START Trial

A Pilot Study on STimulation of ARTeriogenesis Using Subcutaneous Application of Granulocyte-Macrophage Colony-Stimulating Factor as a New Treatment for Peripheral Vascular Disease

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Background—Granulocyte-macrophage colony-stimulating factor (GM-CSF) was recently shown to increase collateral flow index in patients with coronary artery disease. Experimental models showed beneficial effects of GM-CSF on collateral artery growth in the peripheral circulation. Thus, in the present study, we evaluated the effects of GM-CSF in patients with peripheral artery disease.

Methods and Results—A double-blinded, randomized, placebo-controlled study was performed in 40 patients with moderate or severe intermittent claudication. Patients were treated with placebo or subcutaneously applied GM-CSF (10 μg/kg) for a period of 14 days (total of 7 injections). GM-CSF treatment led to a strong increase in total white blood cell count and C-reactive protein. Monocyte fraction initially increased but thereafter decreased significantly as compared with baseline. Both the placebo group and the treatment group showed a significant increase in walking distance at day 14 (placebo: 127±67 versus 184±87 meters, P=0.03, GM-CSF: 126±66 versus 189±141 meters, P=0.04) and at day 90. Change in walking time, the primary end point of the study, was not different between groups. No change in ankle-brachial index was found on GM-CSF treatment at day 14 or at day 90. Laser Doppler flowmetry measurements showed a significant decrease in microcirculatory flow reserve in the control group (P=0.03) and no change in the GM-CSF group.

Conclusions—The present study does not support the use of GM-CSF for treatment of patients with moderate or severe intermittent claudication. Issues that need to be addressed are dosing, the selection of patients, and potential differences between GM-CSF effects in the coronary and the peripheral circulation. (Circulation. 2005;112:1040-1046.)

Key Words: angiogenesis ■ collateral circulation ■ growth substances ■ leukocytes ■ peripheral vascular disease

Arterial occlusion, either acute or chronic, is a final event in the natural course of atherosclerotic disease. In the coronary arteries, such an occlusion may cause refractory angina pectoris, myocardial infarction, or death. However, numerous cases have been documented in which arterial occlusion in the coronary arteries is compensated by augmentation of the capacity of the collateral circulation and subsequent restoration of blood flow to jeopardized myocardial territories.1,2

In the peripheral circulation, arterial occlusion may cause intermittent claudication and in some instances will lead to critical leg ischemia and/or limb loss. However, the compensatory mechanisms in the peripheral circulation are more efficient than in the coronary circulation. In a large proportion of patients with peripheral arterial disease (PAD), the collateral circulation compensates over time almost completely for the impaired tissue perfusion. Nevertheless, a cohort of patients remains in whom symptomatic PAD progresses despite natural compensation, exercise training, and risk factor modulation. In these cases, interventional therapies such as bypass surgery or percutaneous transluminal angioplasty are performed. These interventions show relatively
high rates of reocclusion. Thus, a need exists for alternative, preferentially pharmacological, strategies for symptomatic and functional improvement. Cilostazol is the first substance that has been shown to increase walking distance in patients with PAD, although the exact mechanism of action is unknown. Another potential new treatment modality is the stimulation of arteriogenesis—that is, the development of large collateral conductance arteries. Over the past few years, several substances were shown to induce arteriogenesis in experimental models. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been used in the clinical setting for many years now to treat leucopenia in patients who underwent chemotherapy. GM-CSF also showed a strong proarteriogenic efficacy in rodents. In human beings, Seiler et al reported for the first time the stimulation of arteriogenesis using GM-CSF. In a small, randomized study, he found that coronary collateral flow index was increased in patients with chronic coronary artery disease (CAD), directly on a 14-day treatment with GM-CSF. In addition, GM-CSF treatment led to a decrease in ST-segment changes and episodes of angina during balloon occlusion at the end of the treatment period. In the present study, the effects of GM-CSF were tested for the first time in a group of patients with moderate or severe intermittent claudication (Rutherford grade I, category 2 or 3). Change in walking distance directly after the 14-day treatment served as primary end point.

Methods

Detailed information on the design of the STimulation of ARTerogenesis (START) study has been published. In brief, 40 patients with moderate or severe claudication and a walking distance repeatedly below 200 meters were included. Patients were recruited from the Rijnland hospital (n=10) and the Academic Medical Center Amsterdam (n=20) in the Netherlands and from the University Hospital Freiburg (n=10) in Germany. The presence of a severe occlusion had to be documented by Duplex or angiography, and all patients were candidates for bypass surgery or percutaneous transluminal angioplasty. Exclusion criteria were clinical or laboratory signs of chronic or acute inflammation, previous or current history of neoplasm, diabetes, pregnancy or preserved child-bearing capabilities, and refusal or inability to give informed consent.

Patients were randomly assigned to treatment with either placebo or subcutaneously applied rhGM-CSF in a dosage of 10 μg/kg every other day for a period of 14 days. rhGM-CSF was given either as Leukine (Berlex) or as Leucomax (codistributed by Schering-Plough and Novartis). The use of different suppliers of rhGM-CSF was an unplanned deviation from the protocol, caused by a sudden termination of distribution of Leucomax by Novartis and Schering-Plough per December 1, 2002, throughout Europe. Random assignment was performed by telephone from a central randomization list.

At day 0, day 14, and day 90, walking distance and ankle-brachial index (ABI) were assessed. At day 0, 2, 4, 6, 8, 10, 12, 14, and 90 blood samples were taken to determine total leukocytes, differentiated blood count, creatinine, C-reactive protein, SGOT, SGPT, albumin, triglycerides, total cholesterol, VLDL, LDL, and HDL.

During the course of our study, it was reported by several groups that stem cells potentially are involved in arteriogenesis. We therefore decided to perform CD34+ stem cell measurements in the remaining GM-CSF-treated patients. This resulted in a subset of 7 patients in whom the number of CD34+ cells in peripheral blood was determined by flow cytometric analysis. A sample of 100 μL blood in EDTA was stained with 5 μL of PE-conjugated mouse anti-CD34 MoAb (HPCA-2; Becton Dickinson, San Jose, Calif). In addition, cells were stained with 5 μL of mouse anti-CD16 and anti-CD66 MoAb (Becton Dickinson). PE-conjugated mouse IgG1 MoAb (BD) was used as isotypic control. After incubation at 4°C for 15 minutes in a light-protected area, red blood cells were lysed with lysing solution (FACS, BD) containing 0.83% ammonium chloride at room temperature for 10 minutes in a light-protected area and washed twice with PBS containing 0.1% azide. Analysis was performed on a fluorescence-activated cell sorter (FACScan, Becton Dickinson). A gate was established to include all nucleated cells and to exclude platelets and red blood cells by use of the forward and 90° light scatter. A second gate was established to include only CD45+ cells with the side scatter. The number of events counted was 100,000. The number of bright CD34+ cells with low side scatter was then determined. The percentage of CD34+ cells was calculated by subtracting the number of cells stained with the isotypic control from the number of cells stained with the anti-CD34 antibody and dividing by the number of nucleated cells counted in the first window. White blood cell counts were obtained with an electronic cell counter (Cell Dyn 4000). The number of CD34+ cells was calculated from the number of white blood cells and the percentage of CD34+ cells.

In 30 of 40 patients (those included in the Rijnland Hospital and the Academic Medical Center [AMC]), Laser Doppler flowmetry was performed. This method to noninvasively measure local skin perfusion has proven to be useful as a diagnostic tool in patients with peripheral arterial disease. In the device emits laser light with a wavelength of 780 nm, which penetrates the skin to ~1.5 mm. When reflected by moving particles (mainly erythrocytes), the light undergoes a frequency shift that is proportional to the number and velocity of the moving particles. This flow is expressed in perfusion units (PU), investigated in the supine position after acclimatization for 15 minutes in a temperature-controlled room. The laser Doppler (Periflux 5000, Perimed) probes were attached to the plantar side of the great toes of both legs. A data-acquisition system was used (AcqKnowledge III and MP 100WSW, Biopac System Inc) for the recording and off-line analysis of the data. First, measurements were performed at rest (in volts), which takes 5 to 10 minutes to reach a stable value. Subsequently, a 3-minute arterial occlusion was induced by means of suprasystolic (200 mm Hg) inflation of a cuff around the ankle. During this period, the laser Doppler value reached a nearly zero value, also known as the biological zero. This value was subtracted from the other flow parameters measured, as it does not represent blood flow. After release of the cuff, the postocclusive reactive hyperemia response was recorded, which yields the peak flow (in volts) and time to peak flow (PF, in seconds) parameters. The difference between PF and rest flow (P–RF) can then be calculated, which gives information about the reserve capacity of the local microcirculatory vessels. This parameter is independent of the baseline flow, which is known to be variable.

Occurrence of side effects was documented every other day during the treatment period and at day 90. At days 0, 14, and 90, 4-field fundus photography was performed, including the posterior pole and midperiphery of each eye. An experienced retinal specialist read the fundus photographs in a masked fashion.

For the power calculation, we estimated the placebo effect at 40%. We aimed for a 2.5-fold larger increase in walking distance in the GM-CSF–treated group (100%). This 2.5-fold increase as compared with the placebo group was based on earlier published data from the Therapeutic Angiogenesis with recombinant Fibroblast growth Factor-2 for Intermittent Claudication (TRAFFIC) trial. On the basis of our own data from 3500 patients who visited the vascular laboratory in the AMC, we expected a baseline walking distance of 87 meters, with a standard deviation of 54 meters. To have an 80% power, we calculated a total sample size of 36 patients. With an expected 10% dropout rate, we decided to include 40 patients. For primary analysis, an independent-samples t test was used. The primary analysis excluded patients who were not available for follow-up at day 14. A 1-sample t test was applied to test whether the change in walking distance was statistically significant within each treatment group. Statistical significance was assumed at P<0.05. Analyses involving the secondary end points were carried out as subsidiary analyses. For comparison of end points at day 90 between the GM-CSF–treated and the placebo group, patients who received a
revascularization procedure before day 90 were excluded. For laser Doppler flowmetry (LDF) measurements, a nonparametric Friedman test was performed. Differences between baseline and 14 days and between baseline and 90 days were compared by means of the Wilcoxon test. Differences between patients receiving placebo and those receiving GM-CSF were tested with the use of the Mann-Whitney U test.

**Results**

The distribution of the 40 patients over the 3 centers was well balanced: 10 versus 10 at the AMC, 4 versus 6 at the Rijnland hospital, and 6 versus 4 at the Freiburg University hospital.

Of the 20 patients randomly assigned to the GM-CSF group, in 3 patients, treatment was discontinued because of severe side effects. In 1 patient, sensations of chest pain, shortness of breath, and hypotension occurred 1 hour after the first GM-CSF injection. Such anaphylactic reactions have been described occasionally for GM-CSF. This patient was withdrawn from the study. Two other patients decided to discontinue treatment because of the occurrence of severe chest and/or muscular pain. ECG and laboratory testing did not reveal cardiac ischemia. No long-term effects were observed after abrogation of treatment in these patients.

One patient in the placebo group was withdrawn from the study because of a pulmonary embolism at day 6. These patients were excluded from walking distance and ABI analysis at day 90. For patients who received no intervention therapy between day 14 and day 90, the increase in walking distance prevailed at day 90 in both the placebo group and the GM-CSF–treated group (placebo: 127 ± 67 meters versus GM-CSF: 120 ± 64 meters, P = 0.01). No significant difference in change in pain-free walking distance at day 14 was observed between the placebo group and the GM-CSF treatment group (placebo: 30 ± 43 meters versus GM-CSF: 28 ± 49 meters, P = 0.89). No side effects of GM-CSF such as nausea or muscle pain were reported as a specific cause to abrogate exercise at day 14. All patients abrogated exercise because of occurrence of claudication in the most affected leg.

A total of 7 patients (placebo, n = 4; GM-CSF, n = 3) underwent interventional therapy between day 14 and day 90. These patients were excluded from walking distance and ABI analysis at day 90. For patients who received no interventional therapy between day 14 and day 90, the increase in walking distance at day 14 was observed between the placebo group and the GM-CSF–treated group at day 90 (Figure 2).

**Ankle-Brachial Index**

Depending on whether claudication was unilateral or bilateral, 1 or 2 values of ABI per patient were available for analysis. Only ABIs with a value at day 0 of < 0.95 were included for repeat analysis. At day 14, this resulted in a total of 22 repeat ABI measurements from the GM-CSF treatment group and a total of 27 repeat ABI measurements from the placebo group. A small but significant increase in ABI from day 0 to day 14 was found in the placebo group (day 0, 0.59 ± 0.16 versus day 14, 0.62 ± 0.18; no unit, P = 0.04). No significant changes in ABI were found on GM-CSF treatment (day 0, 0.65 ± 0.18 versus day 14, 0.60 ± 0.20; no unit, P = 0.27). At day 90, a total of 17 repeat ABI measurements were available from the GM-CSF treatment group and a total of 20 from the placebo group. No significant changes in ABI from day 0 to day 90 were found either in the placebo group (day 0, 0.58 ± 0.14 versus day 90, 0.62 ± 0.17; no unit, P = 0.17) or in the GM-CSF treatment group (day 0, 0.61 ± 0.18 versus day 14, 0.63 ± 0.17; no unit, P = 0.42). Also, no statistical significant difference was found at day 90 for the direct comparison between the GM-CSF treatment group and the placebo group.

**Laser Doppler Flowmetry Measurements**

The results of the LDF parameters at the 3 different time points are shown in Table 2.

LDF was performed in 30 patients. Three patients in whom GM-CSF was discontinued because of side effects were...
excluded from analysis, leaving a total of 11 GM-CSF–treated patients as well as 16 placebo-treated patients available for analysis. Three patients from the placebo group as well as 3 patients from the treatment group received bypass surgery between day 14 and day 90.

At baseline, RF and PF were significantly higher in the placebo group than in those treated with GM-CSF (P<0.003 and P=0.015, respectively). This difference disappeared by both follow-up time points. In the patients treated with GM-CSF, no significant differences in time were observed in any of the LDF parameters. In the patients treated with placebo, the TiPF increased significantly (P=0.030), whereas the P-RF decreased significantly over time (P=0.024), indicating a decrease in microcirculatory reserve capacity over time, as opposed to the GM-CSF–treated group. No differences for any of the parameters were observed over time in asymptomatic legs.

**Side Effects**

Less severe but frequently occurring side effects in the treatment group were skin rash, fever, headache, and perspiration (Table 3). The repeated eye examinations by fundus photography showed no induction of retinopathy or other ophthalmologic disorders in any of the patients. No acute cardiac events were reported in any of the patients during the 90-day follow-up period.

**Blood Sampling**

Treatment with GM-CSF led to a gradual increase in total number of leukocytes over the treatment period, resulting in significant differences as compared with day 0 starting at day 10. The percentage of monocytes was significantly increased at day 4 (7.8±1.8% versus 11.0±5.0%, P=0.02). Over time, the percentage of monocytes decreased, resulting in a significantly lower percentage of monocytes at days 12 and 14, as compared with day 0 (7.8±1.8% versus 5.6±2.7% and 5.7±2.5% respectively, P=0.01 for both comparisons). A slight but significant decrease in the percentage of basophiles was found at days 12 and 14. The percentage of granulocytes remained unchanged. Strong changes were found for the percentage of eosinophiles. Their percentage increased from 2.4±1.4% at day 0 to 9.6±4.9% at day 14 (P=0.000002) (Figure 3A). The number of CD34-positive stem cells was significantly increased at days 6 and 8 after initiation of GM-CSF treatment. Thereafter, their number decreased again, returning to baseline values at day 14 (Figure 3B). A rapid increase was found for C-reactive protein (CRP). At day 2, CRP levels were increased from 6.1±3.3 to 43.9±32.6 (P=0.000002) (Figure 3C). At day 90, all of the above-mentioned parameters had returned to baseline levels. In the placebo

<table>
<thead>
<tr>
<th>TABLE 2. Laser Doppler Flowmetry</th>
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<tbody>
<tr>
<td><strong>Peak Flux (V)</strong></td>
</tr>
<tr>
<td><strong>Placebo</strong></td>
</tr>
<tr>
<td>(n=16)</td>
</tr>
<tr>
<td>Baseline</td>
</tr>
<tr>
<td>14 d</td>
</tr>
<tr>
<td>90 d</td>
</tr>
</tbody>
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In placebo patients, peak flux as well as peak minus rest flux decreased over time. In the GM-CSF treatment group, these values remained stable and showed a tendency to increase at day 14.

*P<0.05 compared with baseline.

**Figure 2.** Walking distance in meters. Change in walking time did not differ between groups, either at day 14 (GM-CSF: n=17, placebo: n=19) or at day 90 (GM-CSF: n=14, placebo: n=15). *P<0.05.

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**TABLE 3. Side Effects**

<table>
<thead>
<tr>
<th>Placebo (n=20)</th>
<th>rh-GM-CSF (n=20)</th>
</tr>
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<tbody>
<tr>
<td>Skin rash, %</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>25.2</td>
</tr>
<tr>
<td>Muscle pain, %</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>21</td>
</tr>
<tr>
<td>Fever, %</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>14.3</td>
</tr>
<tr>
<td>Headache, %</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>14.3</td>
</tr>
<tr>
<td>Loss of appetite, %</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5.9</td>
</tr>
<tr>
<td>Perspiration, %</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>6.7</td>
</tr>
<tr>
<td>Nausea, %</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>1.7</td>
</tr>
</tbody>
</table>

The use of GM-CSF led to a large percentage of observed side effects during treatment time. Most reported side effects were skin rash, muscle pain, and fever.
group, all above-mentioned parameters remained unchanged during treatment time as well as at follow-up.

**Discussion**

In the present study, we determined the effects of subcutaneously applied GM-CSF on maximal walking distance in patients with moderate to severe claudication. This is the first randomized, placebo-controlled study aiming specifically at the stimulation of arteriogenesis in patients with PAD. A 14-day treatment schedule did not lead to an increase in maximum walking distance either directly after treatment or at 90 days of follow-up. Also, other parameters such as ABI showed no increase on GM-CSF treatment.

The only observed significant effects of treatment were a tendency to an increase in the peak flow and the peak-minus-rest flow in the GM-CSF group as compared with a significant decrease in the control group. This might indicate a beneficial effect of GM-CSF on the microcirculation, probably secondary to improved endothelial function. However, the clinical relevance of these findings in the present patient population is not clear.

The observed placebo effect was large. In the placebo group, an approximate 50% increase in walking distance was observed at day 14. At day 90, the mean walking distance in the placebo group was still ~50% larger as compared with day 0. Because no change of ABI was observed between day 0 and day 90, it can be concluded that this increase in walking distance is a real placebo effect and is not secondary to actual macrocirculatory changes, for example, as the result of training effect or natural arteriogenesis. Similar placebo effects have been observed in most trials on stimulation of collateral artery growth.

Monocytes are target cells of proarteriogenic strategies because these cells provide growing collateral arteries with the necessary growth factors and cytokines. It is also claimed that the often-debated endothelial progenitor cells are derived from circulating monocytes. Somewhat to our surprise, GM-CSF led to only a temporary increase in monocytes. After day 4, a gradual decrease was observed, finally leading to a level below baseline level both in absolute numbers as well as in percentage of total leukocytes. A similar trend was seen in CD34+ stem cells, indicating that one of the potential explanations for failure of the current treatment strategy is a suboptimal dosing scheme. Potentially, repetitive short periods of treatment are more effective in raising the number of circulating monocytes and stem cells.

Probably, patients with PAD are more difficult to treat than patients with CAD. In PAD, ~60% of patients compensate adequately for vascular obstruction by mechanisms of natural arteriogenesis and changes in metabolism. Moreover, the time between first presentation and interventional therapy is generally long. Thus, patients who were eligible for the present study represent a cohort of patients who have a deficiency in their innate response to vascular obstruction and have atherosclerotic disease for a prolonged period of time. In contrast, in patients with CAD, severe coronary stenoses are left unnoticed only in a minority of patients (although several cases are reported in the literature), and time between first presentation and interventional therapy is generally much shorter than in PAD. This might explain the difference in outcome between the present study and the study by Seiler et al. Another important difference between our study and the study by Seiler is the method to detect collateral artery growth. Seiler used a very sensitive method of intracoronary-derived pressure measurements. Such techniques are not validated for the peripheral circulation, and therefore in our protocol, we were bound to the presumably less sensitive and less objective end point of walking distance. Potentially more sensitive measurements of collateral flow in the peripheral circulation such as MRI flow measurements or invasive pressure and flow measurements would be of great value for future trials in patients with PAD.

Several clinical trials on stimulation of vascular growth, either angiogenesis or angiogenesis/arteriogenesis, were con-
duced in the past few years. Most of these trials included patients with CAD. A minority of studies focused on PAD (for review, also see Schirmer et al14). The first published large, randomized, placebo-controlled trial on stimulation of collateral artery growth in patients with PAD was the TRAFFIC trial.12 In this trial, fibroblast growth factor-2 (FGF-2) protein was intra-arterially infused into the lower extremities of 190 patients with PAD with moderate or severe intermittent claudication. FGF-2 is known for its angiogenic properties but also displays proarteriogenic properties. Patients were randomly assigned to either placebo or to single-dose (30 μg/kg) or double-dose (60 μg/kg) treatment. An increase was found in peak walking time at 90 days (primary end point) in the single-dose group only after secondary intention-to-treat analysis. In the double-dose group, no increase was detected. Also, the subsequent Angiogenesis with Vascular Endothelial Growth Factor in Peripheral Arterial Disease (RAVE) trial, assessing the effects of VEGF in patients with PAD with intermittent claudication, did not meet the high expectations.15 VEGF mainly stimulates angiogenesis and has only a weak proarteriogenic potential. In this trial, 105 patients with intermittent claudication were treated with intramuscular injections of adenoviral VEGF 121. Apart from enhanced peripheral edema, no significant increase in walking time, ABI, or quality of life could be observed. Thus, the present study is the third randomized, placebo-controlled study in patients with PAD in which no increase in walking capacity was found on growth factor therapy.

Nevertheless, growth factor therapy still constitutes a promising therapeutic strategy to treat patients with atherosclerotic disease. A large body of preclinical data is available that unequivocally shows that arteriogenesis alleviates the consequences of arterial obstruction. Several factors involved have been identified, and cellular and molecular mechanisms underlying this process have been unraveled not completely but to a large extent.16–22 The biggest challenge will be the identification of the most optimal factor or combination of factors, as well as delivery platforms (gene therapy versus protein therapy). Also, choice of end points and choice of target population will decide on failure or success of future clinical studies.

Limitations of the Study
Inadequate sample size is a potential problem that might mask smaller beneficial effects of GM-CSF treatment. It should be noted, though, that the observed differences between the 2 groups in either walking distance or ABI are practically zero. Although the present data do not exclude the possibility of a small treatment benefit, they certainly do not support the hypothesis that GM-CSF is beneficial to patients with intermittent claudication.

The change of GM-CSF supplier was an unwanted deviation from the protocol. Data on pharmacokinetics as provided by the manufacturers are comparable. In a subsidiary analysis, no significant differences were found for walking distance, ABI, or laboratory parameters as derived from patients treated with either Leucomax (Novartis/Schering-Plough) or Leukine (Berlex).

The blinding process in the present study was hampered by the strong side effects. In our study, we have sought to prevent observer bias by having an observer without knowledge of the side effects performing the exercise test and the ABI and LDF measurements. Obviously, the patient cannot be prevented from observing his or her own side effects. On the other hand, it can also be argued that the strong side effects had a negative impact on training exercise between day 0 and day 14. Placebo patients potentially did perform more exercise between day 0 and day 14. It should be noted though, that patients were not instructed to perform extra exercise in this period, and, even more importantly, all these patients had been refractory to structured training programs in the past. In any case, such problems with blinding and also the previously mentioned large placebo effect underscore the strong demand for reliable objective end points.

Acknowledgments
This study was supported by Netherlands Heart Foundation, grant 2002B076.

References


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Circulation. 2005;112:1040-1046; originally published online August 8, 2005;
doi: 10.1161/CIRCULATIONAHA.104.529552

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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