  $8.89 \pm 0.98$  pg/ml, respectively;  $P < 0.05$  in both cases).

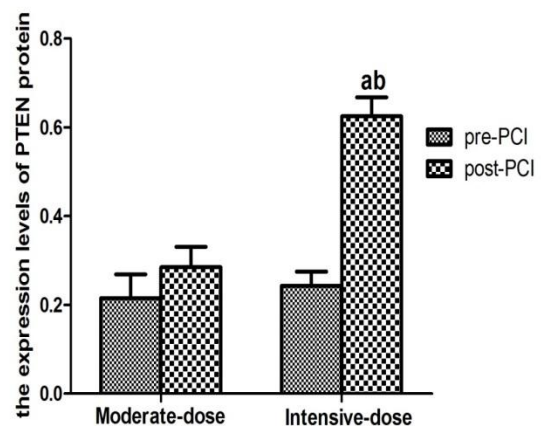
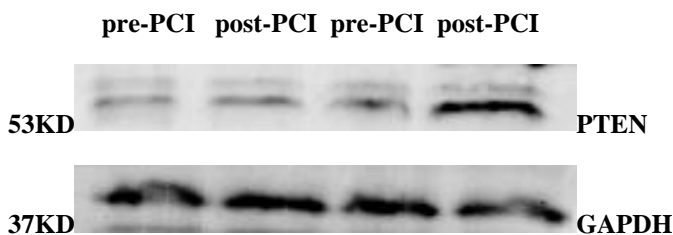
Linear correlation analysis indicated that the level of PTEN was negatively correlated with TNF- $\alpha$  ( $r = -0.672$ ,  $P = 0.000$ ) and hsCRP ( $r = -0.598$ ,  $P = 0.000$ ). Additionally, PTEN was positively correlated with IL-10 ( $r = 0.556$ ,  $P = 0.000$ ) and cTnI ( $r = 0.486$ ,  $P = 0.000$ ).

**Figure 3:** Fluorescence based RT-PCR was used to detect mRNA levels of PTEN



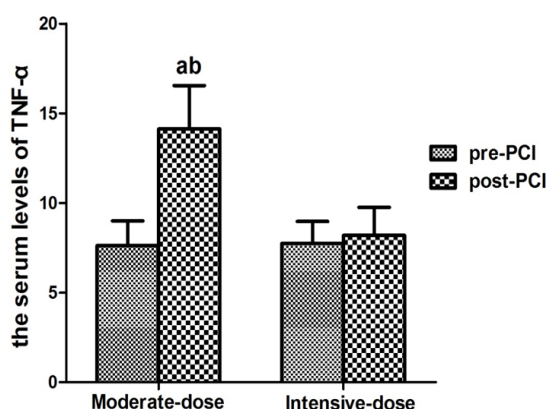
The a indicates a P-value  $< 0.05$  compared with the intensive-dose (pre-PCI) group. The b indicates a P-value  $< 0.05$  compared with the moderate-dose (post-PCI) group.

**Figure 4:** The relative expression of PTEN protein moderate-dose group intensive-dose group



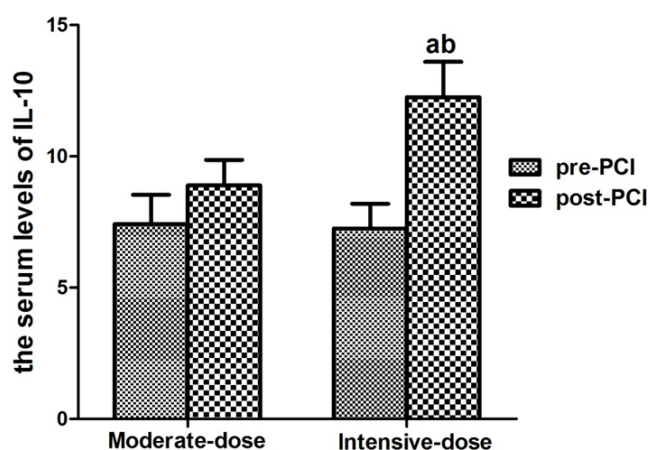
The a indicates a P-value  $< 0.05$  compared with the intensive-dose (pre-PCI) group. The b indicates a P-value  $< 0.05$  compared with the moderate-dose (post-PCI) group.

**Figure 5:** Changes of the serum levels of TNF- $\alpha$  (pg/ml)



The a indicates a P-value < 0.05 compared with the intensive-dose (post-PCI) group. The b indicates a P-value < 0.05 compared with the moderate-dose (pre-PCI) group.

**Figure 6:** Changes of the serum levels of IL-10 (pg/ml)



The a indicates a P-value < 0.05 compared with the intensive-dose (post-PCI) group. The b indicates a P-value < 0.05 compared with the moderate-dose (pre-PCI) group.

## Discussion

This study demonstrates that pretreatment with an 80 mg acute oral loading dose of atorvastatin before elective PCI limits myocardial damage, as shown by the incidence of elevated cTnI levels after PCI. Relative expression of PTEN mRNA and PTEN protein dramatically increased following PCI in CD4+ T lymphocytes of patients receiving intensive-dose atorvastatin treatment prior to PCI versus a conventional treatment. Moreover, serum levels of TNF- $\alpha$  and hsCRP were found to be lower, and IL-10 levels were higher, for the intensive-dose atorvastatin group compared with the control group.

Accumulating evidence indicates that excessive activity of CD4+ T lymphocytes, and increased secretion of various

inflammatory factors, occurs in patients with coronary artery disease [13]. Furthermore, coronary interventional procedures may accelerate deterioration of the inflammatory process induced by CD4+ T lymphocytes [5]. PTEN is a protein that acts as a tumor suppressor, and is a negative regulator of inflammation. Correspondingly, in PTEN-knockdown cells, expression of the immunomodulatory factor (IL-10) was found to decrease [8], and expression of the pro-inflammatory factor, TNF- $\alpha$  markedly increased [14, 15]. In the present study, following a coronary interventional procedure, PTEN mRNA and protein produced by CD4+ T lymphocytes were found to be down-regulated, and serum levels of TNF- $\alpha$  were also significantly elevated. These findings suggest that PTEN may play a role in the inflammatory response induced by CD4+ T lymphocytes in response to a coronary artery interventional procedure.

The ARMYDA study revealed that the average percent increase in CRP levels from baseline was significantly lower in the intensive atorvastatin treatment arm [4], and intensive-dose atorvastatin treatment significantly reduced the release of myocardial damage markers detected following coronary intervention, including myoglobin, cTnI, and CK-MB [3]. Our results found that a 80 mg acute oral loading dose of atorvastatin 12h before PCI can significantly lower incidence of elevated cTnI levels post-PCI, showing intensive-dose atorvastatin pretreatment can reduce post-PCI troponin levels. Several studies have demonstrated that statin agents can directly inhibit the activity of T lymphocytes, and consequently, can inhibit the development of an inflammatory response [16, 17]. Moreover, atorvastatin has been shown to modulate the cellular immune balance between activation and negative regulation on Th1/Th2 cells [18], thereby promoting the expression of anti-inflammatory factor, IL-10, and inhibiting expression of the proinflammatory factor, TNF- $\alpha$  [19]. Indeed, the results of the current study indicate that the administration of an intensive-dose atorvastatin treatment 12h before PCI can significantly up-regulate the expression of PTEN, and also decrease serum levels of TNF- $\alpha$  and hsCRP and increase serum levels of IL-10. The mechanisms that underlie the beneficial effects of intensive-dose atorvastatin treatment remain unclear. However, based on the findings of the present study, we hypothesize that intensive-dose statin treatments are associated with reduced periprocedural myocardial damage following PCI due to an up-regulation of PTEN expression in CD4+ T lymphocytes, which affects the production of pro- and anti-inflammatory factors (e.g., TNF- $\alpha$ , IL-10).

PTEN was known as a tumor suppressor. As a target gene for microRNA-21, PTEN had been widely studied not only in tumor but also in inflammation recently. Kim et al. [20] investigated that activation of PTEN expression and suppression of the inflammatory response in vascular

smooth muscle cells. Blum et al. [21] demonstrated that loss of PTEN and, therefore, sustained PI3-Kinase signaling in myeloid cells especially elevates the osteoclastogenic potential of myeloid cells, leading to enhanced inflammatory local bone destruction. Lin et al. [22] reported Macrophages with PTEN deficiency developed a continuous inflammatory microenvironment, which further aggravated tumor cell growth. From our study, it can also be seen that PTEN as an anti-inflammatory protein may play an important role in post-PCI myocardial inflammatory response in patients with unstable angina. Meanwhile, intensive-dose atorvastatin pretreatment with its anti-inflammatory effect can reduce post-PCI myocardial inflammation by up-regulating PTEN expression in CD4+ T lymphocytes.

Here, a small-scale population was examined, and this may discount the beneficial effects of intensive-dose atorvastatin treatment on periprocedural myocardial damage that were observed. In conclusion, we can also observe that atorvastatin load has a very prompt effect on inflammation markers of these patients. These results identify one of the possible pleiotropic effects of this drug category.

## Study Limitations

This study was based on a limited number of observations made in a small population of patients, potentially diminishing the validity of the drawn statistical inference. The present study results should be applied only with caution to clinical situations in which UA is potentially involved, and further investigation is required. In addition, the incidence of clinical prognostic outcomes, such as cause of death, myocardial infarction, and target vessel revascularization, were not reported. Therefore, a larger study is needed to confirm the observations of the present study and to investigate the clinical endpoints of acute coronary syndrome patients that receive intensive-dose atorvastatin therapy.

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