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**Risk of treatment of peripheral arterial disease is related to
inflammation-sensitive plasma proteins.**

A prospective cohort study.

(short title: ISPs and incidence of PAD)

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Abstract

Background Studies of patients with peripheral arterial disease (PAD) have reported associations between inflammatory markers and severity of disease or worsening of symptoms. However, few have studied the prognostic significance of inflammatory markers in asymptomatic individuals, measured many years before the onset of symptomatic PAD requiring treatment (trPAD).

Materials and Methods Five inflammation-sensitive plasma proteins (ISPs) (fibrinogen, α 1-antitrypsin, haptoglobin, ceruloplasmin, orosomucoid) were determined in 5619 healthy men (mean age: 46.8 \pm 3.7 years) without walking-induced calf pain. Men who subsequently were revascularized due trPAD (intermittent claudication or critical ischaemia) were retrieved from hospital-based registers. Future trPAD was studied in relation to the number of ISPs in the top quartile at the baseline examination.

Results 70 men (1.2%) were revascularized due to trPAD at a mean time of 16.5 years after the baseline examination. The proportion with future trPAD was 0.4%, 1.0%, 1.5% and 3.2%, respectively, for men with 0, 1, 2, and \geq 3 ISPs in the top quartile (trend: $p < 0.0001$). After adjustments for age, screening year, systolic blood pressure, blood pressure medication, cholesterol, diabetes, smoking and tobacco consumption, the corresponding odds ratios (95% CI) were 1.00, 1.5 (CI: 0.7-3.6), 1.9 (CI: 0.8-4.6) and 2.9 (CI: 1.3-6.4), respectively, in these groups (trend: $p = 0.003$).

Conclusion Elevated ISPs, measured 16 years earlier in apparently healthy men without walking-induced calf pain, were associated with an increased risk of developing PAD requiring revascularization.

Introduction

Peripheral arterial disease (PAD) includes a wide range of atherosclerotic manifestations in the lower limbs, from asymptomatic atherosclerosis to symptomatic disease, ranging from intermittent claudication, to critical limb ischaemia, with ulcers, rest pain or gangrene. A minority of those with symptomatic PAD will ultimately develop severe symptoms that require revascularization or amputation; the annual risk has been estimated to approximately 1-2% (1-6).

The relationship between elevated levels of inflammation-sensitive proteins (ISPs) and an increased incidence of myocardial infarction, stroke and cardiovascular death is well established (7-11). Not so many have studied the association of ISPs with the incidence of PAD. Studies of patients with PAD have reported associations between hemostatic or inflammatory markers and severity of disease or worsening of symptoms (12-18). Recent studies have reported an increased incidence of PAD, mostly self-reported intermittent claudication, in men with elevated levels of CRP or fibrinogen (19,20). However, few have studied the relation between elevated ISPs, measured in asymptomatic subjects, and risk of future development of PAD.

Previous studies from the Malmö Preventive Study have shown that the incidences of myocardial infarction (9), stroke (21) and abdominal aortic aneurysms (22) are increased in apparently healthy men with elevated levels of ISPs (fibrinogen, orosomucoid, α 1-antitrypsin, haptoglobin, ceruloplasmin). The present study of middle-aged men from the general population, initially without walking-induced calf pain, was carried out to assess the relation between the ISPs and the future onset of PAD requiring revascularization (trPAD).

Methods

Between 1974 and 1984, 22444 men participated in a screening program for detection of individuals with high risk for cardiovascular diseases (23). Complete birth cohorts from the city of Malmö, Sweden, were invited by a letter. The participation rate was 71%. Determination of the 5 plasma proteins was part of the program for 6193 men, aged 28-61 years, selected at random from cohorts examined between 1974 and 1982. After exclusion of men with a history of myocardial infarction, stroke or cancer (according to questionnaire and the Swedish myocardial infarction register), 6075 men remained. Another 456 men who reported walking-induced calf pain at the baseline screening were also excluded, and separately analyzed and presented (24). Of these, 17 developed trPAD (3.7%). The health service authority of Malmö approved and funded the screening program. All participants gave informed consent.

Baseline examinations

The examinations took place in the morning with the subjects in smoke-free and fasting condition. Subjects were categorized into smokers and non-smokers.

Smokers were subdivided into three groups: less than 10 cigarettes per day, 10-19 cigarettes and daily consumption of 20 cigarettes or more.

Blood pressure (mmHg) was measured twice in the right arm after a 10 minutes rest.

The average of two measurements was used. A sphygmomanometer and a rubber cuff of appropriate size were used. Use of anti-hypertensive medication was assessed in a questionnaire.

Blood samples were taken after an overnight fast and analyzed at the Department of Clinical Chemistry at Malmö University hospital. Serum cholesterol concentrations were analyzed with standard methods at the hospital laboratory. For glucose analysis

a routine hexokinase method was used. Men with fasting whole blood glucose ≥ 6.1 mmol/L, and/or 2-h glucose ≥ 10.0 mmol/L, and men who reported that they had diabetes, were considered diabetic (25).

Physical activity was assessed in a questionnaire. Men who reported that they mostly were sedentary in spare time were considered to be physically inactive.

BMI was calculated as weight/height² (kg/m²).

Inflammation-sensitive plasma proteins (ISPs)

Electroimmuno assay was used to analyze the plasma levels of 5 ISPs (26). The analysis was performed consecutively at the time of screening and was based on one blood draw. The precision of the analysis had a variation <5% (26). The detection limits were 20 mg/L for ceruloplasmin, 50 mg/L for $\alpha 1$ -antitrypsin and 350 mg/L for orosomuroid, haptoglobin and fibrinogen. We have previously reported that the correlation coefficients between the individual proteins range between 0.31 to 0.56 and that the cardiovascular risk increases with the number of ISPs in the top quartile (9,21,22). In accordance with the previous studies, the sample was therefore categorized according to the number of elevated ISPs. The top quartiles in the study population were as follows: fibrinogen >4.0 g/L, orosomuroid >0.93 g/L, $\alpha 1$ -antitrypsin >1.42 g/L, haptoglobin >1.76 g/L, and ceruloplasmin >0.36 g/L (9).

Patients

Patient were retrieved from the local Swedish Vascular registry (SWEDVASC)(27), which systematically has registered all patients with open vascular or endovascular reconstructions since 1987. Patients who underwent revascularization due to intermittent claudication or critical ischaemia between 1987 and 2002 at the Malmö University Hospital or the Lund University Hospital were included in the registry. The population base of these hospitals is approximately 735.000 and covers the

population of Malmö and the surrounding cities and areas. The case-retrieval procedures for incidence of myocardial infarction and stroke have been reported elsewhere (9).

Statistics

One-way ANOVA and Pearson's Chi-square was used to compare risk factors among patients and controls. Logistic regression was performed to investigate the relationships between ISPs and trPAD with adjustments for potential confounders. The Kaplan-Meier method with the log rank statistics was used to compare incidence rates in men with and without trPAD during follow-up.

Results

Patients

A total of 70 men were revascularized due to trPAD. The indications were critical ischaemia (n=32) and intermittent claudication (n=38). The mean time from the baseline examination to the revascularization was 16.5 ± 4.3 years (range 7.8-24.8).

Age at the time of revascularization was 63.9 ± 6.1 years (range 46.1-77.5).

Eleven of the 64 smokers had quit smoking at the time of revascularization. Nine men developed diabetes during the follow-up period.

Thirteen patients (12.3 per 1000 person-years) had non-fatal myocardial infarction and 6 men (5.3 per 1000) had stroke during the period from the screening examination to the vascular reconstruction. In men without trPAD during follow-up (n=5549), incidence of non-fatal myocardial infarction was 3.7 per 1000 person-years, incidence of fatal myocardial infarction was 1.8 per 1000 and incidence of stroke was 2.0 per 1000. Incidence of non-fatal MI ($p < 0.0001$) and stroke ($p = 0.003$) were significantly higher in men who developed trPAD.

Baseline characteristics of men with trPAD and controls

Systolic blood pressure was higher in cases as compared to controls ($p < 0.001$), and prevalence of smoking ($p < 0.001$) and diabetes ($p < 0.001$) was higher. There were no significant differences in BMI, level of physical activity or age at baseline (Table 1). Cholesterol was slightly higher in cases (6.0 ± 0.9 vs 5.7 ± 1.0 mmol/L, $p = 0.03$), but importantly, men who later developed trPAD had higher levels of all ISPs (Table 1).

Incidence of trPAD in relation to ISP levels

The percentage with trPAD during follow-up was 0.4%, 1.0%, 1.5% and 3.2%, respectively, in men with 0, 1, 2, and 3 or more ISPs in the top quartile (p for trend < 0.001). The relation between trPAD and number of elevated ISPs was observed both for trPAD with the indication of critical ischaemia (0.2%, 0.5%, 0.6%, 1.4%, respectively, for men with 0, 1, 2, and 3 and more elevated ISPs) and intermittent claudication (0.2%, 0.6%, 1.0%, 1.7%, respectively).

The relation between the number of elevated ISPs and risk of trPAD remained significant after adjustments for potential confounders (Table 2).

The cohort was subdivided into smokers ($n = 2644$, 64 trPAD) and non-smokers ($n = 2975$, 6 trPAD), and into diabetic ($n = 296$, 15 trPAD) and non-diabetic ($n = 5323$, 55 trPAD) men. Even though few non-smokers and diabetic men developed trPAD, the relations between ISPs and risk of developing trPAD were largely the same in all groups (Table 3).

Incidence of trPAD in relation to individual ISPs

For all individual ISPs, incidence of trPAD was highest in the top quartile of the protein. Before adjustments, all trends were significant. After adjustments for

confounders, only α 1-antitrypsin and orosomucoid showed significant relationships (Table 4).

Discussion

In men from the general population, who initially were without walking-induced calf-pain, the number of elevated ISPs was associated with incidence of PAD requiring revascularization. The ISPs were significantly elevated many years (mean 16.5 yrs) before any surgical or endovascular intervention. This relation remained significant after adjustments for potential confounders, including age, smoking, blood pressure, cholesterol and diabetes.

The ISPs used in our analysis are central elements in the acute and chronic inflammatory response (28,29). The proteins have been used for many years in clinical practice in order to assess the degree of inflammation and to differentiate between inflammatory diseases. The methodology for their analysis is well accepted and accurate (26). However, the measurements were limited to the analyses that were available in routine practice at the time of screening. For example, we had no information about hsCRP or different lipoproteins.

It is now widely accepted that elevated ISPs are associated with an increased incidence of cardiovascular diseases. The causes for this relationship remain unclear. The development of atherosclerosis involves different stages: initiation of the lesion, progression of the lesion and plaque complications (30). The relative contribution of inflammation in these stages is unclear, and the role of inflammation could be different for the various parts of the atherosclerotic process (31). In addition

to the development of atherosclerosis, the final stages of PAD also include critical ischaemia and gangrene. In this study, the ISPs were similarly associated with the risk of developing critical ischaemia and intermittent claudication. Even though fibrinogen has an important role for the thrombogenesis and is a determinant of the blood viscosity, we could not find any support for the hypothesis that these proteins were differentially related to critical ischaemia and intermittent claudication.

Of the individual ISPs, only α 1-antitrypsin and orosomucoid remained significantly associated with trPAD after adjustments for potential confounders. However, the number of elevated ISPs showed stronger associations with incidence of trPAD than any of the individual ISPs. This measure also had an acceptable reliability in terms of internal consistency, i.e., the correlation between each protein and the remaining sum score was fully adequate (Cronbach's $\alpha=0.65$ in this cohort). The present results and several previous studies (e.g., 9,21,22) suggest that a combination of several inflammatory markers is more informative than individual markers in studies of cardiovascular diseases and cardiovascular risk factors.

Elevated ISPs are also strongly associated with incidence of myocardial infarction, stroke and all cause mortality (9,21,22). It can be assumed that many men with PAD and high ISPs died before the vascular disease required surgical treatment. Due to the phenomenon of 'competing causes of deaths', this probably reduced the association between elevated ISPs and trPAD. This assumption is also supported by the fact that incidence of non-fatal myocardial infarction was much higher in men who developed trPAD.

One question is whether atherosclerosis could cause elevated ISPs, i.e., whether elevated ISPs merely reflect the extension and severity of pre-existing atherosclerosis. The degree of pre-existing atherosclerosis is unknown. Men with walking-induced calf pain were excluded from the analysis. Even though this should be adequate for the purpose of excluding subjects with symptomatic intermittent claudication, it is likely that many men had very early, asymptomatic stages of disease. However, previous studies comparing stable and unstable angina pectoris with healthy controls suggest that atherosclerosis per se has small effects on the inflammation (32). The long time period between the baseline examination and the PAD intervention also support the view that severe atherosclerosis was not the main reason for the elevated ISPs.

This population-based study sought to explore the relations between inflammatory markers and the risk of developing PAD in need of revascularization. No attempt was made to study those who had medical treatment or symptoms with low severity. This is both a strength and a weakness of the present study. The accuracy of the diagnosis and the severity of disease is unquestionable in those who underwent surgical intervention for trPAD. As few population-based studies have investigated this group, it is important to specifically study those who ultimately develop trPAD. On the other hand, we cannot make any conclusions about the risk of developing PAD without need of revascularization.

The local Swedish Vascular registry was used for case-retrieval. Because the Swedish Vascular registry started in 1987, cases from the first years of follow-up could not be included. Mean age of this rather young cohort was 46.8 years.

Because PAD is strongly related to age, it is likely that few were revascularized during the first years. It is possible that some cases were lost because the subjects moved out from the area. However, there is no reason to assume that this was related to the ISPs. We do not know if some patients for some reason were more likely to be scheduled for a revascularization procedure and if this could be related to the ISPs at baseline. Even though access to health care is equal and free of charge in Sweden, we cannot completely exclude this possibility. It is also possible that some patients that required amputation were lost. However, in almost all cases, surgical reconstructions have been performed prior to the amputation, and these were included in the registry. Since many other cardiovascular end-points, e.g., the incidences of myocardial infarction, stroke, death, hypertension and abdominal aortic aneurysms, have shown similar relations with the ISPs in this cohort, it seems unlikely that biased case-finding explain the present results.

Elevated ISPs were associated with an increased risk of PAD requiring revascularization. The risk increased progressively with the number of elevated ISPs. In this prospective study of men who initially were without walking-induced calf pain, the ISPs were elevated already 16 years before reconstruction was needed.

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Table 1. Baseline characteristics in men with and without surgical lower limb revascularization during follow-up.

	Revascularization		P
	Yes	No	
N	70	5549	
Age (years)	47.4 \pm 4.0	46.8 \pm 3.7	0.21
Smokers (%)	91	46	<0.001
\geq 20 cig/day (% of smokers)	39	23	0.003
Diabetes (%)	21.4	5.1	<0.001
Systolic BP (mmHg)	137 \pm 19	129 \pm 16	<0.001
Diastolic BP (mmHg)	89 \pm 11	87 \pm 10	0.10
Blood pressure medication (%)	13	4.2	<0.001
Cholesterol (mmol/L)	6.0 \pm 0.9	5.7 \pm 1.0	0.03
BMI (kg/m ²)	24.5 \pm 3.6	24.9 \pm 3.3	0.25
Physical inactivity (%)	56	56	0.99
ISPs (g/L)			
α 1-antitrypsin	1.40 \pm 0.23	1.27 \pm 0.27	<0.001
Ceruloplasmin	0.35 \pm 0.07	0.32 \pm 0.07	<0.001
Fibrinogen	3.89 \pm 0.83	3.50 \pm 0.80	<0.001
Haptoglobin	1.84 \pm 0.94	1.37 \pm 0.67	<0.001
Orosomuroid	0.95 \pm 0.25	0.81 \pm 0.20	<0.001

Table 2. Surgical lower limb revascularization procedures during follow-up in relation to ISPs at baseline in 5619 men who initially were without walking-induced calf pain.

	ISPs in top quartile				P for trend
	0	1	2	3 or more	
All men (n)	2291	1441	840	1047	
Men with revascularization during follow-up n (%)	9 (0.4)	15 (1.0)	13 (1.5)	33 (3.2)	<0.0001
Claudication/critical ischaemia (n/n)	4/5	8/7	8/5	18/15	
Revascularization Adjusted OR* (95% CI)	1.00	1.5 (0.7-3.6)	1.9 (0.8-4.6)	2.9 (1.3-6.4)	0.003

*Odds ratio adjusted for age, screening year, systolic blood pressure, blood pressure medication, cholesterol, diabetes, smoking, tobacco consumption.

Table 3. Surgical lower limb revascularization during follow-up in relation to number of elevated ISPs in categories of smoking and diabetes.

	ISPs in top quartile				P for trend
	0	1	2	3 or more	
trPAD in smokers n (%)	7 (1.0)	14 (2.0)	13 (2.6)	30 (3.9)	<0.001
trPAD in non-smokers n (%)	2 (0.1)	1 (0.1)	0	3 (1.1)	---
trPAD in diabetic men n (%)	2 (2.1)	3 (3.7)	5 (10.9)	5 (6.8)	0.07
trPAD in non-diabetic men n (%)	7 (0.3)	12 (0.9)	8 (1.0)	28 (2.9)	<0.001

The values are number of trPAD (%) out of all men in each group.
 No p-value was computed for non-smokers due to small number of cases.

Table 4. The proportion (%) with revascularization during follow-up in quartiles of 5 ISPs.

	Quartile				P for trend	P for trend*
	1	2	3	4		
α 1-antitrypsin	0.5%	0.8%	1.3%	2.3%	<0.001	0.02
Ceruloplasmin	0.7%	0.8%	1.6%	2.1%	<0.001	0.09
Fibrinogen	0.6%	0.8%	1.2%	2.4%	<0.001	0.11
Haptoglobin	0.5%	1.1%	0.8%	2.6%	<0.001	0.14
Orosomucoid	0.6%	0.5%	1.7%	2.2%	<0.001	0.049

*Adjusted for age, screening year, systolic blood pressure, blood pressure medication, cholesterol, diabetes, smoking, tobacco consumption in a logistic regression model.