Therapeutic arteriogenesis: from experimental observations towards clinical application [cum laude]
van Royen, N.
DESIGN OF THE START-TRIAL: STIMULATION OF ARTERIOGENESIS USING SUBCUTANEOUS APPLICATION OF GMCSF AS A NEW TREATMENT FOR PERIPHERAL VASCULAR DISEASE. A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED TRIAL

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CHAPTER 12

Abstract
Background: Peripheral arterial disease (PAD) affects a large percentage of the elderly population and, apart from risk factor modulation, standard invasive treatment consists of bypass surgery or percutaneous transluminal angioplasty. However, symptomatic recurrence rates are high for both procedures and a substantial part of the patient population with peripheral arterial disease is not a candidate for invasive revascularization due to complexity of the lesion or co-morbidity. Therapeutic arteriogenesis has been proposed as an alternative treatment option. The present manuscript describes the design of the START-trial. This trial aims to determine the potential of the pro-arteriogenic substance GM-CSF to increase maximal walking time in patients with intermittent claudication.

Methods: A double-blinded, randomized, placebo-controlled study is performed in 40 patients with peripheral obstructive arterial disease Rutherford class 1, stage 2 or 3. Based on pharmacokinetic and toxicologic studies, a dose of 10 μg/kg will be used. Patients are treated for a period of 14 days on each consecutive day with the last injection applied on day 12. Primary endpoint will be the change in walking distance from day 0 to day 14 as assessed by exercise treadmill test. Secondary endpoints are ankle-brachial index at rest and after exercise, cutaneous microcirculatory alterations as assessed by laser-doppler fluxmetry and iliac flow and conductance as measured by magnetic resonance imaging.

Conclusion: The design of the START-trial, a placebo-controlled randomized, multi-center and double-blind study, is described. This study evaluates the effects of a 14-day treatment with subcutaneously administered GM-CSF in patients with intermittent claudication and will provide insights in the potential of therapeutic arteriogenesis via GM-CSF.
Introduction
The age-adjusted prevalence of peripheral arterial disease (PAD) is about 12%, of which approximately one third suffers from typical intermittent claudication. Despite secondary prevention and exercise, it is estimated that in about 30% of the cases PAD progresses until percutaneous or surgical intervention becomes a necessity. The mortality and morbidity of percutaneous transluminal angioplasty (PTA) or bypass-surgery are nowadays relative low although peri-operative infection of arterial reconstructions is still associated with high rates of mortality and limb loss. Moreover, a significant number of patients will be rehospitalized due to restenosis of the treated lesion or progression of other lesions. In a recently published study, a total of 46% of all patients required one or more re-interventions within 12 months after femorodistal bypass surgery. Similarly high restenosis rates have been reported after PTA, with actually very poor results for long lesions. Finally, the costs of these procedures is high, warranting the evaluation of alternative treatment strategies.

Pre-existing collateral vessels develop in non-ischaemic tissue upon arterial narrowing or occlusion and connect these regions with under-perfused vascular territories, a process referred to as arteriogenesis. In about 25% of patients with PAD, the peripheral circulation displays a natural ability to adapt very well to arterial obstruction leading to an actual diminishing of physical complaints and it is assumed that the improvement of symptoms in this group of patients is based on an increase of the capacity of the collateral circulation via arteriogenesis. However, in a large group of patients, arteriogenesis is not sufficient enough to restore blood flow and meet oxygen demand of regions distal of the arterial occlusion. A pharmacological therapy, stimulating collateral artery formation would be a valuable adjuvant or an alternative to PTA or bypass-surgery in this group of patients. Several growth factors display arteriogenic properties in experimental animal studies. In the present study we have selected GM-CSF as potential pro-arteriogenic factor. This factor has also been shown to have a pro-arteriogenic effect in an experimental setting. GM-CSF is already available as a pharmaceutical compound. It is used for example to treat leukopenia as observed after chemotherapy. This implies that extensive documentation is available on toxicity, side effects, dosage regimens etc. Another advantage of GM-CSF is the subcutaneous administration, avoiding cumbersome invasive procedures to deliver the substance. GM-CSF is also a good candidate because, in contrast to other angiogenic/arteriogenic factors, it exerts lipid-lowering and anti-atherogenic effects. Moreover, GM-CSF has been shown to inhibit tumor growth. This indicates that atherosclerosis or carcinogenesis are less likely to occur as unwanted side effects of arteriogenic therapy with GM-CSF. Finally, preliminary findings suggest that GM-CSF is effective in stimulating the development of the collateral circulation in patients with coronary artery disease. The primary outcome of the START-trial is the change in mean walking time from day 0 to day 14. In addition, ankle-brachial index is used for evaluation of peripheral dynamics. Monocytes are isolated and changes in expression profiles were determined with the use of gene-arrays. Finally, the feasibility of MRI derived

THE START-TRIAL
measurements of iliac flow reserve and conductance as potential new parameters to
detect changes in the capacity of the peripheral collateral circulation is determined.
For all patients, measurements are repeated at day 90, irrespective of the
performance of an invasive procedure between day 14 and day 90.

Methods

Study population
A total of 40 patients is selected to enter the study, randomly distributed over a
treatment and a control group. Patients are all candidates for PTA or bypass-surgery
and are considered eligible for the study if an obstructive lesion or severe stenosis
(PSV ratio >2.5 on duplex or diameter reduction >50% on angiography) is present in
the peripheral circulation, criteria for Rutherford stage 1, category 2 or 3 are fulfilled
and walking distance at a standard treadmill test examination is repeatedly below
200 meter. Furthermore, walking distance needs to be limited due to specific
symptoms of claudicatio intermittens and not due to shortness of breath, angina,
arthritis or other complaints not directly related to PAD. Patients are excluded in
case of previous, current or suspected malignancy, diabetes, pregnancy or preserved
childbearing capacities, clinical or laboratory signs of chronic or acute inflammation
and refusal or inability to give informed consent (table 1).

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<tr>
<th>Inclusion criteria</th>
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<tr>
<td>- symptomatic patients with PAOD, Rutherford Grade 1, category 2 or 3</td>
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<tr>
<td>- obstructive lesion or severe stenosis (PSV ratio &gt;2.5 on duplex or diameter reduction &gt;50% on angiography)</td>
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<td>- candidates for bypass operation or PTA</td>
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<table>
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<td>- clinical or laboratory signs of chronic or acute inflammation</td>
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<td>- pregnancy or preserved child bearing capabilities</td>
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<td>- refusal or inability to give informed consent</td>
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*Table 1: inclusion and exclusion criteria*
Patients receive appropriate medication and risk factor modulation is installed. Patients are recruited at the Academic Medical Center Amsterdam and the Rijnland Hospital Leiderdorp in the Netherlands as well as at the University Clinic Freiburg in Germany.

Study design and intervention
The study is conducted in accordance with the principles of the “Declaration of Helsinki” and is designed as a double-blinded, randomized and placebo-controlled trial comparing GM-CSF treatment with placebo in a 1:1 ratio. Patients are treated with rGM-CSF at a dosage of 10 μg/kg per injection (either Leukine, Berlex or Leucomax, Scheringh-Plough) or with placebo. The use of different suppliers of rGM-CSF was an unplanned deviation from the protocol, caused by a sudden termination of distribution by Schering-Plough per Dec. 1, 2002. Placebo consists of 0.1% albumin in aqua ad injectionem. Injections are subcutaneously applied at day 0, 2, 4, 6, 8, 10 and 12. In the weeks following termination of treatment, patients are seen by a physician and a decision is made whether or not to perform PTA or bypass surgery. Both physician and patient are still blinded to the medication at the time of this decision.

Hypothesis
In the current study, we test the hypothesis that repetitive subcutaneous application of GM-CSF increases maximal walking distance in patients with intermittent claudication, 14 days after initiation of treatment.

Measurements
Measurements are performed at baseline and day 14. The primary endpoints consist of maximum walking distance at day 14. Therefore, a treadmill walking test is performed with the speed set at 3.2 km/h and the angle at 8%. Additional secondary endpoints consist of pain-free walking distance and ankle-brachial index (ABI) at rest and after exercise. With the use of a pneumatic cuff and a continuous-wave doppler system, ABI is derived from the highest measured arm pressure divided by the highest measured leg pressure. ABI measurements are performed both at rest and directly following exercise. All measurements are repeated at day 90. Figure 1 shows a flow chart for the entire study.

In a sub-group of patients, skin microcirculatory perfusion is assessed by means of laser Doppler (Periflux 4001®, Perimed, Sweden) as previously described. The laser Doppler probe (PF 408 standard probe, Perimed) is attached to the pulp of the hallux using its probe holder and double-sided adhesive tape. LDF is measured on the pulp of the toes, since this area has a large number of arteriovenous anastomoses as opposed to the dorsum of the foot. Measurements are performed at rest with the patient in the supine position and the foot at heart level. In addition, LDF is recorded during reactive hyperemia, after a 3 min. arterial occlusion induced by inflating a cuff around the ankle. The biological zero flux (the basal LDF during arterial occlusion) is subtracted from all LDF values measured.
In another subset of patients, volume flow measurements are performed using non-contrast Magnetic Resonance Imaging (MRI) with 2D-phase contrast technique. Total inflow is measured at level of the A. Iliaca Communis, both at rest and during reactive hyperemia. To induce hyperemia, a pneumatic cuff is applied around the upper leg and inflated to 200 mmHg for 1 minute. Flow measurements are then performed directly following release of the cuff. Iliac flow reserve is calculated by dividing baseline flow and hyperemic flow. Volume flow measurements and ABI are combined to calculate conductance. Conductance is the reciprocal value of resistance and reflects the maximal flow capacity of a circulatory system. Formulas for iliac flow reserve and conductance are shown in figure 2. Feasibility of iliac flow reserve measurement was tested in a small group of healthy volunteers as well as patients suffering from unilateral PAD. Results of a representative subject from each group are shown in figure 3. Figure 4 shows the measurements of conductance of the non-affected normal leg versus the affected leg in a patient suffering from PAD.

Bloodsamples are collected at day 0, 2, 4, 6, 8, 10, 12 and 14 and at 3 months follow-up for measurements of total leukocytes, differentiated blood count, creatinine, C-reactive protein, SGOT, SGPT, albumin, triglycerides, total cholesterol, VLDL, LDL, HDL and homocystein.

Finally, repeat eye examinations are performed to exclude induction of retinopathy by rGM-CSF treatment. Therefore, a digital photo of the retina is performed under conditions of pupil dilation. Of each eye, two photos are made under opposite angles of 55 degrees and then scored for absence or presence of retinopathy. In case of presence of retinopathy, worsening, stability or improvement of disease are documented.

Sample size
Sample size is set at 40 patients. Placebo effect in this group of patients is approximately 40%. In the recently published TRAFFIC trial a 2.5 increase in walking distance was found as in patients treated with b-FGF as compared to the placebo group. We therefore aimed at a comparable effect of treatment in the present study. Mean walking distance in patients with Rutherford stage 2 or 3 is 87 meters with a standard deviation of 54 meters (own data, based on observations in 3,500 patients that visited the Vascular Laboratory in the AMC). A 2.5 increase in mean walking distance would result in 217.5 meters. Thus 20 patients per group were required (alpha: 0.95, 1-beta: 0.80).

Randomization
Patients are divided over the groups with the use of a computer generated randomization list in a 1:1 ratio. Randomization is primarily performed by BA and reported to JO for patients included in the Rijnland Hospital and to SHS for patients included in the University Clinic Freiburg.
Inclusion: 40 patients Rutherford 1. class 2 or 3

Randomization:

Day 0:
- Treadmill Walking Test
- Ankle Brachial Index

Treatment:
- Placebo day 0, 2, 4, 6, 8, 10 & 12
- GM-CSF, 10 µg/kg day 0, 2, 4, 6, 8, 10 & 12

Day 14:
- Treadmill Walking Test
- Ankle Brachial Index

Day 90:
- Treadmill Walking Test
- Ankle Brachial Index

Figure 1: Flowchart of the START-trial

Figure 2: Formulas for iliac flow reserve and conductance. Flow is assessed with MRI.
Figure 3: Figure 3A shows iliac flow data for a normal healthy control. An iliac flow reserve of about 2.2 is found. In a patient with unilateral PAD, iliac flow reserve is strongly reduced in the affected left leg. The non-affected leg still shows a relative normal hyperemic response (3B).

Conductance normal leg:
\[
\frac{100}{(100 \text{ mmHg} - 36 \text{ mmHg})} \times 6.03 \text{ ml/s} \times 60 = 9045 \text{ ml/min/100 mmHg}
\]

Conductance affected leg:
\[
\frac{100}{(100 \text{ mmHg} - 28 \text{ mmHg})} \times 2.23 \text{ ml/s} \times 60 = 185 \text{ ml/min/100 mmHg}
\]

Figure 4: Conductance is strongly reduced in the presence of extensive unilateral PAD as compared to the non-affected leg.
BA, JO and SHS were responsible for application of the substance but were not involved in data analysis or manuscript preparation. All other study personnel and participants were blinded to treatment assignment for the duration of the study.

**Statistics**
For primary analysis, an independent samples t-test was used to test whether the change in log-transformed maximal walking distance (between baseline and day 14) was different between the two treatment groups. The primary analysis excluded patients that were not available for follow-up. A one-sample t-test was applied to test whether the change in log-transformed walking distance was statistically significant within each treatment group. Statistical significance was assumed at $p < 0.05$. Analyses involving the secondary endpoints were carried out as subsidiary analyses.

**Discussion**
Our own experimental data on the arteriogenic potential of GM-CSF, in combination with the clinical data derived from the study by Seiler et al. in patients with CAD, prompted us to conduct these experiments in patients with PAD. All patients are candidates for PTA or bypass surgery. It was not our intention to withhold patients standard interventional therapy and therefore the decision to perform such therapy following the 14-day treatment period and assessment of our primary endpoint is left at the discretion of the patient and the treating physician. Patients as well as physicians are blinded to treatment during the complete follow-up period of 90 days. The present study is merely designed as a proof of the principle study whether the course of PAD can be modified pharmacologically via stimulation of collateral artery growth using subcutaneously applied GM-CSF.

Non-interventional treatment of PAD is mainly limited to risk factor modulation via smoking cessation, treatment of hyperlipidemia, anti-hypertensive drugs, anti-platelet drugs like aspirin and clopidogrel and the tight control of serum glucose. Cilostazol is currently the only drug available that actually improves functional status and maximal walking time although the exact working mechanism of this drug is not known.

Growth of collateral arteries upon arterial obstruction is a naturally occurring phenomenon in patients with both peripheral as well as coronary artery disease and the importance of collateral flow under ischaemic conditions has been recognized in both experimental and clinical studies. After birth, blood vessels can grow either via the process of angiogenesis or via the process of arteriogenesis. Angiogenesis is the formation of capillary networks via the sprouting of endothelial cells and is mediated via hypoxia and the release of hypoxia inducible factor-1 (HIF-1). The term arteriogenesis was introduced by Schaper and is now generally accepted as a process distinct from angiogenesis. Arteriogenesis is the proliferation of pre-existing arteriolar connections into functional collateral conduit arteries. According to the law of Hagen-Poiseuille, maximal flow depends on vessel diameter to the fourth potency, which means that small changes in vessel diameter result in large
changes in blood flow. This process is far more efficient in restoring bulk blood flow than the development of numerous small-diameter capillaries as observed during angiogenesis. The main stimulus for arteriogenesis is an increase in shear stress due to the development of a pressure gradient across the collateral pathways upon arterial occlusion. This increase in shear stress leads to an upregulation of adhesion molecules (ICAM, VCAM, Selectins) on the endothelium. Circulating monocytes are attracted and migrate into the vessel wall, giving rise to the production of various cytokines and growth factors.

In a large group of patients with PAD, the formation of collateral arteries is insufficient to restore blood flow and to meet oxygen demand of regions distal of a vessel occlusion under working conditions, giving rise to intermittent claudication. Therefore, positive modulation of collateral artery growth constitutes a promising concept for treatment of arterial occlusive diseases. However, although the first uncontrolled studies reported positive results, confirmation in randomized trials is still awaited. The TRAFFIC-trial showed beneficial effects for b-FGF treatment, but only in a secondary intention-to-treat analysis. Other randomized trials like the non-published VIVA-trial using VEGF or the AGENT and FIRST-trial using b-FGF were also negative with regard to their primary outcomes.

We have shown that intra-arterial infusion of GM-CSF for a period of one week caused a marked enhancement of collateral growth in a rabbit hindlimb model. In contrast to other arteriogenic substances, GM-CSF remains arteriogenically active when administered subcutaneously or intravenously since it acts upon circulating monocytes, increasing their life-span via inhibition of apoptosis. GM-CSF is already clinically available to treat chemotherapy-induced neutropenia and, moreover, it exhibits anti-atherogenic properties and inhibits tumor growth. Seiler showed that GM-CSF increases collateral flow index (ratio of distal and proximal pressure during maximal balloon inflation) in patients with coronary artery disease and this was actually the first randomized study showing pharmacological modulation of collateral artery growth as assessed with a validated and objective intracoronary-derived haemodynamic measurement.

Based upon pharmacokinetical and toxicological studies a dose of 10 µg/kg has been selected for current clinical applications and therefore we have chosen for the same dosage for this clinical study. Subcutaneous application was selected since it was shown to have an improved toxicity as well as efficacy profile as compared to intravenous administration.

This trial serves to determine whether subcutaneous treatment with GM-CSF has a beneficial effect on maximal walking distance of patients with intermittent claudication. The study is designed as a "proof-of-the-principal" study and therefore the walking distance at day 14 serves as the primary endpoint. In addition, new parameters are introduced that might serve as additive measurements to detect the influence of substances on collateral flow in the peripheral circulation.
References


