# Inflammatory mediators are associated with 1-year mortality in critical limb ischemia

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***Objective:*** **The atherosclerotic process has inflammatory features. Patients with peripheral atherosclerosis and critical limb ischemia have a poor prognosis. This study evaluated the hypothesis that inflammatory markers are associated with mortality among patients admitted to the hospital because of critical limb ischemia.**

***Methods:*** **This was a prospective, single-center, 1-year, follow-up study of 259 consecutive patients with critical limb ischemia who were admitted to a secondary referral center of vascular diseases. Interventions included evaluation of intercurrent disease, ankle and arm blood pressures, plasma glucose and lipid levels, plasma homocysteine, cardiolipin antibodies, resistance to activated protein C, plasma endothelin-1, and the inflammatory mediators tumor necrosis factor-α, interleukin-6, neopterin, high-sensitivity C-reactive protein, CD40 ligand, and 8-iso-prostaglandin $F_{\alpha}$ in plasma. The main outcome measure was total mortality and causes of death assessed 1 year after admission.**

***Results:*** **During the first year after admission, 61 patients (24%) died. These patients were older ($P < .0001$), showed a higher leukocyte count ($P = .0011$) and levels of serum creatinine ($P < .0001$), lower levels of high-density lipoprotein (HDL) cholesterol ($P = .003$) and frequency of active treatment ($P = .014$) than the 198 (76%) survivors. More nonsurvivors had gangrene ($P < .0001$), and fewer ($P = .004$) had lipid-lowering treatment. The plasma levels of interleukin-6 ($P < .0001$), tumor necrosis factor-α ($P < .0001$), neopterin ($P < .0001$), and high-sensitivity C-reactive protein ($P = .002$) at admission for critical limb ischemia were all significantly lower in the survivors, whereas there was no difference concerning CD40 ligand. In logistic regression adjusted for age, sex, lipid-lowering therapy, active treatment, gangrene, leukocyte count, creatinine, and serum HDL cholesterol, the inflammatory mediators tumor necrosis factor-α ($P = .0084$), neopterin ($P = .0035$), but not interleukin-6 ($P = .585$) or high-sensitivity C-reactive protein ($P = .314$) were independent risk variables of death within 1 year.**

***Conclusions:*** **Increased age, leukocyte count, creatinine, and inflammatory mediators, together with gangrene, were associated with 1-year mortality despite intervention in critical limb ischemia. For tumor necrosis factor-α and neopterin in plasma, this association was independent of the other parameters. (J Vasc Surg 2005;42:75-80.)**

Epidemiology and pathophysiology are less well studied in critical limb ischemia than in atherosclerotic manifestations in the coronary and precerebral arteries. In particular, the importance of leukocytes and inflammatory reactions for the pathophysiology of ischemic vascular disease[1,2] has been studied predominantly in subjects with coronary artery disease. Although inflammatory markers have been evaluated among asymtomatic[3-5] and symtomatic[3,5,6] subjects with peripheral arterial disease, their prognostic importance in critical limb ischemia is not established.

The importance of inflammatory reactions may be more difficult to clarify in critical limb ischemia because patients with this condition, by definition,[7] often have ischemic leg ulcers featuring inflammatory processes that might affect the patterns of inflammatory mediators. Concerning risk factors of atherosclerosis, patients with critical limb ischemia are also often inadequately treated compared with patients with coronary or precerebral atherosclerosis.[8-10]

Tumor necrosis factor-α (TNF-α),[11] neopterin,[12] and interleukin-6 (IL-6) are inflammatory mediators reported to be involved in atherogenesis.[13] The expression of the immune mediator CD40 ligand is also increased in cells involved in atherogenesis.[14,15] Endothelin-1 (ET-1), a vasoconstrictive factor produced by the endothelium that promotes the initiation and progression of atherosclerosis,[16] is another substance involved in the pathogenesis of atherosclerosis.[17] Isoprostanes, including 8-epi-prostaglandin (PG)$F_{2\alpha}$, are prostaglandin $F_2$-like compounds[18,19] measured as markers for lipid peroxidation that are believed to contribute to atherosclerosis and thrombosis.[20]

The 1-year mortality in patients with critical limb ischemia is about 20%[7,21,22] and is influenced by features of inflammation such as leukocyte count and fibrinogen.[23] The aim of the present observational, hypothesis-generating study was to evaluate whether, at admission, plasma levels of TNF-α, neopterin, IL-6, CD40 ligand, ET-1, or 8-epi-prostaglandin (PG)$F_{2\alpha}$ were associated with total 1-year mortality in consecutive patients admitted to hospital because of critical limb ischemia.

## METHODS

**Patients.** The Department of Vascular Diseases at Malmö University Hospital is the single referral center for

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all patients with critical limb ischemia in the three southernmost health-care districts in Sweden (723,750 inhabitants in 2001). During a 14-month period, 316 consecutive patients were referred to the department with a confirmed diagnosis of critical limb ischemia, and 259 (82%) consented to be in the present study. With respect to age, sex, or blood pressure, they did not differ from patients who declined to participate. The study protocol conformed to the Declaration of Helsinki, and all participants gave written informed consent. The Lund University Regional Ethical Review Board approved the study.

The diagnosis of critical limb ischemia was made in accordance with TransAtlantic Inter-Society Consensus scientific criteria[7] of ulceration, gangrene, or rest pain caused by peripheral arterial disease proven by ankle pressure (<50 to 70 mm Hg), reduced toe pressure (<30 to 50 mm Hg), or reduced transcutaneous oxygen tension ($TCPO_2$). Diagnosis was confirmed by an experienced vascular surgery consultant and toe pressure measurements in those patients where the arteries in the affected leg were noncompressible and the ankle pressure was >50 to 70 mm Hg.[7] One-year mortality after admission and causes of death were assessed from the Swedish Board of Health and Welfare.

**Patient variables.** Body mass index was calculated as weight in kilograms/height in $m^2$. We measured arm and ankle blood pressures with a sphygmomanometer with the patient in the supine position. Gangrene was defined as visible tissue loss on clinical examination.

Venous blood glucose levels were determined by a routine hexokinase method. Serum levels of triglycerides and total, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol were determined by a DAX 48 automatic analyzer (Bayer AB, Gothenburg, Sweden).[24]

Plasma homocysteine was analyzed by a high-performance liquid chromatographic assay.[25-27] The reference values were <18 μmol/L and the interassay coefficient of variation was 3.7%. We tested resistance to activated protein C (APC) with a predilution of sample plasma in factor V-deficient plasma, CV was 7%. The method[28] has a specificity and sensitivity of near 100% for the factor $V:Q^{506}$ allele, which is the mutation responsible for APC resistance with so-called low APC ratio.

Anticardiolipin antibodies were evaluated in serum by enzyme-linked immunosorbent assay (ELISA).[29] The reference values were <20 IgG phospholipid units, and values >40 IgG phospholipid units were considered positive.

**Analysis of inflammatory mediators.** Plasma TNF-α and IL-6 were measured by ELISA using commercially available test kits (Pharmingen, San Diego, Calif). The detection limits were 0.12 pg/mL and 0.70 pg/mL, respectively.

Plasma neopterin was determined by ELISA (Henning, Berlin, Germany). The detection limit was 2 nmol/L, and the intra- and interassay coefficients of variation were 1.7% and 8.2%.

Serum high-sensitivity C-reactive protein (hs-CRP) was measured by rate turbidimetry at the Department of Clinical Chemistry, Malmö University Hospital. The detection limit was 0.2 mg/L, and the interassay coefficients of variation were 6% at 15 mg/L and 5 % at 85 mg/L.

We measured plasma ET-1 by radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, Calif). The detection limit was 1.0 pg/mL, the intra-assay coefficient of variation based on pooled samples was 11.3%, and the interassay variation was 22%.

Plasma 8-epi-$PGF_{2\alpha}$ was measured by an enzyme immunoassay using commercially available test kits (Cayman Chemical Company, Ann Arbor, Mich). The detection limit was 3.9 pg/mL.

Serum CD40 ligand was analyzed by an immunoassay using commercially available test kits (R & D Systems Inc, Minneapolis, Minn). The detection limit was 2.1 pg/mL.

**Statistics.** We evaluated the differences between survivors and nonsurvivors with the Mann-Whitney *U* test and the $\chi^2$ test. The independent predictive effect of inflammatory mediators for 1-year mortality was tested in step-wise logistic regression adjusted for all variables that were found to differ between survivors and nonsurvivors. All tests were two-tailed, and $P < .05$ was considered significant. The results are presented as mean ± SD. All variables were recorded in a database, and statistical analyses and calculations were performed with the statistical software StatView 5.0 (SAS Institute, Cary, NC).

## RESULTS

During the first year after admission because of critical limb ischemia, 80 (25%) of the 316 patients died. Among the 57 patients who declined study participation, 19 (33%) died; 61 (24%) of the included 259 patients died ($P = .0310$). The cause of death was cardiac or vascular disease in 45 (74%). Included patients who died within 1 year after admission were older ($P < .0001$). They showed a higher leukocyte count ($P = .0011$) and serum creatinine levels ($P < .0001$), and lower levels of HDL-cholesterol ($P = .003$) than did the 198 (76%) surviving patients (Table I). Of the five patients undergoing dialysis, four died during the 1-year follow-up.

The nonsurvivors had a higher prevalence of gangrene ($P < .0001$) and a lower prevalence of lipid-lowering treatment (statins in 59 of 61 patients with lipid-lowering treatment) ($P = .004$).

On the other hand, no differences were noted between the survivors and the nonsurvivors regarding sex, blood pressures, prevalence of diabetes mellitus (Table I), or the frequency of endovascular, surgical, or medical treatment of critical ischemia during the 1-year follow-up (Table II). The total frequency of any active surgical, endovascular, or medical treatment was higher ($P = .014$) in survivors, however (Table I).

Plasma levels of the inflammatory mediators IL-6 ($P < .0001$), TNF-α ($P < .0001$), neopterin ($P < .0001$), and hs-CRP ($P = .0024$) at hospital admission because of critical limb ischemia were all significantly lower in the survivors than in the nonsurvivors (Fig). However, plasma

**Table I.** Results in patients who survived or died 1 year after admission for critical limb ischemia

| | *Survivors* (n = 198)* | *Nonsurvivors* (n = 61)* | P |
|---|---|---|---|
| Demographic data | | | |
| Age (years) | 74 ± 10 | 80 ± 10 | <.0001† |
| Male sex | 99 (50) | 39 (64) | .081 |
| BMI (kg/m$^2$) | 25.1 ± 4.7 | 24.8 ± 4.0 | .903 |
| Hemoglobin (g/l) | 123 ± 18 | 120 ± 19 | .206 |
| Leukocyte count | 9.2 ± 3.1 | 10.8 ± 4.2 | .011† |
| Platelet count | 302 ± 112 | 329 ± 134 | .067 |
| Glucose (mmol/L) | 5.5 ± 4.4 | 5.9 ± 4.3 | .516 |
| Serum creatinine (μmol/L) | 118 ± 81 | 183 ± 175 | <.0001† |
| Ulceration | 120 (61) | 35 (57) | .653 |
| Gangrene | 16 (8) | 17 (28) | <.0001† |
| Aortoiliacal AS | 18 (9) | 1 (2) | .051 |
| Multisegmental AS | 30 (15) | 13 (21) | .258 |
| Infrainguinal AS | 149 (75) | 48 (79) | .582 |
| Ankle-brachial index | 0.31 ± 0.21 | 0.32 ± 0.26 | .994 |
| Ankle BP affected leg (mm Hg) | 45 ± 31 | 44 ± 31 | .851 |
| Risk factors and treatment | | | |
| Current smoker | 69 (35) | 15 (25) | .134 |
| Diabetes mellitus | 100 (51) | 35 (57) | .434 |
| Arterial hypertension | 141 (71) | 40 (66) | .313 |
| Systolic BP (mm Hg) | 149 ± 25 | 142 ± 28 | .576 |
| Diastolic BP (mm Hg) | 76 ± 15 | 73 ± 12 | .236 |
| Cholesterol (mmol/L) | 4.83 ± 1.24 | 4.57 ± 1.11 | .140 |
| LDL (mmol/L) | 2.90 ± 1.06 | 2.82 ± 0.93 | .629 |
| HDL (mmol/L) | 1.18 ± 0.43 | 1.01 ± 0.44 | .003† |
| Triglycerides (mmol/L) | 1.69 ± 0.93 | 1.64 ± 1.10 | .838 |
| Lipid-lowering drugs | 55 (28) | 6 (10) | .004† |
| Active treatment | 168 (85) | 38 (62) | .014† |
| Homocysteine (μmol/L) | 16.7 ± 6.8 | 18.3 ± 7.2 | .078 |
| Cardiolipin antibodies | 60 (30) | 11 (18) | .131 |
| Factor V Leyden | 27 (14) | 6 (10) | .283 |
| Inflammatory markers | | | |
| Endothelin-1 (pg/mL) | 1.63 ± 1.11 | 1.65 ± 0.72 | .583 |
| 8-epi-PGF$_{2\alpha}$ (pg/mL) | 243 ± 219 | 295 ± 269 | .059 |
| High-sensitivity CRP (mg/L) | 33.6 ± 56.5 | 49.4 ± 62.1 | .002† |
| IL-6 (pg/mL) | 21.5 ± 37.5 | 40.5 ± 60.0 | <.0001† |
| TNF-α (pg/mL) | 1.73 ± 1.24 | 3.71 ± 6.57 | <.0001† |
| Neopterin (nmol/L) | 19.1 ± 20.6 | 49.7 ± 66.2 | <.0001† |
| CD 40 ligand (pg/mL) | 644 ± 1034 | 1136 ± 3126 | .643 |

*BMI,* Body mass index; *BP*, blood pressure; *AS,* atherosclerosis; *LDL,* low-density lipoprotein; *HDL,* high-density lipoprotein; *CRP,* C-reactive protein.
*Data are mean ± SD or n (%).
†Value is significant.

levels of ET-1, CD40 ligand, or 8-epi-PGF$_{2\alpha}$ did not differ between the groups (Table I).

In logistic regression adjusted for age, sex, lipid-lowering therapy, gangrene, leukocyte count, and serum levels of creatinine and HDL cholesterol, plasma levels of the inflammatory mediators TNF-α ($P = .0084$), neopterin ($P = .0035$), but not serum hs-CRP ($P = .314$), or p-IL-6 ($P = .586$) were independently associated with death within 1 year. Because of their high internal correlation, however, this association disappeared for TNF-α ($P = .486$) and neopterin ($P = .272$) when all inflammatory variables were adjusted for each other (Table III).

To exclude the possibility that differences in inflammatory mediators between the survivors and the nonsurvivors were explained by gangrene, the two groups were compared after patients with gangrene were excluded. This analysis revealed that the differences between the survivors and the nonsurvivors persisted for IL-6 ($P = .0006$), TNF-α ($P = .0004$), neopterin ($P = .0002$), and hs-CRP ($P = .227$). Antibiotic treatment was given on admission to 12 (36%) of the patients with gangrene and to 56 (25%) of the patients without gangrene ($P = .1577$).

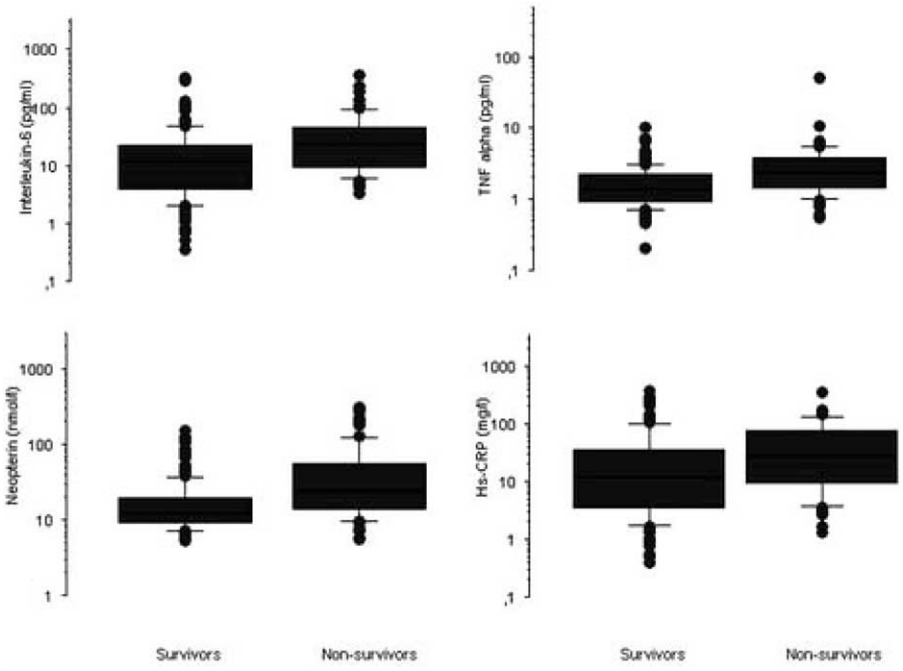
## DISCUSSION

Our study confirmed the ominous prognosis for patients with critical limb ischemia, with a one-year mortality of 26% mainly due to cardiovascular disease, comparable to figures previously published.[21,22] The mortality was slightly higher among the patients not consenting to study inclusion, probably because some critically ill patients with high mortality were not able to sign written consent. Furthermore and most importantly, our study showed that the inflammatory mediators IL-6, TNF-α, neopterin, and hs-CRP were associated with 1-year mortality in subjects with critical limb ischemia. For TNF-α and neopterin, this association was independent of other variables such as age, sex,

**Table II.** One-year outcome related to different types of treatment in 259 patients with critical limb ischemia during 1-year follow-up

| *Type of treatment* | *N (%)* | *1-year mortality (% subgroup)* | *Amputation during 1 year (% subgroup)* | *Amputated-alive at 1 year (% amputees)* |
|---|---|---|---|---|
| Femoropopliteal or distal bypass | 46 (18) | 11 (24) | 8 (17) | 7 (88) |
| Endarterectomy, X-over, Y-graft | 22 (8) | 4 (18) | 5 (24) | 4 (80) |
| PTA | 34 (13) | 7 (21) | 4 (12) | 3 (75) |
| Endovascular stenting | 33 (13) | 5 (15) | 1 (3) | 1 (100) |
| SAP | 54 (21) | 10 (19) | 3 (6) | 3 (100) |
| Nonsuccessful SAP | 11 (4) | 4 (36) | 4 (36) | 4 (100) |
| Iloprost infusion | 6 (2) | 2 (33) | 1 (17) | 1 (100) |
| Conservative | 53 (20) | 18 (34) | 7 (13) | 2 (29) |
| Total | 259 | 61 (24) | 33 (13) | 25 (76) |

*PTA,* Percutaneous transluminal angioplasty; *SAP,* subintimal angioplasty.




Increased levels of the inflammatory mediators interleukin-6 ($P < .0001$), tumor necrosis factor alpha (*TNF*) ($P < 0.0001$), and neopterin in plasma ($P < 0.0001$) and high sensitivity C-reactive protein (*Hs-CRP*) ($P = .002$) in serum among patients with critical limb ischemia who did not survive 1 year after hospital admission compared with surviving patients

gangrene, lipid-lowering therapy, leukocyte count, renal function, and HDL-cholesterol levels.

A limitation of our study, however, is that we cannot draw any conclusions about possible relationships between the levels of inflammatory mediators and the efficacy of interventions in our patients. Further studies are needed, and as we have made only a predictive sampling showing the associations between inflammatory mediators and mortality, our results cannot be used to determine the best treatment of a certain patient. Elevated levels of inflammatory mediators are only indicators of a more severe prognosis.

Other limitations of the study are that it was a descriptive, observational, hypothesis-generating study performed at a single center. It lacked a case-control design and a healthy comparison group.

Among patients with critical limb ischemia, inflammatory mediators can be hypothesized to originate partly from ulcers and gangrene of the ischemic limb, but also from the general atherosclerotic process. Furthermore, the IL-6 gene is upregulated in the hypoperfused musculature of subjects with critical limb ischemia,[30] and IL-6 has been reported to be increased in symptomatic peripheral artery disease.[3] Surprisingly, in a small group of eight patients

**Table III.** Logistic regression analysis with all variables differing significantly between survivors and nonsurvivors after 1 year

| | | | 95% CI | |
|---|---|---|---|---|
| *Significance* | (P) | *OR* | *Lower* | *Upper* |
| Male sex | .525 | 1.347 | 0.537 | 3.378 |
| Age* | .0005 | 1.095 | 1.041 | 1.151 |
| Gangrene | .059 | 2.870 | 0.961 | 8.570 |
| Lipid lowering drugs | .115 | 0.375 | 0.111 | 1.271 |
| No active treatment | .341 | 1.546 | 0.631 | 3.786 |
| Leukocyte count* | .006 | 1.202 | 1.054 | 1.370 |
| Serum creatinine* | .653 | 1.002 | 0.995 | 1.008 |
| HDL* | .614 | 0.727 | 0.211 | 2.507 |
| HS-CRP* | .174 | 0.993 | 0.984 | 1.003 |
| IL-6* | .496 | 1.004 | 0.992 | 1.016 |
| TNFα* | .486 | 1.126 | 0.806 | 1.574 |
| Neopterin* | .272 | 1.013 | 0.990 | 1.035 |

*OR,* Odds ratio; *CI,* confidence interval; *HDL,* high-density lipoprotein; *HS-CRP,* high-sensitivity C-reactive protein; *IL-6,* interleukin-6; *TNF,* tumor necrosis factor.
*Continuous variables, odds ratio is shown for an increase of one unit.

with critical limb ischemia, including five with ischemic lesions,[31] neither TNF-α nor IL-6 differed from values in healthy controls.

Because the relationships between inflammatory mediators and mortality in our study persisted in the logistic regression analysis and after exclusion of patients with gangrene, these relationships may only partly be explained by the fact that patients with inflammatory processes such as gangrene of the extremities showed a high mortality. Thus, our results indicate that the atherosclerotic process in itself might explain part of the release of inflammatory mediators.

Not surprisingly, age, leukocyte count, and serum creatinine levels were also associated with 1-year mortality in our patients. This has been reported previously[23] for leukocyte count and creatinine. Furthermore, neutrophil count has previously been shown to be associated with a need for amputation in critical limb ischemia.[32] This outcome was not separately assessed in our study, however. Well-established conventional risk factors for atherosclerosis such as blood pressure, diabetes mellitus, and smoking showed no relationships with mortality in this aged population already showing widespread atherosclerotic disease.

Concerning lipids, only a low serum level of HDL cholesterol was associated with the 1-year mortality, and other investigators have presented evidence for a similar relationship with lipoprotein (a)[33] that was not assessed in our study. In this context, however, it has to be remembered that the serum lipid levels often are falsely low among patients with critical limb ischemia,[34] and our lipid results must therefore be interpreted with some caution.

The outcome of critical limb ischemia has previously been reported to be worse in diabetic patients than in nondiabetic patients,[35] but surprisingly, the presence of diabetes mellitus did not affect the 1-year mortality in our patients. The duration of diabetes mellitus was not assessed, so we cannot exclude the possibility that this lack of relationship was due to a short duration of diabetes mellitus.

Neither did we see any relationships between homocysteine, p-8-epi-$PGF_{2\alpha}$, factor V Leyden, cardiolipin antibodies, and 1-year mortality in our patients with critical limb ischemia. In contrast to newly presented data[36] concerning the importance of the CD40 ligand in acute coronary heart disease, we did not find any difference concerning this variable between the survivors and the nonsurvivors 1 year after admission. In this context it must be remembered that we, unlike Aggarwal et al,[36] did not measure CD40 ligand in coronary artery blood, and that this marker might have different relevance in coronary and peripheral atherosclerosis.

Apart from their well-documented effects upon the mortality among patients with vascular disease, including peripheral artery disease,[37] statins favorably influence the leg function[38] and are associated with graft patency and limb salvage after infrainguinal bypass surgery[10] among patients with peripheral arterial disease. It is therefore interesting to note that statin use was more common in survivors. This can hardly be interpreted as a causal relation, however, because this was not a randomized study, and statin treatment might have been prescribed mainly to younger patients considered to have a better prognosis. These results must therefore be evaluated with great caution.

It is also important to emphasize that we only correlated laboratory variables recorded at admission to hospital to 1-year mortality in this study. Possible effects on mortality because of medications added later, amputations, or later surgical or endovascular interventions cannot be evaluated from this study but might be elucidated by further follow-up. The mortality among amputees was surprisingly low, however.

In conclusion, high plasma levels of the inflammatory mediators TNF-α and neopterin were associated with increased 1-year mortality in subjects with critical limb ischemia. This effect was only partly caused by gangrene of the affected extremity.

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