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Inflammatory Mediators Across the Spectrum of Ankle-Brachial Index

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Aim: Peripheral artery disease (PAD) is a manifestation of atherosclerosis with poor prognosis. It is generally complicated by vascular calcification, which is located either in the intima as patchy infiltrates; or circumferentially in the media, also known as medial arterial calcification (MAC). Obstructive PAD is reflected by low ankle-brachial index (ABI ≤ 0.9), whereas MAC is revealed by high ABI (ABI >1.4). Considering the increase in cardiovascular mortality at both ends of the ABI spectrum, this study aimed to explore the underlying pathology through cytokines with established prognostic significance; namely pentraxin-3(PTX3), high sensitivity C-reactive protein (hsCRP), copeptin, soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), NT-proBNP, and neopterin.

Methods: We categorized 180 patients with previous multivessel coronary artery bypass grafting surgery into three groups based on their ABI measurements; 60 patients with ABI ≤ 0.9, 60 patients with ABI within 0.91 and 1.4 (normal ABI), and 60 patients with ABI >1.4 constituted the "PAD," "normal," "MAC" groups, respectively. The circulating levels of the biochemical markers were determined.

Results: In the PAD group, the cytokine levels with predominantly proatherogenic actions such as PTX3, hsCRP, copeptin, and sTREM-1 were increased and these cytokine levels declined as the ABI increased. In the MAC group, the cytokine concentrations with pleiotropic actions such as NT-proBNP and neopterin increased and; NT-proBNP and neopterin concentrations decreased as ABI decreased. The linear regression analysis revealed that neopterin ($\beta = 0.72$), PTX3 ($\beta = -0.32$), and copeptin ($\beta = -0.48$) were independent predictors of ABI.

Conclusions: These findings suggest that different inflammatory pathways influence the pathology at the opposing ends of the ABI spectrum. Consequently, we suggest that PTX3, copeptin, and neopterin are promising biomarkers for future research.

Key words: Peripheral artery disease, Ankle-brachial index, Pentraxin-3, Copeptin, Neopterin

Introduction

Peripheral artery disease (PAD) is a distinct manifestation of atherosclerosis, characterized by severely calcified, extensive atherosclerotic involvement of arterial tree[1]. It is generally accompanied by other atherosclerotic manifestations, wherein the prevalence of concomitant coronary artery disease (CAD) is reported between 60% and 90%[2, 3]. PAD is an independent predictor of mortality and morbidity in CAD[4].

Vascular calcification, which is a prominent feature of atherosclerosis, settles at different layers of the arterial wall[5, 6]. It may be localized either in the intima

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or the media, depending on the underlying pathology. In general, the atherosclerotic process in obstructive PAD causes patchy calcium infiltrates in the intima[7]. Medial arterial calcification (MAC), in contrast, is a circumferential calcification of the arterial media resulting in noncompressible arteries[8]. The clinical expression of two entities depends on the net effect of the predominant pathology; a decreased ankle-brachial index (ABI ≤ 0.9) in predominantly atherosclerotic obstructive PAD but an increased ABI (ABI >1.4) in predominant MAC[9]. Regardless of the cause, an abnormal ABI is a strong predictor of mortality in CAD[10].

Experimental studies have provided evidence on the relation of a wide range of cytokines revealing mortality with the cardiovascular diseases. These include the proatherogenic cytokines such as pentraxin-3 (PTX3), high sensitivity C- reactive protein (hsCRP), and soluble triggering receptor expressed on myeloid cells-1 (sTREM-1); pleiotropic cytokines like neopterin; and biomarkers of hemodynamic stress such as copeptin and N terminal probrain natriuretic peptide (NT-proBNP)[11-16]. PTX3 and hsCRP are the two most characteristic acute phase proteins from the pentraxin family; sTREM-1 is a recently identified cell surface receptor that propagates the proinflammatory cytokines, all of which are mediated through the nuclear factor kappa beta (NF-κB)signaling, the key inflammatory pathway in the pathogenesis of atherosclerosis (**Fig. 1A**)[11, 12]. Neopterin is a pteridine derivative that transduces signals of cell differentiation, migration, proliferation, and apoptosis thorough Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (**Fig. 1B**)[15].

Aim

Considering the increase in cardiovascular mortality at both ends of the ABI spectrum, we hypothesized that the distinct inflammatory features in pathogenesis define the deviations in ABI and prognosis. We, therefore, studied the circulating levels of cytokines with established diagnostic and prognostic significance in patients with CAD across the ABI spectrum. We evaluated PTX3, hsCRP, copeptin, sTREM-1, NT-proBNP, and neopterin concentrations in a patient population with previous coronary artery bypass grafting (CABG) who were stratified by ABI.

Methods

Between June 2016 and July 2017, we prospectively enrolled patients with previous CABG surgery due to multivessel CAD who presented to the outpatient clinic for a routine follow-up visit at our institution. A detailed cardiovascular examination and transthoracic echocardiography were performed and the demographic data on cardiovascular risk factors such as hypertension, diabetes, hyperlipidemia, family history of cardiovascular disease, and present smoking habits were recorded for every patient during this visit. ABI was measured in all patients. Fasting blood samples were collected in the morning (9–11 am) following the ABI measurement. Each patient was asked if he or she had any leg discomfort on exertion suggesting intermittent claudication, and the presence of symptoms was recorded accordingly.

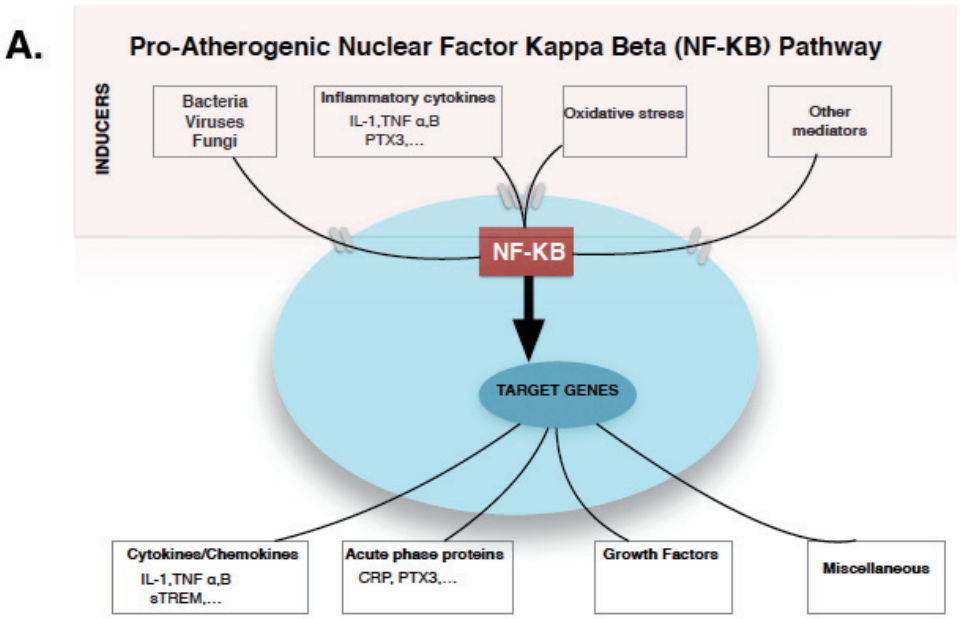
The following patient groups were excluded from the study: patients with symptoms of stable or unstable angina pectoris; patients with an acute coronary event within the past 6 months, patients with severe systolic dysfunction with an left ventricular ejection fraction (LVEF) of <50%, severe valvular heart disease, chronic inflammatory disorders, acute infection, known malignancy, chronic liver disease, and those with an estimated glomerular filtration rate <60 mL/min/1.73 m^2. Patients with a history of carotid atherosclerotic diseases identified in the interview or routine preoperative Duplex ultrasound evaluation were also excluded from the study (**Fig. 2**; flow diagram).

Of the consecutive patients fulfilling the above criteria, we recruited 60 patients with ABI ≤ 0.9, 60 patients with ABI within 0.91–1.4, and 60 patients with ABI >1.4 into the "*low ABI or PAD*," "*normal ABI*," and "*high ABI or MAC*" groups, respectively. We stopped recruiting patients for a specific group once the target of 60 patients was achieved. The study protocol was approved by the local Ethics committee and written informed consent was obtained from all participants (Registration number: 2015/121/11/04).

ABI Measurements

ABI was measured by Doppler technique using a 8–10 MHz Doppler ultrasound device (Huntleigh Healthcare limited, Wales, UK) by an experienced physician[9]. Following a 15-min rest period in supine position, the brachial and ankle systolic pressures were determined using appropriately sized cuffs placed on the brachial artery and on the ankle above the malleoli. To calculate the ABI of each leg, higher ankle pressure measured on dorsalis pedis and tibialis posterior for each limb was used in the numerator, and higher brachial pressure measured from both arms was used in the denominator.

Using G*Power version 3.1 for Mac software, the minimum total sample size of 159 patients provided an effect size of 0.25 with an alpha error of 0.05 and power of 0.80 in three groups.



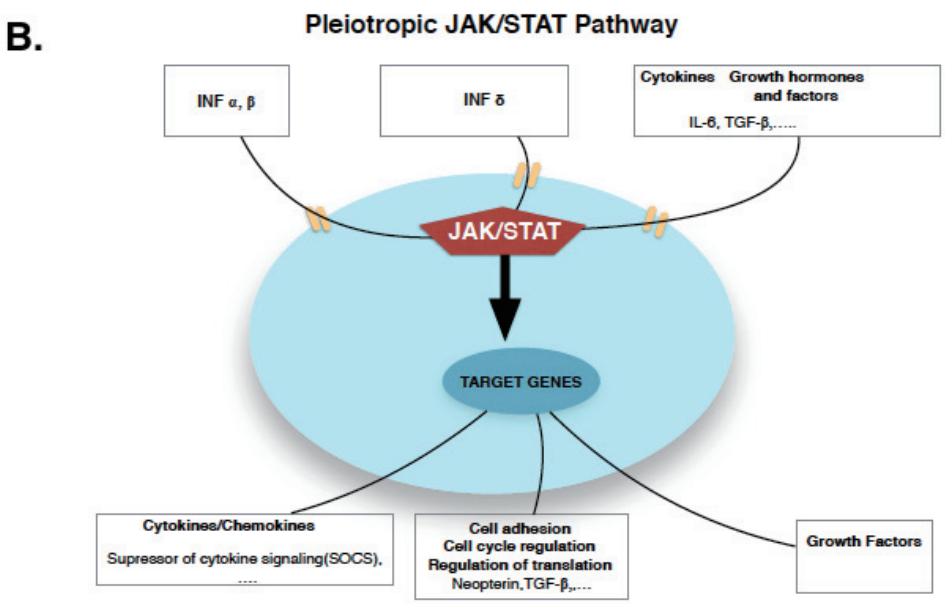


Fig. 1. Overview of the inflammatory mediators

A: Inflammatory mediators with mainly proatherogenic effects are mediated by interleukin-1(IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) through nuclear factor kappa beta (NF-κB) signaling cascade. B: Inflammatory mediators with pleiotropic effects like cell differentiation, migration, proliferation, and apoptosis are mediated through Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway.

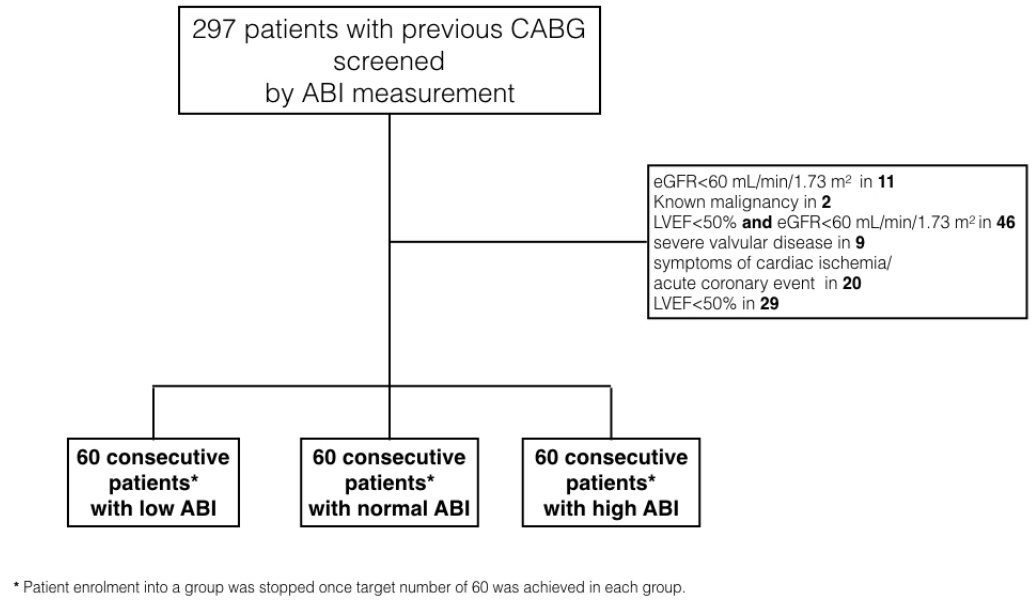


Fig. 2. Flow diagram of the patient enrollment

Biochemical Analyses

Fasting blood samples were collected from a vein in the antecubital fossa without venous occlusion. The whole blood samples were placed into specimen tubes containing EDTA and were immediately analyzed by ABX Pentra DX 120 blood counter (ABX-Horiba, Montpellier, France). For the biochemical analyses, samples collected into plain tubes were centrifuged at 3000 rpm for 10 min. Creatinine concentrations were determined by Jaffe method and Roche Cobasc501 analyzer (Roche Diagnostics, Indianapolis, USA). Serum samples were then stored at −86℃ until further analyses. Copeptin, neopterin, pentraxin3, hsCRP, NT-proBNP, and sTREM-1 concentrations were determined by enzyme-linked immunosorbent assay (ELISA) method based on the competition principle and microtiter plate separation.

PTX3 kit (Human Pentraxin 3 ELISA kit, SunRed Biotechnology Co. Ltd. Shangai, PCR) revealed the inter- and intra-assay CV% as <10%; and the minimum detectable dose of human PTX3 was 0.051 ng/mL. Copeptin kit (Human CCP ELISA kit, SunRed Biotechnology Co. Ltd. Shangai, PCR) and sTREM-1 kit (Human sTREM-1 ELISA kit, SunRed Biotechnology Co. Ltd. Shangai, PCR) revealed an inter- and intra-assay CV% as <12% and <10%, respectively. The minimum detectable dose of human copeptin was 0.067 ng/mL and that of human sTREM-1 was 3.102 pg/mL. hsCRP kit (C-reactive protein HS ELISA kit, DRG International, Inc. USA) revealed the inter- and intra-assay CV% as <4.5% and <7.5 %, respectively; and the minimum detectable dose of hsCRP was 0.1 mg/L. Neopterin (Human neopterin ELISA kit, SunRed Biotechnology Co. Ltd. Shangai, PCR) and NT-proBNP kit (Human NT-proBNP ELISA kit, SunRed Biotechnology Co. Ltd. Shangai, PCR) revealed the inter- and intra-assay CV% as <12% and <10 %, respectively. The minimum detectable doses of human neopterin and NT-proBNP were 0.117 nmol/L and 1.117 pg/mL, respectively.

Statistical Analysis

The continuous variables were presented either as mean ± standard deviation or median (min–max); categorical variables were presented as the absolute and relative frequencies (*n*, %). The variables were tested for the normality of distribution by the Kolmogorov Smirnov test. The three patient groups were compared by ANOVA in normally distributed variables and the Kruskal–Wallis test in abnormally distributed variables. *Post hoc* analysis in case of significant deviations in ANOVA, was performed using Tukey or Tamhane's test depending on the homogeneity of variances. Similarly, Dunn's test was used in the nonparametric pairwise multiple comparisons procedure following the Kruskal–Wallis test. The categorical variables were compared by chi square test. A p value of 0.017 adjusted by the Bonferroni method was used in the pairwise comparisons of categorical vari-

Table 1. The comparison of demographic characteristics of the study population

Characteristic	PAD (ABI ≤ 0.9) $n=60$	Normal ABI $n=60$	MAC (ABI > 1.4) $n=60$	p value	p^a	p^b	p^c
Age, years	64.8 ± 10.8	63.0 ± 8.7	64.2 ± 8.9	0.61	0.57	0.85	0.88
Male Gender, *n* (%)	50 (83.3%)	50 (83.3%)	53 (88.3%)	0.67	1	1	0.43
Smoking, *n* (%)	19 (31.7%)	13 (21.7%)	15 (20%)	0.28	0.23	0.78	0.14
Family History, *n* (%)	26 (43.3%)	15 (25%)	26 (43.3%)	0.05	0.03	0.034	0.93
Diabetes mellitus, *n* (%)	34 (56.7%)	26 (43.3%)	27 (45%)	0.28	0.14	0.85	0.20
Hypertension, *n* (%)	47 (78.3%)	52 (86.7%)	46 (76.7%)	0.33	0.23	0.15	0.82
Hyperlipidemia, *n* (%)	41 (68.3%)	48 (80%)	49 (81.7%)	0.17	0.14	0.817	0.09
ABI	0.71 (0.4-0.9)	1.17 (0.92-1.31)	1.5 (1.41-2.0)	< 0.001	< 0.001	< 0.001	< 0.001
Creatinine	0.86 (0.59-1.2)	0.86 (0.58-1.25)	0.85 (0.5-1.2)	0.72	0.43	0.38	0.92
eGFR	86 (60-121)	90 (60-117)	87 (62-120)	0.32	0.14	0.45	0.43
LVEF, %	64 (50-67)	65 (60-69)	64 (50-68)	0.63	0.44	0.38	0.91
WBC, x10^3	6.7 (4.8-10.4)	6.9 (4.4-9.1)	7.3 (4.4-9.5)	0.98	0.88	0.87	0.97
Medications (%)							
Beta-blockers	34 (56.7%)	47 (78.3%)	42 (70%)	0.04	0.011	0.29	0.13
ASA	31 (51.7%)	13 (21.7%)	17 (28.3%)	0.002	0.001	0.43	0.009
Clopidogrel	37 (61.7%)	46 (76.7%)	45 (75%)	0.11	0.053	0.70	0.11
Statin	33 (55%)	43 (71.7%)	44 (73.3%)	0.06	0.058	0.83	0.036
ACEi	12 (20%)	14 (23.3%)	16 (26.7%)	0.68	0.65	0.67	0.38
ARB	15 (25%)	15 (25%)	14 (23.3%)	0.97	1	0.83	0.83
Insulin	13 (21.7%)	4 (6.7%)	7 (11.7%)	0.048	0.018	0.34	0.14
Metformin	9 (15%)	12 (20%)	9 (15%)	0.69	0.47	0.47	1
OAD	6 (10%)	5 (8.3%)	3 (5%)	0.59	0.65	0.47	0.31
Symptom	35 (58.3%)	11 (18.3%)	13 (21.7%)	< 0.001	< 0.001	0.64	< 0.001

ABI: ankle-brachial index, ASA: acetylsalicilic acid, ACEi: angiotensin converting enzyme inhibitor, ARB: angiotensin receptor blocker, eGFR: estimated glomerular filtration rate, LVEF: Left ventricular ejection fraction, OAD: oral antidiabetic drugs, MAC: medial arterial calcification, PAD: peripheral arterial disease, WBC: white blood cell

The categorical variables are compared by chi square, continuous variables are compared by ANOVA or Kruskal–Wallis test.

p^a: low ABI vs. normal ABI, $p<0.017$ as the level of significance

p^b: normal ABI vs. high ABI, $p<0.017$ as the level of significance

p^c: low ABI vs. high ABI, $p<0.017$ as the level of significance

ables. The Spearman correlation coefficients were calculated to evaluate the continuous and noncontinuous relationships among the biomarkers and other variables. With setting ABI as the dependent variable, multivariate linear regression analysis was conducted, and the variables with a p value ≤ 0.1 were included into the model using stepwise method to determine the predictors of ABI. The potential predictors identified by univariate model were smoking; hyperlipidemia; medications like acetylsalicylic acid, clopidogrel, betablocker, statin, and biochemical markers like PTX3, hsCRP, copeptin, NT-proBNP, and neopterin. As clinically important parameters, eGFR, LVEF, and WBC counts were also included into the final model as the potential confounders. A p value of <0.05 was considered as statistically significant. Statistical analyses were performed using IBM® SPSS® Statistics for Mac, Version 20 software (IBM Corp., Armonk, NY).

Results

A total of 180 patients with prior CABG and mean age of 63.8 ± 9.4 years (85% male) were enrolled into the study. The demographic characteristics of the patients stratified by ABI are presented in **Table 1**. The frequency of the cardiovascular risk factors was similar within the study groups except for the family history of CAD, which was less common in normal ABI group compared to low and high ABI groups (25% vs. 43.3% vs. 43.3%; $p=0.05$). Similarly, no significant difference was observed among the study groups in terms of serum creatinine, eGFR, LVEF, and WBC counts. Use of most of the medications was similar within the groups except for those of beta blocker, acetylsalicylic acid, and insulin. Patients with low ABI were less frequently on beta-blockers (56.7% vs. 78.3% vs. 70%; $p=0.04$) and more frequently on

Table 2. The comparison of biochemical markers among the study groups

Biomarkers	PAD (ABI ≤ 0.9) $n=60$	Normal ABI $n=60$	MAC (ABI > 1.4) $n=60$	p	p^a	p^b	p^c
PTX3, ng/mL	4.6 (0.19-12.5)	3.2 (0.2-12.6)	2.6 (1.63-6.7)	<0.001	0.002	0.03	<0.001
hsCRP, mg/L	5.2 (0.5-10.5)	3.8 (0.1-10.5)	2.9 (0.3-10.2)	<0.001	0.024	0.609	<0.001
Copeptin, ng/mL	4.0 (0.9-12.6)	3.0 (0.03-12.6)	1.9 (0.9-9.6)	<0.001	0.69	0.004	<0.001
sTREM-1, pg/mL	146.9 (1.0-434.2)	135.7 (1.01-584.6)	105.3 (20.7-578.9)	0.037	1.0	0.226	0.037
NT-ProBNP, pg/mL	232.0 (115.9-1038.3)	257.9 (45.1-1046.9)	317.8 (128.7-902.1)	0.004	1.0	0.045	0.004
Neopterin, nmol/L	5.0 (0.9-20.9)	9.1 (0.3-20.9)	9.4 (0.3-20.9)	<0.001	<0.001	0.197	<0.001

PTX3: pentraxin 3, hsCRP: high sensitivity C-reactive protein, sTREM-1: soluble triggering receptor expressed on myeloid cells-1, NT-proBNP: N terminal probrain natriuretic peptide, PAD: peripheral artery disease, MAC: medial arterial calcification.
The groups are compared using Kruskal–Wallis test; the *post hoc* analyses of pairwise comparisons were performed using Dunn's test.

acetylsalicylic acid (51.7% vs. 21.7% vs. 28.3%) and insulin therapy (21.7% vs. 6.7% vs. 11.7%; $p=0.048$) when compared to the normal and high ABI patients. Table 2 and Fig. 3 indicate the circulating concentrations of the biochemical markers with respect to the ABI groups.

The highest levels of PTX3 were in patients with PAD (4.6 [0.19–12.5] ng/mL) followed by those with normal ABI (3.2 [0.2–12.6] ng/mL) and with MAC (2.6 [1.63–6.7] ng/mL, overall $p<0.001$). The short pentraxin, hsCRP, was higher in the PAD group when compared to the normal ABI and MAC groups (5.2 [0.5–10.5] in PAD vs. 3.8 [0.1–10.5] in normal ABI vs. 2.9 [0.3–10.2] mg/L in MAC, overall $p<0.001$). The difference between the normal and MAC groups, however, was not statistically significant ($p=0.609$). Copeptin levels in the PAD patients were 4.0 [0.9–12.6] ng/mL versus 3.0 [0.03–12.6] ng/mL in those with normal ABI versus 1.9 [0.9–9.6] ng/mL in patients with MAC (overall $p<0.001$). For sTREM-1, another inflammatory cytokine of atherosclerosis, levels were highest in patients with PAD and gradually decreased with increasing ABI (146.9 [1–343.2] in PAD, 135.7 [1.01–584.6] in normal ABI, and 105.3 [20.7–578.9] pg/mL in MAC); among these, only the difference between PAD and MAC groups was statistically significant ($p=0.037$). NT-proBNP concentrations were higher in the MAC patients (317.8 [128.7–902.1] pg/mL) when compared to the normal ABI patients (257.9 [45.1–1046.9] pg/mL) or PAD patients (232.0 [115.9–1038.3] pg/mL). The difference between the normal and PAD patients did not reach statistical significance. The neopterin levels were higher in MAC and normal ABI groups than the PAD group (9.4 [0.3–20.9] in MAC, 9.1 [0.3–20.9] in normal ABI, and 5.0 [0.9–20.9] nmol/L in PAD).

Correlation analysis was performed to determine the strength of correlations between ABI and biochemical markers (Table 3). The biochemical markers that efficiently correlated with ABI were neopterin ($r=0.40$, $p<0.001$) and PTX3 ($r=-0.40$, $p<0.001$). Notably, significant correlations of proatherogenic cytokine PTX3 with copeptin ($r=0.65$, $p<0.001$), sTREM-1 ($r=0.49$, $p<0.001$), and hsCRP ($r=0.22$, $p<0.001$), were observed.

The linear regression analysis revealed that PTX3 ($\beta=-0.32$, $p=0.005$), copeptin ($\beta=-0.48$, $p<0.001$), and neopterin ($\beta=0.72$, $p<0.001$) were independent predictors of ABI; in which the increase in neopterin was associated with high ABI, whereas increase in PTX3 and copeptin was associated with low ABI (Table 4).

Discussion

In the present study, the study population comprised considerably homogenous group of patients with advanced coronary atherosclerosis stratified by ABI. The deviations in concentrations of the biomarkers were therefore observed over CAD, representing a patient population close to the clinical practice. In this context, we majorly revealed the convergence of proatherogenic mediators such as PTX3, hsCRP, sTREM-1, and copeptin on low ABI; and pleiotropic/hemodynamic mediators like NT-proBNP and neopterin on higher ABI values. Our findings provided evidence that obstructive PAD and MAC are entities with distinct cytokine pattern wherein; (1) an increase in PTX3 and copeptin was independently related to low ABI; and (2) an increase in neopterin was independently related to high ABI.

In the present study, the results indicated that the levels of inflammatory cytokines associated with NF-κB signaling; namely PTX3, hsCRP, copeptin, and sTREM-1, were elevated in patients with PAD and declined as the ABI increased. The activation of this pathway, consequently, induces transcription of numerous proatherogenic mediators (Fig. 1A)[12].

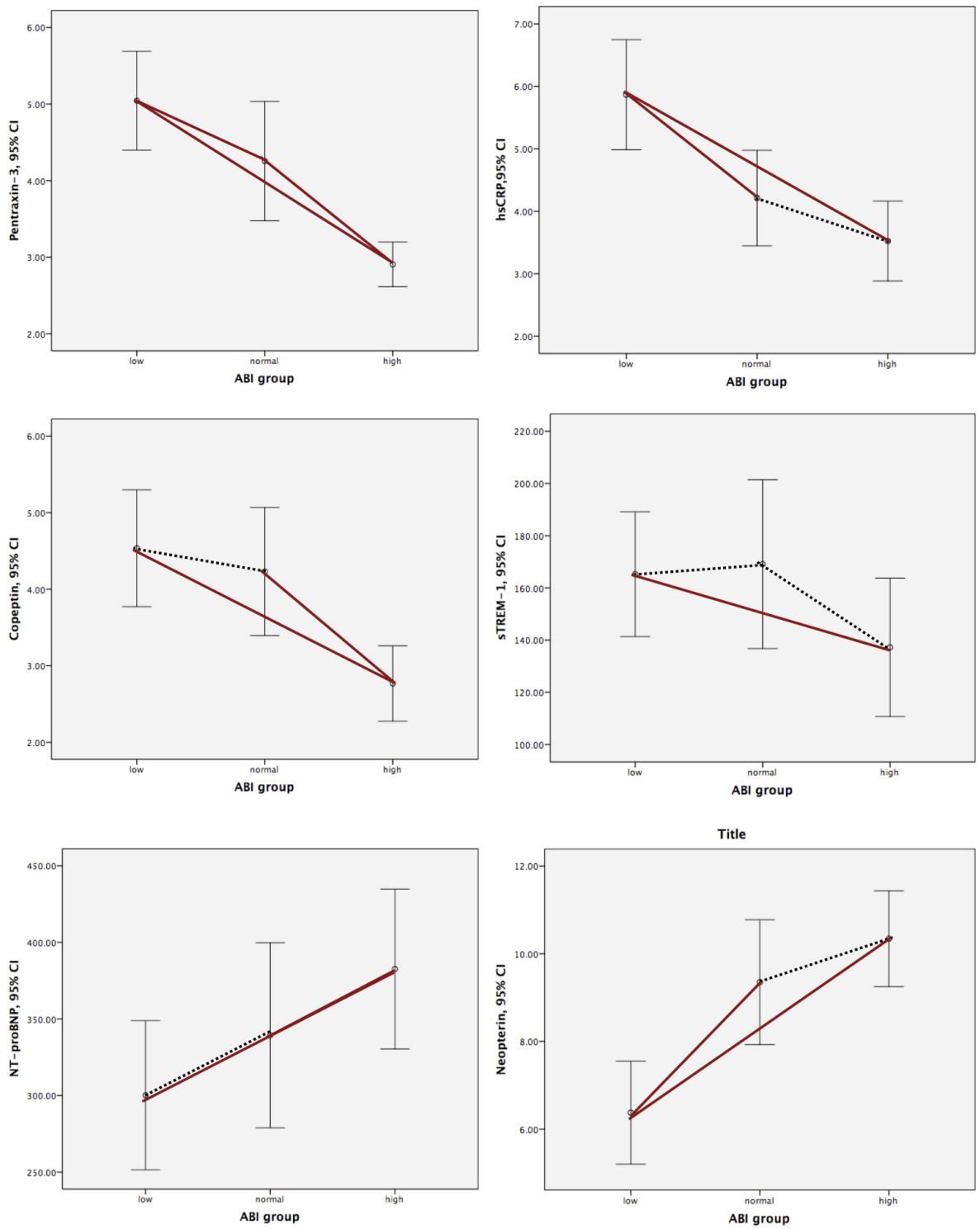


Fig. 3. Comparison of biochemical markers among the study groups

Red interpolation lines represent statistically significant difference; dotted lines represent differences without statistical significance

CI: Confidence interval, PTX3: pentraxin 3, hsCRP: high sensitivity C-reactive protein, sTREM-1: soluble triggering receptor expressed on myeloid cells-1, NT-proBNP: N terminal probrain natriuretic peptide

Table 3. Spearman correlations among the ABI and biochemical markers

	ABI	PTX3	Copeptin	hsCRP	sTREM	Neopterin	NT-ProBNP
ABI	1	−0.40**	−0.29**	−0.23**	−0.18*	0.40**	0.21**
PTX3	−0.40**	1	0.65**	0.22**	0.49**	NS	0.17*
Copeptin	−0.29**	0.65**	1	NS	0.34**	0.25**	NS
hsCRP	−0.23**	0.22**	NS	1	NS	−0.26**	NS
sTREM	−0.18*	0.49**	0.34**	NS	1	NS	0.24**
Neopterin	0.40**	NS	0.25**	−0.26**	NS	1	0.56**
NT-ProBNP	0.21**	0.17*	0.21**	NS	0.24**	0.56**	1

PTX3: pentraxin 3, hsCRP: high sensitivity C-reactive protein, sTREM-1: soluble triggering receptor expressed on myeloid cells-1, NT-proBNP: N terminal probrain natriuretic peptide
NS: nonsignificant
**indicates r values with $p<0.01$
*indicates r values with $p<0.05$

Table 4. Univariable and multivariable models for the predictors of ABI

Univariable model				
	β	95%CI		p value
Smoking	−0.14	−0.24	0.006	0.06
Diabetes mellitus	−0.096	−0.17	0.036	0.19
Hyperlipidemia	0.15	0.008	0.255	0.037
ASA	−0.22	−0.27	−0.057	0.003
Clopidogrel	0.13	−0.008	0.225	0.067
Beta blocker	0.13	−0.009	0.216	0.072
Statin	0.14	−0.004	0.218	0.059
PTX3	−0.30	−0.063	−0.023	<0.001
hs-CRP	−0.24	−0.045	−0.012	0.001
Copeptin	−0.23	−0.048	−0.011	0.002
sTREM-1	−0.1	−0.001	0.00	0.18
NT-ProBNP	0.14	0.00	0.00	0.059
Neopterin	0.30	0.012	0.032	<0.001
eGFR	0.018	−0.003	0.004	0.820
LVEF	0.072	−0.007	0.018	0.363
WBC	0.021	−0.049	0.034	0.719
Multivariable Model				
PTX3	−0.32	−0.075	−0.013	0.005
Copeptin	−0.48	−0.087	−0.030	<0.001
Neopterin	0.72	0.041	0.067	<0.001

ASA: acetyl salicilic acid, PTX3: pentraxin 3, hsCRP: high sensitivity C-reactive protein, sTREM-1: soluble triggering receptor expressed on myeloid cells-1, NT-proBNP: N terminal probrain natriuretic peptide, eGFR: estimated glomerular filtration rate, LVEF: left ventricular ejection fraction, WBC: white blood cell count. eGFR, LVEF, WBC count, and the variables in the univariable model are used as possible confounders to construct multivariable model by stepwise method.

PTX3 and hsCRP are the prototypes of acute phase proteins: PTX3 is the long and hsCRP is the short form of soluble pattern recognition molecules or pentraxins[13]. The association of pentraxins with CAD is well established. More recent studies have reported that PTX3 could predict endothelial dysfunction and PAD more accurately than CRP[17, 18]. In accordance with these reports, the correlation of PTX3 with ABI, in our study, was stronger than that of hsCRP. Moreover, PTX3 was an independent predictor of ABI even

when adjusted for hsCRP and WBC count in multivariate analyses. These findings suggest that PTX3 is a better indicator for obstructive PAD than hsCRP. Copeptin, the C terminal fragment of proarginine vasopressin, is a stress hormone released in response to various stimuli[16]. The stimulation of hypothalamopituitary axis by several inflammatory mediators such as TNF-α, IL-1, and IL-6 is known to increase the copeptin levels[16]. Herein, we demonstrated that copeptin was negatively correlated with ABI and was a strong determinant of obstructive PAD. To the best our knowledge, no such association of copeptin with ABI was reported in the literature to date.

sTREM-1 is a recently identified receptor on granulocyte and macrophages whose activation triggers the synthesis of proinflammatory cytokines like TNF-α[14]. Although the elevated plasma levels were reported in infectious diseases, its involvement in atherosclerosis was recently revealed by Rao *et al.* who demonstrated the expression of sTREM-1 on carotid plaques[19]. As discussed in this study, it represents the activation of proatherogenic cycle and acts in the same manner as PTX3 and copeptin; adding fuel to fire. The significant positive correlations between sTREM-1 and PTX3 (r=49) and copeptin (r=0.34) support the basic science studies.

MAC, in contrast, is considered as a nonatheromatous lesion[19]. Deposition of calcium at the extracellular matrix of vascular media is an active process mediated by vascular smooth muscle cells; however, data on the underlying pathophysiological mechanism is scarce. MAC clinically presents as calcified arteries usually apparent on plain X-ray[20]. It is a prevalent entity in PAD, evidenced by O'Neill *et al.*, who reported MAC in 72% of the histological examinations of the amputated limbs[6, 21].

In the present analysis, the proatherogenic cytokines were decreased in patients with MAC; however, cytokines with pleiotropic effects like neopterin and NT-proBNP were increased. Moreover, neopterin was found to be an independent determinant of increasing ABI. Low grade systemic inflammation is the hallmark of MAC in which the relatively low plasma expression of proatherogenic cytokines was due[22]. Neopterin is produced by the activated macrophages upon stimulation by interferon δ (INF δ)[15]. Unlike PTX3, hsCRP, copeptin, and sTREM-1, INF δ mediated inflammation inhibits IL-1 receptor, inactivating the downstream signaling cascade[23]. Meanwhile, it utilizes the JAK-STAT signaling pathway instead of NF-KB pathway[24]. INF δ was also revealed to have contrasting roles with IL-6[25]. It is therefore obvious to have neopterin concentrations in the opposite direction to those of the proatherogenic cytokines. In the clinical CAD studies, neopterin concentrations were associated with plaque vulnerability and mortality, but not with the anatomical extent of coronary lesions[26, 27]. These concentrations are considered as an activation marker of macrophages with important prognostic information[28]. Notably, several studies suggest an association between neopterin and vascular calcification. Macrophages activated by INF-δ, express an enzyme that converts vitamin D into calcitriol; which in turn stimulates dystrophic calcification[29]. Similarly, Naito et al. have reported increased serum neopterin levels in patients with calcific aortic valve stenosis[30]. Hence, high neopterin levels in MAC may be a marker of the ongoing medial calcification. In the present study, although a positive linear relationship with ABI and neopterin levels was evident; we could not demonstrate a significant increase in the neopterin levels, when the normal and high ABI were compared (**Table 2**). This may be due to the PAD patients in high ABI group who were masked by severely calcified, noncompressible arteries. This finding suggests a possible association not only with high ABI and increased mortality but also with vascular calcification through neopterin. This is the first study to demonstrate the influence of neopterin on ABI.

In our study, NT-proBNP, which is an established biomarker of cardiovascular disease, was decreased in the PAD group, and increased in the MAC group. Previous studies had also reported that NT-proBNP levels were 2.5-fold higher in the patients with MAC than those with PAD, which was explained by the increased hemodynamic stress and arterial stiffness[31]. Although we did not use an objective surrogate of arterial stiffness such as pulse wave velocity (PWV) to provide an evidence for the increased stiffness in the present study, data suggest that adverse hemodynamic effect of incompressible arteries was the reason of increased NT-proBNP[32]. In the previous studies of ankle-brachial PWV, a clear positive association of NT-proBNP and PWV was manifested[33]. Decreased levels of NT-proBNP in PAD are intriguing. Published data refers to the antiatherogenic, proregenerative, and pleiotropic effects of natriuretic peptides[34]. We, therefore, suppose that loss of these effects characterizes the cytokine pattern in PAD.

There are several limitations of this study. First, it has a cross-sectional design, limiting one to draw conclusions on the causality of observed differences in the cytokine patterns. Second, involvement of CABG patients with advanced atherosclerosis can complicate the interpretation of results. Having CABG population, however, has also provided a homogenous study group limiting the uncontrolled involvement of coronary and carotid atherosclerosis into any specific sub-

set. Third, the potential overlap of obstructive PAD and MAC is another limitation. Nevertheless, there is no recommended diagnostic method for setting the MAC diagnosis other than ABI measurement[9, 20]. It is, therefore, important to observe this distinct pattern of cytokines, despite the possible involvement of obstructive PAD patients into the high ABI group. Fourth, lack of PWV analysis as a surrogate of hemodynamic stress can be considered as a limitation; however, PWV can be underestimated when ABI <0.9[35].

In conclusion, we demonstrated a remarkable increase in the proatherogenic cytokines such as PTX3, hsCRP, copeptin, and sTREM-1 concentrations in patients with low ABI, wherein increase in the PTX3 and copeptin was the independent predictor of obstructive PAD, expressed by low ABI. The concentrations of pleiotropic mediators like neopterin and NT-proBNP elevated with increasing ABI; thus, neopterin was the determinant of increasing ABI. These results suggest the influence of distinct inflammatory pathways at the opposing ends of ABI spectrum. This study has implications to clinical practice since the identified cytokine patterns may guide future research for diagnosis, prevention, or treatment of different forms of vascular disease such as PAD and MAC. .

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Conflicts of Interest

The authors declare that there is no conflict of interest.

References

1) Hussein AA, Uno K, Wolski K, Kapadia S, Schoenhagen P, Tuzcu EM, Nissen SE, Nicholls SJ. Peripheral arterial disease and progression of coronary atherosclerosis. J Am Coll Cardiol, 2011; 57: 1220-1225
2) Dormandy J, Mahir M, Ascady G, Balsano F, De Leeuw P, Blombery P, Bousser MG, Clement D, Coffman J, Deutshinoff A. Fate of the patient with chronic leg ischaemia. A review article. J Cardiovasc Surg (Torino), 1989; 30: 50-57
3) Cirqui MH. Peripheral arterial disease-epidemiological aspects. Vasc Med, 2001; 6: 3-7
4) Welten GM, Schouten O, Hoeks SE, Chonchol M, Vidakovic R, van Domburg RT, Bax JJ, van Sambeek MR, Poldermans D. Long-term prognosis of patients with peripheral arterial disease: a comparison in patients with coronary artery disease. J Am Coll Cardiol, 2008; 51: 1588-1596
5) Demer LL and Tintut Y. Vascular calcification, pathobiology of a multifaceted disease. Circ, 2008; 117: 2938-2948
6) Nakamura E, Sato Y, Iwakiri T, Yamashita A, Moriguchi-Goto S, Maekawa K, Gi T and Asada Y. Asymptomatic plaques of lower peripheral arteries and their association with cardiovascular disease: An autopsy study. J Atheroscler Thromb, 2017; 24: 921-927
7) Rocha-Singh KJ, Zeller T and Jaff MR. Peripheral arterial calcification: Prevalence, mechanism, detection, and clinical implications. Catheter Cardiovasc Interv, 2014; 83: 212-220
8) Ho CH and Shanahan CM. Medial arterial calcification: An overlooked player in peripheral arterial disease. Arterioscler Thromb Vasc Biol, 2016; 36: 1475-1482
9) Aboyans V, Criqui MH, Abraham P, Allison MA, Creager MA, Diehm C, Fowkes FG, Hiatt WR, Jönsson B, Lacroix P, Marin B, McDermott MM, Norgren L, Pande RL, Preux PM, Stoffers HE, Treat-Jacobson D. Measurement and interpretation of the ankle-brachial index. A scientific statement from the American Heart Association. Circ, 2012; 126(24): 2890-2909
10) Criqui MH, McClelland RL, McDermott MM Allison MA, Blumenthal RS, Aboyans V, Ix JH, Burke GL, Liu K, Shea S. The ankle-brachial index and incident cardiovascular events in the MESA (Multi-Ethnic Study of Atherosclerosis). J Am Coll Cardiol, 2010; 56: 1506-1512
11) Lawrence T. The nuclear factor NF-κB pathway in inflammation. Cold Spring Harb Perspect Biol, 2009; 1: a001651
12) Pashar Y, Bias SR, Gill NS. Emerging role of various signaling pathways in the pathogenesis and therapeutics of atherosclerosis. Rev Cardiovasc Med, 2017; 10: 10-12
13) Vilahur G and Badimon L. Biological actions of pentraxins. Vasc Pharmacol, 2015; 73: 38-44
14) Bouchon A, Dietrich J and Colonna M. Cutting edge inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. J Immunol, 2000; 16410: 4991-4995
15) Zuo H, Ueland PM, Ulvik A, Eussen SJ, Vollset SE, Nygård O, Midttun Ø, Theofylaktopoulou D, Meyer K, Tell GS. Plasma biomarkers of inflammation, the kynurenine pathway, and risks of all-cause, cancer, and cardiovascular disease mortality. The Hordaland Health Study. Am J Epidemiol, 2016; 183: 249-258
16) Paraskevas KI, Briana DD and Malamitsi-Puchner A. Copeptin for all: A biomarker from infant pathology to adult cardiovascular disease. Angiology, 2016; 67: 894-895
17) Zhou Y, Ni Z, Zhang J, Zhang W, Wu Q, Shen G, Wang Y, Qian J. Plasma pentraxin 3 may be a better marker of peripheral artery disease in hemodialysis patients than C-reactive protein. Vasc Med, 2013; 18: 85-92
18) Igari K, Kudo T, Toyofuku T, Inoue Y. Relationship of inflammatory biomarkers with severity of peripheral arterial disease. Int J Vasc Med, 2016; 6015701
19) Rao VH, Rai V, Syoupa S, Subramanian S, Agrawal DK.

Tumor necrosis factor-α regulates triggering receptor expressed on myeloid cells-1 dependent matrix metalloproteinases in the carotid plaques of symptomatic patients with carotid stenosis. Atherosclerosis, 2016: 248: 160-169
20) Lanzer P, Boehm M, Sorribas V, Thiriet M, Janzen J, Zeller T, St Hilaire C, Shanahan C. Medial vascular calcification revisited: review and perspectives. Eur Heart J, 2014; 35: 1515-1525
21) O'Neill WC, Han KH, Schneider TM, hennigar RA. Prevalence of nonatheromatous lesions in peripheral arterial disease. Arterioscler Thromb Vasc Biol, 2015; 35: 439-447
22) Shao JS, Cheng SL, Sadhu J, Towler DA. Inflammation and the osteogenic regulation of vascular calcification a review and perspective. Hypertension, 2010 Mar; 55(3): 579-592
23) Hu X, Ivashkiv LB. Cross-regulation of signaling pathways by interferon-δ: implications for immune responses and autoimmune diseases. Immunity, 2009 16; 31: 539-550
24) Rawlings JS, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. J Cell Sci, 2014; 117: 1281-1283
25) Qi YF, Huang Y, Wang H, Zhang Y, Bao YL, Sun LG, Wu Y, Yu CL, Song ZB, Zheng LH, Sun Y, Wang GN, Li YX. Elucidating the crosstalk mechanism between IFN-gamma and IL-6 via mathematical modelling. BMC Bioinformatics, 2013; 14: 14-41
26) Sugioka K, Naruko T, Matsumura Y, Shirai N, Hozumi T, Yoshiyama M, Ueda M. Neopterin and atherosclerotic plaque instability in coronary and carotid arteries. J Atheroscler Thromb, 2010; 17(11): 1115-1121
27) Sun Y, He J, Tian J, Xie Z, Wang C, Yu B. Association of circulating levels of neopterin with non-culprit plaque vulnerability in CAD patients an angiogram, optical coherent tomography and intravascular ultrasound study. Atherosclerosis, 2015; 241: 138-142
28) De Rosa S, Cirillo P, Pacileo M, Petrillo G, D'Ascoli GL, Maresca F, Ziviello F, Chiariello M. Neopterin: from forgotten biomarker to leading actor in cardiovascular pathophysiology. Curr Vasc Pharmacol, 2011; 9: 188-199
29) Johnson RC, Leopold JA and Loscalzo J. Vascular calcification, pathobiological mechanisms and clinical implications. Circ Res, 2006; 99: 1044-1059
30) Naito Y, Tsujino T, Akahori H, Matsumoto M, Ohyanagi M, Mitsuno M, Miyamoto Y, Masuyama T. Increased serum neopterin in patients with nonrheumatic aortic valve stenosis. Int J Cardiol, 2010; 145: 360-361
31) Jouni H, Rodeheffer RJ and Kullo IJ. Increased serum N-Terminal Pro-B-Type Natriuretic Peptide levels in patients with medial arterial calcification and poorly compressible leg arteries. Arterioscler Thromb Vasc Biol, 2011; 31: 197-202
32) Tomiyama H, Matsumoto C, Shiina K, Yamashina A. Brachial-ankle PWV: Current status and future directions as a useful marker in the management of cardiovascular disease and/or cardiovascular risk factors. J Atheroscler Thromb, 2016; 23(2): 128-146
33) Kimura K, Tomiyama H, Matsumoto C, Odaira M, Shiina K, Nagata M, Yamashina A. Correlations of arterial stiffness/central hemodynamics with serum cardiac troponin T and natriuretic peptide levels in a middle-aged male worksite cohort. J Cardiol, 2015; 66(2): 135-142
34) Hua BC, Li Y, Liu M, Sheng CS, Wang JG. Ankle-brachial index in relation to the natriuretic peptide system polymorphisms and urinary sodium excretion in Chinese. Atherosclerosis, 2013; 230: 86-91.
35) Matsushima H, Hosomi N, Hara N, Yoshimoto T, Neshige S, Kono R, Himeno T, Takeshima S, Takamatsu K, Shimoe Y, Ota T, Maruyama H, Ohtsuki T, Kuriyama M, Matsumoto M. Ability of the ankle brachial index and brachial-ankle pulse wave velocity to predict the 3-month outcome in patients with non-cardioembolic stroke. J Atheroscler Thromb, 2017; 24(11): 1167-1173