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Inflammatory markers and IL-6 polymorphism in peripheral arterial disease with and without diabetes mellitus

P Danielsson^a, L Truedsson^b, K-F Eriksson^a and L Norgren^a

Abstract: Peripheral arterial disease (PAD) is a manifestation of systemic atherosclerosis, recognized as an inflammatory disease of the vessel wall, probably accelerated by diabetes mellitus (DM). Elevated interleukin (IL-6) levels have been associated with increased cardiovascular morbidity and a common polymorphism has been identified in the promoter region of the IL-6 gene. The aim of this prospective study was to investigate inflammatory mediators in PAD patients (\pm DM) and to investigate a possible relationship to the IL-6 gene polymorphism. Five groups of patients (DM, intermittent claudication \pm DM, critical limb ischemia (CLI) \pm DM) and a control group of 20 individuals each were included. Hemoglobin, high sensitive C-reactive protein (hsCRP), creatinine, blood lipids, white blood cells (WBC); CD11b/CD18; vascular cell adhesion molecule (sVCAM-1), intercellular adhesion molecule (sICAM-1), sE-selectin, sP-selectin; IL-6, IL-8, tumour necrosis factor (TNF) α , sTNF α -R1 and sTNF α -R2 were analysed. The IL-6 gene polymorphism was determined in all groups and also compared with 200 healthy controls from a larger study of blood donors. In a multiple regression analysis, adjusted for gender, smoking and age, the effect of CLI was significantly ($p < 0.05$) associated with elevated levels of the WBC count, hsCRP, proinflammatory cytokines (IL-6, TNF α -R1–2) and endothelial (sICAM, sVCAM) and WBC (CD11b gran) markers. The effect of less advanced PAD (intermittent claudication) was related to an increased concentration of sVCAM-1 and the number of monocytes and granulocytes. DM or leg ulcers were not significantly related to any of the markers. No significant difference in frequency of the various IL-6 genotypes was found between the groups or when compared with the group of 200 blood donors ($p > 0.3$). Activation of cytokines, endothelial cells and WBC was related to the Fontaine stage of PAD but not to the presence of DM or ulcers. No association was found between the polymorphism in the IL-6 promoter region and PAD.

Key words: diabetes mellitus; inflammation; interleukin-6; peripheral arterial disease; polymorphism

Introduction

Peripheral arterial disease (PAD) is a manifestation of atherosclerosis, recognized as a chronic inflammatory disease of the vessel wall,^{1,2} involving endothelial cells, white blood cells (WBC), platelets and inflammatory mediators. The symptomatic manifestation of PAD is characterized either by intermittent claudication (IC) or critical limb ischemia (CLI).

Parameters reflecting the inflammatory response in PAD, although not extensively investigated, are increased compared with controls^{3–5} but also compared with patients suffering from coronary artery disease.⁶

To study the inflammatory response, abundant markers are available, but proinflammatory cytokines (interleukin (IL)-1, IL-6, tumour necrosis factor (TNF) α , adhesion molecules on WBC (CD11b/CD18) interacting with endothelial cell markers (ICAM, VCAM, selectins) are relevant and well described.⁷ Elevated levels of cytokines in the circulation have been found in several situations where inflammation is an important part of the pathology (e.g. elevated IL-6 levels have been associated with increased cardiovascular risk⁸). IL-6 is a multifunctional proinflammatory cytokine that mediates many functions in host defence. It is also an important

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stimulus to the production of C-reactive protein (CRP),⁹ which is a relatively well established predictor for cardiovascular disease.¹⁰ The concentration of IL-6 is likely to be influenced by several environmental and genetic factors. Such a genetic factor may be the polymorphic site (-174G/C) in the promoter region of the IL-6 gene.¹¹ However, the significance of this polymorphism and its relationship to cardiovascular risk is unclear. In previous studies, the G allele has been reported to be associated with PAD¹² and the C allele with increased IL-6 production and high cardiovascular mortality.¹³

In PAD, the clinical situation is often complicated and several sources of inflammatory response can be distinguished besides the atherosclerosis per se, such as ulcers, gangrene, infections, ischemic stress or the effect of diabetes mellitus (DM).^{14,15} Patients suffering from DM usually present an aggressive form of atherosclerosis with early and severe PAD. The increased metabolic stress is suggested to be responsible for the accelerated atherosclerotic process,¹⁶ however this is not predictable, since the mechanisms are still unknown.

The aim of this study was to characterize the inflammatory response in different stages of PAD in populations with and without DM by studying some of the most important inflammatory mediators and also to investigate a possible relationship to polymorphism in the promoter region of the IL-6 gene.

Patients and methods

Patients

Demographic data are presented in Table 1. Five groups of 20 patients each and one control group,

also of 20 patients, were included. All groups had a comparable age range. The group size was chosen before the start of the study. Recruitment was from patients referred to the Department of Vascular Diseases because of vascular symptoms; diabetic patients and male controls were participants in a health control study¹⁷ and the female controls were from a health control program at the hospital. Samples were collected between November 2001 and February 2003. In addition, DNA samples from 200 healthy blood donors were available.

The study was approved by the Ethics Committee of Lund University and the participants gave their informed consent.

Blood sampling

All blood sampling was performed in the morning with patients and controls fasting. The samples were collected in pyrogen-free tubes with and without ethylene diamine tetra-acetic acid (EDTA). EDTA-blood was prepared for cell and DNA analysis and serum was separated from coagulated blood and frozen at -20°C within 4 hours of collection.

Routine blood analysis

Hemoglobin, high sensitive CRP (hsCRP), cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG), glycated hemoglobin (HbA1c) and creatinine were analysed.

Analysis of cell activation

Full and differential WBC counts were performed. Activation of granulocytes and monocytes was assessed by flow cytometry measurement of CD11b and

Table 1 Clinical characteristics of the participants stratified into six groups, each comprising 20 individuals; ^amedian values and ranges.

Characteristic	Group 1: controls	Group 2: DM	Group 3: IC	Group 4: DM + IC	Group 5: CLI	Group 6: CLI + DM
Age (years) ^a	75 (69–87)	74 (73–76)	67.5 (40–86)	73 (59–82)	77 (60–94)	71.5 (61–91)
Men	12	20	11	13	12	15
Women	8	0	9	7	8	5
Current smoker	1	2	7	5	3	3
Former smoker	6	14	11	14	13	13
DM (insulin dependent)	0 (0)	20 (4)	0 (0)	20 (7)	0 (0)	20 (10)
Hypertension	5	14	13	11	10	14
Angina pectoris	1	6	4	6	5	4
Heart failure	0	1	1	5	3	3
Stenosis of a carotid artery	0	2	1	1	2	0
Cerebrovascular disease	2	2	2	6	1	1
Lung disease	0	1	0	2	0	0
Renal failure	0	0	0	1	1	4
Ulcer/gangrene	0	0	0	0	9	16
Weight (kg) ^a	72 (44–83)	84 (63–107)	75 (59–107)	78 (60–113)	71 (50–95)	82.5 (44–112)
Blood pressure (mmHg) ^a	130 (120–160)	145 (110–180)	145 (120–180)	150 (120–170)	140 (100–180)	165 (110–190)
Ankle pressure (mmHg) ^a	135 (120–160)	150 (110–160)	110 (50–150)	90 (50–140)	75 (0–150)	55 (0 ≥ 200)

DM, diabetes mellitus; IC, Intermittent claudication; CLI, critical limb ischemia.

CD18 expression. The different cell populations were identified using their morphological characteristics (forward versus side scatter). The cell concentration was determined by adding a known volume and concentration of Flow-Count Fluorospheres (Beckman Coulter, Brea, CA, USA) to the blood sample prior to flow cytometry analysis. CD11b and CD18 adhesion molecules were measured on the surface of granulocytes and monocytes using monoclonal r-phycoerythrin-conjugated anti-CD11b and fluorescein isothiocyanate-conjugated anti-CD18 (Dako A/S, Glostrup, Denmark). The analyses were made by a Coulter Epics XL-MCL (Coulter Electronics, Hialeah, FL, USA) at 488 nm. To keep a constant signal level, the fluorescence intensity was adjusted before analysis according to results obtained using Flow-Set Fluorospheres (Beckman Coulter). The mean channel fluorescence values for CD11b and CD18, respectively, were recorded.

Analysis of cytokines and soluble adhesion molecules and receptors

The serum concentrations of IL-6, IL-8 and TNF α were measured using an automated immunoassay system, Immulite[®] (DPC, Los Angeles, CA, USA). The concentrations of the soluble adhesion molecules sE-selectin, sP-selectin, vascular cell adhesion molecule (sVCAM-1), intercellular adhesion molecule (sICAM-1) and the soluble TNF α receptors sTNF α -R1 and sTNF α -R2 were measured by enzyme-linked immunosorbent assay using kits from R&D Systems (Minneapolis, MN, USA).

Analysis of the -174 G/C IL-6 gene promoter polymorphism

Extraction of genomic DNA from peripheral blood cells was performed according to the procedure described by Miller et al.¹⁸ A nested polymerase chain reaction (PCR) was used to amplify the 5'-flanking region of the IL-6 gene, identifying the -174G/C polymorphism. Primers used for the outer PCR were 5'-CAG AAG AAC TCA GAT GAC TGG-3' and 5'-GCT GGG CTC CTG GAG GGG-3' amplifying a 611 bp fragment.¹¹ Denaturation at 95°C for 5 minutes was carried out before the cycling program consisting of 25 cycles with denaturation at 95°C for 1 minute, annealing at 63°C for 1 minute, and extension at 72°C for 2 minutes. The program ended with a prolonged extension at 72°C for 10 minutes. The inner PCR amplified a 196 bp fragment using 5'-CGC TTC TTA GCG CTA GCC TC-3' and biotinylated 5'-GAG ACT CTA ATA TTG AGA CTC-3' primers. The amplification started with denaturation at 95°C for 5 minutes before the cycling program, which consisted of 50 cycles of denaturation at 95°C for 1 minute, then annealing using a touchdown program starting at 57°C for 2 minutes for 10 cycles, and then at 54°C for 40 cycles and 72°C of extension for 1 minute, and ending

with an extension at 72°C for 5 minutes. The PCR reactions were performed in 25 μ l volumes in a Gene Amp PCR system 2400 thermocycler (Applied Biosystems, Foster City, CA, USA). The outer reaction mixture contained 0.5 μ g of genomic DNA and the inner 0.4 μ l of the product from the outer PCR reaction. Both PCR mixes contained 1.5 mM MgCl₂, 0.2 mM dNTP (Amersham Biosystem, NJ, USA), 0.5 μ M of each primer (MVG Biotech, Ebersberg, Germany) and 0.5 units of AmpliTaq Gold[™] (Applied Biosystems, Foster City, CA, USA).

The -174G/C polymorphism was detected using the pyrosequencing technique.¹⁹ The primer used for sequencing was 5'-CCC TAG TTG TGT CTT GC-3' and the reaction was carried out as described by the manufacturer on a PSQ[™]96MA System (Pyrosequencing AB, Uppsala, Sweden).

Statistics

The Kruskal–Wallis test was used to compare the tested variables between the six groups. However, the groups differed with respect to potential confounders, namely sex, age and smoking habits (Table 1). Moreover, the Kruskal–Wallis test cannot separate the effect of PAD from the effect of DM on the tested variables. Therefore, a multivariate linear regression analysis was also conducted, with age, gender and smoking habits as covariates in order better to assess relationships between the clinical stage of PAD, DM and the tested variables. The dependent variables were log-transformed in order to obtain regression residuals that conformed more closely to the normal distribution. The primary predictors in the regression analysis were binary markers of DM, IC and CLI. The dataset was considered too small to allow for interaction between clinical stage of PAD and DM in the regression modelling. The regression analysis permitted comparison of the tested variables among patients with and without DM, while controlling for differences in age, sex and PAD. It was also used to compare patients with IC and CLI with those without PAD, respectively, while controlling for differences in age, sex and DM. The allele distribution between the groups was analyzed by a chi-squared test, using an exact method for *p*-value calculation available in StatXact-5 (Cytel Software Corporation, Cambridge, MA, USA). All other statistical analyses were performed using SPSS for Windows, release 11.5.1 (SPSS Inc., Chicago, IL, USA). *P*-values less than 0.05 were considered significant.

Results

Routine blood analysis (Tables 2 and 3)

Hemoglobin, hsCRP, creatinine, HDL, TG and HbA1c differed significantly between the groups (Table 2).

Table 2 Variation of standard laboratory parameters; median values, ranges and *p*-values for testing the hypothesis of no difference between the six groups (Kruskal–Wallis test) are presented; *n* = 20 in each group.

Parameter	Group 1: controls	Group 2: DM	Group 3: IC	Group 4: DM + IC	Group 5: CLI	Group 6 CLI + DM	<i>p</i> -value
Hb (g/l)	136.5 (94–143)	136.5 (112–156)	144 (130–165)	132.5 (100–148)	120 (78–146)	127.5 (96–152)	<0.001
hsCRP (mg/l)	4.5 (0.3–246)	0.9 (0.3–2.1)	4.8 (0.5–14.9)	5.1 (0.5–43.7)	6.0 (0.3–97.6)	11.5 (1.1–114)	<0.001
Creatinine (μ mol/l)	79 (11–157)	91 (79–174)	81.5 (58–241)	104 (54–221)	105 (58–344)	108 (60–644)	0.02
Cholesterol (mmol/l)	5.3 (3.7–7.6)	4.8 (3.7–6.6)	4.93 (3.6–8.4)	5.3 (3.0–7.4)	5.2 (3.7–7.4)	5.25 (3.3–7.35)	0.79
LDL (mmol/l)	3.35 (1.9–4.8)	2.9 (1.7–4.6)	3.1 (1.6–6.7)	3.5 (0.1–4.2)	3.1 (1.8–5.7)	2.8 (1.6–5.1)	0.82
HDL (nmol/l)	1.46 (0.6–2.7)	1.2 (0.5–2.5)	1.04 (0.7–2.5)	1.1 (0.4–3.5)	1.3 (0.65–2.1)	0.97 (0.49–1.85)	0.01
TG (mmol/l)	0.95 (0.5–1.9)	1.22 (0.4–4.6)	1.3 (0.6–3.0)	1.4 (0.8–8.2)	1.2 (0.6–3.4)	2.1 (0.6–5.0)	0.02
HbA1c (%)	4.65 (4.0–4.9)	6.75 (5.1–8.7)	4.6 (4.1–5.6)	6.1 (4.3–9.1)	4.6 (3.8–5.3)	6.5 (4.6–10.9)	<0.001

DM, diabetes mellitus; IC, intermittent claudication; CLI, critical limb ischemia; Hb, hemoglobin; hsCRP, high sensitive C-reactive protein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; HbA1c, glycated hemoglobin..

Table 3 Standard laboratory parameters: *p*-values for the effect of DM compared with all patients without DM and the effect of IC and CLI, respectively, compared with patients without PAD in a multiple regression analysis adjusted for age, gender and smoking habits.

Parameter	DM	IC	CLI
Hb	>0.30	0.15	<0.001
hsCRP	>0.30	0.093	<0.001
Creatinine	>0.30	0.027	<0.001
Cholesterol	>0.30	>0.30	>0.30
HDL	0.21	0.020	0.016
LDL	0.29	>0.30	>0.30
TG	0.011	0.055	0.006
HbA1c	<0.001	>0.30	>0.30

Abbreviations: refer to Table 2.

No significant differences was found in cholesterol or LDL concentrations. In the regression analyses, adjusted for age, gender and smoking habits, hsCRP, creatinine and TG were significantly higher and hemoglobin and HDL significantly lower in CLI patients than in patients without PAD (Table 3). In IC patients compared with patients without PAD, the concentration of creatinine was significantly higher and HDL significantly lower. In the same analyses, DM was significantly associated with increased levels of TG and HbA1c.

Cell activation and cytokines (Tables 4 and 5)

Significant differences between the six groups were recorded for IL-6, TNF α , sTNF α -R1, sTNF α -R2, sVCAM-1, sICAM-1, WBC count, and granulocyte and monocyte counts (Table 4). By contrast, no statistically significant differences between the six groups were discerned with respect to IL-8, sE-selectin, sP-selectin, and CD11b and CD18 on monocytes or granulocytes. In the regression analyses adjusted for

age, gender and smoking habits, patients with advanced PAD (CLI) had, compared with those without PAD, significantly higher concentrations of IL-6, TNF α , sVCAM-1, sICAM-1, sTNF α -R1, sTNF α -R2, and of WBC, monocyte and granulocyte counts and CD11b on granulocytes (Table 5). The results were not dependent on the presence of ulcers among the CLI patients (*p* > 0.12 for all tested variables; Kruskal–Wallis test comparing CLI patients with ulcers (*n* = 25) with CLI patients without ulcers (*n* = 15)). Significantly elevated levels of sVCAM-1, monocyte and granulocyte counts were also recorded in IC patients. No clear association between diabetes and any of these markers was found in the regression analyses.

IL-6 polymorphism (Table 6)

No significant differences in the frequency distribution of any of three genotypes (GG,CC,GC) were found between the six groups (*p* > 0.3; chi-squared test for a 3 \times 6 cross-table). Neither was any significant difference found when CLI and IC patients (groups 3–6 collated into a single group) were compared with a control group of 200 healthy blood donors (*p* > 0.3; chi-squared test for a 3 \times 2 cross-table).

Discussion

The main finding in this study was a significant inter-relationship between advanced PAD and increased inflammatory activity. By contrast, DM was not found to be associated with increased levels of any of the studied inflammatory markers, indicating that DM alone, if well regulated as it was in these patients, does not cause significant endothelial or inflammatory cell activation. In less advanced PAD, represented by

Table 4 Variation of laboratory parameters reflecting leukocyte and endothelial cell activation; median values, ranges and *p*-values for testing the hypothesis of no difference between the six groups (Kruskall-Wallis test); *n* = 20 in each group.

Parameter	Group 1: controls	Group 2: DM	Group 3: IC	Group 4: IC + DM	Group 5: CLI	Group 6: CLI + DM	<i>p</i> -value
IL-6 (pg/ml)	2.5 (2.5-168)	2.5 (2.5-5)	2.5 (2.5-6)	2.5 (2.5-19)	2.5 (2.5-32)	5 (2.5-159)	0.005
IL-8 (pg/ml)	2.5 (2.5-17)	2.5 (2.5-6)	2.5 (2.5-10)	2.5 (2.5-8)	2.5 (2.5-25)	2.5 (2-17)	0.7
TNF α (pg/ml)	2 (2-6)	2 (2-33)	2 (2-12)	2 (2-6)	2 (2-13)	3 (2-17)	<0.001
sTNF α -R1 (pg/ml)	1370 (960-3720)	1260 (940-3160)	1290 (850-5320)	1650 (760-5450)	1765 (820-9370)	2700 (910-15 530)	<0.001
sTNF α -R2 (pg/ml)	2535 (1900-4320)	2120 (1470-5750)	2540 (1530-6210)	3310 (2150-6450)	3845 (1500-7090)	4075 (1760-7060)	<0.001
sVCAM-1 (ng/ml)	595 (360-900)	590 (270-1090)	650 (350-1030)	620 (380-1480)	805 (350-1810)	895 (440-2380)	<0.001
sICAM-1 (ng/ml)	215 (160-360)	190 (150-730)	260 (130-390)	245 (150-640)	290 (190-450)	255 (200-1590)	<0.001
sE-selectin (ng/ml)	33 (18-83)	42.5 (11-100)	41 (20-85)	36.5 (16-77)	38.5 (19-96)	49 (23-320)	0.09
sP-selectin (ng/ml)	100 (46-370)	145 (23-480)	150 (52-380)	120 (38-330)	165 (69-450)	175 (110-310)	0.13
WBC count (10 ⁹ /l)	6 (4.1-14.7)	6.35 (3.1-12)	7.4 (4.3-10.7)	7.4 (0.54-10.1)	7.25 (4.6-14.3)	8.85 (5.9-11.5)	0.006
Granulocytes (10 ⁹ /l)	3.35 (0.78-11.4)	3.4 (1.4-6.5)	4.4 (2.5-6)	4.2 (2.7-9.1)	4.3 (2.8-9.3)	5.5 (3.2-7.4)	<0.001
Monocytes (10 ⁹ /l)	0.48 (0.24-3.3)	0.46 (0.19-0.85)	0.56 (0.38-0.9)	0.6 (0.38-6)	0.57 (0.34-1)	0.68 (0.44-1.4)	0.005
CD18, granulocytes (MCF)	3.5 (2.5-5)	4 (3-6.5)	4.4 (2.6-8.3)	3.45 (2.2-7)	4.15 (2.9-6.8)	4.2 (2.5-9.6)	0.06
CD11b, granulocytes (MCF)	12.5 (5.3-21)	12 (6.1-25)	16 (5.7-26)	12.5 (6-40)	14.5 (7.8-39)	15 (7-34)	0.2
CD18, monocytes (MCF)	7.4 (5.5-9.2)	7.85 (5.8-12)	8.4 (6-15)	7.75 (4.5-15)	8.5 (5.6-14)	7.9 (5.4-17)	0.1
CD11b, monocytes (MCF)	20 (8.7-29)	16 (13-27)	18 (8-36)	18 (11-51)	23.5 (9.8-35)	22 (8.7-41)	0.4

DM, diabetes mellitus; IC, intermittent claudication; CLI, critical limb ischemia; IL, interleukin; TNF, tumor necrosis factor; sVCAM-1, vascular cell adhesion molecule; sICAM-1, intercellular adhesion molecule; WBC, white blood cell; MCF, mean channel fluorescence.

Table 5 Laboratory parameters reflecting leukocyte and endothelial cell activation: *p*-values for the effect of DM compared with all patients without DM and the effect of IC and CLI, respectively, compared with patients without PAD in a multiple regression analysis adjusted for age, gender and smoking habits.

Parameter	DM	IC	CLI
IL-6	0.12	>0.30	0.022
IL-8	0.29	>0.30	0.30
TNF α	>0.30	>0.30	0.003
sTNF α -RI	0.06	>0.30	0.002
sTNF α -R2	0.11	>0.30	0.007
sVCAM-1	>0.30	0.026	<0.001
sICAM-1	>0.30	>0.30	0.001
sE-selectin	0.12	>0.30	0.15
sP-selectin	>0.30	>0.30	0.08
WBC count	>0.30	>0.30	0.010
Granulocyte count	0.22	0.006	<0.001
Monocyte count	>0.30	0.027	0.011
CD18, granulocytes	>0.30	>0.30	>0.30
CD11b, granulocytes	>0.30	>0.30	0.008
CD18, monocytes	0.13	>0.30	0.26
CD11b, monocytes	>0.30	>0.30	0.11

Abbreviations: refer to Table 4.

patients with IC (groups 3 and 4), associations were found with increased levels of sVCAM-1 and monocyte and granulocyte counts. This response may depend on the inflammatory activity in atherosclerotic plaques, a pattern demonstrated in several studies.^{20–22} Such association, but also high levels of IL-6, were found in patients with advanced PAD but no relationship to polymorphism in the promoter region of the IL-6 gene was observed in any group.

It may be argued that the statistical results are hampered by the fact that normality of the regression residuals cannot be checked properly, given the large number of parameters ($n = 6$) in the regression model in relation to the total number of observations ($n = 120$). However, it should be noted that the results of the regression analyses (Tables 3 and 5) and the Kruskal–Wallis test (Tables 2 and 4), with one exception (CD11b on granulocytes), are entirely consistent with respect to variables that differ significantly between the groups. The inconsistency for CD11b and granulocytes is most likely due to the fact that regression analysis is more sensitive than the

Kruskal–Wallis test in situations when only few groups differ markedly (see CLI patients, $p = 0.008$, Table 5) from the others. Several markers have been analysed in this study and the elevated risk of false positive results must be considered, especially for a marker such as CD11b on granulocytes, where the Kruskal–Wallis test failed to reveal differences between the groups. However, overall, the results were consistent and not likely to be explained by chance alone.

In group 2 (DM) all the patients were men, which could be a confounding factor but, in groups 4 and 6, there were seven and five women with DM respectively.

Regarding the effect of renal insufficiency, small molecules such as IL-6 are partly eliminated by glomerular filtration (<70 kDa) and renal failure may therefore influence the results. In the present series, however, there were no patients on dialysis and creatinine levels ranged from 221 to 644 $\mu\text{mol/l}$ among the six patients classified as having renal failure. Most studies on inflammatory markers and endothelial cell activation have focused on coronary artery disease. There is, however, a considerable difference between coronary artery disease and PAD patients. The latter are usually older and more commonly have DM. A major difference is probably the more advanced and generalized atherosclerosis occurring in PAD, indicated by higher levels of WBC activation and expression of endothelial cell markers.⁶ Only a few studies have explored inflammatory markers in PAD but the results demonstrate a correlation between the clinical stage of PAD and WBC/endothelial cell activation, which is in line with our results.^{6,23–27} No elevated levels of pro-inflammatory cytokines have been described in PAD patients, but raised baseline levels of CRP²⁸ and inflammatory markers (soluble TNF and IL-6 receptors, alpha 1-antitrypsin and haptoglobin) suggest increased inflammatory activity and activation of a regulatory feedback system.²³

In this study, advanced-stage PAD (groups 5 and 6) was related to increased expression of the majority of the tested inflammatory markers. Possible sources for the elevated inflammatory response in more advanced PAD could be the atherosclerosis per se, tissue ischemia or activity from existing ulcers. When the critically ischemic patients with and without ulcers

Table 6 Distribution of the –174G/C IL-6 promoter genotypes in the controls, patient groups and 200 healthy blood donors; *n* (%) for each group.

Genotype	Blood donors	Group 1: controls	Group 2: DM	Group 3: ^a IC	Group 4: ^a IC + DM	Group 5: ^a CLI	Group 6: ^a CLI + DM
GG	53 (26)	5 (25)	2 (10)	7 (37)	4 (21)	5 (26)	4 (22)
CC	48 (24)	6 (30)	6 (30)	5 (26)	1 (5)	4 (21)	5 (28)
GC	99 (50)	9 (45)	12 (60)	7 (37)	14 (74)	10 (53)	9 (50)

^aDNA was not available from all patients in these groups.

were compared, it could be seen that the results did not depend on the presence of ulcers. In another study, yet to be published, we have demonstrated that revascularization in CLI did not significantly influence the inflammatory activity at 4 weeks after successful surgery. These findings suggest that the increased expression of inflammatory markers in CLI depends on advanced and active atherosclerosis and not on tissue ischemia or ulcers. Previous studies have also shown a correlation between inflammatory activity and the stage of PAD.^{3,6,29} In the current study, this trend was most obvious for sVCAM-1 and granulocyte and monocyte counts. Atherosclerotic lesions can be very quiescent, with low inflammatory activity, for long periods but if there is plaque rupture or thrombosis formation the inflammatory response will increase. Such activity is more likely to be found in the most advanced stages of PAD, but high activity in atherosclerotic lesions can also be expected in patients with IC.

Other research has revealed elevated levels of soluble endothelial markers and increased activation of WBC in patients with DM³⁰ and macrovascular complications.³¹ The response is, however, of low intensity and related to metabolic control.^{32,33} The interaction of WBC and endothelial cells is probably required for the accelerated atherogenesis observed in DM, but the coexistence of other mechanisms is most likely. Impaired endothelial cell regulation by nitric oxide³⁴ and endothelin-1³⁵ and the modification of LDL by glycosylation³⁶ are other features of DM that are responsible for accelerated atherogenesis.

In this study, no increased expression of WBC or endothelial cell markers was found as an indication of increased WBC/endothelial cell interaction in DM. The lack of response in the DM patients in this study could be explained by reasonably good metabolic control (HbA1c: 6.10–6.75%) and low cholesterol levels. In turn this may be an effect of previous participation in a lifestyle and cardiovascular risk factor modification program.¹⁷ Variations in the IL-6 response, suggested to cause variations in cardiovascular risk, have been postulated to be related to a polymorphism at position –174 in the promoter region of the IL-6 gene. The significance of this polymorphism is not unambiguous and may be of other importance in different vascular areas. In the UK small aneurysm study,¹³ the CC genotype was associated with the highest IL-6 levels and highest mortality risk. In a recent study by Flex and coworkers,¹² the GG genotype was noted to be significantly more common in PAD patients compared with controls.

In this study, high levels of IL-6 were associated with CLI, reflecting augmented inflammatory activity in advanced atherosclerosis. However, no difference in the IL-6 genotype was found in these patients compared with the controls (Table 6). The results are based on a relatively small population, but they contradict a strong association between genotype and PAD.

The high IL-6 levels in advanced PAD patients probably depend more on other stimuli than the genotype of the IL-6 gene.

Conclusions

In conclusion, the results of this study confirm the correlation between advanced PAD, but not DM with good metabolic control, and an increased inflammatory response and may suggest a relationship depending on advanced atherosclerosis but not on the presence of ulcers. High IL-6 levels correlated with advanced PAD, but the –174 IL-6 gene promoter polymorphism was not found to be associated with PAD. The clinical implication, based on these findings, is that it is of the greatest importance to address the general atherosclerotic process in PAD patients by prevention and treatment options, in addition to treating the reduced blood flow that is causing the PAD symptoms.

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