DOI: 10.4149/BLL_2018_114

Bratisl Med J 2018; 119 (10)
646 – 650

CLINICAL STUDY

Humoral predictors of ankle-brachial index in patients with peripheral arterial disease and controls

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ABSTRACT

INTRODUCTION: Peripheral arterial disease (PAD) is a common condition due to atherosclerosis with high prevalence in population over 55 years. Although its pathophysiology is well recognized, the role of inflammatory markers is still not fully known.

OBJECTIVES: The aim of the study was to assess the relation of C-reactive protein (CRP), tumor necrosis factors-alpha (TNF-alpha) and interleukin-6 (IL-6) to ankle-brachial index (ABI) and metabolic variables in patients with PAD. The second aim was to find the most significant humoral predictor of ABI.

PATIENTS AND METHODS: The study groups consisted of 55 patients (36 men and 19 women) diagnosed with PAD (age 63.65 ± 6.11 years) and 34 control subjects (7 men, 27 women) of average age 59.88 ± 6.10 years with ABI > 0.9. Blood samples were analyzed for glycaemia, lipid profile and inflammatory markers (CRP, TNF-alpha and IL-6).

RESULTS: A significantly higher serum total cholesterol ($p = 0.04$), triglycerides ($p = 0.005$) and lower HDL cholesterol ($p < 0.0001$) were found in the PAD group as compared to controls. Patients with PAD had significantly higher serum glucose ($p = 0.008$), CRP ($p = 0.0044$), IL-6 ($p < 0.0001$) and TNF-α ($p < 0.0001$) in comparison to controls. In a multiple linear regression analysis among variables log IL-6 and log HDL cholesterol were most significantly related to ABI (LW 4.75 for log IL-6, LW 4.016 for log HDL cholesterol, respectively, $p < 0.01$) in all subjects.

CONCLUSIONS: We conclude that among traditional and humoral risk factors IL-6 is the strongest predictor of ABI. HDL cholesterol is also significant and strong predictor of decreased ABI and could be a potential biomarker of PAD in patients using lipid lowering drugs *(Tab. 1, Ref. 31).*

KEY WORDS: peripheral arterial disease, CRP, TNF-alpha, interleukin-6, lipid profile.

Introduction

Peripheral arterial disease (PAD) is a common condition due to atherosclerosis with the prevalence of around 10–25% in population aged over 55 years (1). Current evidence suggests that PAD is a significant predictor of cardiovascular morbidity and mortality and requires aggressive medical management (2–4). Approximately 70 % of PAD cases can be explained by traditional risk factors such as age, hypertension, dyslipidemia, diabetes mellitus, metabolic syndrome and cigarette smoking (5). Although pathophysiology of PAD is intensively studied and relatively well recognized, the role of inflammatory markers is still not fully recognized, especially from the viewpoint of potential diagnostic use or treatment target for medical therapy. Cigarette smoking and diabetes mellitus, the strongest predictors of developing PAD, promote oxidative stress which directly and indirectly enhances inflammatory pathways. In addition, dyslipidemia may activate inflammatory process by modifying the oxidation of low-density lipoproteins and very low-density lipoproteins (6, 7). There is evidence that PAD patients exert worse lipid profile as compared to those without PAD. Increased total cholesterol (TC), LDL cholesterol (LDL-C) and decreased HDL-cholesterol (HDL-C) have been demonstrated to be most common findings in these subjects (8).

Over last twenty years a number of inflammatory and humoral biomarkers have been identified and several studies tried to recognize a typical humoral profile or single marker as a predictor of PAD. However, lot of studies yielded conflicting results and various markers of atherosclerosis, arterial stiffness, inflammation, angiogenesis, remodeling, endothelial dysfunction etc. have been reported to be increased in PAD subjects. Among these biomarkers, high sensitivity CRP, tumor necrosis factor alpha (TNF-alpha) and interleukin-6 (IL-6), seem to be mostly associated with PAD and their measurement significantly improved the ability to identify patients at high or very high risk PAD (9). In addition the high sensitivity CRP (hs-CRP) has been recognized to be a very

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Acknowledgement: This study was supported by grant VEGA No 1/0364/17.

sensitive marker of increased cardiovascular risk in patients with PAD (10). Further biomarkers such as IL-8, selectins, matrix metalloproteinases 2,9 (MMP 2,9), intercellular adhesion molecule (ICAM), vascular endothelial growth factor (VEGF), selectins, vascular adhesion molecule (VCAM), natriuretic peptides and other have been also identified to be significantly associated with PAD (9, 11). Higher levels of inflammatory biomarkers are also associated with poorer outcomes after lower extremity revascularization, including graft restenosis and mortality (12). Despite many studies and various results, no single biomarker has been found to be a significant predictor of PAD and to be a sensitive marker for early diagnosis of the disease.

The primary aim of the study was to assess the relationship of inflammatory markers (CRP, TNF-alpha, IL-6) and metabolic variables to ankle-brachial index (ABI) in patients with PAD. The secondary aim was to determine the most significant predictor of ABI in the model of multivariate regression analysis.

Materials and methods

The study group consisted of 55 patients (36 men and 19 women) diagnosed with peripheral arterial disease (mean age 63.65 ± 6.11 years (median 63, range 53–75 years). Diagnosis of PAD was based on a careful history of intermittent claudication, clinical findings and ABI measurements. All patients were hospitalized in the Department of Angiosurgery of the East Slovak Institute of Cardiovascular Diseases in Košice. According to Fontaine´s classification the first stage of PAD had 18 % of patients, the second stage was diagnosed in 42 % of patients, the third stage in 16 % and fourth stage in 24 % of patients, respectively.

The control group consisted of 34 subjects (7 men, 27 women) of average age 59.88 ± 6.10 years (median 60, range 50–75 years), without severe chronic diseases and without clinical symptoms of PAD. All subjects had normal ABI that was higher than 0.9. Exclusion criteria for both study groups were severe systemic disorders, such as acute and chronic heart failure, acute coronary syndrome, renal and liver failure or severe rheumatic diseases. In addition, acute viral or bacterial infection was ruled out in all subjects included in the study. All participants signed a written informed consent and the study was approved by the Ethics Commission of the Faculty Hospital in Košice.

Methods

Clinical covariate assessment

Covariates were defined at the time of investigation. Arterial hypertension was defined as an average blood pressure of systolic 140 or diastolic 90 mmHg or use of antihypertensive drugs. Body mass index (BMI) was calculated as weight divided by the height in meters squared (kg/m^2). Dyslipidemia was defined based on TC level ≥ 5.2 mmol/l, triglycerides level ≥ 1.7 mmol/l, HDL-C level ≤ 1.0 mmol/l in men and ≤ 1.3 mmol/l in women, LDL-C ≥ 2.5 mmol/l or use of hypolipidemic medication in history taking. Diabetes mellitus was defined by careful history or by standard criteria at the time of investigation. Current smoking history was based on self report.

Laboratory investigations

Venous blood samples were collected between 7 and 8 o´clock am after an overnight fasting on the same day as ABI was measured, into EDTA tubes (Beckton-Dickenson) and immediately iced. Tubes were spun at 3,000 revolutions per minute for 20 minutes at 4 degrees Celsius a refrigerated centrifuge. Serum was stored at –70 degrees Celsius until final analysis. Blood samples were analyzed for glycaemia, lipid profile (TC, LDL-C, HDL-C and triglycerides) and inflammatory markers (CRP, TNF-alpha and IL-6). Serum glucose, lipids and high sensitive CRP were analyzed routinely using autoanalyser (Roche Diagnostics GmbH). Serum IL-6 and TNF-α were measured using ELISA method (Enzyme-linked immunosorbent assay) with commercially available kits BioAim Scientific. Measurement was performed on the ULTRA MICROPLATE READER (BIO-TEK INSTRUMENTS. INC. SA). The intra individual variation was < 10 % and inter individual variation was < 15 % for both inflammatory markers.

Measurement of ankle-brachial index (ABI)

Ankle-brachial systolic blood pressure measurements were obtained using an ultrasonic Doppler flow detector (Super Dopplex II, Huntleigh Healthcare comp) by the single and trained physician. Limb blood pressures were repeated, and if the initial and repeat blood pressures differed by more than 10 mmHg at any one site, a third measurement was obtained. Measurements were obtained from the arteria dorsalis pedis only if the posterior tibial pulse could not be located by palpation or with Doppler probe. For this study, the ABI was defined as the ratio of the average systolic blood pressure in the ankle divided by the average systolic blood pressure in the higher arm. The lower ABI was used for analysis. ABI value ≤ 0.9 indicated PAD.

Measurement of intima-media thickness

All subjects underwent ultrasonographic evaluation of peripheral arteries by B mode using ESAOTE MyLab 70XVG and linear tranducer of 7.5 MHz. Intima media thickness (IMT) of a common carotic artery (CCA) and common femoral artery (FCA) bilaterally were measured. Measurements were performed under standard condition in longitudinal projection. Four measurements were realized on each side and mean IMT on the right and left CCA and FCA were calculated. An average value of both right and left sides represented a final result of IMT. Value of 0.9 mm or more has been considered pathologic.

Statistical analysis

SAS JMP version 13.0.0 (USA) software was used for statistical analysis. Data are presented as mean ± SD regardless of their distribution. Data with not normal distribution were initially logarithmically transformed for further evaluation. For normally distributed variables Student´s T test was used to compare means between groups, whereas for non-normally distributed data the non-parametric Mann–Whitney test was used to compare means among two groups. Linear regression analysis was used to assess correlations between variables. Values were considered to be statistically significant at $p \leq 0.05$. Analysis of variance (ANOVA)

was used to assess differences between parameters according to stage of PAD.

Results

Mean values of anthropometric parameters in the group of patients and controls are demonstrated in the Table 1. Although the age difference between PAD and controls was small, it reached statistical significance (p = 0.0061). Number of cigarettes was also statistically significant as compared both groups (p = 0.0013). However, both PAD and control groups were weight matched, i.e. there were no significant difference in weight, BMI and waist circumference between the groups.

A significantly higher serum total cholesterol (p = 0.04), TG (p = 0.005) and significantly lower HDL cholesterol (p < 0.0001) were found in the PAD group as compared to controls. However there was no significant difference in the serum LDL cholesterol between both groups. Patients with PAD had significantly higher serum glucose (p = 0.008) than control group. When compared serum levels of inflammatory markers PAD group had significantly higher CRP (p = 0.0044), IL-6 (p < 0.0001) and TNF-α (p < 0.0001) in comparison to control group.

Using univariate linear regression analysis there was found a significant positive correlation between ABI and HDL cholesterol (r = 0.54, p < 0.0001) and negative correlation between IMT AFC vs HDL cholesterol (r = 0.55, p < 0.0001). In addition both IMT AFC and IMT ACC were in significant positive correlation with TNF-α (r = 0.55, p < 0.0001 for IMT AFC, r = 0.48, p = 0.0002 for IMT ACC, respectively).

Using model of multiple regression analysis an influence of various independent variables on ABI was analyzed. A prediction profiler demonstrates relationship of each independent variable to ABI provided that values of other variables are stable.

Tab. 1. Anthropometric and laboratory parameters in the peripheral arterial disease group and controls.

	PAD	controls	P value
Age, years	63.65 (6.11)	59.88 (6.10)	0.0061
Cigarettes, number per day	11.04 (13.69)	2.26 (8.72)	0.0013
Weight, kg	81.12 (14.13)	84.47 (17.69)	0,33
BMI, kg/m ^{2}	28.23 (3,98)	29.19 (5.68)	0,35
WC, cm	100.93 (11.75)	98.67 (13.76)	0,42
TC, mmol/l	5.2 (0.73)	4.82 (1.35)	0.043
TG, mmol/l	2.11 (1.43)	1.47 (0.59)	0.0051
HDL, mmol/l	0.95 (0.25)	1.26 (0.27)	<0.0001
LDL, mmol/l	3.22 (0.99)	3.47 (0.57)	0,13
Glycaemia, mmol/l	5.4 (1.66)	4.59 (0.63)	0.008
ABI	0.59 (0.19)	1.07 (0.07)	<0.0001
IMT AFC, mm	1.32 (0.41)	0.75 (0.064)	<0.0001
IMT ACC, mm	1.12 (0.194)	0.76 (0.07)	<0.0001
CRP, mg/l	13.13 (20.59)	4.51 (5.19)	0.0044
TNF alpha, ng/l	20.47 (14.79)	6.49 (3.59)	<0.0001
IL-6, ng/l	26.52 (21.19)	6.92 (5.21)	<0.0001

PAD – peripheral arterial disease, BMI – body mass index, WC – waist circumference, TC – total cholesterol, TG – triglycerides, HDL – high density lipoproteins, LDL – low density lipoproteins, ABI – ankle brachial index, IMT – intima media thickness, CRP – C reactive protein, TNF alpha – tumor necrosis factor alpha, IL-6 – interleukin 6

Discussion

Early diagnosis of PAD is very crucial because it is an independent risk factor for cardiovascular morbidity and mortality and also represents a high risk for cardiovascular events (13). It is well known that inflammation is an integral component of atherosclerosis. In patients with coronary artery disease increased levels of inflammatory biomarkers and pro-inflammatory cytokines are associated with an increased incidence of adverse outcomes (14). In addition, there is also evidence that PAD subjects have higher levels of various inflammatory biomarkers than those without PAD, as was demonstrated by numerous observational studies (15). In our study patients with PAD had significantly higher glycaemia, serum levels of TC, TG and significantly lower HDL-cholesterol as compared with controls. They also had significantly higher serum CRP, TNF-alpha and IL-6, which is in agreement with previous studies, although not all these biomarkers were found to be elevated in all previously published papers. Higher levels of CRP, especially hs-CRP were found by many authors (16, 17). Increased levels of CRP have been demonstrated to represent a higher risk of cardiovascular events in the group of patients with PAD (14).

Numerous studies have also studied the impact of other inflammatory biomarkers with different results. Among them IL-6 seems to be most commonly identified pro-inflammatory marker associated with PAD and its functional outcomes (11, 15, 18–22)). Besides CRP, IL-6, TNF-alpha some other markers have been found to be associated with PAD, namely VCAM-1, ICAM, selectins, IL-8, fibrinogen, natriuretic peptides etc. (23, 24). Study of Wozniak et al demonstrated that serum concentrations of inflammatory biomarkers are similar to that observed in the chronic obstructive pulmonary disease (COPD). Thus the patients with PAD exert similar inflammatory status that can be seen in COPD (23). Unfortunately despite many studies no single biomarker has been identified to be a strong predictor of atherosclerosis and PAD as well. In our study we chose three most important inflammatory biomarkers, such as CRP, TNF-alpha, IL-6 and serum lipids and we try to assess which marker could be the strongest humoral predictor of PAD (defined by ABI) in the concert with covariates, mostly traditional risk factors. According to published data ABI is still considered to be a sensitive and specific for diagnosis of the disease with approximately 95% sensitivity in detecting angiographic-positive PAD. In addition ABI has also been recognized an independent predictor of vascular events (24, 25).

To obtain relevant data we used the high value JMP SAS program, version 9.0 for multivariate analysis. We demonstrated that in concert of various risk factors, log IL-6 and log HDL cholesterol were the strongest and most significant predictors of ABI in all subjects. This significant relationship was observed also after age and BMI adjustment. These data are in agreement with some other studies. In the study of Murabito among 12 biomarkers only IL-6 and TNF-alpha were significantly associated with PAD (16). In multivariate analysis of Cauley et al, IL-6, TNF-alpha and CRP were most significant predictors of PAD after adjustment of smoking (26). In the recently published study 47 candidate biomarkers representing various pathways in etiology of AS have been

studied. After adjustment of covariates nine biomarkers were associated with lower ABI including IL-6 and TNF-alpha (24). Data are also in correlation with Edinburgh study in which CRP, IL-6 and SIAM-1 levels were significantly associated with ABI decrease independently of CV risk factors (20). In the Rotterdam study, logarithmically transformed CRP and IL-6 were inversely related to ABI, after adjustment for age, smoking, BMI and DM. Moreover IL-6 independently correlated with functional disturbance (21). All these results may indicate that inflammation may be involved in the pathogenesis and also in the development and severity of PAD symptoms.

It remains to be clarified whether primary systemic inflammation is responsible for more severe claudication or whether extensive atherosclerosis may contribute to the increased inflammatory status. However, inflammatory markers could not be only sensitive early marker of PAD but reducing its levels and activity may serve as a new approach for the prevention and simultaneously for the treatment of the disease. Until this time no clinical trials have been performed to establish whether therapies that specifically block or lower inflammatory biomarkers improve outcomes in patients with PAD. Our results indicate that IL-6 may be the first target in the blocking therapy, but it requires further experimental and clinical studies.

We also confirmed a worse lipid profile in PAD persons, i.e. higher TC and TG and lower HDL cholesterol in PAD patients as compared to controls. Groups did not significantly differed in the LDL-C levels, because of the fact that many subjects took statins that could influence relationship between LDL-C and ABI. This fact was clearly shown in the study of Daskalopoulou et al (25). Using univariate linear regression analysis we found a significant positive correlation between ABI and HDL-C and negative correlation between IMT AFC vs. HDL-C. Moreover HDL-C was the second strongest predictor of ABI in all subjects. Decreased levels of HDL-C and significant positive correlation with carotic and femoral IMT as a marker of peripheral atherosclerosis PAD were demonstrated by some studies (27–29). Ridker et al found significantly lower HDL cholesterol in PAD patients. Moreover TC/HDL-C ratio was the strongest predictor of ABI in PAD community (30). It has been confirmed by the recent study of Zhang et al (31). Our results indicate that not only TC/HDL-C but also HDL-C alone may be associated with lower ABI and it could be a potential biomarker for early screening of PAD in patients on lipid lowering drugs. That is one of advantages of our study. This study has several limitations. The main limitation of the study is relatively small group of subjects. However, use of a high level statistic program allows to postulate relevant conclusions.

Conclusion

We can conclude that among traditional and humoral risk factors IL-6 is the strongest predictor of ABI in PAD patients and all evaluated subjects. HDL-C is also significant and strong predictor of decreased ABI and could be an early potential biomarker of PAD in patients using lipid lowering drugs. Further studies with the clinical use of such biomarkers are needed.

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Received April 7, 2018.
Accepted June 8, 2018.