Chapter 9

Treatment with Intra-muscular Vascular Endothelial Growth Factor Gene Compared to Placebo for Patients with Diabetes Mellitus and Critical Limb Ischemia: A Double Blind Randomized Trial


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Human Gene Therapy, accepted for publication
ABSTRACT

Aims. Despite advances in re-vascularization techniques limb salvage and relief of pain cannot be achieved in many diabetic patients with diffuse peripheral vascular disease. Our objective was to determine the effect of IM administration of phVEGF\textsubscript{165} (VEGF gene containing plasmid) on Critical Limb Ischemia (CLI) compared to placebo (0.9% NaCl).

Results. A double blind placebo controlled study was performed in 54 adult diabetic patients with CLI. The primary endpoint was the amputation rate at 100 days. Secondary endpoints were a 15% increase in pressure indices (ABI/TBI), clinical improvement (skin, pain and quality of life [QOL]) and safety.

In patients (n=27) treated with placebo versus the phVEGF\textsubscript{165} treated patients (n=27) the following results were found: 6 amputations versus 3 (n.s.), hemodynamic improvement in 1 versus 7 ($P=0.05$), improvement in skin ulcers 0 versus 7 ($P=0.01$), decrease in pain 2 versus 5 (n.s.), and overall 3 versus 14 responding patients ($P=0.003$). No grade 3 and 4 adverse effects were seen in these patients.

Conclusions. This small, randomized gene therapy study, failed to meet the primary objective of significant amputation reduction. However, significant and meaningful clinical and hemodynamic improvement was found in patients treated with a VEGF\textsubscript{165} containing plasmid. There were no substantial adverse events.
OVERVIEW/ SUMMARY

In patients with diabetes mellitus and CLI surgical re-vascularization is often impossible due to the predominance of micro-vascular occlusions and these patients have limited options beyond amputations. Angiogenesis of the lower extremity remains therefore an important area of therapeutic investigation. Initial approaches in small studies have shown beneficial clinical effects by using the VEGF_{165} gene. This trial represents the first randomized placebo versus phVEGF_{165} controlled gene therapy trial to be published in diabetic patients with CLI. These patients received phVEGF_{165} injected IM into the most ischemic limb. This injection was repeated once at 4 weeks, resulting in a total of 4000 ug of phVEGF_{165}. In a matching placebo procedure patients received 0.9% NaCl. In this study significant clinical and hemodynamic improvements were found in patients treated with phVEGF_{165} without adverse events. However, the primary endpoint of the study, a significant reduction in amputation rate, was not met. This small randomized study contributes to the data on clinical gene therapy in CLI and could serve to restart the interest in the angiogenic approach in patients with CLI.
INTRODUCTION

CRITICAL Limb Ischemia (CLI) is a disease manifested by sharply diminished blood flow to the legs; it is the most common cause of non-traumatic amputation in diabetes. This condition is responsible for 70% of the 150 lower limb amputations per million in the population. While a combination of neuropathy, obstructive macro-vascular disease and/or micro-vascular changes is usually pivotal in the development of the diabetic foot, the contribution of micro-vascular occlusions is predominant in the diabetic subgroup with CLI and is not accessible for surgical re-vascularization. Amputation is unavoidable in 0.7 per 10,000 patients with diabetes mellitus. In CLI patients who already have had surgical re-vascularization, amputation is inevitable in approximately 50% of the patients. The median survival of patients with CLI is approximately 3 years. The QOL during this period is limited.

New methods of treatment of CLI have been recently explored. Pre-clinical studies have defined a role for vascular growth factors in neo-angiogenesis in animal models of peripheral ischemia. The most potent angiogenic factor affecting endothelial cell proliferation is VEGF, an endothelial cell specific mitogen from a family of 6 isoforms. However as a protein its short half life and its effects on vascular permeability have limited its clinical application. Its use in the form of gene therapy, either as naked plasmid or in a viral vector, has only been reported in small studies showing beneficial clinical effects in some but not all trials.

If the clinical benefits originally seen in the studies by Isner and Baumgartner, using intra-muscular (IM) injections of naked VEGF plasmid DNA (phVEGF) could be reproduced in a well defined patient group in a randomized study, this would, in our opinion, redefine the place of this form of gene therapy for CLI.

The primary aim of our study was to assess the effects of phVEGF, in addition to maximal standard treatment, on the amputation rate in a placebo-controlled randomized study, in a group of diabetic patients with CLI. The secondary objectives were hemodynamic improvement, clinical improvement and safety.

METHODS

Study design

The study was a two center, randomized, double blind, controlled study comparing phVEGF with placebo (0.9% NaCl), with limb survival and/or predefined changes in pressure indices as primary measure of effect. The study was approved by the Centrale Commissie Mensgebonden Onderzoek (CCMO: central medical ethical committee) in the Netherlands and performed in two centers (University Medical Center Groningen, Leiden University Medical Center, The Netherlands).
Patients received phVEGF\textsubscript{165} or placebo by computerized block randomization, without stratification or matching. Block randomization was performed by the department of pharmacy of the University Medical Center Groningen. Patients were assigned either to receive 2000 \textmu g of phVEGF\textsubscript{165} or placebo on day 0 and day 28. Follow-up evaluation was performed on days 7, 14, 35, 42, 72, and 100, with registration of clinical symptoms, wound status and hemodynamic condition. In addition, routine hematologic, chemical, urinalysis, anti double-strand DNA antibodies and circulating VEGF and phVEGF\textsubscript{165} levels were determined. Ophthalmologic examination was performed before treatment, on day 28 and at the conclusion of the study.

**Patients**

Patients were recruited from a large number of academic and non-academic hospitals in the Netherlands by approaching their departments of vascular surgery. Patients with either type I or type II diabetes mellitus according to current American Diabetes Association (ADA) criteria were eligible. Evidence of critical limb ischemia had to be present including rest pain and/or nonhealing ulcers for a minimum of 2 weeks despite conventional therapy. Patients with compressible vessels had to have a resting ankle systolic blood pressure < 50 mmHg, or toe systolic blood pressure < 30 mmHg in the affected limb. Patients had to be no suitable candidates for surgical or percutaneous re-vascularization as judged after contrast angiography by the vascular surgeon and intervention radiologist. Further exclusion criteria were active proliferative diabetic retinopathy, a history of malignancy, severe co-morbidity and/or compromising co-medication. Patients gave written informed consent for their participation.

**Gene product and administration**

The plasmid containing the human VEGF\textsubscript{165} gene (Genbank accession no. AB021221) which is transcriptionally regulated by the cytomegalovirus promoter/enhancer, was manufactured under Good Manufacturing Practices guidelines according to Sarkar et al.\textsuperscript{10,18} The plasmid was a gift from professor Isner, and was the same as has been used by his group. Patients received four aliquots each containing 500 \textmu g of phVEGF\textsubscript{165} diluted in a volume of 1.0 mL of 0.9% NaCl (total 2000 \textmu g) injected IM (26G needle) into the thigh (two aliquots) and calf muscles (two aliquots) of the most ischemic limb. The injection sites were arbitrarily selected according to available muscle mass as described in the protocol from Baumgartner et al.\textsuperscript{14} This procedure was repeated once at 4 weeks, resulting in a total of 4000 \textmu g of phVEGF\textsubscript{165} being administered into the ischemic leg. In a matching placebo procedure patients received 4 times 1.0 mL of 0.9% NaCl. No difference between the phVEGF\textsubscript{165} and placebo could be seen or felt by the physician who performed the injection.
Procedures

Ischemic skin defects were copied onto a transparent sheet to calculate the ulcer surface area. In addition, ischemic skin defects were documented by color photography. Assessment of ischemic rest pain was performed using a visual analogue scale (VAS) for pain scores and by the documentation of the daily use of analgesics.

Ankle pressure was measured according to conventional procedures with the patient at rest in a semi-supine position. Measurements were performed by an experienced vascular technician using an 8 MHz Parkes Doppler with the occluding cuff around the ankle, unless wounds extended to the proximal foot or ankle, in which case the cuff was placed around the upper leg. Toe pressures were measured after at least 10 minutes of warming using a photoplethysmographic diode on the pulp area and a small occluding cuff at the base of the toe. The pressure at which pulsatile signals reappeared upon cuff release was noted. If a skin defect was present in the dig. I-II, toe pressures were not measured. Ankle to brachial index (ABI) and toe to brachial index (TBI) were calculated as the quotient of the absolute ankle or toe pressures, and the simultaneously measured brachial pressure.

In accordance with the literature our trained vascular technicians scored a coefficient of variation of ABI in patients with CLI of 3.2% and the interobserver difference did not exceed this.

QOL assessment using the RAND-36 questionnaire was performed to determine if a clinical response had a positive effect on the QOL.

Ophthalmologic examination at baseline and each subsequent follow-up visit included best-corrected visual acuity measurement and intra-ocular pressure, slitlamp biomicroscopy, indirect ophthalmoscopy and fundus photography. fluoresceïn angiography (with intravenous administration of 5 mL of 10% sodium fluoride) was performed as a baseline and after 100 days of the study. Diabetic retinopathy was classified as follows: no retinopathy, background retinopathy, pre-proliferative and proliferative diabetic retinopathy.

Systemic VEGF levels were determined using the Quantikine Human VEGF enzyme-linked immunosorbent assay (ELISA; R&D Systems Inc. Minneapolis, MN). Whole blood (in citrate-theophyllin-adenosin-dipyridamole [CTAD] tube) was diluted 3 times with PBS. In order to damage the membranes the cellular suspension was frozen and thawed twice. The serum (coagulation for at least 1 hour) samples were centrifuged for 15 minutes at 3000 g at room temperature. The samples were stored in aliquots at –80°C until analysis. Results were compared with a standard curve of human VEGF with a detection limit of 5 pg/mL.

Analysis of systemic phVEGF_{165} in whole blood was performed by PCR after isolation of the DNA according to the Boom procedure. This method allowed a detection limit of 2.0 fg phVEGF_{165}/µl blood.

Definitions of measures of effect

Response was defined as limb survival, hemodynamic improvement of ABI or TBI at two different time points, or improvement of skin ulcers and rest pain. Limb survival was defined as the absence of a major amputation. A major amputation is an amputation proximal to the
level of the ankle. A hemodynamic improvement is defined as an absolute increase of >15% in ABI or TBI. This increase is considered as a significant clinically relevant improvement.\textsuperscript{19,24,25} Ischemic wound response was defined as a decrease in wound surface area of >60%. Improvement of pain was defined as >50% decrease in pain scores as assessed using the VAS at different time-points (baseline to day 28, 72, and day 100).

Safety was assessed by incidence and severity of adverse events: vital signs (i.e. fever, or hypotension defined as systolic blood pressure < 90 mm Hg) during and after administration of IM injections, diabetic retinopathy, edema, anti ds DNA (Farr assay), proteinuria, teleangiectasia, circulating phVEGF\textsubscript{165} and analysis of survival.

\textbf{Statistical analysis}

The target number of patients was determined based on the expected incidence of amputations in the control group and the foreseen success rate of the intervention. The incidence of amputation in diabetic patients is 0.7/10,000. The data from studies by Klevsgard et al\textsuperscript{4} and da Silva et al\textsuperscript{3} give indirect indications of the incidence of amputation in end stage CLI. In both studies the patients are still amenable to surgical intervention, and therefore constitute an earlier disease state than our patients. Nevertheless, amputation occurs in 40% in the patients from da Silva and at least 40% in the study by Klevsgard. We therefore projected an amputation rate of 50% in the control group. In diabetic patients with critical ischemia in whom surgical options are exhausted this rate is at least 50%.\textsuperscript{3,4}

The expected success rate of the intervention was estimated from the available clinical data on VEGF gene therapy at that time.\textsuperscript{12-14,26} About three quarters of these patients were either rescued from imminent amputation or showed substantial improvement in parameters such as ABI, that can be considered to be directly relevant to the chance of avoiding amputation in the near future.

Based on this success rate 54 patients were considered to be needed to be able to demonstrate the expected reduction from 50 to 25% in the amputation rate (power 0.85, $P=0.05$).

Baseline characteristics and response rate comparisons between the groups were analyzed using the chi-square test, corrected for continuity. For continuous variables the independent T-test was used. For QOL analysis changes within the group between baseline, 28 day and 100 day assessments were analyzed using the Friedman's test. Differences between the groups were measured using the Mann-Whitney U test. The same tests were used for analysis of laboratory parameters. Survival analysis was calculated according to Kaplan Meier. A $P$-value <0.05 was considered statistically significant.
RESULTS

In the period between February 2000 and January 2004, ninety-seven patients were screened: 54 were found to be eligible and were randomized. Patient refusal and surgical alternatives were the most common exclusion grounds. Five patients were excluded because of proliferative diabetic retinopathy. Basic demographic characteristics were similar in both groups (see Table 1).

<table>
<thead>
<tr>
<th>Table 1. Baseline characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GENERAL</strong></td>
</tr>
<tr>
<td>Age (years; mean [range])</td>
</tr>
<tr>
<td>Women</td>
</tr>
<tr>
<td>Diabetes type 1/2</td>
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<tr>
<td>ID*</td>
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<td>HbA1C (%; mean [range])</td>
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<td>Diabetes duration (years; mean [range])</td>
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<td>Pain</td>
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<tr>
<td>Skin ulcer</td>
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<tr>
<td>Concomitant cardiovascular:</td>
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<tr>
<td>• Hypertension</td>
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<tr>
<td>• Hypercholesterolemia</td>
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<td>• CAD**</td>
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<tr>
<td>• Duration of leg ischemia symptoms (months; mean [range])</td>
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<tr>
<td>• Prior vascular reconstruction / percutaneous angioplasty</td>
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<tr>
<td>• Prior amputation</td>
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</tbody>
</table>

Data are numbers, unless otherwise stated. No significant differences were found (by chi-square corrected for continuity, for continuous variables the independent t - test was performed).

*: insulin dependent

**: coronary artery disease
Treatment results (figure 1, table 2a and 2b).

All patients were evaluated for all endpoints (pressure, skin ulcers, and pain). Usually wounds precluded measuring of pressure.

A major amputation was performed in 6 of the control patients and 3 of the phVEGF$_{165}$ treated patients (n.s, table 2b). The mean time to amputation was 78 days in the phVEGF$_{165}$ treated patients and 25.5 days in the control arm ($P=0.11$). The amputation rate in the control group was therefore 25%.

For hemodynamic assessment 16 patients were not evaluable because of incompressible vessels or extensive ulceration which made ankle or toe pressure assessment not feasible. An absolute increase of > 15% in ABI or TBI on at least 2 time points occurred in 7 of 21 evaluable phVEGF$_{165}$ patients. In the control group only one patient of the 17 evaluable patients showed a hemodynamic increase of >15%. phVEGF$_{165}$ treatment tended to improve pressure parameters ($P=0.05$). Median time to improvement (day 0 to first increase of > 15%) was 4 weeks and this improvement was still present at day 100.

Skin-ulcers were evaluated in all the 38 patients with ulcers (see Table 1). Of 21 evaluable phVEGF$_{165}$ patients there were 7 responders, whereas none of the control patients showed an improvement of ulceration ($P=0.01$). Ulcer healing of more than 60% occurred after a median 5 weeks after injection and was still present at day 100 (Figure 1).

Figure 1. Patient with CLI and a not closing wound two months after surgery. Treatment with phVEGF$_{165}$ started two month after surgery. Pictures at day 0, day 28 and day 100. At day 100 the wound is nearly closed.
One phVEGF\textsubscript{165} treated patient presented with ischemic ulceration in both legs. Skin ulcers in the injected as well as in the opposite leg showed a clinically relevant improvement with a decrease of >60\% in ulcer surface.

In 7 patients there was no rest pain. Five patients were not evaluated for pain because of minor surgical intervention, i.e. amputation of the toe or extensive debridement of skin defects, shortly prior to the first IM injection. In 10 patients, who could not understand the VAS scale, pain data were incomplete. However, in 5 of these patients there was no change in the VAS performance as determined on the basis of anamnesis and of use pain medication. Two of 11 control patients had a > 50\% decrease in pain score versus 5 of 21 phVEGF\textsubscript{165} patients (n.s.).

Overall there were 17 responding patients (Table 2a). As some patients responded in more than one category (hemodynamic, skin ulcers or pain) there were a total of 22 responses. In the phVEGF\textsubscript{165} treated patients there were 4 patients with improvement in more than one category as opposed to none in the control group. Three responders received placebo and 14 received phVEGF\textsubscript{165}. The advantage for phVEGF\textsubscript{165} compared with placebo was significant ($P=0.003$, table 2b).

**Table 2a. An overview of the responding patients.**

<table>
<thead>
<tr>
<th>Responding Patients(^*))</th>
<th>Improvement in ABI (^1)</th>
<th>Improvement in skin ulcer(^†)</th>
<th>Decrease in pain(^‡)</th>
<th>Hemodynamic responder</th>
<th>Clinical responder</th>
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<tbody>
<tr>
<td>1</td>
<td>x</td>
<td>x</td>
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<td>10</td>
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<td>C</td>
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</table>

\(^*)\) 1-14: PhVEGF\textsubscript{165} treated patients, A-C: controls. x = \(^1\) absolute increase of > 15\% in ankle to brachial index or toe to brachial index, \(^†\) decrease in ulcer surface of > 60\%, \(^‡\) > 50\% decrease in rest pain on the VAS.
Table 2b. Treatment results

<table>
<thead>
<tr>
<th>ENDPOINT</th>
<th>CONTROL n/TOTAL (%)</th>
<th>phVEGF165 n/TOTAL (%)</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major amputations</td>
<td>6/27 (22 %)</td>
<td>3/27 (11 %)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hemodynamic improvement1)</td>
<td>1/17 (6 %)</td>
<td>7/21 (33 %)</td>
<td>0.05</td>
</tr>
<tr>
<td>Improvement in skin ulcer†</td>
<td>0/17 (0 %)</td>
<td>7/21 (33 %)</td>
<td>0.01</td>
</tr>
<tr>
<td>Decrease in pain‡</td>
<td>2/11 (18 %)</td>
<td>5/21 (24 %)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Responding patients          3/27(11 %) 14/27 (52 %) 0.003

Data in number of patients (percentage of evaluable patients).
1) absolute increase of > 15 % in ankle to brachial index or toe to brachial index,
† decrease in ulcer surface of > 60 %, ‡ > 50 % decrease in rest pain on the VAS.

Quality of life assessment (table 3).

QOL assessment was performed in 46 patients, 8 patients did not provide sufficient data. At the baseline the only imbalance between the control and phVEGF165 treated group was in health experience (data not shown). Overall there was no improvement in QOL with phVEGF165 treatment as compared to placebo treatment (data not shown). However, clinical and/or hemodynamic responders showed improved physical, social functioning, and health experience as compared to non-responders (P=0.002, P=0.045, P=0.05 respectively).

Table 3. Quality of life

<table>
<thead>
<tr>
<th></th>
<th>BASELINE</th>
<th>100 DAYS</th>
<th>NON-RESPONDERS</th>
<th>RESPONDERS 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL</td>
<td>phVEGF165</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical function</td>
<td>15.9 ± 11.7</td>
<td>19.1 ± 15.4</td>
<td>16.1 ± 10.3*</td>
<td>31.6 ± 14.5)*</td>
</tr>
<tr>
<td>functioning</td>
<td>40.6 ± 19</td>
<td>42.3 ± 22.0</td>
<td>44.7 ± 19.4†</td>
<td>61.7 ± 25.2†</td>
</tr>
<tr>
<td>Social functioning</td>
<td>4.3 ± 16.3</td>
<td>7.0 ± 10.8</td>
<td>0 ± 0</td>
<td>18.7 ± 34.8</td>
</tr>
<tr>
<td>Physical role</td>
<td>22.7 ± 39.0</td>
<td>19.3 ± 35.7</td>
<td>25.6 ± 43.5</td>
<td>35.4 ± 44.7</td>
</tr>
<tr>
<td>Emotional role</td>
<td>52.2 ± 11.9</td>
<td>51.4 ± 11.0</td>
<td>55.1 ± 10.3</td>
<td>55.0 ± 14.3</td>
</tr>
<tr>
<td>Mental health</td>
<td>42.1 ± 13.3</td>
<td>42.4 ± 14.0</td>
<td>44.8 ± 10.9</td>
<td>50.6 ± 11.8</td>
</tr>
<tr>
<td>Vitality</td>
<td>24.9 ± 13.5</td>
<td>33.8 ± 121.5</td>
<td>37.6 ± 19.9‡</td>
<td>58.9 ± 17.0‡</td>
</tr>
<tr>
<td>Pain</td>
<td>36.1 ± 19</td>
<td>42.6 ± 19.5</td>
<td>42.1 ± 15.7§</td>
<td>47.2 ± 24.5§</td>
</tr>
<tr>
<td>Health experience</td>
<td>33.3 ± 27.3</td>
<td>34.5 ± 29.0</td>
<td>36.6 ± 28.4</td>
<td>59.4 ± 31.5</td>
</tr>
</tbody>
</table>

Data in mean (SD). Score range: 0-100, higher scores indicating better quality of life.
*: P=0.002, †: P=0.045, ‡: P=0.073, §: P=0.05 (Mann-Whitney test)
1) Responder = a patient with a response in any category (pain, ulcer, hemodynamic)
**VEGF blood levels**

Median serum VEGF levels at baseline was 321 pg/mL (range 53-1677) in control patients and 275 pg/mL (range 53-1103) in phVEGF<sub>165</sub> treated patients (n.s.). Median whole blood VEGF levels were 846 pg/mL (range 199-1963) in the control group compared to 911 pg/mL (range 365-1843) in the phVEGF<sub>165</sub> patients (n.s.). There was no transient increase in circulating VEGF after IM treatment in either group. In individual patients, 10% showed a 50% increase of VEGF levels within fourteen days after intramuscular injection in both groups.

**Safety (table 4).**

PhVEGF<sub>165</sub> was well tolerated. No changes in systolic or diastolic blood pressure were observed. Edema was already present before injection in 11 patients of the control group and in 10 patients of the phVEGF<sub>165</sub> treated patients. The edema increased in these 21 patients and new formation of edema occurred in 3 phVEGF<sub>165</sub> treated patients, and in 4 control patients (n.s.).

New telangiectasias were found in 2 patients: in one phVEGF<sub>165</sub> treated and one control patient. They occurred within 14 days after IM injection, and persisted thereafter. Otherwise unexplained hypoglycemia (< 3 mmol/L) occurred in 2 phVEGF<sub>165</sub> treated patients in the first 2 to 3 weeks after intra-muscular injection.

Microalbuminuria (30 to maximal 300 mg/day) was detected in the majority of the 37 patients measured, and remained stable during follow-up in both groups without significant variation. In the phVEGF<sub>165</sub> treated group no anti dsDNA was detected. The analysis for the presence of phVEGF<sub>165</sub> in peripheral blood was possible in 20 phVEGF<sub>165</sub> patients, phVEGF<sub>165</sub> could actually be detected in 4 patients within the first 3 days after injection.

There were 4 deaths within the follow-up period of 100 days. These deaths were not related to the treatment: two patients died in the phVEGF<sub>165</sub> treated group and 2 patients in the placebo group. In the phVEGF<sub>165</sub> treated group one patient died during a sepsis, 3 weeks after a major amputation of the leg, and one patient died in the postoperative period after a total hip replacement. In the control arm 2 patients died: one patient died 2 weeks after a major amputation, and the other patient died due to a protracted Staphylococcus Aureus sepsis caused by an infected hip prosthesis.

The 1-year survival in non-amputated patients was in the control and phVEGF<sub>165</sub> treated group, respectively 60 and 84% (n.s.). The progression from no diabetic retinopathy to minimal background retinopathy (less than 10 red dots) was 15% in both treatment arms, no progression to proliferative diabetic retinopathy was seen. Other ophthalmologic parameters remained stable during the whole study. There was no diagnosis of cancer.
Table 4. Safety analysis during follow-up period of 100 days

<table>
<thead>
<tr>
<th></th>
<th>CONTROL (N=27)</th>
<th>PHVEGF165 (N=27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemodynamic complications</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ankle edema</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Increase in proteinuria</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Teleangiectasia</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Anti dsDNA (&gt; 2 IU/mL)</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Circulating phVEGF165*</td>
<td>–</td>
<td>4*</td>
</tr>
<tr>
<td>Deaths</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Proliferative retinopathy</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diagnosis of cancer</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data in number of patients, no significant differences detected (by Fischer exact test).
* In 20 evaluable samples of 20 patients within day 1-3 after IM.

**DISCUSSION**

Once the options for surgical intervention have been exhausted few treatment alternatives remain for patients with end stage CLI. In this study we did not meet the primary endpoint of a reduced amputation rate. We did, however, demonstrate that IM injections of the naked plasmid DNA encoding VEGF165 (phVEGF165) significantly improve wound healing and reduce hemodynamic insufficiency compared to placebo. Importantly, in the responders these clinical improvements resulted in improved physical functioning (mobility, daily activities such as washing, dressing or cleaning) and improved social functioning as detected by the RAND 36 questionnaire for QOL. Therefore, a “response” as defined in this study seems to be a meaningful notion.

Despite the rigorous entry criteria applied, a placebo effect, either a symptom of natural variation and fluctuation in the degree of ischemia, or an effect of intensified care in the study patients, is undeniable as three of the responders were among the placebo treated patients. In contrast significant clinical improvement was seen in 14 of the 27 treated with the naked plasmid DNA. This 50% success rate is in agreement with the pioneering study of Baumgartner et al.14 Our results further confirm more recent data from a study in a Chinese and Korean cohort, in which an even higher response rate was achieved.15,16 On the other hand no clinical improvement was found in a study by the group of Makinen, although angiography measurements suggested improvement.17 However, their patient group was not well defined and the plasmid was given intra arterially instead of IM. Table 5 shows an overview of gene therapy studies in patients with CLI, treated with a plasmid containing VEGF<sub>165</sub>. The amputation rate in these series confirms ours of approximately 16%.

In a phase II randomized, double blind controlled study in patients with intermittent claudication VEGF<sub>121</sub> in an adenoviral vector as a single IM injection was used,27 instead of phVEGF<sub>165</sub>. This trial was, in contrast to our study, not associated with an increase in
ABI/TBI, improved wound healing or QOL. Besides as a result of a different study design, a
difference in local VEGF concentrations might be responsible for the observed difference in
clinical results. There is a major difference between these VEGF proteins as VEGF_{121} diffuses
easily while VEGF_{165} binds to matrix components.

Our primary aim was limb salvage or a reduction in major amputation rate. However,
our estimate of expected amputations (50%) proved to be too pessimistic. The small number
of amputations (17%) occurring ultimately in the present study precludes any conclusions
with regard to treatment efficacy in terms of limb salvage. Although improvements in surgery
have in the past influenced the need for amputation in this patient group,\textsuperscript{38} in our patient who
were beyond surgical intervention this can not have played a role. In a more recent Danish
study, a further decline in amputations followed the institution of dedicated multidisciplinary
foot clinics.\textsuperscript{29} As these clinics have been instituted in the Netherlands in recent years, they may
in part be responsible for the low incidence of amputations in our patient cohort. An

\begin{table}
\centering
\caption{Studies with phVEGF165 in CLI}
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
\textbf{STUDY DESIGN} & \textbf{TREATED LIMBS (CLI)} & \textbf{TREATED LIMBS (CLI) (IMPROVEMENT/TOTAL)} & \textbf{SYSTEMIC VEGF LEVEL (INCREASE/TOTAL)} \\
 & & & & ULCER & REST & ABI/TBI & ANGIO & AMPUTATION \\
\hline
IM Isner 1996 & n=1 & & & 1/1 & 1/1 & & & \\
Phase I/II & n=10 limbs (9 pts) & 3/6 & 3/3 & 7/9 & 7/10 & 2/7 & 3/7 & \\
IM Baumgartner & 6/9: ischemic ulcers & & & & & & & \\
Phase I/II & n=7 (6 pts) & 3/5 & 2/5 & 3/7 & 6/6 & 2/6 & 3/7 & \\
IM Isner 1998 & 5/6: ischemic ulcers & & & & & & & \\
 & 1/6: isolated rest pain & & & & & & & \\
Phase I/II & n=24 (21 pts) & 11/15 & 18/21 & P<0.001 & 19/24 & 2/21 & 8/21 & \\
IM Skyu 2003 & 16/21: ischemic ulcers & & & & & & & \\
 & 21/21: rest pain & & & & & & & \\
Phase II & n=4 control & 2/4 & 1/4 & P<0.05‡ & 3/17 & 0/4 & No increase & \\
Makenen 2002 & & & & & & & in mean value & \\
Intra arterial* & n=6 VEGF† & 3/6 & 0/6 & P<0.05‡ & 10/16 & 0/6 & & \\
Phase I & n=9§ (9 pts) & 4/6 & 6/7 & 6/9 & No increase & & & \\
IM Kim 2004 & & & & & & & in mean value & \\
\hline
\end{tabular}
n: number of treated limbs with critical limb ischemia.
Improvement of ischemic ulcers indicates complete or partial response, improvement in ABI / TBI indicates
>10 % absolute increase, *: only patients with CLI mentioned and treated with placebo or VEGF, †: VEGF-
plasmid/liposome, §: patients with claudication and CLI, §: one patient with intermittent claudication not
included in this report.
alternative possibility would be that our patient cohort has been included too early in the course of their disease. This explanation is unlikely in view of the rigorous entry criteria, the limited median survival of the whole group and the severely affected QOL at the beginning of the study. In a cohort of ambulant diabetic patients in the same hospital area with early peripheral artery disease, published by Meijer et al, the QOL scores were between 50 to 90% in relevant domains compared to 5 to 50% in the present study.\(^\text{30}\)

In contrast to other studies but in agreement with a recent phase I study in non-diabetic patients and the phase II study of Makinen we found no evidence of increased levels of systemic VEGF.\(^\text{13-17}\) Therefore, the discussion whether local or systemic effects, or both, contribute to the response remains open. There do not seem to be easy ways to clarify this problem in the clinical situation. Although amputation material is occasionally available for analysis, results would be unlikely to give information on successful interventions. Biopsies to clarify the mechanism in responding patients who will have severe compromised wound repair are undesirable.

Intra-muscular phVEGF\(_{165}\) was well tolerated. With the possible exception of hypoglycemia in 2 patients no side effects occurred.

This small randomized study could serve to restart the interest in the angiogenic approach in CLI. In a disease with an intrinsic placebo effect, the first priority obviously would be to confirm these results in a larger study. The number of patients to be studied should permit rather low numbers of amputations in the control group. Also the use of completely different endpoints, preferably of a non-invasive nature, should be considered. In such a study the theoretical possibility of a therapeutic effect of an empty plasmid could also be excluded. Improvements in the treatment scheme would thereafter include changes in the duration of treatment, the possible combined application of multiple effective genes, and the use of alternative transfection methods.

Acknowledgements

This study was supported by a grant from Fornix BioSciences NV, Vijzelweg 11, 8243 PM Lelystad, The Netherlands. We thank Mrs. W.A. Dam, technician, for measuring circulating VEGF\(_{165}\) and phVEGF\(_{165}\) levels.
REFERENCES


Diagnostic and Therapeutic Aspects


