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The Evaluation of New Biomarkers of Inflammation and Angiogenesis in Peripheral Arterial Disease

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Additional information is available at the end of the chapter

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1. Introduction

Peripheral artery disease is a clinical manifestation of atherosclerosis with significant morbidity and mortality (Sharma Sharma & Aronow, 2012; Resnick et al. 2004; Diehm et al. 2009). Despite well-recognized significance of traditional risk factors in the initiation and progression of the disease, not all causes and mechanisms leading to disease development have been identified so far. Inflammation, angiogenesis, and endothelial activation are important processes contributing to the pathogenesis of peripheral arterial disease which are related in a complex and interdependent manner (Li et al., 2007; Brevetti et al., 2010; Brevetti et al., 2003; Brevetti et al., 2008; Findley et al., 2008).

Pathophysiologic events in peripheral artery disease are represented by ischaemic tissue damage, and the severity of clinical presentation depends on the site and extent of stenosis and availability of collateral circulation (Meru et al., 2006; Cooke 2008). Angiogenesis and arteriogenesis (collateral growth) are different forms of vessel growth, which contribute to the compensation for an occluded artery. Hypoxia is known to trigger angiogenesis in the setting of ischaemia, whereas fluid shear stress might be the most important stimulus for initiation of collateral growth. Besides these specific initial triggers, angiogenesis and collateral growth share growth factors, chemokines, proteases, and inflammatory cells, which play different roles in promoting and refining these processes (Silvestre et al., 2008).

During an tissue ischemia, hypoxia-inducible factor 1 (HIF-1) drives transcriptional activation of hundreds of genes involved in vascular reactivity, angiogenesis, arteriogenesis, the mobilization of bone marrow-derived angiogenic cells (Rey & Semenza 2010). The current evidence suggests considerable overlap between the molecular mechanisms and

physical stimuli that trigger angiogenesis and inflammation (Costa et al., 2007). Furthermore, there is compelling evidence that HIF-1 contributes to both processes by regulating angiogenesis and functions of inflammatory cells. Many inflammatory stimuli can activate the angiogenic programme of endothelial cells. Inflammatory cells, especially monocytes/macrophages secrete many angiogenic factors such as vascular endothelial growth factor (VEGF), CXCL8 (interleukin-8), granulocyte colony stimulating factor, transforming growth factor- α and β , platelet-derived growth factor, tumor necrosis factor- α , and prostaglandins. The angiogenic factors bind to cognate receptors which are expressed on the surface of vascular endothelial cells and vascular pericytes/smooth muscle cells. Receptor-ligand interaction activates these cells and promotes the angiogenic response. Communication between endothelial cells and monocytes/macrophages appears to be bidirectional, because endothelial cell-secreted factors also induce chemotaxis and increased angiogenic activity in monocytes/macrophages, thus initiating a positive feedback cycle (Shireman, 2007).

The angiogenesis are tightly regulated in a complex balance between pro- and anti-angiogenic mechanisms (Carmeliet, 2003; Otrock et al., 2007). The most important proangiogenic growth factors are VEGF and angiopoietins. VEGF and angiopoietins, acting as the modulators of endothelial activation via receptor tyrosine kinase Tie-2, are important for angiogenesis and vascular remodeling. VEGF increases microvascular permeability and induces the proliferation, migration, and differentiation of endothelial cells (Hoebe et al., 2009; Stüttgen & Ballmer-Hofer, 2009; Olsson et al., 2006). Angiopoietin-2 is a natural endogenous antagonist of the Tie-2, which acts as an autocrine negative regulator of endothelial function (Augustin et al., 2009; Scharpfenecker et al., 2004; Fiedler & Augustin, 2006; Fukuhara et al., 2010). In the presence of VEGF, it mounts an inflammatory response by endothelial activation and induction of permeability, and in the absence of VEGF, it destabilizes the existing vessels and leads to vascular regression. Soluble receptors of angiogenic growth factors which are being released to circulation can act as the inhibitors of angiogenesis and, in some cases, may correlate with the disease severity independently of altered haemodynamics (Findley et al., 2008).

The findings of the large prospective investigations have confirmed the significance of high-sensitivity C-reactive protein (hs-CRP) as a marker of progression, functional activity, and adverse cardiovascular outcome in patients with peripheral artery disease (Abdellaoui & Al-Khaffaf, 2007).

Platelet activating factor acetylhydrolase (PAF-AH; E.C. 3.1.1.47) also named lipoprotein-associated phospholipase A(2) (Lp-PLA(2)) is a novel inflammatory biomarker that has an active role in atherosclerotic development and progression. This enzyme is characterized by its ability to specifically hydrolyze the short acyl group at the sn-2 position of the phospholipids in oxidized LDL, which leads to production of the pro-inflammatory, atherogenic by-products lysophosphatidylcholine and oxidized nonesterified fatty acids. These bioactive lipid mediators act as chemoattractants for monocytes, impair endothelial function, disrupt plasma membranes, and induce apoptosis in smooth muscle cells and macrophages. Epidemiologic studies demonstrate that elevated circulating levels of PAF-AH predict an increased risk of myocardial infarction and stroke, whereas histologic ex-

amination of diseased human coronary arteries reveals intense presence of the enzyme in atherosclerotic plaques that are prone to rupture. The biological role of PAF-AH in the development of peripheral arterial disease is controversial because substrates and products of the catalytic reactions implicating PAF-AH have proatherogenic properties (Zalewski & Macphee, 2005; Gazi et al., 2005; Srinivasan & Bahnson, 2010; Tsimikas et al., 2007; Münzel & Gori, 2009; Ballantyne et al., 2007; Daniels et al., 2008; Garza et al., 2007; Koenig et al., 2004).

The hypothesis set out in this investigation is that PAF-AH, as a novel biomarker of inflammation, and VEGF, Ang-2, and its receptor Tie-2, as new biomarkers of angiogenesis, play a significant role in the development and progression of peripheral artery disease. The aim of this study was to investigate the association of the catalytic concentrations of platelet activating factor acetylhydrolase (PAF-AH), the concentrations of VEGF, angiopoietin 2 (Ang-2) and its receptor Tie-2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains), as novel biomarkers of inflammation and angiogenesis with the lipid status and CRP, as a nonspecific marker of inflammation and cardiovascular risk factor in patients with peripheral arterial disease and matched control group. In the group of patients with peripheral arterial disease, the relationship between the biochemical parameters under study and the anatomical extent of peripheral arterial atherosclerotic changes, will be explored, and those will be evaluated through their potential clinical utility as novel diagnostic and prognostic tools in peripheral arterial atherosclerosis.

2. Patients and methods

2.1. Patients

The study included 110 patients, 19 women and 91 men, with clinically and angiographically confirmed diagnosis of peripheral arterial disease. The study population was referred to the Digital subtraction angiography (DSA) in order to determine the precise extent and localization of peripheral limb atherosclerosis and assess the technical possibility to perform percutaneous transluminal angioplasty (PTA). Based on the angiographic findings, for the purpose of the present investigation the angiographic score was assessed for each patient. The angiographic score takes into consideration the extent (percentage of vessel lumen reduction) and diffusion of peripheral arterial disease (involved segments of vascular tree). The distal aorta plus 10 segments (common iliac artery, external iliac artery, common femoral artery, profunda femoral artery, superficial femoral artery, popliteal artery, truncus tibiofibularis, anterior tibial artery, posterior tibial artery and fibular artery) on each side were scored on the basis of vessel lumen reduction: 1 if stenoses involved a reduction in the vessel lumen of <50%, 2 if stenoses involved 50 to 99% reduction, and 3 if total occlusion was present. The sum of the points assigned to each of these arteries was called the angiographic score.

The control group consisted of 118 patients, 61 female and 57 male with suspected symptoms of peripheral arterial disease referred to Doppler examination. At the Doppler examination, all of them had normal triphasic waveforms of the peripheral arteries.

All Doppler and DSA procedures were performed at the Institute for Diagnostic and Interventional Radiology of the Merkur University Hospital. Doppler examinations were performed at a center of excellence with more than 3,000 examinations performed per year. DSA was performed by an experienced vascular interventional radiologist. All participants gave their informed written consent. This study was approved by the Ethics Committee of the Merkur University Hospital, Zagreb, Croatia.

2.2. Samples

Blood samples were taken under controlled pre-analytical conditions in the morning after 12-h fast. Serum was separated by centrifuging the samples at 4°C at 3000 rpm for 15 minutes.

2.3. Methods

2.3.1. The lipid status and CRP

Analytical methods for measurement of the lipid status, including serum triglyceride, total cholesterol, LDL and HDL-cholesterol concentrations as well as CRP used in this study have been accredited according to ISO 15189, Medical laboratories - Particular requirements for quality and competence (ISO 15189, 2008) (Flegar-Meštrić et al., 2010). All measurements were performed on fresh sera on the day of blood collection using standard commercial kits (Olympus Diagnostic GmbH, Hamburg, Germany) on the Olympus AU 600 analyzer (Olympus Mishima Co., Ltd., Shizuoka, Japan). Serum triglyceride and total cholesterol were measured by enzymatic PAP- method. HDL cholesterol was measured with direct method based on selective inhibition of the non-HDL fractions by means of polyanions. A homogeneous assay for the selective measurement of LDL cholesterol in serum was used. The index of atherosclerosis and the established risk factor were calculated as the ratio of LDL cholesterol to HDL cholesterol and total cholesterol to HDL cholesterol. CRP concentrations were determined by high-sensitivity latex-enhanced immunoturbidimetric assay.

2.3.2. The catalytic concentrations of PAF-AH

The catalytic concentrations of PAF-AH were determined in serum by spectrophotometric method described by Kosaka T. et al. (2000) using the AZWELL Auto PAF-AH Assay Kits (AZWELL Inc., Osaka, Japan) on a biochemical analyzer Olympus AU600 (Olympus Mishima Co., Ltd., Shizuoka, Japan). Serum samples were kept frozen at -80°C until the day of analysis. PAF-AH hydrolyzes the sn-2 position of the substrate (1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine), producing 4-nitrophenyl succinate. This compound immediately degrades in aqueous solution and liberates 4-nitrophenol. In the first phase, 2 µL of serum was added to 240 µL of 200 mmol/L HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Reagent 1), pH 7.6 and pre-incubated at 37°C for 5 min. The reaction was started by adding 80 µL of 20 mmol/L citric acid monohydrate buffer, pH 4.5 containing 90 mmol/L 1-myristoyl-2-(4-nitrophenylsuccinyl)phosphatidylcholine (Reagent 2). The liberation of 4-nitrophenol was measured by reading differences in absorbance at 405 nm (main wavelength) and 505 nm (subwavelength) between 1 and 3 minutes after addition of the substrate. The

catalytic concentrations of PAF-AH are expressed in international units per liter of serum and standardized against concentration of LDL-cholesterol.

2.3.3. The concentrations of angiogenesis biomarkers: VEGF, Ang-2 and Tie-2 receptor

Commercially available ELISA kits for VEGF (DVE00), Ang2 (DANG 20) and Tie2 (DTE 200) were purchased from R&D Systems (Minneapolis, MN, USA) and used according to the manufacturer's instruction. Serum samples were kept frozen at -80°C until the day of analysis. Briefly, the microtitre plates were coated with monoclonal antibodies specific for either VEGF-A, Ang-2 or Tie-2 and the first step was to add standards and samples to the wells. During the following incubation period, the VEGF-A, Ang-2 or Tie-2 present in standards and samples were bound to the immobilized antibody. After a thorough wash, an a horseradish peroxidase-linked polyclonal antibody specific for VEGF, Ang-2 or Tie-2 was pipetted into the wells and following a second incubation and wash step a substrate solution was added and colour developed in proportion to the amount of VEGF-A, Ang-2 or Tie-2. After further washings to remove any unbound antibody-enzyme reagent, tetramethylbenzidine was added. The colour development was subsequently stopped and the intensity of colour was measured by using Stat Fax®2100, Microplate reader, Awareness Technology Inc., Palm City, FL, USA. The values were calculated using a standard curve generated with specific standards provided by the manufacturer. The detection limit for VEGF, Ang-2 and Tie-2 was 9 ng/L, 8,3 ng/L, and 14 ng/L, respectively. The intra-assay and interassay coefficients of variation were in the range given by the manufacturer <6,7% and <8,8% for VEGF, < 6,9% and <10,4% for Ang-2 and < 5,3% and <8,5% for Tie-2 receptor.

2.4. Statistical analysis

Statistical analyses were performed using the SPSS software package for Windows, version 13 (SPSS Inc, Chicago, IL, USA). Descriptive analyses were performed and data were presented as mean, median, S.D. and percentile. Normal distribution of the study variables was tested using Kolmogorov-Smirnov test. Student *t* test and Mann-Whitney U test or the Kruskal-Wallis test applied according to the normal or non-normal distribution. Spearman coefficient of correlation was calculated to evaluate relationships between different variables.

3. Results

3.1. Patients

Demographic and clinical characteristics of the participants are shown in Table 1.

3.2. The lipid status and CRP

The patients had significantly higher concentrations of CRP, triglyceride, index of atherosclerosis, the ratio of total and HDL cholesterol, and lower concentrations of total, LDL and HDL-cholesterol (Table2).

Parameter	Patients with PAD (N=110)	Control subjects (N=118)	P
Age (years), x±sd	64,33 ± 9,79	59,11± 7,31	P<0,001
Male sex, n (%)	91 (83%)	57 (48%)	P<0,001
Body mass index (kg/m ²), x±sd	26,63 ± 4,04	26,51± 3,08	P = 0,795
Systolic blood pressure >140 mm Hg, n(%)	74 (67%)	0	P<0,001
Diastolic and systolic blood pressure >90 mm Hg, n (%)	30 (27%)	0	P<0,001
Diabetes, n (%)	39 (35%)	0	P<0,001
Active smokers, n (%)	49 (45%)	13 (11%)	P<0,001
Hypolipemic therapy, n (%)	64 (58%)	0	P<0,001
Antihypertensive therapy, n (%)	69 (63%)	0	P<0,001
Cerebrovascular simptoms, (%)	11 (10%)	0	P<0,001
Coronary artery disease symptoms, n (%)	26 (24%)	0	P<0,001

Table 1. Demographic and clinical characteristics of the study groups: patients with peripheral arterial disease (PAD) and controls. Data are given as mean ± standard deviation, unless otherwise stated.

Biochemical parameters (units)	Patients with PAD (N=110)	Control subjects (N=118)	P
Triglyceride (mmol/L)	1,89 (1,30-2,36)	1,42 (1,03-1,76)	<0,001
Total cholesterol (mmol/L)	5,45 (4,68-6,10)	6,35 (5,66-6,89)	<0,001
HDL-cholesterol (mmol/L)	1,10 (0,98-1,30)	1,60 (1,39-1,81)	<0,001
LDL- cholesterol (mmol/L)	3,30 (2,70-3,90)	3,96 (3,40-4,55)	<0,001
CRP (mg/L)	3,70 (1,78-7,40)	1,40 (0,60-2,43)	<0,001

Mann–Whitney's tests

Table 2. The lipid status and CRP concentrations in the patients with peripheral arterial disease (PAD) and controls. Data are given as median (interquartile range).

3.3. The catalytic concentrations of PAF-AH

The catalytic concentrations of PAF-AH did not differ between the two groups, while LDL standardized catalytic concentrations of PAF-AH (U/mmol) showed significant difference (Table 3). The catalytic concentrations of PAF-AH were higher in men than in women in control subjects (Table 4.), whereas no gender difference was observed in patients with peripheral arterial disease (Table 5.).

A significant difference in the catalytic concentrations of PAF-AH was found between subjects on lipolythic therapy and subjects off therapy (P=0,032), with the median concentration of PAF-AH in subjects off therapy being higher than that observed in subjects on lipolythic therapy: 425, interquartile range 351-494 U/L vs 364, interquartile range, 316-427 U/L. There was no difference in catalytic concentrations of PAF-AH between smokers and non-smokers, diabetic and nondiabetic subjects nor between the subjects on antihypertensive therapy and subjects off therapy.

A statistically significant correlation was found between the catalytic concentration of PAF-AH and the concentration of triglycerides, total and LDL-cholesterol in both groups studied (Table 6.).

Biochemical parameters (units)	Patients with PAD (N=93)	Control subjects (N=64)	P
PAF-AH (U/L)	405 (330-471)	406 (359-479)	0,591
PAF-AH/LDL (U/mmol)	121 (107-139)	98 (86-120)	<0,001

Mann–Whitney's tests

Table 3. The catalytic concentrations of PAF-AH in the patients with peripheral arterial disease (PAD) and control subjects. Data are given as median (interquartile range).

Biochemical parameters (units)	Male (N=28)	Female (N=36)	P
PAF-AH (U/L)	459 (383- 519)	385 (319-437)	0,005
PAF-AH/LDL (U/mmol)	121 (95-137)	92 (79-103)	<0,001

Mann–Whitney's tests

Table 4. The catalytic concentrations of PAF-AH in the male and female control subjects. Data are given as median (interquartile range).

Biochemical parameters (units)	Male (N=75)	Female (N=18)	P
PAF-AH (U/L)	405 (331- 477)	409 (329-442)	0,722
PAF-AH/LDL (U/mmol)	123 (108-141)	110 (100- 119)	0,031

Mann–Whitney's tests

Table 5. The catalytic concentrations of PAF-AH in male and female patients with peripheral arterial disease. Data are given as median (interquartile range)..

	Correlation coefficient			
	Patients with PAD (N=93)		Control subjects (N=64)	
	r	P	r	P
Triglyceride (mmol/L)	0,33	0,001	0,41	0,001
Total cholesterol (mmol/L)	0,70	<0,001	0,32	0,010
HDL-cholesterol (mmol/L)	-0,22	0,035	-0,33	0,009
LDL- cholesterol (mmol/L)	0,70	<0,001	0,33	0,009
CRP (mg/L)	-0,09	0,371	-0,06	0,617

Table 6. Relationships between the catalytic concentrations of PAF-AH and serum lipids parameters and CRP concentrations in the study groups: patients with peripheral arterial disease (PAD) and controls

3.4. Serum VEGF, Ang-2 and Tie-2 concentrations

The concentration of VEGF did not differ significantly between groups (Figure 1., Table 7.). The patients had higher concentrations of Ang-2 and Tie-2 receptor. (Figure 2.,3., Table 7.).

A significant difference in the concentrations of VEGF was found between diabetic and nondiabetic subjects ($P=0,006$), with the median (interquartile range) concentration of VEGF in diabetics being higher than that observed in nondiabetic subjects: 358 (210-463) vs. 197 (130-335) ng/L. There was no difference in concentrations of VEGF, Ang-2, and Tie-2 receptor between smokers and non-smokers, nor between the subjects on lipolytic and antihypertensive therapy and subjects off therapy. All three serum biomarkers of angiogenesis correlated with the CRP concentrations (Table 8). The concentrations of HDL- cholesterol, VEGF, Ang-2, and Tie-2 were statistically significantly different among the subjects with various cardiovascular risk according to CRP concentrations (Table 9.). Post hoc tests (Mann–Whitney's test) suggested a significant difference in HDL -cholesterol values between the low risk subjects ($CRP < 1,0$ mg/L) compared with the moderate (CRP between 1,0-3,0 mg/L) ($P=0,004$) and high risk ($P=0,011$) subjects ($CRP > 3,0$ mg/L). The subject groups of moderate and high cardiovascular risk did not differ significantly in the HDL cholesterol concentration ($P=0,666$). Statistically significant difference was found in the concentrations of VEGF ($P=0,011$), Ang-2 ($P < 0,001$),

and Tie-2 receptor (P=0,005) between low and high risk subjects, as well as in the concentrations of VEGF (P=0,012), Ang-2 (P<0,001), and Tie-2 receptor (P=0,02) between the moderate and high cardiovascular risk subjects, whereas there were no statistically significant differences in the concentrations of VEGF (P=0,377), Ang-2 (P=0,438), and Tie-2 receptor (P=0,673) between the groups of low and moderate cardiovascular risk subjects.

Biochemical parameters (units)	Patients with PAD (N=110)	Control subjects (N=54)	P
VEGF (ng/L)	263 (142-403)	287 (115-483)	0,983
Ang-2 (ng/L)	2018 (1613-2689)	1603 (1452-2138)	0,001
Tie-2 (µg/L)	21,4 (18,6-23,9)	19,6 (18,1-22,2)*	0,049

Mann–Whitney's tests, *N=43

Table 7. Biochemical parameters in the patients with peripheral arterial disease (PAD) and controls. Data are given as median (interquartile range)..

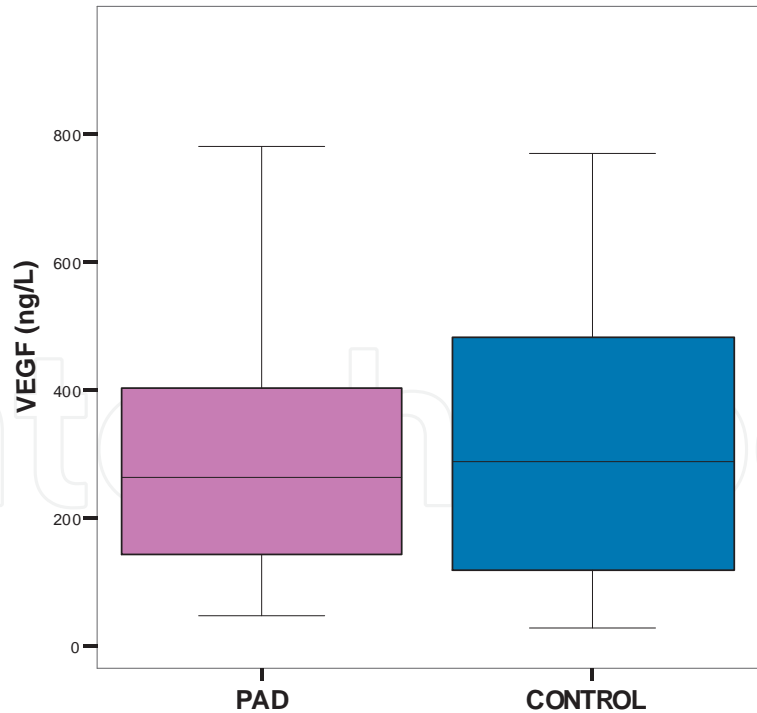


Figure 1. Comparison of VEGF concentrations (median, interquartile range) in the patients with peripheral arterial disease (PAD) and control subjects.

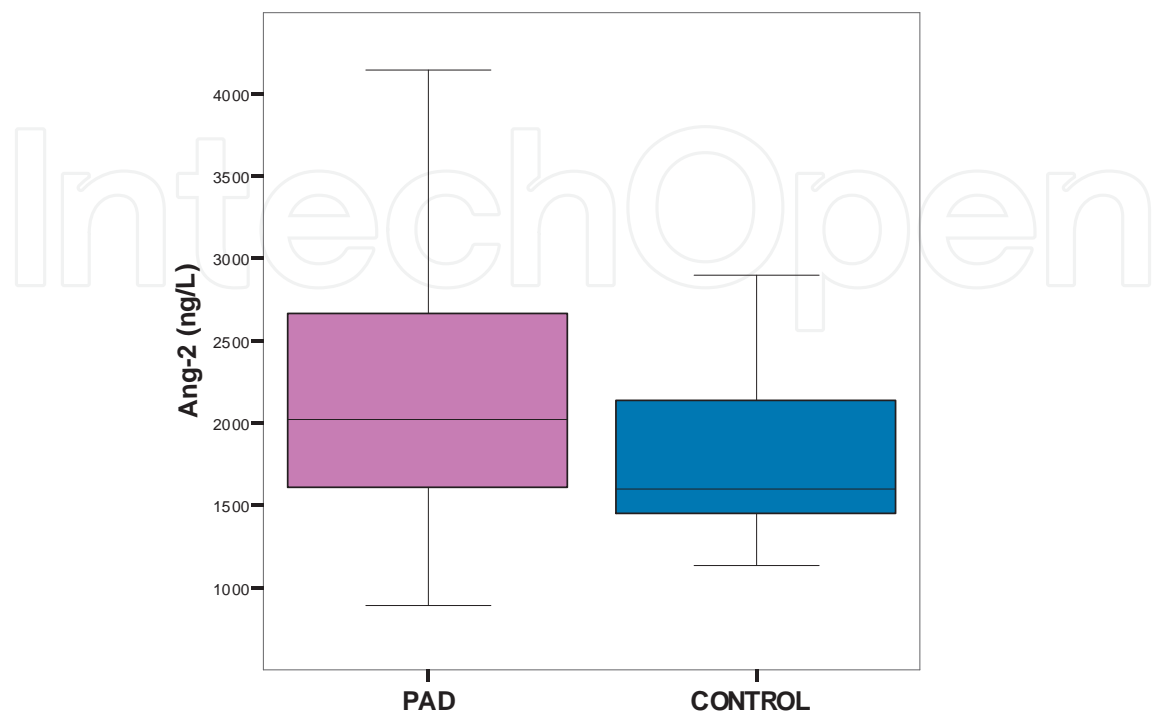


Figure 2. Comparison of Ang-2 concentrations (median, interquartile range) in the patients with peripheral arterial disease (PAD) and control subjects.

Biochemical parameters (units)	VEGF(ng/L)		Ang-2 (ng/L)		Tie-2 (µg/L)	
	r	P	r	P	r	P
Triglyceride (mmol/L)	0,01	0,955	-0,07	0,489	0,02	0,861
Total cholesterol (mmol/L)	-0,13	0,182	-0,02	0,839	0,08	0,424
HDL-cholesterol (mmol/L)	-0,26	0,006	-0,14	0,134	0,03	0,735
LDL- cholesterol (mmol/L)	-0,05	0,581	0,01	0,955	0,01	0,909
CRP (mg/L)	0,45	<0,001	0,36	<0,001	0,25	0,008

Table 8. Spearman coefficient of correlation between the lipid profile, CRP and biomarkers of angiogenesis in patients with peripheral arterial disease (n=110).

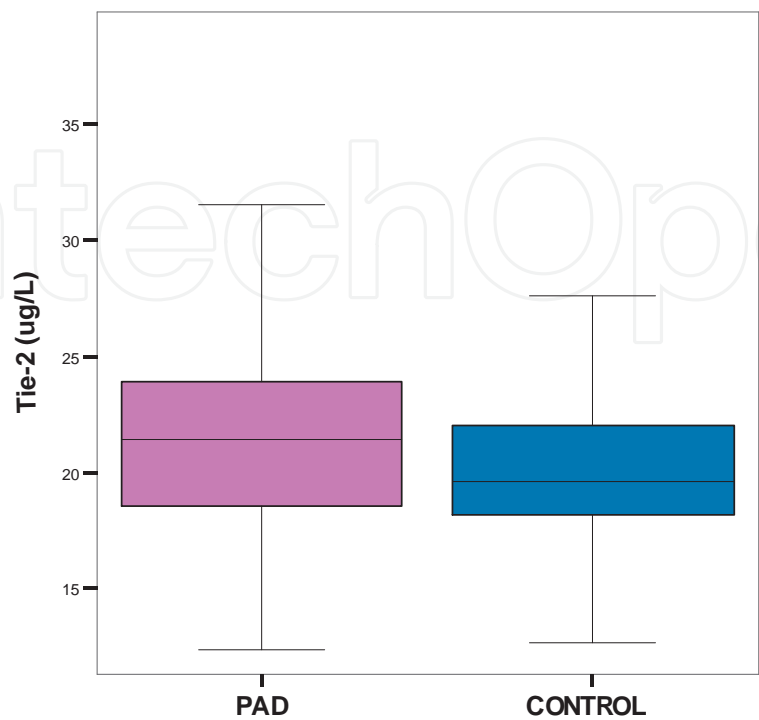


Figure 3. Comparison of Tie-2 concentrations (median, interquartile range) in the patients with peripheral arterial disease (PAD) and control subjects.

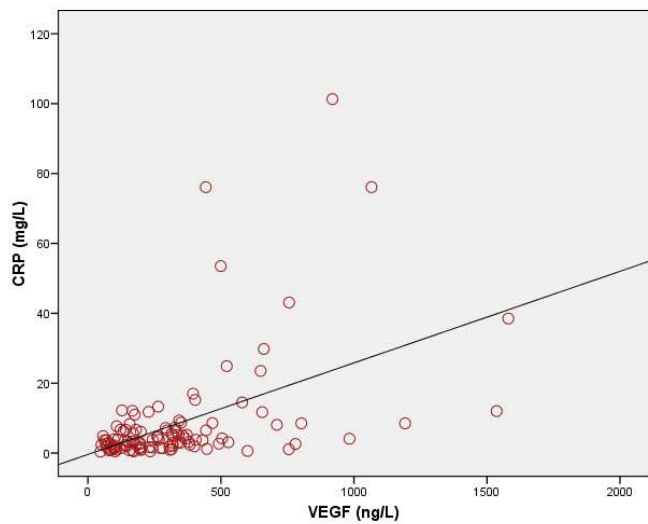


Figure 4. Correlation between serum concentrations of VEGF and CRP in patients with peripheral arterial disease. Sperman coefficient of correlation $r=0,45$; $P<0,001$.

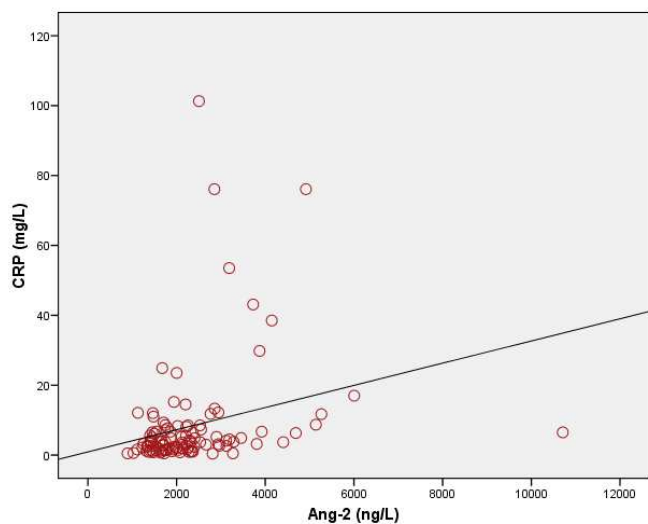


Figure 5. Correlation between serum concentrations of Ang-2 and CRP in patients with peripheral arterial disease. Sperman coefficient of correlation $r=0,36$; $P<0,001$.

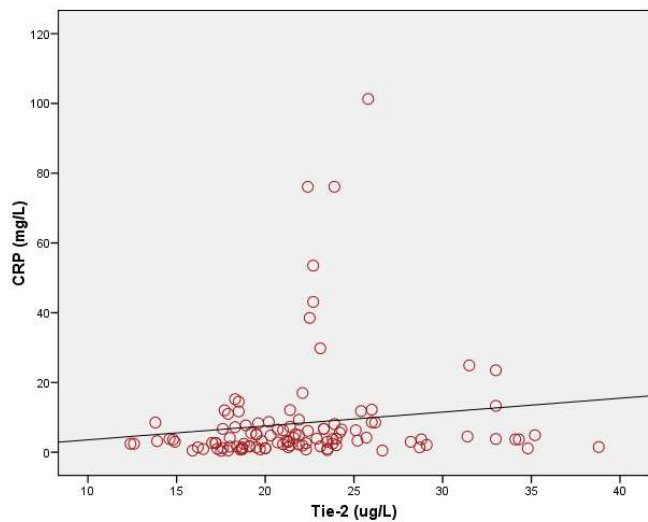


Figure 6. Correlation between serum concentrations of Tie-2 and CRP in patients with peripheral arterial disease. Sperman coefficient of correlation $r=0,25$; $P=0,008$.

3.5. The relationship between the biochemical parameters under study and the anatomical extent of peripheral arterial atherosclerotic changes

None of the biochemical parameters investigated correlated with the angiographic score as a measure of the anatomic extent of atherosclerotic alterations in the peripheral arteries.

(Table 10). From among the traditional risk factors, only the subject age correlated significantly with the angiographic score ($r=0.33$; $P<0,001$). The patients with diabetes had a statistically

Biochemical parameters (units)	Patients with PAD (N=110)			P
	low risk	moderate risk	high risk	
Triglyceride (mmol/L)	1,59 (1,32-2,21)	1,97 (1,39-2,40)	0,87 (1,19-2,38)	0,674
Total cholesterol (mmol/L)	5,60 (4,50-6,00)	5,55 (4,83-6,10)	5,40 (4,50-6,00)	0,732
HDL-cholesterol (mmol/L)	1,40 (1,10-1,60)	1,10 (0,93-1,30)	1,10 (0,90-1,30)	0,017
LDL- cholesterol (mmol/L)	3,40 (2,40-3,80)	3,45 (2,80-4,18)	3,20 (2,70-3,90)	0,549
VEGF (ng/L)	167 (88-234)	197 (100-319)	332 (170-504)	0,002
Ang-2 (ng/L)	1663 (1379-2279)	1803 (1527-2216)	2256 (1707-3185)	0,003
Tie-2 (µg/L)	18,7 (17,5-22,3)	20,4 (17,7-23,4)	22,4 (19,5-25,2)	0,018
PAF-AH (U/L)	350 (294-458)	417 (355-468)	399 (322-480)	0,452

Table 9. Biochemical parameters in the patients with peripheral arterial disease (PAD) according to CRP concentrations as a cardiovascular risk marker. Levels of CRP below 1mg/L are considered low; levels of 1 - 3 mg/L are considered moderate and levels greater than 3 mg/L are considered high risk. Data are given as median (interquartile range).

significant increase in the score compared with nondiabetic subjects (13.77 ± 6.67 compared with 11.02 ± 5.50 ; $P=0,023$).

Biochemical parameters (units)	Angiographic score	
	r	P
Triglyceride (mmol/L)	-0,13	0,167
Total cholesterol (mmol/L)	-0,14	0,156
HDL-cholesterol (mmol/L)	0,06	0,539
LDL- cholesterol (mmol/L)	-0,12	0,208
CRP (mg/L)	0,07	0,461
VEGF (ng/L)	0,08	0,406
PAF-AH (U/L)	-0,08	0,450
Ang-2 (ng/L)	0,04	0,684
Tie-2 (µg/L)	0,13	0,171

Table 10. Spearman coefficient of correlation between the biochemical parameters and angiographic score in patients with peripheral arterial disease (n=110).

4. Discussion

Peripheral artery disease is a systemic manifestation of atherosclerosis with significant morbidity and mortality. Pathophysiological processes implicated in the development, progression, and complications of the disease are complex and interdependent and include interactions between genetic and environmental factors. Pathophysiological events associated with peripheral artery disease include tissue ischaemia, and the severity of clinical presentation is dependent of the site and extent of peripheral arterial stenotic-occlusive changes and the availability of collateral circulation. Ischaemia incites a cascade of biochemical reactions, leading directly or indirectly to endothelial homeostasis disturbance. Dysfunctional endothelium is incapable of maintaining adhesiveness coagulation neutrality within the circulating blood, or regulating tonic arterial activity. In addition to disturbing vessel movements and promoting atherosclerosis formation, endothelium actively modulates the architecture of already present atherosclerotic plaques and increases vulnerability of the lesions which thus become prone to rupture and lead directly to the development of thromboembolic incidents. The role of the new biomarkers of inflammation, thrombosis, lipoprotein metabolism and oxidative stress, which are involved in the regulation of vascular homeostasis, is under an intensive investigation aimed at earlier detection and better understanding of the aetiology and progression of peripheral artery disease, as well as development of new therapeutic possibilities.

The catalytic concentrations of PAF-AH did not differ significantly between the subjects and the control group, contrary to their standardized catalytic concentrations (PAF-AH/LDL) which were statistically significantly higher ($<0,001$) in the subjects analyzed compared with the control group. The catalytic levels of PAF-AH were significantly different between the genders in the control group, females ($n=36$) having lower values than males ($n=28$), which is consistent with the literature data (Winkler et al., 2005; Iribarren, 2010). Moreover, females also had lower PAF-AH standardized catalytic concentrations in both groups studied. Changes in the PAF-AH catalytic levels depend on the concentrations of lipid status parameters, whereat the PAF-AH catalytic concentrations show a statistically significant positive correlation with the concentration of triglycerides, total and LDL cholesterol, the atherosclerosis index, and the total/HDL cholesterol ratio. Statistically significant negative correlation was found between the catalytic concentration of PAF-AH and the concentration of HDL cholesterol in the control group, which is consistent with literature data (Winkler et al., 2005; Flegar-Meštrić et al., 2003; Kamisako et al., 2003; Flegar-Meštrić et al., 2008; Flegar-Meštrić et al., 2012). PAF-AH catalytic levels did not correlate with the CRP concentration in either of the groups examined.

The results of the present study are consistent with our previous results obtained for the patients with lesions of the cerebral arteries (Flegar-Meštrić et al., 2003; Flegar-Meštrić et al., 2008; Flegar-Meštrić et al., 2012). However, in this investigation, we failed to confirm our previous results in 182 patients with peripheral arterial disease in whom PAF-AH catalytic concentrations were significantly higher compared with the control group (Perkov et al., 2010). The differences in the results obtained can be explained by the differences in the number of patients included in the analysis. Furthermore, the PAF-AH catalytic concentrations are in

a significant positive correlation with the concentrations of triglycerides, total and LDL cholesterol. Thus, changes in enzymatic activities may also result from the changed concentrations of lipid parameters, particularly if standardized catalytic PAF-AH concentrations are observed in relation to LDL cholesterol.

The development of vascular endothelial dysfunction is a key mechanism linking the risk factors and atherosclerosis, and it plays an important role in the pathophysiology of peripheral artery disease (Brevetti et al., 2010). Vascular remodeling, as an adaptive response to haemodynamic and biochemical stressors, is characterized by progressive structural and functional alterations in blood vessel walls, preceding the development of a cardiovascular disease. Recent investigations suggest that a crucial role in the regulation of vascular homeostasis is played by the Tie ligand receptor system. Some smaller scale clinical trials have revealed that the concentrations of Ang-2, Tie-2, or both, are found in the patients with peripheral arterial disease (Findley et al., 2008), congestive heart failure (Chong et al., 2004), acute coronary syndrome (Lee et al., 2004), hypertension (Lim et al., 2004), and that they have a predictive ability for myocardial infarction (Patel et al., 2005).

In our investigation, the serum concentrations of Ang-2 and its tyrosine kinase receptor, Tie-2, in the subjects analyzed were statistically significantly higher compared with those in the control subjects, which is in agreement with the results by Findley et al., (2008) (8). However, contrary to their results, the VEGF concentrations were not found to be statistically significantly different between our groups. The above mentioned differences in the results may be accounted for by the great biological variability observed for VEGF. In fact, it is well known that interindividual and intraindividual variability of VEGF differ significantly depending on the kind of material used. Analysis samples include serum, whole blood, and plasma. The intraindividual variation of VEGF in serum, plasma, and whole blood is 10.7%, 14.1%, and 14.1%, respectively, and the interindividual variation of VEGF in serum, whole blood, and plasma is 47.6%, 28.8%, and 18.1%, respectively (Meo et al., 2005). The greater intraindividual variability in the whole blood is impacted by the release of VEGF from lymphocytes, granulocytes, monocytes, and megakaryocytes, variability also being dependent on the process of leukocyte lysis, irrespective of the use of standardized methods (Meo et al., 2005). In light of the potential clinical utility of VEGF in the prognosis, patient selection, and follow-up of anti-VEGF therapeutic effects, Kong et al., (2008) (49) have constructed the reference intervals for VEGF in the serum and plasma of the population of the Republic of North Korea using the ELISA method with R&D Systems reagents. The reference intervals were calculated in 131 subjects, aged 20 to 78 years (68 males and 63 females). Reference intervals differ considerably in serum and plasma, whereat the values in serum are ten- to twenty eight- fold higher than those in plasma.

Moreover, plasma concentrations of VEGF depend on the kind of anticoagulant, with the values being considerably higher when determined by EDTA as an anticoagulant than when determined using heparin as an anticoagulant. In addition to VEGF, concentrations of Ang-2 and Tie-2 also statistically significantly differ according to gender and kind of material used (Lieb et al., 2010).

From among the parameters analyzed, only VEGF showed a statistically significant negative relationship with age in the control subjects. The Ang-2 concentrations were statistically significantly higher in the control group females. Other parameters were not statistically significantly different between male and female subjects of the groups studied.

The levels of VEGF, Ang-2, and Tie-2 determined in the serum of the control group were within the value range for healthy individuals set out by the manufacturer and other authors using the same method and reagent from the same manufacturer (Lieb et al., 2010; Nylaende et al., 2006).

A significant difference in the concentrations of VEGF was found between diabetic and nondiabetic subjects, with the median concentration of VEGF in diabetics being higher than that observed in nondiabetic subjects. There was no difference in concentrations of VEGF, Ang-2, and Tie-2 receptor between smokers and non-smokers, nor between the subjects on lipolytic and antihypertensive therapy and subjects off therapy.

In the patients with peripheral arterial disease, VEGF significantly correlated with CRP ($r=0,45$, $P<0,001$) and HDL cholesterol ($r=-0,26$, $P=0,006$). Angiopoietin-2 significantly correlated with CRP ($r=0,36$, $P<0,001$), as well as Tie-2 which showed a weak but significant association with CRP ($r=0,25$, $P=0,008$).

Because all three markers of angiogenesis correlated with the CRP concentration in the group studied, compared with the controls, and a correlation between the concentrations of VEGF and HDL cholesterol was found, we examined whether the concentrations of the biochemical parameters under study differed depending on the CRP concentration as a cardiovascular risk factor. The concentrations of HDL-cholesterol, VEGF, Ang-2, and Tie-2 were statistically significantly different among the subjects with various cardiovascular risk profiles, with the HDL-cholesterol values being significantly higher in the low risk subjects ($CRP<1,0$ mg/L) compared with the moderate (CRP between $1,0-3,0$ mg/L) ($P=0,004$) and high risk ($P=0,011$) subjects ($CRP>3,0$ mg/L). The subject groups of moderate and high cardiovascular risk did not differ significantly in the HDL cholesterol concentration ($P=0,666$). Statistically significant difference was found in the concentrations of VEGF ($P=0,011$), Ang-2 ($P<0,001$), and Tie-2 receptor ($P=0,005$) between low and high risk subjects, as well as in the concentrations of VEGF ($P=0,012$), Ang-2 ($P<0,001$), and Tie-2 receptor ($P=0,02$) between the moderate and high cardiovascular risk subjects, whereas there were no statistically significant differences in the concentrations of VEGF ($P=0,377$), Ang-2 ($P=0,438$), and Tie-2 receptor ($P=0,673$) between the groups of low and moderate cardiovascular risk subjects. The results are suggestive of an association between inflammation and angiogenesis in peripheral arterial disease.

In this investigation, no association was found of the biochemical parameters under study, namely, triglycerides, total, HDL-, LDL-cholesterol, CRP, and novel biomarkers of inflammation (PAF-AH) and angiogenesis (VEGF, Ang-2, and Tie-2 receptor) with the angiographic score as a measure of the anatomic extent of atherosclerotic alterations in the peripheral arteries.

It has been well documented that inflammation is implicated in all stages of the atherosclerotic process. The role of CRP, as a nonspecific marker of inflammation and cardiovascular risk

factor in the development and progression of atherosclerosis, is extensively investigated. Tzoulaki et al., (2005), in a large prospective trial nested within the Edinburgh Artery Study confirmed the role of CRP, interleukin-6 (IL-6), and intercellular adhesion molecule (ICAM) in the progression of peripheral artery disease in the general population. The trial included 1582 individuals, ranging in age 55 to 75 years, and atherosclerotic progression was defined as reduction in the ankle brachial index (ABI) over the period of 5 and 12 years. In the investigation of the patients with peripheral arterial disease who had $ABI < 0.90$, the CRP levels greater than 3.0 mg/L had an additive predictive value to risk assessment for adverse cardiovascular events (Khawaja & Kullo, 2009). Although in the clinical practice, ABI measurement is considered a simple method of assessing peripheral artery disease progression, and the ABI values correlate well with the degree of peripheral arterial atherosclerotic changes as measured using the digital subtraction angiography method, these two methods represent different aspects of severity assessment of peripheral arterial disease, and cannot be directly compared (Nylaende et al., 2006). Nylaende et al., (2006) evaluated the relationship between inflammatory markers and the severity of peripheral artery disease assessed on the basis of the angiographic score and ABI determined with and without Treadmill test. The study was conducted in 127 patients, range 45-79 years, with the symptoms of intermittent claudication in whom the angiographic score was determined based on the angiographic criteria for haemodynamically significant stenosis. The results of their study demonstrated significant associations of MCP-1, CD40L, IL-6, and TNF-alpha with the angiographic score contrary to the concentrations of CRP, IL-10, E-selectin, P-selectin, ICAM-1, and VCAM-1 for which no significant associations with the angiographic score were observed. ICAM-1 and IL-6 showed a statistically significant correlation with the maximum walking distance on the treadmill, and neither of the markers under study correlated with the ABI. Based on the available data, this is the only investigation into the association between the inflammation marker and the extent of angiographically detected atherosclerotic alterations in the patients with peripheral atherosclerosis. A substantial number of studies have investigated the correlation between the marker of inflammation and the degree of angiographically demonstrated atherosclerotic changes in cerebral and coronary atherosclerosis. Flegar-Meštrić et al., (2007), in a study of 119 patients, age range between 43 and 80 years, with stenosis of extracranial cerebral arteries found a significant association between the CRP level and stenotic extent greater than 70% compared with the control group with normal-appearing cerebral arteries on ultrasonography. The association with CRP of angiographically confirmed coronary atherosclerosis is controversial. Coronary disease and CRP are considered to independently and additively contribute to the risk for adverse cardiovascular events. Angiographic imaging seems to detect stable and instable plaques, and the value of CRP lies in its ability to predict myocardial infarction or fatal outcome independently of the result of angiography (Niccoli et al., 2008; Geluk et al., 2008). Niccoli et al., (2008), in an investigation of 97 patients with unstable angina, failed to demonstrate any correlation between the basal CRP values and the severity of angiographic changes. In a prospective study within the Prevention of Renal and Vascular Endstage Disease (PREVEND) trial, including 8,139 individuals with no presence of coronary artery disease, Geluk et al., (2008) found weak correlations between the basal CRP concentrations and the degree of alterations demonstrated on angiogram in 216 patients who developed coronary disease over a 5-year period.

In our investigation, we found no evidence of associations between the CRP level and the extent of peripheral arterial changes on angiography, which is consistent with the results by Nylaende M. et al. (2006). It is also possible that some of the biomarkers for which a difference in concentrations between the groups studied has been found are involved in other mechanisms of vascular homeostasis regulation, and that they have importance in earlier phases of development of peripheral arterial atherosclerotic changes, which evade detection by the digital subtraction angiography method.

5. Conclusion

This study confirmed the role of hypertriglyceridemia and CRP as risk factors in the development of peripheral arterial disease. Lower concentrations of HDL cholesterol in patients could indicate its reduced protective role in preventing the atherogenic process. PAF-AH can not be considered a reliable diagnostic indicator of peripheral arterial disease since the changes in enzyme activity may reflect the altered lipid parameters. Correlation between the CRP concentrations and the concentrations of VEGF, Ang-2 and its receptor Tie-2 appears to suggest an association between inflammation and angiogenesis in the development of peripheral arterial disease. An increased concentration of Ang-2 and Tie-2 receptor could indicate increased vascular remodeling in response to the presence of risk factors and could be considered new biomarkers of angiogenesis which indicate the presence of peripheral arterial disease. The absence of significant correlation between the concentrations of the biochemical parameters investigated and the angiographic score suggests that other factors play a more important role in the progression of the disease. Further research is needed on larger groups of subjects to confirm the value of PAF-AH, VEGF, Ang-2 and Tie-2 receptor, as new diagnostic indicators of atherosclerosis of peripheral arteries.

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