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Original Article

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Neopterin and Cardiovascular Events Following Coronary Stent Implantation in Patients with Stable Angina Pectoris

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Aim: Neopterin is an activation marker for monocytes/macrophages. We prospectively investigated the predictive value of plasma neopterin levels on 2-year and long-term cardiovascular events in patients with stable angina pectoris (SAP) undergoing coronary stent implantation.

Methods: We studied 123 consecutive patients with SAP who underwent primary coronary stenting (44 patients with bare metal stent: BMS group and 79 with drug-eluting stent: DES group). Plasma neopterin levels were measured on admission using HPLC. Moreover, one frozen coronary artery specimen after DES and three frozen coronary specimens after BMS were obtained by autopsy or endarterectomy, followed by immunohistochemical staining for neopterin.

Results: Plasma neopterin levels were significantly higher in patients with cardiovascular events than in those without them ($P<0.001$). In subgroup analyses, higher levels of plasma neopterin in patients with cardiovascular events ($P<0.001$) and a positive correlation between neopterin levels and late lumen loss after stenting ($P=0.008$) were observed in the BMS group but not in the DES group ($P=0.53$ and $P=0.17$, respectively). In long-term cardiovascular events, multivariate Cox regression analysis identified the significance of the high-neopterin group as independent determinants of cardiovascular events (hazard ratio, 2.225; 95% CI, 1.283−3.857; $P=0.004$). Immunohistochemical staining showed abundant neopterin-positive macrophages in the neointima after BMS implantation but no neopterin-positive macrophages in the neointima after DES implantation.

Conclusion: These findings suggest that neopterin is associated with cardiovascular events after coronary stent implantation in patients with SAP. However, there might be a strong association between neopterin and cardiovascular events after BMS but not after DES in these patients.

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Key words: Neopterin, Coronary disease, Stent, Restenosis

Introduction

Macrophage activation plays a significant role in the inflammatory process associated with atherosclerosis and plaque vulnerability[1)]. Neopterin is an activation marker for monocytes/macrophages system[2)] and has been reported to be associated with plaque instability in coronary and carotid arteries[3-6)]. Previous coronary angiographic studies have shown that serum neopterin levels are associated with rapid progression of coronary artery disease (CAD) in patients with stable angina pectoris (SAP)[3)] and with complex coronary lesions in

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patients with acute coronary syndrome (ACS)[4]. Furthermore, prospective studies have demonstrated that serum neopterin levels are an independent predictor of major adverse cardiovascular events both in patients with SAP[7] and in those with ACS[8, 9]. Coronary vascular inflammation and macrophage activation may play an important role not only in the progression of CAD but also in the mechanism of restenosis after percutaneous coronary intervention (PCI) including in-stent restenosis after stent implantation. Although coronary stent implantation has now become widespread for the treatment of SAP, there are no data regarding an association between neopterin and cardiovascular events after coronary stent implantation in patients with SAP. It is, therefore, important to clarify a possible relation of neopterin to in-stent restenosis as well as CAD progression after PCI.

In the present study, we measured plasma neopterin levels in patients with SAP undergoing coronary stent implantation and investigated whether there is a relationship between preprocedural plasma neopterin levels and subsequent cardiovascular events after coronary stent implantation (group I). Moreover, one frozen coronary artery specimen after drug eluting stent (DES) and three frozen coronary specimens after bare metal stent (BMS) were obtained by autopsy or endarterectomy, followed by immunohistochemical staining for neopterin (group II).

Methods

The study protocol was approved by the hospital ethics committee, and written informed consent was obtained from each patient.

Study Population

Group I (plasma neopterin)

The study population consisted of 234 consecutive patients with SAP who underwent coronary stenting and in whom successful coronary reperfusion between March 2004 and December 2007. SAP was defined as effort-related angina without any change in clinical pattern in the preceding 2 months. In 181 of 234 patients, follow-up data could be obtained at 2 years following the index PCI procedure. Of 181 patients, 123 patients were eligible for study inclusion (**Fig. 1**). Exclusion criteria included a history of coronary stent implantation ($n=40$), and several factors that might influence plasma neopterin levels such as intercurrent inflammatory, infectious diseases, neoplastic diseases likely to be associated with an acute-phase response ($n=6$), renal dysfunction (serum creatinine levels >1.2 mg/dl; $n=5$)[10], and low left ventricular ejection fraction $<40\%$ ($n=7$)[11, 12].

Of 123 patients enrolled in this study, 44 patients underwent PCI with BMS and 79 patients with DES. For each study patient, clinical data and history regarding risk factors such as age, diabetes mellitus, hypertension, hypercholesterolemia, and smoking were obtained.

Furthermore, we examined the association between plasma neopterin levels and long-term cardiovascular events (**Fig. 1**).

Coronary Stenting Procedure

All procedural decisions, including device selection and adjunctive pharmacotherapy, were made at the discretion of the individual PCI operator. Procedural success was defined as residual stenosis $<20\%$ without major complications. All patients received 81 or 100 mg/day of aspirin for at least 24 h before the procedure. Dual antiplatelet therapy (aspirin [81 or 100 mg] and 200 mg of ticlopidine or 75 mg of clopidogrel) was given to all patients treated with BMS for 4 weeks and in those treated with DES for at least 12 months. Glycoprotein (GP) IIb/IIIa inhibitors were not used, because they had not been approved in Japan. The following types of BMS were implanted: Multi-Link ZETA (Abbott Vascular, Santa Clara, CA) 4 patients; Duraflex (Avantec Vascular, Sunnyvale, CA) 9 patients; Driver (Medtronic, Shoreview, MN) 19 patients; and Express (Boston Scientific Corporation, Natick, MA) 12 patients. In the DES group, Cypher (Cordis, Johnson & Johnson, Miami Lakes, FL) was the only type of DES used.

Quantitative Coronary Angiography

In 123 patients after stenting, off-line quantitative coronary angiography was conducted as previously described[13]. The reference diameter, diameter stenosis (DS), and minimal lumen diameter (MLD) were measured before and after stenting and at the time of the follow-up coronary angiography. On the basis of these measurements, we obtained the value of acute gain (MLD after stenting minus MLD before stenting) and late lumen loss (MLD after stenting minus MLD at follow-up angiography) for the lesions. Angiographic restenosis was defined as $>50\%$ DS at follow-up angiography.

Biochemical Analysis

Venous blood samples were obtained from all patients before PCI after an overnight fast. The following measurements were performed: serum levels of total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, serum high sensitivity C-reactive protein (hs-CRP) levels, leukocyte count, neutrophil count, and plasma neopterin levels. Serum hs-CRP levels were assayed with the use

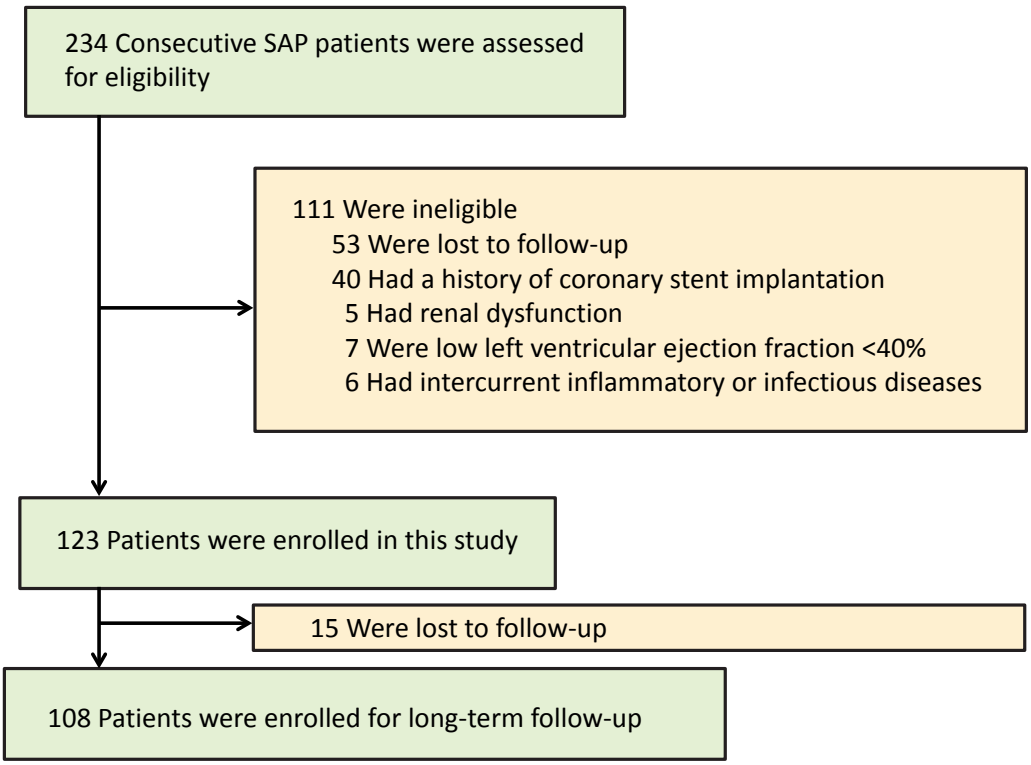

Fig. 1. Flowchart of the study.

of latex-enhanced immunonephelometric assays on a BN II analyzer (Dade Behring, Newark, DE, USA). Plasma neopterin levels were determined by the method described by Fukushima and Nixon[14] using high-performance liquid chromatography with fluorimetric detection. The neopterin measurement was performed within 12 h after the blood was drawn from each patient before PCI. Intra-assay coefficient of variation for the measurement of plasma neopterin levels was <6.3%, and inter-assay coefficient of variation was <7.9%.

Definition of End Points

At 2 years following the index PCI procedure, cardiovascular events including cardiac death, acute coronary syndrome (ACS), ischemia-driven target-lesion revascularization (TLR), and non-TLR were documented in all patients. ACS was defined as either UAP or acute myocardial infarction (MI). Urgent angiography was performed if the patient developed symptoms of angina within the follow-up period. All other patients underwent follow-up angiography at 6−8 months after PCI. TLR was defined as revascularization of a lesion previously treated by PCI during index hospitalization. Non-TLR was defined as revascularization of lesions and vessels that had not been treated by PCI during index hospitalization.

Patients: Group II (neopterin expression in pathological study)

One coronary artery specimen was obtained by autopsy from an 80-year-old man who died due to sudden cardiac death 6 months after sirolimus-eluting stenting. Another coronary artery specimen was obtained by autopsy from a 46-year-old man who died due to congestive heart failure 3 months after BMS. In the post-PCI arteries, the site of the stent implantation that contained the culprit lesion was established by comparing the heart specimens with the clinical angiograms. The site containing the stent was cut into 5-mm segments. In the two autopsy cases, the first segment of stented arteries was fixed in 20% buffered formalin and submitted for plastic embedding and stained with Cole's hematoxylin-eosin solution. The other segments were snap-frozen and stored at −80℃.

Moreover, two coronary artery specimens were obtained from two patients, ranging from 3 to 6 months after BMS using endarterectomy, followed by immunohistochemical staining for neopterin. Endarterectomy was performed due to stent restenosis when bypass surgery was performed. All these patients had symptom of, or signs related to, restenosis of the dilated artery. Immediately after endarterectomy, the tissue specimens were carefully oriented along their longest axis, snap-frozen, and stored at −80℃.

Table 1. Baseline Clinical and Angiographic Characteristics Stratified by Plasma Neopterin Levels

	Lowest ($n=41$)	Intermediate ($n=41$)	Highest ($n=41$)	*P*
Age, years	66 ± 9	65 ± 9	69 ± 8	0.15
Male, n (%)	33 (80)	36 (88)	30 (73)	0.25
Hypertension, n (%)	25 (61)	26 (63)	26 (63)	0.97
Diabetes mellitus, n (%)	15 (37)	8 (20)	17 (41)	0.08
Smoking, n (%)	26 (65)	31 (75)	26 (65)	0.49
Hypercholesterolemia, n (%)	24 (58)	26 (63)	22 (54)	0.67
HDL cholesterol, mg/dL	54 ± 19	50 ± 15	48 ± 14	0.13
LDL cholesterol, mg/dL	99 ± 56	85 ± 54	104 ± 54	0.29
Medications				
Antiplatelets, n (%)	40 (98)	40 (98)	41 (100)	0.60
ACE inhibitors/ARB, n (%)	14 (34)	16 (39)	14 (34)	0.87
Beta-blockers, n (%)	14 (34)	13 (32)	14 (34)	0.96
Calcium antagonists, n (%)	17 (41)	21 (51)	21 (51)	0.60
Nitrates, n (%)	8 (20)	16 (39)	10 (24)	0.12
Statin, n (%)	21 (51)	25 (61)	22 (54)	0.73
PCI-related artery, n (%)				0.24
LAD	21 (51)	24 (58)	19 (46)	
RCA	17 (42)	9 (22)	16 (39)	
LCX	3 (7)	8 (20)	6 (15)	
Multivessel disease, n (%)	15 (37)	18 (44)	21 (51)	0.41
DES implantation, n (%)	27 (66)	28 (68)	23 (56)	0.48
No. of stents per lesion	1.05 ± 0.22	1.12 ± 0.40	1.05 ± 0.22	0.90
Stent size, mm	3.17 ± 0.43	3.10 ± 0.44	3.17 ± 0.44	0.72
Stent length, mm	16.5 ± 7.7	18.2 ± 8.7	17.2 ± 7.7	0.75
QCA analysis (baseline)				
Reference diameter, mm	2.90 ± 0.54	2.91 ± 0.50	3.02 ± 0.54	0.53
MLD, mm	0.49 ± 0.31	0.38 ± 0.30	0.47 ± 0.36	0.24
% DS	83.8 ± 11.2	87.1 ± 10.2	83.9 ± 11.1	0.23
QCA analysis (after stening)				
MLD, mm	2.71 ± 0.44	2.66 ± 0.51	2.82 ± 0.46	0.18
Acute gain, mm	2.22 ± 0.51	2.28 ± 0.54	2.35 ± 0.47	0.43
% DS	5.2 ± 9.6	6.5 ± 10.8	7.8 ± 7.6	0.79
Leukocyte count, /mm^3	6190 ± 1453	6413 ± 1658	6469 ± 1614	0.79
Neutrophil count, /mm^3	3753 ± 1316	4022 ± 1351	3943 ± 1202	0.61
hs-CRP, mg/L	0.73 (0.36-1.60)	0.96 (0.60-1.64)	1.02 (0.57-2.61)	0.16
Creatinine, mg/dL	0.83 ± 0.16	0.84 ± 0.25	0.81 ± 0.18	0.88

Data are expressed as mean ± SD, n (%) or median (interquartile ranges) as appropriate. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; ACE, angiotensin- converting enzyme; ARB, angiotensin Ⅱ type 1 receptor blockers; LAD, left anterior descending coronary artery; RCA, right coronary artery; LCX, left circumflex artery; DES, Drug-eluting stents; QCA, quantitative coronary angiography; MLD, minimal lumen diameter; DS, diameter stenosis; hs-CRP, high sensitivity C-reactive protein.

The snap-frozen samples, obtained either by autopsy or endarterectomy, were subsequently serially sectioned at 7-μm thickness and fixed in acetone. Every first section was stained with hematoxylin-eosin; the other sections were used for immunohistochemical staining for the purpose of investigating the cellular component and characteristics of the stented segments.

Immunohistochemical Examination

The cellular components were analyzed by the use of monoclonal antibodies against smooth muscle cells (SMCs) (1A4, DAKO, Glostrup, Denmark), macrophages (EBM11, DAKO), T cells (SK7, Becton Dickinson, San Jose, CA), a protein specific to erythrocyte membranes (glycophorin A, DAKO), and neopterin (Biogenesis Inc., NH). To identify neutrophils, the fol-

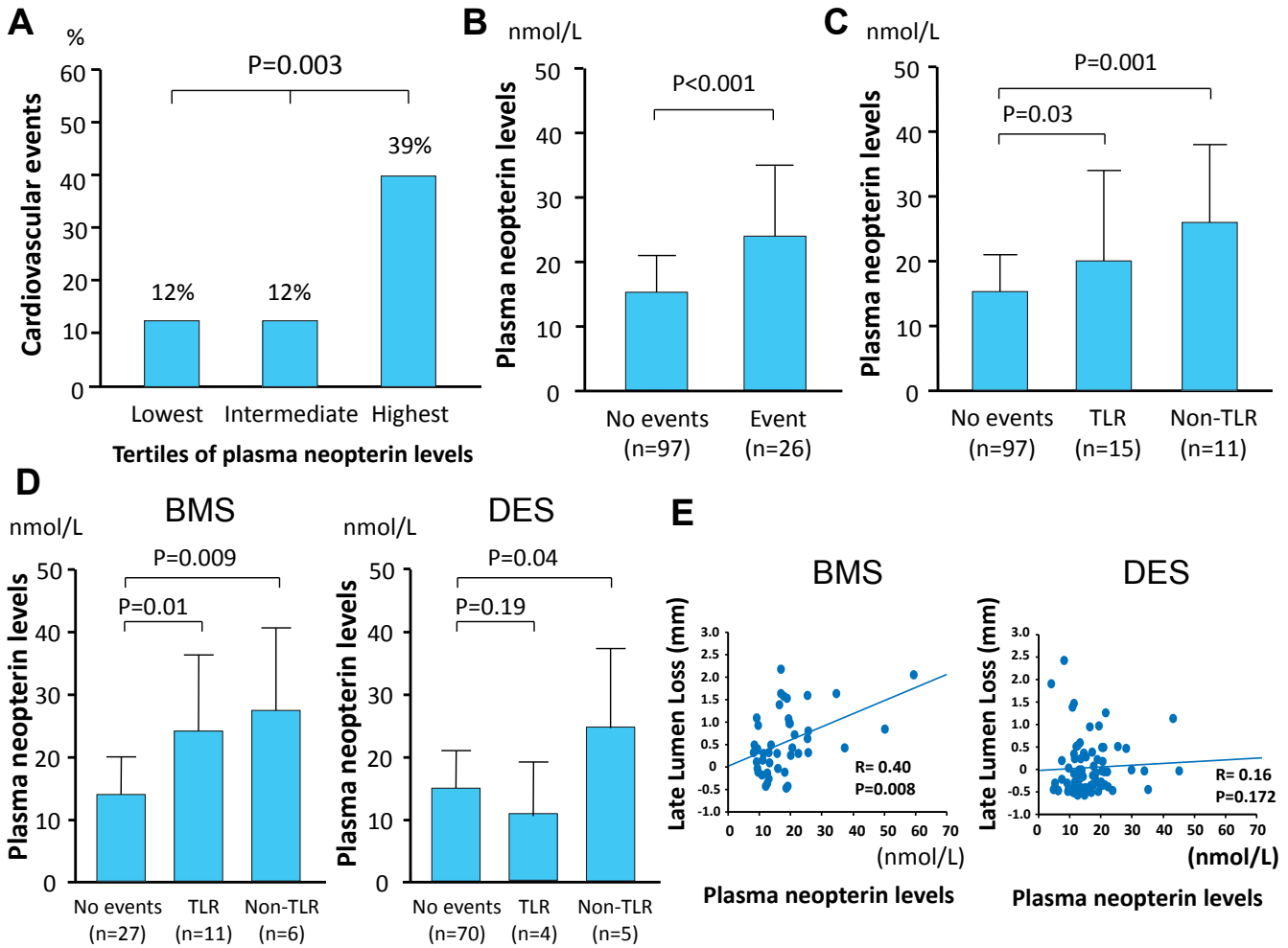

Fig. 2. (A) Prevalence of cardiovascular events by neopterin tertile. (B) Plasma levels of neopterin in patients without cardiovascular events and in those with events. (C) Plasma levels of neopterin in patients without cardiovascular event, with needed TLR and with needed non-TLR. (D) Plasma levels of neopterin in patients without cardiovascular events, with needed TLR and with needed non-TLR in the BMS and the DES groups. (E) Relationship between plasma neopterin levels and late lumen loss at follow-up angiography after stenting in SAP patients with the BMS group and DES group.

lowing antibodies were used: CD66b (80H3, Beckman Coulter, Fullerton, CA) and myeloperoxidase (MPO-7, DAKO). Moreover, platelets were detected with a monoclonal antibody against GP Iib/IIIa (CD41, DAKO). Microvessels of the tissue sections were assessed using antibodies for CD31 (DAKO) and von Willebrand factor (vWF) (DAKO). To investigate the phenotypes of macrophages within the neointima, the following antibodies were used: CD 163 (Santa Cruz, CA) to identify "M2" macrophages and iNOS (BD Biosciences, San Jose, CA) to identify "M1" macrophages[15].

Nonimmune mouse IgG serum (DAKO) served as a negative control. Sections were incubated at 4℃ overnight or 1 h at room temperature and then subjected to a three-step staining procedure. Peroxidase activity was visualized with 3-amino-9-ethyl-carbazole (10 minutes, room temperature), and the sections were faintly counterstained with hematoxylin. Furthermore, for the simultaneous identification of SMCs and macrophages, two primary monoclonal antibodies (1A4 and CD68) were performed, as previously reported[16]. The enzymatic activity of β-galactosidase for 1A4 was visualized in turquoise (BioGenex Kit; San Ramon, CA) and that of alkaline phosphatase for CD68 was visualized in red (New Fuchsin Kit; DAKO). To identify types of cells stained for neopterin, we performed double-immunostaining for macrophages and neopterin according to the method previously reported with minor procedural modifications[16]. With this method of staining, alkaline phosphatase was visualized with fast blue BB (blue, macrophages) and peroxidase with 3-amino-9-ethycarbazole development (red, neopterin).

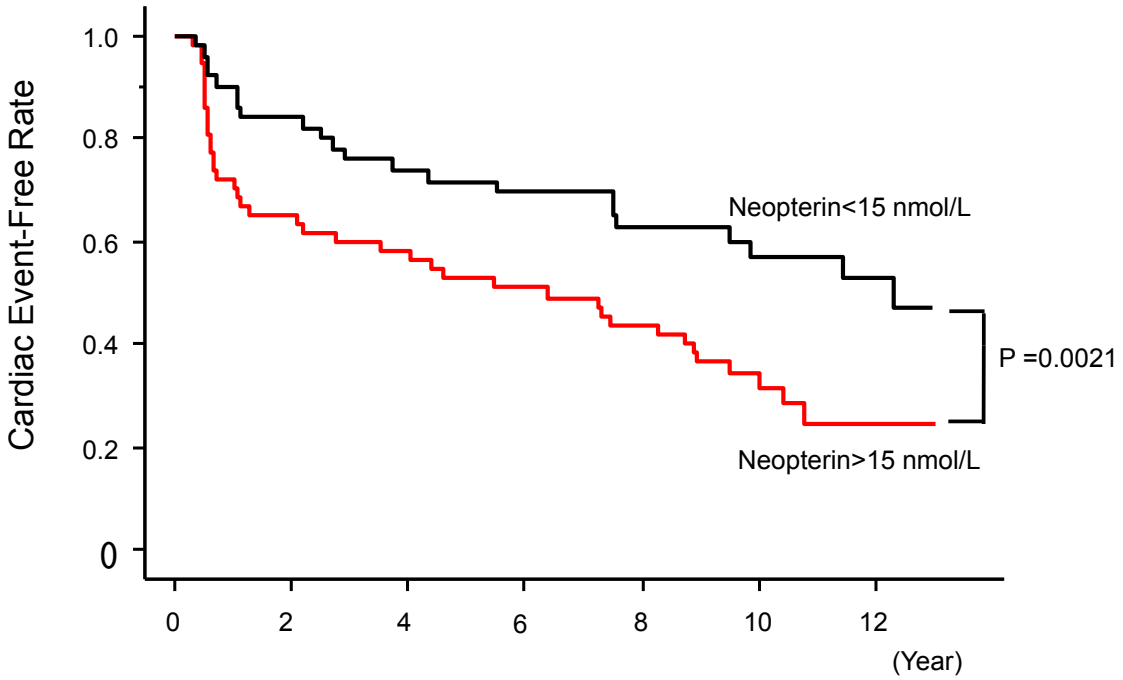

Fig. 3. Kaplan–Meier curves for cardiovascular events in patients in the high-neopterin group (>15.0 nmol/L) and the low-neopterin group (<15.0 nmol/L).

Statistical Methods

Continuous variables are expressed as mean ± SD and categorical variables are expressed as percentages. A Mann–Whitney *U* test was used to compare two groups and a one-way analysis of variance (ANOVA) was used to compare three groups. If a significant difference was found with the ANOVA, then a Mann–Whitney *U* test was used to compare each pair of groups. Categorical variables were compared using a chi-square test or Fisher exact test. Correlation between plasma neopterin levels and late lumen loss were assessed using simple linear regression. Multivariate logistic regression analysis was then applied to individuate the variables independently associated with cardiovascular events. Only variables with a value of $P<0.1$ on univariate analysis and hs-CRP >2.0 mg/L were included in the multivariate model. Age was entered as a continuous variable in regression analyses. Moreover, univariate and multivariate Cox regression analyses were performed for various parameters that might affect long-term cardiovascular events in patients with SAP. Values of $P<0.05$ were considered significant.

Results

Baseline Characteristics

Enrolled 123 patients were grouped into tertiles according to preprocedural plasma neopterin levels (the lowest tertile ([<12.0 nmol/L], intermediate tertile [12.1–17.9 nmol/L], and the highest tertile [>18.0 nmol/L]). The baseline characteristics are shown in Table 1. There were no significant differences among the three groups with respect to clinical factors and biochemical or angiographic parameters.

Plasma Neopterin Levels and Cardiovascular Events at 2 Years Follow-up

Twenty-six of 123 patients had cardiovascular events during the 2 years of follow-up. No cardiac death occurred, but ACS occurred in two patients and non-TLR was needed in these two patients. In the remaining 24 patients, TLR was needed in 15 patients and non-TLR was needed in 9 patients because of symptoms of effort angina. Cardiovascular events occurred more frequently in patients in the highest tertile of plasma neopterin levels than in those in the intermediate or lowest tertiles (12% vs. 12% vs. 39%; $P=0.003$; Fig. 2A). Plasma neopterin levels were significantly higher in patients with cardiovascular events than in those without them (22.9 ± 13.2 vs.14.6 ± 6.3 nmol/L, $P<0.001$, Fig. 2B). Furthermore, they were significantly higher in patients with TLR (20.1 ± 13.3 nmol/L, $P=0.03$) and non-TLR (26.6 ± 12.6 nmol/L; $P=0.001$) than in those without any cardiovascular events (Fig. 2C). Univariate logistic regression analyses revealed that smoking (HR 3.07, 95% CI 0.98–9.63; $P=0.055$), statin treatment (HR 0.42, 95% CI 0.17–1.02; $P=0.056$), and high levels of neopterin levels (HR 4.61,

Table 2. Cox Proportional Hazard Analysis for Predicting Cardiovascular Events

	Univariate		Multivariate	
	Hazard ratio (95% Confidence Interval)	*P*	Hazard ratio (95% Confidence Interval)	*P*
Age	1.002 (0.973-1.032)	0.876		
Male	0.975 (0.518-1.838)	0.938		
Hypertension	0.786 (0.456-1.356)	0.387		
Hypercholesterolemia	0.914 (0.543-1.540)	0.737		
Diabetes Mellitus	1.131 (0.665-1.923)	0.650		
Smoking	0.785 (0.451-1.365)	0.391		
Multivessel disease	1.730 (1.038-2.884)	0.035	1.505 (0.856-2.646)	0.156
DES implantation	1.752 (1.051-2.919)	0.031	1.219 (0.633-2.347)	0.553
No. of stents per lesion	1.219 (0.570-2.609)	0.610		
Stent size	3.056 (1.652-5.655)	0.0005	2.654 (1.326-5.312)	0.006
Stent length	0.999 (0.967-1.032)	0.956		
Reference diameter	1.612 (0.972-2.674)	0.064		
Post MLD	1.682 (0.970-2.919)	0.063		
Creatinine	0.613 (0.157-2.406)	0.485		
hs-CRP >0.1 ng/mL	1.703 (1.012-2.865)	0.045	1.526 (0.883-2.637)	0.130
Neopterin >15.0 nmol/L	2.281 (1.330-3.913)	0.003	2.225 (1.283-3.857)	0.004

DES, Drug-eluting stents; MLD, minimal lumen diameter; hs-CRP, high sensitivity C-reactive protein

95% CI 1.85−11.47; $P=0.001$) were associated with cardiovascular events ($P<0.1$). Multiple logistic regression analysis after adjustment for smoking, statin treatment, and hs-CRP >2.0 mg/L showed that the elevated neopterin levels (the highest tertile of neopterin levels) were an independent predictor of cardiovascular events (OR 5.20, 95% CI 1.94−13.98; $P=0.001$).

Subgroup Analyses in the BMS Group and in the DES Group

Seventeen of 44 patients in the BMS group and 9 of 79 in the DES group had cardiovascular events during the 2 years of follow-up. ACS occurred in two patients in the BMS group. TLR was needed in 11 patients in the BMS group and 4 patients in the DES group. Non-TLR was needed in six patients in the BMS group and five patients in the DES group. In the BMS group, higher levels of plasma neopterin were observed in patients with cardiovascular events than in those without them (13.9 ± 5.1 vs. 25.0 ± 13.2 nmol/L; $P<0.001$) and both in patients with TLR and non-TLR (23.6 ± 13.5 nmol/L; $P=0.01$ and 27.5 ± 13.5 nmol/L; $P=0.009$; **Fig. 2D**). In the DES group, however, there was no difference between in patients with cardiovascular events and in those without them (18.9 ± 12.8 vs. 14.9 ± 6.7 nmol/L; $P=0.53$). Plasma neopterin levels were higher in patients with non-TLR than in those without cardiovascular events (25.5 ± 12.8 nmol/L; $P=0.04$), but similar in patients with TLR in the DES group (10.7 ± 7.6 nmol/L; $P=0.19$; **Fig. 2D**).

Follow-up angiography was accomplished in 121 patients (98%), for which 2 patients refused to undergo. Restenosis occurred in 23 of the 121 patients. Restenosis occurred in 15 of the 43 patients in the BMS group and 8 of the 78 patients with the DES group. Plasma neopterin levels showed a positive correlation ($R=0.40$, $P=0.008$) with late lumen loss after stenting in the BMS group, while there was no correlation between plasma neopterin levels and late lumen loss in the DES group ($R=0.16$, $P=0.17$; **Fig. 2E**).

Plasma Neopterin Levels and Long-term Cardiovascular Events

Follow-up was obtained for 108 patients (88%), for which 15 patients were lost to follow-up. Over a mean follow-up period of 6.5 years (range, 118 days to 13 years), cardiovascular events occurred in 34 patients (31%) starting at 2 years post PCI onward. Sudden cardiac death occurred in one patient in the BMS group. ACS occurred in three patients in the DES group, and TLR was needed in these two patients, and non-TLR was needed in one patient. In the remaining 30 patients, TLR was needed in 4 patients in the DES group and non-TLR was needed in 26 patients, because of symptoms of effort angina. Kaplan−Meier analysis showed that the high-neopterin group (>15.0 nmol/L) had significantly worse outcomes than the low-neopterin group ($P=0.0021$ by log-rank test), with a hazard ratio of 2.281 (95% CI, 1.330−3.913; $P=0.003$; **Fig. 3**). The results of univariate and multivariate analyses of factors associated with cardiovascular events are listed in **Table 2**. Multivariate Cox regression analysis

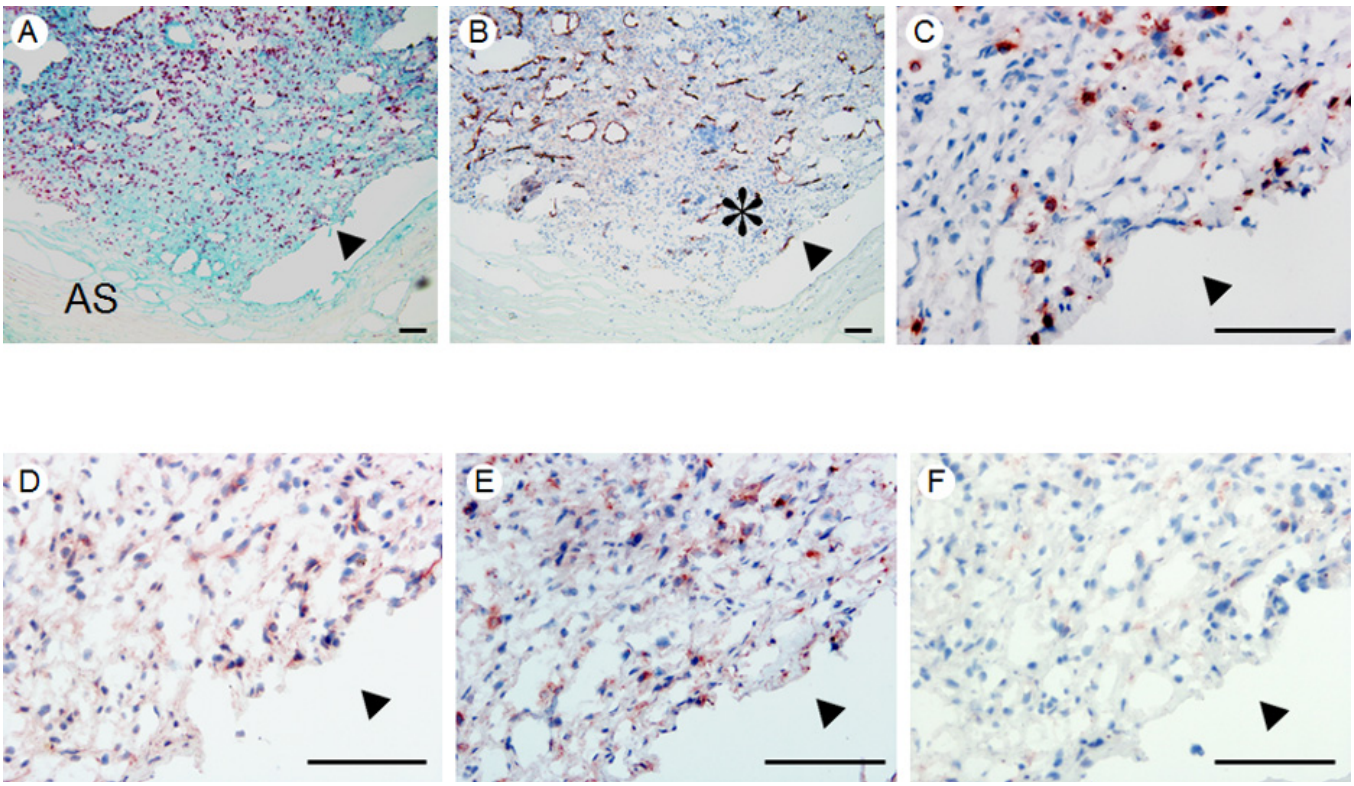

Fig. 4. Micrographs of a coronary endarterectomy specimen taken from a patient who had undergone BMS implantation and exhibited symptoms related to restenosis of the dilated artery 3 months after BMS implantation. (A) Double immunostaining (smooth muscle cell, turquoise/macrophage, red) reveals abundant macrophages in the neointima. AS, atherosclerotic plaque. Stent struts (arrowhead). (B) The anti-vWF antibody reveals vWF -positivity of endothelial cells. The asterisk area is enlarged in C - F. (C) The anti-T cells CD3 antibody shows a large number of T cells. (D) The adjacent section stained with anti-neopterin antibody reveals the presence of abundant neopterin-positive cells. (E) The anti-iNOS antibody shows most macrophages are positive for iNOS. (F) The anti-CD163 antibody shows most macrophages are negative for CD163. Bar: A−E, 100 μm.

identified the significance of the high-neopterin group, in addition to other established predictors (stent size), as independent determinants of cardiovascular events (hazard ratio, 2.225; 95% CI, 1.283−3.857; $P=0.004$).

Immunohistochemical Examination

In the patient undergoing endarterectomy at 3 months after BMS, the neointima consisted of SMCs, abundant macrophages, and microvessels. The neointima also contained abundant T-lymphocytes and neopterin-positive macrophages. Most macrophages in the neointima were positive for iNOS. However, only a few macrophages were positive for CD 163 in the neointima (**Fig. 4**). In the autopsy specimen obtained 3 months after BMS, abundant macrophages were also seen in the deep layer of the neointima around the stent struts. Moreover, the neointima also contained abundant T-lymphocytes and neopterin-positive macrophages. The majority of these macrophages showed staining positivity for iNOS, but only occasional macrophages revealed CD163 positivity. Double immunostaining for macrophages (blue) and neopterin (red) distinctly revealed that most cells showed double staining (purple), indicating that the vast majority of neopterin-positive cells were macrophages (**Fig. 5**). At 6 months after BMS, the neointima composed predominantly of SMCs was found, and the number of macrophages and neutrophils were decreased. However, T-lymphocytes and neopterin-positive macrophages were still found in the luminal site (**Fig. 6**). In the autopsy specimen obtained 6 months after DES, a totally occlusive thrombus was found. In this lesion, neointimal proliferation was found at the edges of the stent. The neointima was composed of actin-negative spindle-shaped cells and abundant macrophages, with only a small number of SMCs and only a few T-lymphocytes. In the neointma, there was no positivity for neopterin (**Fig. 7**).

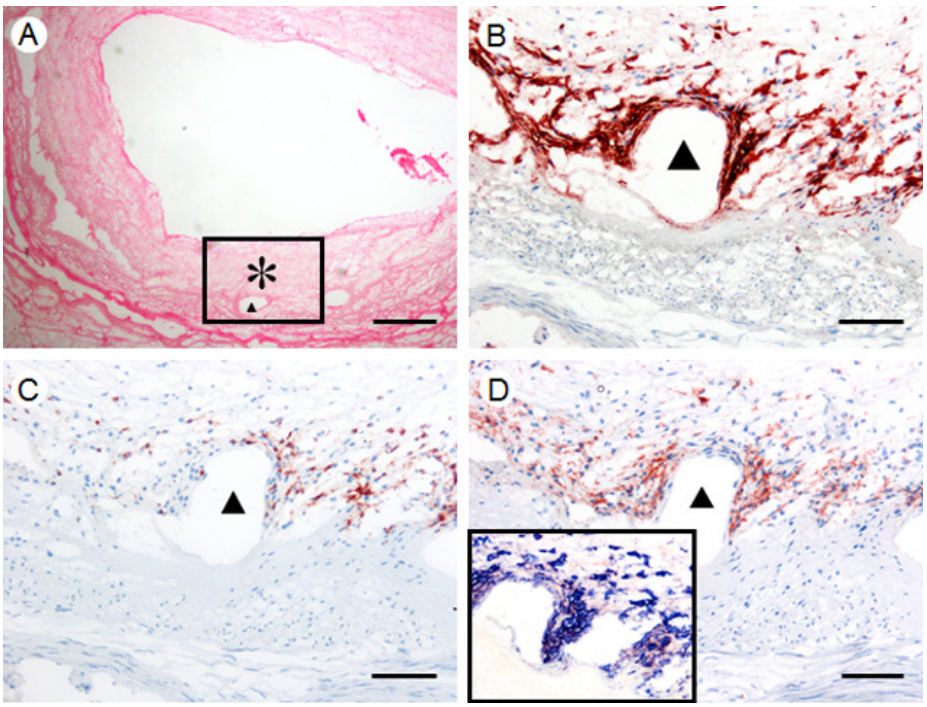

Fig. 5. Micrographs of a post-PCI injury site, 3 months after BMS stenting. (A) Hematoxylin-eosin stain shows neointimal proliferation (asterisk) around the stent struts (arrowhead). The boxed area is enlarged in B–D. (B) The anti-CD68 antibody shows macrophage accumulation in the deep layer of the neointima around the stent struts (arrowhead). (C) The anti-T cells CD3 antibody shows a large number of T cells. (D) The adjacent section stained with anti-neopterin antibody reveals the presence of neopterin-positive cells. Inset: Double immunostaining for macrophages (blue) and neopterin (red) reveals that most cells show double staining (purple), indicating that the vast majority of neopterin-positive cells are macrophages. Bar: A: 500 μm and B–D: 100 μm.

Discussion

Several markers were reported regarding the progression of CAD but also restenosis after PCI including in-stent restenosis after stent implantation[17, 18]. To the best of our knowledge, this study is the first to evaluate the relationship between preprocedural plasma neopterin levels and cardiovascular events after coronary stent implantation in patients with SAP and to investigate the immunohistochemical staining for neopterin at the sites following stenting. The present study showed that preprocedural plasma neopterin levels are an independent predictor for cardiovascular events after coronary stent implantation. In the subgroup analyses, higher plasma neopterin levels were significantly associated with cardiovascular events only in the BMS group but not in the DES group. Despite a limited number of cases studied, our immunohistochemical study, based on frozen sections of human coronary arteries at 3–6 months after BMS, revealed the distinct presence of neopterin in macrophages within the neointima. These findings suggest that neopterin plays a role in the development of neointimal proliferation after BMS.

Intravascular ultrasound[19] and histopathological studies[20-24] have identified restenosis caused by neointimal proliferation. Our group[20-24] and others[25] have shown an important role for infiltration of monocytes and neutrophils in the development of exuberant neointimal proliferation of in-stent restenosis. Previously, we have demonstrated that persistence of an increased level of plasma ox-LDL at discharge is a strong independent predictor of stent restenosis at 6-month follow-up in patients with AMI[13]. Infiltrated monocytes and neutrophils and activated platelets have been shown to promote LDL oxidation[26], which stimulates proliferation and migration of SMCs via induction of platelet-derived growth factor[27]. In the present study, we have demonstrated that neopterin levels showed a positive correlation with late lumen loss after stenting in the BMS group. Additionally, immunohistochemical staining showed that accumulation of T-lymphocytes, neopterin, and extensive neovascularization at sites of in-stent restenosis at 3 months after BMS implantation, and neointimal proliferation with accumulation of SMCs at the luminal side at 6 months after BMS implantation. These findings support that the T-lym-

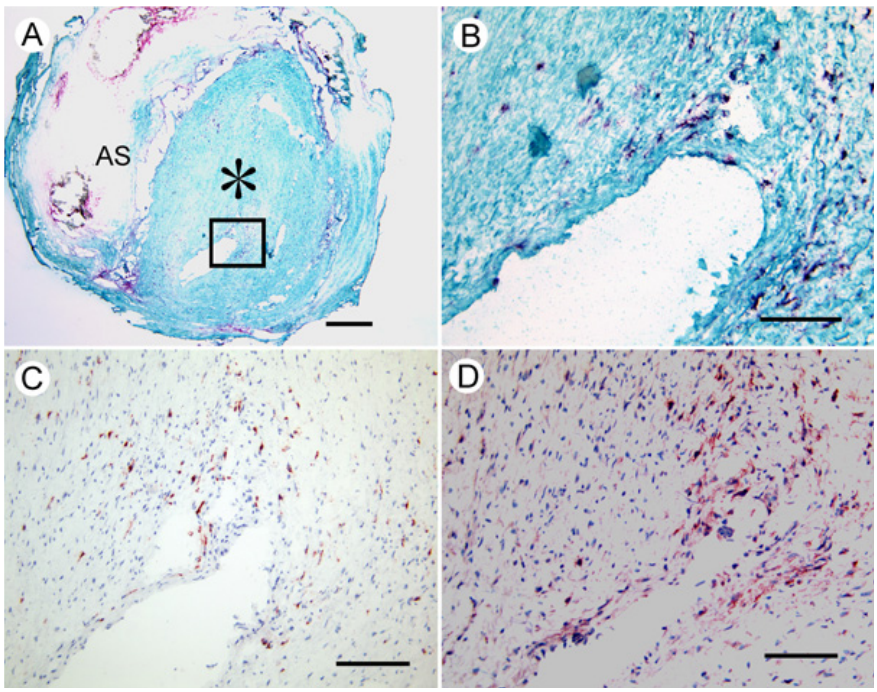

Fig. 6. Micrographs of a coronary endarterectomy specimen taken from a patient who had undergone BMS implantation and exhibited symptoms related to restenosis of the dilated artery 6 months after BMS implantation. (A) Double immunostaining (smooth muscle cell, 1A4; turquoise/macrophage; red) shows that there is extensive neointimal tissue (asterisk), composed predominantly of smooth muscle cells. AS: atherosclerotic plaque. The boxed area is enlarged in B–D. (B) Double immunostaining (smooth muscle cell, turquoise/macrophage, red) reveals numerous macrophages. (C) The anti-T cells CD3 antibody shows a large number of T cells. (D) The adjacent section stained with anti-neopterin antibody reveals the presence of neopterin-positive cells. Bar: A: 500 μm and B–D, 100 μm.

phocyte-monocyte/macrophage system could play a significant role in in-stent restenosis after BMS implantation. In several studies, elevated neopterin levels may reflect atherosclerotic disease activity and vulnerability to the development of ACS[3-8]. In the BMS group, when the vessel wall was injured by stent implantation, activated macrophages in the vessel wall could promote production of neopterin. Interestingly, neopterin itself has the capacity to further enhance oxidative stress[28]. In addition, neopterin stimulates nuclear factor-κB translocation to the nucleus[29], promoting the expression of proinflammatory genes, adhesion molecules, tissue factor, and other substances implicated in the inflammatory processes. Thus, neopterin may represent not only a clinical maker for risk stratification in patients with SAP but also play a pathogenic role in restenosis after BMS implantation.

In contrast, in the DES group, there were no differences in plasma neopterin levels between patients with cardiovascular events and those without them. Sirolimus (Rapamune), a natural macrocyclic lactone, is a potent immunosuppressive agent that inhibits the activation of T-lymphocytes and approved for the prophylaxis of renal transplant rejection[30, 31]. Several experimental studies have shown that the sirolimus inhibits the proliferation of both rat and human SMCs *in vitro* and reduces intimal thickening in models of vascular injury[32]. Actually, we have demonstrated only spars neointimal proliferation and only a few T-lymphocytes at post-PCI injury site, 6 months after DES implantation. Moreover, there was no positivity for neopterin in this lesion. These observations provide hypothesis that inflammatory response and oxidative stress caused by T-lymphocytes, macrophages, and neopterin may be suppressed by the local elution of sirolimus after DES implantation.

A close relationship between serum neopterin levels and coronary complex lesions or rapid progression of CAD has been shown in the previous studies[3, 4]. Furthermore, Avanzas *et al.*[7] demonstrated that higher neopterin levels were independent predictor of cardiovascular events in 297 patients with SAP who did not undergo PCI at 1-year follow-up. Recently, Sun *et al*[6] have reported that higher neopterin levels were notably associated with more vulnerable plaque characteristics such as thin-cap fibroatheroma, thinner fibrous cap thickness, greater plaque burden, frequent plaque rupture, and microvessel occurrence in nonculprit

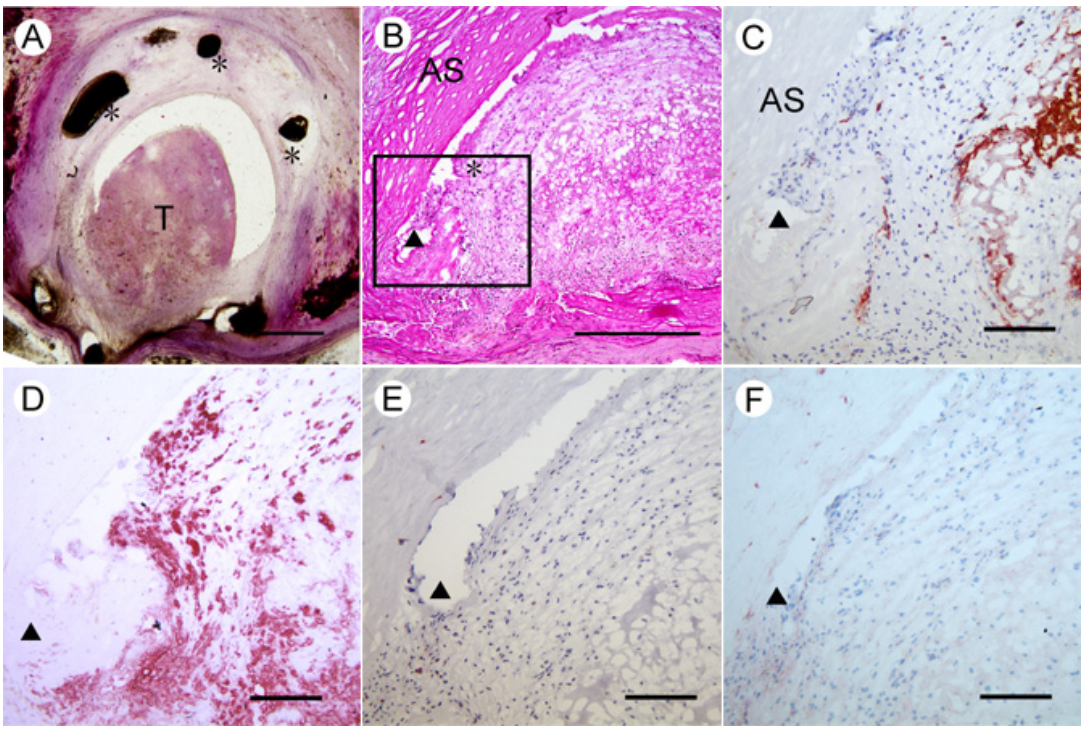

Fig. 7. Micrographs of a post-PCI injury site, 6 months after DES implantation. (A) Hematoxylin-eosin stain shows neointimal (asterisks) proliferation around the stent struts (plastic embedding and Cole's hematoxylin-eosin stain). The lumen contains thrombi (T). (B) The adjacent frozen section shows neointimal (asterisk) proliferation around the stent struts (hematoxylin-eosin stain). Stent struts (arrowhead). The boxed area is enlarged in C–F. (C) Immunostaining for glycophorin A. AS, atherosclerotic plaque. (D) Immunostaining for macrophages (CD68). (E) Immunostaining for T cells. (F) Immunostaining for neopterin. Bar: A and B: 500 μm; C–F, 100 μm.

plaques of patients with CAD using optical coherence tomography. Our results showing that neopterin levels were significantly higher in patients with SAP with non-TLR both in patients with BMS and DES are consistent with results by these previous studies. Moreover, previous large prospective studies in patients with ACS by Ray *et al.*[6] and Kaski *et al.*[9] showed that higher neopterin levels were a strong independent predictor of cardiovascular events. However, only few data are available regarding the relation between neopterin levels and cardiovascular events after PCI. Previously, Eber *et al.*[33] have reported that serum neopterin levels were not suitable as markers for restenosis following PCI; however, the study population was small and included only patients with balloon angioplasty not those after stenting. Furthermore, Dominguez-Rodriguez *et al.*[12] demonstrated that high neopterin levels were independently associated with left ventricular remodeling in patients with ST-segment elevation MI undergoing primary PCI. In addition to their results, we have shown that higher neopterin levels are an independent predictor of cardiovascular events in patients with SAP after stent implantation. In the subgroup analyses, we have also found that there are differences in the prognostic value of plasma neopterin levels between patients with BMS and those with DES, and that the enhanced activation of T-lymphocytes-monocyte/macrophage system could play a significant role in cardiovascular events only after BMS implantation.

Recently, the second-generation DES, including Xience/Promus everolimus- eluting stent, which consists of a thin strut platform coated with durable fluorinated copolymer and everolimus, were be in widespread use. In several pathological studies[34, 35], the second-generation DES showed greater strut coverage with less inflammation, less fibrin deposition, and less late and very late stent thrombosis compared with the first-generation DES. In our present study, there were no differences in plasma neopterin levels between patients with cardiovascular events and those without them in the first-generation DES. Future studies should confirm an association between plasma neopterin levels and cardiovascular events in the second-generation DES.

The neoatherosclerosis, characterized by a relatively thin fibrous cap and large volume of yellow-lipid accumulation after DES implantation, has attracted much attention owing to its close relationship with late complications, such as revascularization and late

stent thrombosis[36, 37]. In the long-term follow-up of the present study, TLR due to late restenosis was needed in six patients (two patients with ACS and four patients with effort angina), and all these six patients were in the DES group. Although these cases lacked a contemporary assessment of plaque morphology by IVUS or OCT, these cases may be associated with neoatherosclerosis after DES implantation.

Recently several classes of macrophages have been described based on their expression of markers, the production of specific factors, and their biological functions[38]. Classically activated (M1) macrophages are typically induced by interferon-γ and tumor necrosis factor. To protect against such tissue damage, the inflammatory response is spatially and temporally counter balanced by regulatory mechanisms driven by alternatively activated (M2) macrophages. In the present study, most neopterin-positive macrophages in the neointima, at 3−6 months after BMS, were positive for iNOS but negative for CD163. These findings strongly suggest that these macrophages within the neoitima at 3− months after BMS are predominatory M1 macrophages. These findings are consistent with the previous reports that neopterin stimulates nuclear factor-κB translocation to the nucleus[29], promoting the expression of proinflammatory genes in the inflammatory processes.

The study population was relatively small because of our strict screening criteria to exclude the many factors that might influence plasma neopterin levels. Moreover, we immunohistochemically examined the neopterin expression using only four frozen samples within 6 months after stenting, because the antibody against neopterin works well on frozen sections only. Further studies with large numbers of cases are needed to validate our observations.

Conclusion

The present study revealed that preprocedural plasma neopterin levels are independently associated with cardiovascular events after coronary stent implantation in patients with SAP. However, higher plasma neopterin levels significantly related to cardiovascular events only in the BMS group but not in the DES group. These findings suggest that neopterin is closely associated with coronary plaque instability and restenosis after BMS, but there might be differences in neopterin activities at the site of the stent implantation between BMS and DES.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

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