Serum Vitamin C Concentration Is Low in Peripheral Arterial Disease and Is Associated With Inflammation and Severity of Atherosclerosis

Michel Langlois, MD, PhD; Daniel Duprez, MD, PhD; Joris Delanghe, MD, PhD; Marc De Buyzere, PhD; Denis L. Clement, MD, PhD

Background—Peripheral arterial disease (PAD) is a severe atherosclerotic condition frequently accompanied by inflammation and oxidative stress. We hypothesized that vitamin C antioxidant levels might be low in PAD and are related to inflammation and disease severity.

Methods and Results—We investigated vitamin C (L-ascorbic acid) levels in 85 PAD patients, 106 hypertensives without PAD, and 113 healthy subjects. Serum L-ascorbic acid concentrations were low among PAD patients (median, 27.8 μmol/L) despite comparable smoking status and dietary intake with the other groups (P<0.0001). Subclinical vitamin C deficiency (<11.4 μmol/L), confirmed by low serum alkaline phosphatase activity, was found in 14% of the PAD patients but not in the other groups. Serum C-reactive protein (CRP) concentrations were significantly higher in PAD patients (P<0.0001) and negatively correlated with L-ascorbic acid levels (r=−0.742, P<0.0001). In stepwise multivariate analysis, low L-ascorbic acid concentration in PAD patients was associated with high CRP level (P<0.0001), smoking (P=0.0009), and shorter absolute claudication distance on a standardized graded treadmill test (P=0.029).

Conclusions—Vitamin C concentrations are lower in intermittent claudicant patients in association with higher CRP levels and severity of PAD. Future studies attempting to relate vitamin C levels to disease occurrence should include in their analysis an inflammatory marker such as CRP. (Circulation. 2001;103:1863-1868.)

Key Words: arteries ■ atherosclerosis ■ antioxidants ■ inflammation ■ claudication

Peripheral arterial disease (PAD) is a severe atherosclerotic condition causing intermittent claudication and is associated with increased cardiovascular and cerebrovascular morbidity and mortality.1,2 Moreover, it has a major impact on the quality of life because of the reduction in walking ability. In clinical practice, PAD is assessed noninvasively by the determination of the ankle/brachial systolic blood pressure index (ABI) and absolute claudication distance (ACD) on a treadmill test, a parameter of functional adaptation to the disease.3-5

Smoking, diabetes mellitus, hyperlipidemia, and hypertension are the key risk factors for PAD,6 but it is now equally recognized that low-grade inflammation contributes importantly to the initiation and the progression of the vascular atherosclerotic lesions. Specifically, slightly elevated levels of C-reactive protein (CRP), an acute phase reactant, are associated with PAD and are found among apparently healthy subjects at risk for developing future PAD.7,8 It has been suggested that inflammation within the atherosclerotic lesions and the subsequent release of free radicals by phagocytes result in oxidative stress that further enhances vascular damage.9

In this study, we assessed whether inflammation is associated with antioxidant status in patients with intermittent claudication. Vitamin C (L-ascorbic acid) is a water-soluble antioxidant capable of scavenging free radicals and is the first-line defense in the control of the redox state, sparing other endogenous antioxidants from consumption.10,11 We tested the hypothesis that vitamin C status might be low in PAD and that it correlates with measures of inflammation and functional status in patients with intermittent claudication.

Methods

Study Groups

We investigated 85 PAD patients (Fontaine stage II) who were regularly followed for at least 12 months at our department, 106 patients with essential hypertension without PAD (to account for the effect of pulse pressure), and 113 healthy volunteers of the same region. Hypertensives were defined along the WHO/ISH criteria for at least 12 months and were receiving treatment with antihypertensive drugs.12 Patients with either surgical revascularization or percutaneous transluminal angioplasty procedures at the lower limb arteries were excluded, as well as patients with acute myocardial infarction, angina pectoris, heart failure, coronary revascularization procedures, or cerebrovascular events during the last 6 months. Other
Fasting blood samples were taken between 8:00 and 9:00 AM. None of the subjects took any medication during the last 12 hours before sampling. Within 30 minutes, serum vitamin C (L-ascorbic acid) concentrations were measured by the ascorbate oxidase method on a Hitachi 747 analyzer (Roche). Serum CRP concentrations were measured by high-sensitive latex-enhanced immunonephelometry (BN II nephelometer, Dade Behring). Plasma fibrinogen was assayed by prothrombin time–derived measurements on a Hitachi 911 analyzer (Roche). Serum alkaline phosphatase activity, concentrations were measured by the ascorbate oxidase method on a Hitachi 747 analyzer (Roche).18

Table 1 summarizes clinical characteristics of the study groups. A dietary history was taken to estimate the amount of vitamin C ingested by means of a food frequency questionnaire according to Donnan et al.15 Intensity and duration of physical activity were also assessed with a questionnaire.16 Smoking status was classified as current smokers and nonsmokers; individuals who stopped smoking within 4 weeks before enrollment in the study were excluded to account for the effect of smoking cessation on vitamin C status.17 Informed consent was obtained from all subjects, and the study was approved by the ethics committee of Ghent University Hospital.

### Biochemical Investigations

Fasting blood samples were taken between 8:00 and 9:00 AM. None of the subjects took any medication during the last 12 hours before sampling. Within 30 minutes, serum vitamin C (L-ascorbic acid) concentrations were measured by the ascorbate oxidase method on a Hitachi 911 analyzer (Roche).18 Serum alkaline phosphatase activity, a biochemical marker of vitamin C deficiency,19 was measured with commercial reagents on a Hitachi 747 analyzer (Roche). Serum CRP concentrations were measured by high-sensitive latex-enhanced immunonephelometry (BN II nephelometer, Dade Behring). Plasma fibrinogen was assayed by prothrombin time–derived measurements on an ACL–200 analyzer (Instrumentation Laboratory). Serum total cholesterol, HDL cholesterol, and triglycerides were assayed with commercial reagents (Hitachi 747, Roche); LDL cholesterol concentration was calculated by the Friedewald formula. Diabetes control status was assessed by hemoglobin A1c (HbA1c) with a Variant II chromatographic system (Bio-Rad). Intra-assay coefficients of variation were <5.0% for L-ascorbic acid, <1.3% for alkaline phosphatase, <3.8% for CRP, <4.4% for fibrinogen, <1.8% for lipids, and <1.5% for HbA1c.

### Vascular Investigations

After blood sampling, all subjects underwent clinical examinations including blood pressure measurements at the left and right brachial artery by sphygmomanometry (3 times in sitting position with 2-minute intervals) and 12-lead ECG. In PAD patients, arterial systolic blood pressures at the left and the right brachial and posterior tibial arteries were measured by a Doppler 8-MHz ultrasound device (Scimed Digitop 840S). ABI was calculated for both legs, and the lowest ABI was taken as the study parameter. Thereafter, PAD patients (n=70) who did not have contraindications to performing a walking test (chronic obstructive pulmonary disease, cardiac rhythm disturbances, orthopedic or neurological conditions, ulcer) underwent a standardized graded treadmill protocol with a speed of 3 km/h and adjustable grade. For the first 5 minutes, the patients had to walk at a grade of 0%, then the grade increased every 5 minutes by 5% up to 15%.20,21 All patients were accustomed to treadmill tests and had at least 3 tests during the last 12 months. ACD on the present treadmill test was taken as study parameter. Clinical characteristics of patients who participated in the treadmill protocol were age, 69±9 years; male sex, 77%; body mass index, 26±4 kg/m²; smokers, 36%; diabetes mellitus, 11%; hypertension, 53%; pulse pressure, 59±19 mm Hg; ABI, 0.60±0.14; and ACD, 389±165 m.


**Statistics**

Values are expressed as mean±SD or median and interquartile ranges. Statistical differences were evaluated with the Wilcoxon test for comparison between 2 groups, the Kruskal-Wallis test for comparison between >2 groups, or the χ² test. PAD patients were stratified for L-ascorbic acid levels with the lower reference limit (28.4 μmol/L) and the limit for vitamin C deficiency (11.4 μmol/L). The median CRP and fibrinogen levels were used to categorize PAD patients above and below these levels. Correlations between data were examined by Spearman rank correlation coefficients. Stepwise multivariate analysis was performed with MEDCALC and SPSS 9.0 software. Probability values <0.05 were considered statistically significant.

**Vitamin C Levels**

Table 2 summarizes biochemical data of the study groups. Serum L-ascorbic acid concentrations were comparable among healthy subjects and hypertensives without PAD but were much lower in the PAD group (P<0.0001). 52% of the PAD patients had L-ascorbic acid levels below the lower reference limit (28.4 μmol/L), and 14% showed biochemical evidence of vitamin C deficiency (<11.4 μmol/L). In the latter subgroup of patients, serum alkaline phosphatase activity was very low compared with PAD patients with L-ascorbic acid levels ≥11.4 μmol/L (Figure 1): 21.3 (9.1 to 45.4) U/L versus 64.8 (50.0 to 76.7) U/L, respectively (P<0.0001).

Smoking was comparable between the study groups and was associated with ~40% lower serum L-ascorbic acid concentrations in PAD patients (P=0.0005, Table 3), hypertensives (P=0.0013), and healthy subjects (P=0.0002). No significant associations of vitamin C levels with age, sex, body mass index, physical activity, HbA1c, or blood pressures or smoking were observed in the study groups. Serum L-ascorbic acid concentration correlated with vitamin C intake only in healthy subjects (r=0.297, P=0.004) and hypertensives without PAD (r=0.261, P=0.006).

**Inflammatory Markers**

Biochemical markers of inflammation (CRP, fibrinogen) were higher in PAD patients than in the other study groups (P<0.005, Table 2). In the PAD group, serum CRP correlated with plasma fibrinogen (r=0.600, P<0.0001); no significant associations were observed with age, sex, body mass index, physical activity, HbA1c, blood pressures, or smoking status of the PAD patients. Serum CRP and plasma fibrinogen were less elevated in PAD patients taking aspirin (Table 3).

Serum L-ascorbic acid concentration in PAD patients showed a negative correlation with serum CRP (r=−0.742, P<0.0001) (Figure 2) and plasma fibrinogen (r=−0.387, P<0.0001) levels. After adjustment for smoking and aspirin intake, a relative risk for vitamin C deficiency of 1.68 (95% CI, 1.27 to 2.21) was calculated among PAD patients with serum CRP concentration >4.80 mg/L (median value). Serum L-ascorbic acid levels in the PAD subgroup with CRP concentration ≤4.80 mg/L were comparable to those in hypertensives without PAD and healthy subjects.

**Results**

Table 2 shows box-and-whisker plots of serum alkaline phosphatase activity in PAD patients (n=85) according to serum L-ascorbic acid concentration, categorized by lower reference limit (28.4 μmol/L) and limit for subclinical vitamin C deficiency (11.4 μmol/L). Boxes show median and quartiles; whiskers are 5th and 95th percentiles.

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### Table 2. Vitamin C Levels, Inflammatory Markers, Lipids, and Diabetes Control

<table>
<thead>
<tr>
<th></th>
<th>Healthy Subjects (n=113)</th>
<th>Hypertension Without PAD (n=106)</th>
<th>PAD (n=85)</th>
<th>Wilcoxon Test* (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Ascorbic acid, μmol/L</td>
<td>51.7 (42.6–63.5)</td>
<td>49.6 (38.1–62.5)</td>
<td>27.8 (15.8–42.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>2.51 (0.78–4.16)</td>
<td>2.32 (1.28–3.58)</td>
<td>4.80 (2.00–9.55)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fibrinogen, g/L</td>
<td>3.12 (2.35–3.86)</td>
<td>3.64 (2.99–4.84)</td>
<td>4.95 (3.99–5.73)</td>
<td>0.002</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.21 (4.58–6.20)</td>
<td>5.22 (5.15–6.55)</td>
<td>5.78 (5.19–6.87)</td>
<td>0.003</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.52 (1.21–1.82)</td>
<td>1.32 (1.15–1.62)</td>
<td>1.04 (0.79–1.30)</td>
<td>0.0004</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.47 (2.51–4.04)</td>
<td>3.60 (2.98–4.22)</td>
<td>4.30 (3.60–5.05)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.11 (0.88–1.71)</td>
<td>1.48 (1.05–1.92)</td>
<td>2.20 (1.27–2.86)</td>
<td>0.0003</td>
</tr>
<tr>
<td>HbA1c † %</td>
<td>...</td>
<td>7.6 (6.4–9.3)</td>
<td>7.4 (7.0–8.8)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are median (interquartile range).

*Kruskal-Wallis test: P<0.05 for L-ascorbic acid, inflammatory parameters, and lipids. Subtesting PAD vs hypertension without PAD (Wilcoxon test).

†Hemoglobin A1c in diabetic patients (20 hypertensives without PAD, 14 PAD patients).
Serum Lipids

Serum concentrations of total cholesterol, LDL cholesterol, and triglycerides were higher \((P<0.005)\) among PAD patients, whereas HDL cholesterol levels were lower \((P<0.0005)\) compared with the other groups (Table 2). In the PAD group, serum HDL cholesterol positively correlated with serum L-ascorbic acid \((r=0.362, P=0.0001)\) and negatively with serum CRP \((r=-0.437, P=0.003)\). Serum L-ascorbic acid concentration did not correlate with other lipids (total and LDL cholesterol, triglycerides).

L-Ascorbic Acid Stability

In serum from male nonsmokers (15 PAD patients, 10 healthy control subjects), vitamin C (Fluka) was added to increase its concentration by 114 \(\mu\text{mol/L} (20 \text{ mg/L})\). The sera were then incubated at 37°C for 4 hours and L-ascorbic acid concentrations were measured (in triplicate) at baseline and at the end of the experiment. Baseline L-ascorbic acid concentrations in the spiked sera were 127.5 \(\pm 5.8 \mu\text{mol/L (PAD)}\) and 132.4 \(\pm 6.2 \mu\text{mol/L (control subjects)}\). After 4 hours, the decrease of L-ascorbic acid concentration was significantly higher in sera from PAD patients \((57 \% \text{ (PAD)}, 40 \% \text{ (control subjects)})\) \((P=0.0005)\). The L-ascorbic acid decrease was even more striking \((68 \% \text{ (PAD)})\) in serum from PAD patients with high CRP concentration \((8.1 \text{ to } 12.3 \text{ mg/L})\), whereas depletion of the vitamin in patient sera with lower CRP level \((1.5 \text{ to } 4.2 \text{ mg/L})\) was not significantly different than in control sera (Figure 3).

Vascular Investigations

Table 4 illustrates the effect of vitamin C levels and inflammation on ABI and ACD. PAD patients with serum L-ascorbic acid concentrations <28.4 \(\mu\text{mol/L} (\text{lower reference limit})\) were characterized by a lower ABI \((P=0.0007)\) and a shorter ACD \((P=0.0011)\). These differences were significant in both smoking and nonsmoking patients. Elevated inflammatory markers were also associated with low ABI and ACD; no effects of serum lipids were observed. ABI correlated with serum L-ascorbic acid \((r=0.406, P=0.001)\), serum CRP \((r=-0.429, P=0.0001)\), and plasma fibrinogen \((r=-0.397, P=0.009)\). Similarly, ACD correlated with serum L-ascorbic acid \((r=0.552, P=0.0001)\), serum CRP \((r=-0.476, P<0.0001)\), and plasma fibrinogen \((r=-0.426, P=0.006)\).

Pulse pressure of PAD patients showed a weak negative correlation with serum L-ascorbic acid \((r=-0.247, P=0.048)\). In stepwise multivariate analysis \((r=0.798, P=0.0001)\) for the model), only CRP, smoking, and ACD were significantly related to vitamin C levels in PAD (Table 5).

Discussion

We found that circulating vitamin C (L-ascorbic acid) concentrations are depleted in PAD patients, whereas levels of the antioxidant are comparable between hypertensives without PAD and healthy subjects. Vitamin C is the most effective defense against free radicals in the blood and is the first antioxidant to be used up during oxidative stress, thereby sparing other endogenous antioxidants.\(^{10,11}\) Therefore, our finding suggests a higher oxidative stress in patients with
Vitamin C depletion affects the functional state of the peripheral circulation, as evidenced by low ACD. Smoking has been demonstrated to affect walking distance, but we found that the relation between vitamin C depletion and low ACD was also consistent among nonsmoking PAD patients. A number of studies have compared the reproducibility of the initial and absolute claudication distance, with most demonstrating that ACD is more reproducible and therefore presumably the more appropriate measurement to use as a primary end point. ACD also has the theoretical justification that it probably more truly represents real life, in which the patient is likely to walk even after the first appearance of claudication discomfort.

In stepwise multivariate analysis, ACD is a determinant of vitamin C concentration in PAD patients, whereas other vascular parameters (ABI, pulse pressure) failed to enter the model. We found that low ABI is related to inflammation, similar to what has been observed in previous studies. The question arises whether antioxidant vitamin supplements would be useful to address reduction of oxidative stress in PAD. A randomized controlled trial has suggested that antioxidants may prevent cardiovascular events in PAD patients but do not improve lower limb function. Recent trials showed no benefit of vitamin E in the treatment for intermittent claudication. An ongoing multicenter European trial, the Critical Leg Ischemia Prevention Study (CLIPS), investigates the effectiveness of low-dose aspirin and antioxidant vitamins (vitamin E, vitamin C, β-carotene) with a 2×2 factorial design. However, vitamin C administered as a dietary supplement to humans has been shown to exhibit pro-oxidant properties, which may give rise to paradoxical effects in clinical intervention trials.

Summary

Vitamin C concentrations are low in PAD and are associated with inflammation and the patient’s functional state. Low-
grade inflammation in atherosclerosis may be associated with oxidative stress and the resultant decrease in antioxidants such as vitamin C. Future studies attempting to relate circulating vitamin C concentrations to disease occurrence should include in their analysis a marker of inflammatory response such as CRP.

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