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Therapeutic Angiogenesis With Intramuscular NV1FGF Improves Amputation-free Survival in Patients With Critical Limb Ischemia

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This study evaluated the efficacy and safety of intramuscular administration of NV1FGF, a plasmid-based angiogenic gene delivery system for local expression of fibroblast growth factor 1 (FGF-1), versus placebo, in patients with critical limb ischemia (CLI). In a double-blind, randomized, placebo-controlled, European, multinational study, 125 patients in whom revascularization was not considered to be a suitable option, presenting with nonhealing ulcer(s), were randomized to receive eight intramuscular injections of placebo or 2.5 ml of NV1FGF at 0.2 mg/ml on days 1, 15, 30, and 45 (total 16 mg: 4 × 4 mg). The primary end point was occurrence of complete healing of at least one ulcer in the treated limb at week 25. Secondary end points included ankle brachial index (ABI), amputation, and death. There were 107 patients eligible for evaluation. Improvements in ulcer healing were similar for use of NV1FGF (19.6%) and placebo (14.3%; $P = 0.514$). However, the use of NV1FGF significantly reduced (by twofold) the risk of all amputations [hazard ratio (HR) 0.498; $P = 0.015$] and major amputations (HR 0.371; $P = 0.015$). Furthermore, there was a trend for reduced risk of death with the use of NV1FGF (HR 0.460; $P = 0.105$). The adverse event incidence was high, and similar between the groups. In patients with CLI, plasmid-based NV1FGF gene transfer was well tolerated, and resulted in a significantly reduced risk of major amputation when compared with placebo.

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INTRODUCTION

Peripheral arterial disease is a progressive illness, and the most severe manifestation is termed "critical limb ischemia" (CLI).[1] It is estimated that each year there are ~500 new cases of CLI per million individuals in the USA and Europe.[2] These patients experience chronic ischemic rest pain, ulcers, or gangrene in the lower limbs, and have a very poor prognosis. Many will undergo amputation, and their quality of life has been described as being similar to that in patients with critical- or terminal-stage cancer.[3–5]

The current goals of management of CLI are to relieve ischemic pain, heal ischemic ulcers, prevent amputation, reduce cardiovascular mortality, and improve the patient's functioning and quality of life.[6,7] There is no effective pharmacologic therapy available, and treatment consists primarily of percutaneous or surgical revascularization. However, restenosis rates are high, and peripheral bypass surgery is associated with significant morbidity and mortality. Importantly, in a considerable proportion of patients (10–15%), revascularization is not a suitable option. Of these patients, >40% will require a major amputation, and 20% will die within 6 months.[5,6]

Therapeutic angiogenesis is a novel strategy for the treatment of ischemic vascular disease. It uses angiogenic factors to increase blood perfusion in ischemic tissues through various mechanisms of action.[8–23] Gene transfer of fibroblast growth factor 1 (FGF-1) has been used successfully to promote angiogenesis.[16–18] NV1FGF (Centelion SAS, Vitry sur Seine, France) is a novel pCOR DNA plasmid–based gene delivery system for local expression of FGF-1.[24] The pCOR plasmid backbone has several features that increase biosafety. These attributes include replication in only a narrow host range of laboratory, but not wild type, *Escherichia coli* strains, absence of an antibiotic-resistance gene, and lack of the

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potentially immunostimulatory sequence motifs found in ColE1 plasmids.[24] Preclinical studies of intramuscular administration of NV1FGF have demonstrated that local expression of FGF-1 persists for several weeks. The pharmacological efficacy has been demonstrated based on morphological and functional end points in two experimental animal models of hindlimb ischemia, the rabbit model[25] and the hypercholesterolemic hamster model, that exhibit sustained defects in collateral vessels and arteriole formation after induction of ischemia.[19] Intramuscular administration of pCOR plasmid expressing FGF-1 in the rabbit model resulted in a statistically significant improvement in the formation of capillaries and collateral vessels as well as tissue perfusion in the treated limb. Improvement was statistically significant in a dose-dependent manner at all the end points when compared with the effects produced by vehicle or plasmid controls.[25] The angiogenic effect of NV1FGF was confirmed in the more severe animal model. The administration of NV1FGF in the hypercholesterolemic hamster model of hindlimb ischemia significantly enhanced the formation of large conductance vessels as well as small resistance arteries in ischemia-injured muscles.[19]

In an open-label Phase I trial involving 51 patients, single and repeated (two) intramuscular doses of NV1FGF in patients with CLI were well tolerated.[20] In another open-label study, six CLI patients who were to undergo planned amputation were administered eight intramuscular injections of NV1FGF, four in the calf and four in the thigh. Muscle samples collected from the injected sites and from distant sites of the amputated legs showed that myofibers of CLI patients were capable of being transfected with NV1FGF and of expressing NV1FGF-derived messenger RNA and FGF-1 protein. NV1FGF-derived FGF-1 expression was localized to the injection site and not detected in muscles distant from the injection site. The local expression of FGF-1 and the presence of FGF receptors (1–4) in ischemic muscles of all the tested patients provide a rationale for using NV1FGF in peripheral arterial disease patients.[26]

This phase II study was designed to evaluate the efficacy and safety of NV1FGF versus placebo in CLI patients in whom revascularization was not considered to be a suitable option.

RESULTS

A summary of the patients' characteristics is shown in **Figure 1**. A total of 205 patients were screened, and 125 patients who met the inclusion and exclusion criteria were randomized to NV1FGF (n = 59) or placebo (n = 66). In this multinational study, only eight of the sites were able to enroll complete blocks, and this led to a slight imbalance in numbers between the placebo and NV1FGF groups. Randomization to NV1FGF or placebo was well balanced (**Figure 1**).

The modified intention-to-treat (MITT) population included 107 patients receiving NV1FGF (n = 51) or placebo (n = 56) (**Figure 1**). The rate of study discontinuation was 45.5% of the patients in the placebo group and 30.5% in the NV1FGF group. The two main reasons for discontinuation were adverse events and death.

The mean age of the patients was 72 years, and most of the patients were men (71%). Other baseline characteristics common in this type of population were relatively well balanced between the two groups (all P values > 0.1) (**Table 1**).

The mean aggregate ulcer size (in square centimeters) was similar in the two groups, ranging from very small ulcers to extensive lesions: mean size of 21.80 cm^2 (0.09–148.14) in the placebo group and 21.72 cm^2 (0.35–483.00) in the NV1FGF group.

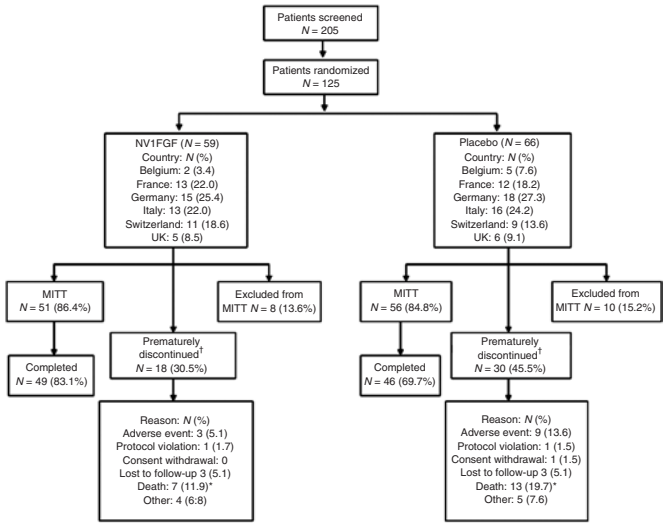

Figure 1 Patient disposition. Completed: MITT patients who had received their last injection (visit 5, week 7) even if previous injections had been missed. Dagger represents data calculated from randomized population. Asterisk represents the number of deaths as a reason for discontinuation is lower than the total number of deaths observed in the randomized population (placebo, N = 16; NV1FGF, N = 10). This is because a patient could discontinue the study for a reason other than death (*e.g.,* an adverse event), with death occurring later but before the end of the trial. MITT, modified intention-to-treat.

Primary end point

Ischemic ulcers. Similar rates of ulcer healing occurred with NV1FGF (19.6%) and placebo (14.3%; P = 0.514) (**Table 2**). There were also no differences between the treatment groups in aggregate ulcer size or ulcer depth.

Secondary end points

In addition to the analysis of data from the MITT population, the robustness of the findings in relation to the occurrence of amputation, death, and combined major amputation and death was confirmed in the total randomized population (data not shown).

Amputation. The use of NV1FGF reduced by twofold the risk of all amputations [hazard ratio (HR) 0.498; P = 0.015] and major amputations (HR 0.371; P = 0.015) in the MITT study population, as shown in **Figure 2a** and **b**, and **Table 2**. This finding was confirmed by post-hoc multivariate analysis (Cox model) of the potential influence of prognostic factors for the disease, including diabetes status, smoking status, hypertension, prior amputation, initial risk of amputation, ankle brachial index (ABI), and transcutaneous oxygen pressure ($TcPO_2$) (**Table 3**).

Death. There was no statistically significant trend to suggest that the use of NV1FGF reduces the risk of death (HR 0.460, P = 0.105; **Figure 2c** and **Table 2**).

Combined major amputation and death. The risk of combined major amputation and death was significantly reduced in the

Table 1 Patient demographics and disease status at baseline

	Placebo group (*N* = 56)	NV1FGF group (*N* = 51)	*P*
Age—years, mean ± SD	73.3 ± 9.8	71.1 ± 10.4	0.249
Male sex	42 (75.0)	33 (64.7)	0.245
Diabetes	28 (50.0)	19 (37.3)	0.185
Hypertension	48 (85.7)	38 (74.5)	0.145
Hyperlipidemia	28 (50.0)	25 (49.0)	0.919
Smoking status			
Nonsmoker	14 (25.0)	16 (31.4)	
Ex-smoker	39 (69.6)	25 (49.0)	0.464
Current smoker	3 (5.4)	10 (19.6)	
Carotid disease[a]	17 (30.4)	16 (31.4)	0.910
Coronary artery disease	6 (10.7)	11 (21.6)	0.125
Previous treatment in the treated limb			
Amputation	14 (25.0)	10 (19.6)	0.504
Skin grafting	4 (7.1)	2 (3.9)	0.472
Angioplasty	22 (39.3)	15 (29.4)	0.284
Bypass surgery	27 (48.2)	27 (52.9)	0.625
Critical limb ischemia disease status			
Ulcers			
Number per patient (*n*, min–max)	2.0 (1–5)	1.8 (1–6)	0.379
Aggregate size—cm^2 (mean ± SD)	21.8 ± 35.2	21.7 ± 68.7	0.994
Aggregate size—(min–max) cm^2	0.09–148.14	0.35–483.00	
Pain visual analog scale—mm (mean ± SEM)	47.4 ± 24.5	44.2 ± 30.1	0.552
Ankle brachial index[b] (mean ± SEM)	0.4 ± 0.3	0.4 ± 0.2	0.756
$TcPO_2$—mm Hg (mean ± SD)			
Dorsal surface of the foot[c]	16.3 ± 15.8	21.2 ± 19.1	0.161
Upper surface of the calf[d]	41.8 ± 23.2	44.6 ± 25.7	0.581

Abbreviation: $TcPO_2$, transcutaneous oxygen pressure.
Data are presented as percent of patients, mean value ± SD, or mean (minimum–maximum) as appropriate. Pain visual analog scale: 0 = no pain to 100 = worst pain.
[a]Carotid disease was defined as a history of stroke or a documented carotid stenosis in medical records (degree of stenosis ≥50%); [b]Patient numbers for ankle brachial index are placebo, *N* = 46; NV1FGF, *N* = 46; [c]Patient numbers for transcutaneous oxygen pressure at the dorsal surface of the foot are placebo, *N* = 54; NV1FGF, *N* = 50; [d]Patient numbers for transcutaneous oxygen pressure at the upper surface of the calf are placebo, *N* = 52; NV1FGF, *N* = 47.

NV1FGF versus placebo group (HR 0.435; *P* = 0.009) (**Figure 2d** and **Table 2**). Kaplan–Meier curves suggested a further increased benefit for patients with diabetes relating to reduction in the risk of combined major amputation and death, but this additional benefit was not statistically significant (**Figure 2e**).

Hemodynamic parameters. The hemodynamic parameters ABI and toe brachial index (TBI) increased marginally over time as compared to baseline values, and were similar in both groups **Table 2**). $TcPO_2$ also increased in both NV1FGF and placebo groups.

Table 2 Efficacy of NV1FGF versus placebo in patients with critical limb ischemia

	Placebo (*N* = 56)	NV1FGF (*N* = 51)	Hazard ratio	*P* value
Primary end point				
Complete healing of at least one ulcer selected at baseline at week 25	8 (14.3)	10 (19.6)	–	0.514[a]
Secondary end points over 52 weeks				
Amputation rate				
All	31 (55.4)	19 (37.3)	0.498	0.015[b]
Major	19 (33.9)	8 (15.7)	0.371	0.015[b]
Death rate	13 (23.2)	6 (11.8)	0.460	0.105[b]
Combined major amputation and death rates	29 (51.8)	14 (27.4)	0.435	0.009[b]
Hemodynamic parameters at week 25				
Adjusted mean change from baseline ± SEM				
Ankle brachial index	0.01 ± 0.04	0.05 ± 0.04	–	0.45
Toe brachial index	0.03 ± 0.02	0.04 ± 0.02	–	0.84
$TcPO_2$ at the dorsal surface of the foot—mm Hg (mean ± SD)	9.81 ± 3.15	8.55 ± 3.41	–	0.79

Abbreviation: $TcPO_2$, transcutaneous oxygen pressure.
Major amputation was defined as through or above the ankle and significantly affecting the functionality of the limb. Minor amputation was defined as below the ankle and having little or no effect on the functionality of the limb. One patient can have more than one amputation.
[a]Glimmix model; [b]log-rank test.

Pain. Ischemic rest pain visual analog scale was decreased in both NV1FGF and placebo groups. At week 52, the adjusted mean change ± SEM from baseline was –10.32 ± 22.83 in the placebo group versus –22.28 ± 22.53 in the NV1FGF group.

Adverse events. The safety population included 118 patients receiving placebo (*n* = 61) or NV1FGF (*n* = 57). The incidence of adverse events is reported in **Table 4**. Patients receiving NV1FGF experienced significantly lower rates of severe adverse events compared with those receiving placebo (*P* = 0.025).

Adverse events that occurred in ≥10% of the patients in either treatment group were: peripheral edema (placebo 21%, NV1FGF 28%, *P* = 0.522), pain in extremity (placebo 15%, NV1FGF 10%, *P* = 0.586), gangrene (placebo 13%, NV1FGF 7%, *P* = 0.365), anemia (placebo 13%, NV1FGF 5%, *P* = 0.207), and diarrhea (placebo 5%, NV1FGF 10%, *P* = 0.311).

Table 4 also shows the incidences of selected adverse events. There was a trend for cardiac events occurring more frequently with the use of NV1FGF than with placebo (24.6% versus 11.5%, *P* = 0.091), as was the case with renal/urinary events as well (15.8% versus 8.2%, *P* = 0.259; renal events were mainly hematuria, clinically not relevant). The incidence of potentially angiogenesis-related adverse events (neoplasm and retinopathy) was similar between groups (cancer and retinopathy were reported in 3/61 and 0/61 patients, respectively, in the placebo group and in 3/57 and 1/57 patients, respectively, in the NV1FGF group). No impairment of either renal or liver functions was observed under treatment in the two groups.

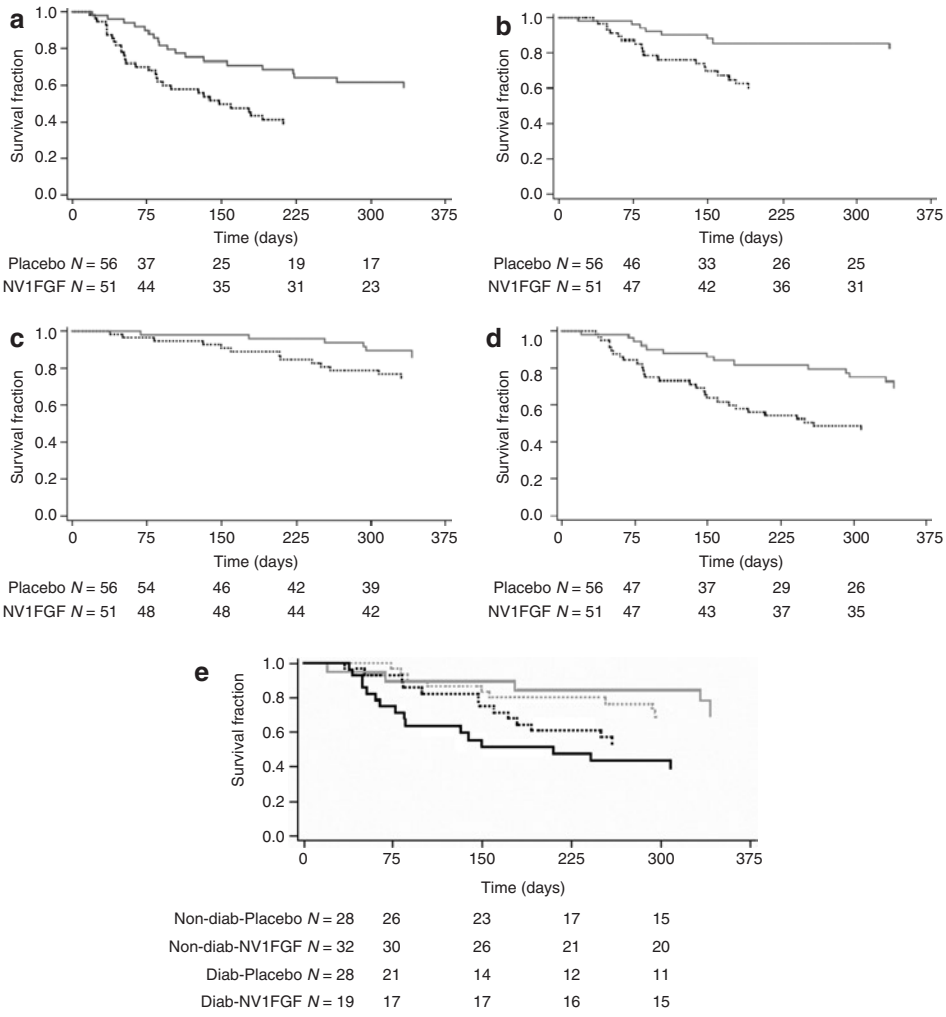

Figure 2 Kaplan–Meier curves for amputation and death (1-year follow-up). (**a**) Kaplan–Meier curve of time to first major amputation after baseline in the modified intention-to-treat (MITT) population (maximum follow-up 375 days). (**b**) Kaplan–Meier curve of time to first amputation after baseline in the MITT population (maximum follow-up 375 days). (**c**) Kaplan–Meier curve of time to death after baseline in the MITT population (maximum follow-up 375 days). (**d**) Kaplan–Meier curve of time to death or major amputation after baseline in the MITT population (maximum follow-up 375 days). (**e**) Kaplan–Meier curve of time to death or major amputation after baseline in the diabetic and non-diabetic subgroup populations of the MITT population (maximum follow-up 375 days). Kaplan–Meier survival curves stop at the time the last event occurred. In **a–d**, dotted lines represent placebo-administered patients, while solid lines represent NV1FGF-administered patients. In **e**, dotted lines represent nondiabetic patients administered with placebo, and dotted gray lines represent nondiabetic patients administered with NV1FGF; solid lines represent diabetic patients administered with placebo and gray solid lines represents diabetic patients administered with NV1FGF. Diab, diabetic; non-diab, nondiabetic.

DISCUSSION

This phase II study is the first double-blind, randomized, placebo-controlled, multinational trial using a therapeutic angiogenesis agent in patients with CLI. It was designed to explore the effects of the novel plasmid-based gene delivery system NV1FGF on recommended[2,7] clinical (mortality, amputation, ulcer healing, pain) and hemodynamic (ABI, TBI, $TcPO_2$) end points. Although prevention of amputation is a goal in the management of patients with CLI, previous clinical angiogenesis trials have not designated amputation as an efficacy end point.[9–15,17,20–23] In contrast to earlier randomized trials with angiogenic agents, the present trial includes multiple administrations of the angiogenic agent. This is made possible by the nonimmunogenic profile of the drug and the selection of a seriously ill population of patients with CLI.

The study led to three main findings. First, the use of NV1FGF did not demonstrate a statistically significant improvement in ulcer healing. Second, the use of NV1FGF significantly reduced the risk of all and major amputations. Third, there was a trend (although not statistically significant) toward a decrease in mortality with the use of NV1FGF. The lack of any significant effect on ulcer healing may be explained by the great heterogeneity and severity of baseline skin lesions. Eligible patients had heterogeneous ulcers of various sizes ranging from 0.09 to 148 cm^2 in the placebo group, and from 0.35 to 483 cm^2 in NV1FGF group. Similarly, some patients had severe disease with fully necrotic distal tissue that would have been impossible to heal, even with increased blood flow. Moreover, wound care, which varies widely in clinical practice, was not standardized across investigational sites. Most important, a significantly higher amputation rate in

Table 3 Multivariate analysis of predictors of the risk of first major amputation (Cox analysis)

	N (%)	Hazard ratio (95% CI)	*P* value
Treatment (*N*[a])			
Placebo (*N* = 46)	15 (32.6)		
NV1FGF (*N* = 45)	7 (15.6)	0.35 (0.13, 0.94)	0.036
Amputation as previous peripheral arterial disease treatment			
No amputation (*N* = 72)	17 (23.6)		
Amputation (*N* = 19)	5 (26.3)	0.92 (0.31, 2.77)	0.889
Diabetic status			
Nondiabetic (*N* = 54)	13 (24.1)		
Diabetic (*N* = 37)	9 (24.3)	1.75 (0.66, 4.59)	0.257
Smoking status			
Nonsmokers (*N* = 26)	2 (7.7)		
Current and ex-smokers (*N* = 65)	20 (30.8)	6.29 (1.41, 27.95)	0.016
Hypertension			
No hypertension (*N* = 16)	5 (31.2)		
Hypertension (*N* = 75)	17 (22.7)	0.48 (0.14, 1.62)	0.234
High risk of amputation			
No high risk of amputation (*N* = 38)	9 (23.7)		
High risk of amputation (*N* = 53)	13 (24.5)	1.49 (0.55, 3.99)	0.424
Ankle brachial index			
≥0.4 (*N* = 41)	3 (7.3)		
<0.4 (*N* = 50)	19 (38.0)	10.54 (2.96, 37.46)	<0.001
$TcPO_2$ dorsal surface of the foot (mm Hg)[b]			
≥30 (*N* = 17)	1 (5.9)		
≥20 and <30 (*N* = 14)	2 (14.3)		
≥10 and <20 (*N* = 21)	5 (23.8)	1.77 (1.05, 2.99)	0.032
<10 (*N* = 39)	14 (35.9)		

Abbreviations: ABI, ankle brachial index; CI, confidence interval; $TcPO_2$, transcutaneous oxygen pressure.
Multivariate analysis was performed including treatment allocation, diabetic status, smoking status, hypertension, prior amputation, initial risk of amputation, ABI, and $TcPO_2$.
[a]*N* corresponds to the total number of study patients with available data on risk factors; [b]risk is given for an increase of 1 unit.

Table 4 Adverse events of NV1FGF versus placebo in patients with critical limb ischemia in the "safety" population (*n* = 118)[a]

	Placebo (*N* = 61)	NV1FGF (*N* = 57)	*P*
All	56 (91.8)	53 (93.0)	1.000
Serious	49 (80.3)	40 (70.2)	0.285
Severe	42 (68.9)	27 (47.4)	0.025
Possibly related to study drug	13 (21.3)	15 (26.3)	0.666
Leading to study discontinuation	10 (16.4)	4 (7.0)	0.156
Selected			
Cardiac	7 (11.5)	14 (24.6)	0.091
Renal and urinary[b]	5 (8.2)	9 (15.8)	0.259
Neoplasm (all)	5 (8.2)	5 (8.8)	1.000
Malignant neoplasm	3	3	
Retinopathy	0	1 (1.8)	0.483

[a]The "safety" population (*n* = 118) consists of patients who have received at least one injection of the study drug or placebo; [b]The majority of renal/urinary disorders represented hematuria.

the placebo group influenced the statistical analysis, in that ulcers that increased in size were more often censored by amputation in the placebo arm. Finally, this trial was insufficiently powered to demonstrate a significant difference in complete wound-healing between treatment groups, given the sample size.

In this study, NV1FGF significantly reduced the risk of all and major amputations, and there was a no statistically significant trend toward decrease in mortality. Because major amputation is associated with high peri-operative mortality, the reduction in amputations in the NV1FGF arm may have had an impact on mortality rates in the trial. A similar benefit was observed for the population of patients with diabetes, in respect of all and major amputations. The trial missed its primary efficacy end point, but a significantly reduced risk for all and major amputations, allowing for the preservation of functional limbs in the NV1FGF group is clinically extremely relevant to the management of patients with CLI.[6,7]

Interestingly, the benefit relating to the amputation rate observed with angiogenic therapy in the present study was not associated with a benefit in respect of hemodynamic end points (ABI, TBI), and no significant differences in these were observed between the two groups. These data indicate that ABI and TBI, as well as ulcer healing and ulcer size, may not be useful markers of NV1FGF efficacy in patients with CLI, in view of the hypothesis that the benefit of NV1FGF may be primarily because of the effects at the microvascular level (FGF-1 has been shown to lead to arteriole formation and maturation[27]). However, further studies in experimental models are needed to explain the mechanisms underlying the observed improvements in patient outcomes.

Both placebo- and NV1FGF-treated patients experienced a high rate of adverse events (including cardiovascular events), reflecting the severity of the underlying disease in this trial. The patients' arterial disease had led to tissue necrosis (ischemic ulcer or gangrene), and most of those enrolled had been expected to require a major amputation within the subsequent 6 months to 1 year in the absence of revascularization to improve blood flow. In addition, peripheral arterial disease is often associated with significant comorbidities such as coronary artery disease, cerebral artery disease, diabetes, and renal impairment. Importantly, the incidence of adverse events (including potentially angiogenesis-related adverse events such as cancer and proliferative retinopathy) was no higher with the use of NV1FGF treatment than with the use of placebo. The rate of occurrence of adverse events that were serious or severe enough to result in discontinuation of the study was significantly lower in the NV1FGF group than in the placebo group, and this may reflect the efficacy of NV1FGF.

These data also confirm the finding from earlier studies that NV1FGF is well tolerated.[20]

Previous trials of therapeutic angiogenesis have investigated angiogenic recombinant protein, or gene therapy, with FGFs, vascular endothelial growth factors, and hepatocyte growth factor.[9–15,17,20–22] These agents have been generally well tolerated, and the results of some studies have suggested therapeutic benefit. In a small open-label trial in patients with CLI, intramuscular administration of naked plasmid DNA encoding human vascular endothelial growth factor significantly improved ABI and healed or improved ulcers in comparison with baseline data.[11] An alternative method of implanting bone marrow–derived or peripheral blood mononuclear cells in the legs of patients with CLI has also been investigated and shown evidence of benefit.[23,28]

The discrepancy between the positive result obtained in this study and the negative results in earlier studies of protein or gene therapy may be explained by the fact that the use of NV1FGF enables re-administration of pCOR plasmid–based therapy. Multiple administrations of NV1FGF may allow sustained local exposure to the expressed FGF-1 at the site of administration compared with the more transient exposure obtained from a single administration.

These data indicate that NV1FGF therapy may offer the potential for effective management of CLI, and have provided the basis for initiation of the phase III study TAMARIS (Therapeutic Angiogenesis for the Management of Arteriopathy in a Randomized International Study) designed to assess world-wide, in 490 similar patients, the efficacy of NV1FGF versus placebo with the combined end point, amputation or death, as the primary end point of the study.

MATERIALS AND METHODS

The study was designed by the sponsor (Centelion SAS, a wholly owned subsidiary of Sanofi-Aventis SA, Paris, France) in collaboration with experts in the field of peripheral arterial disease. The sponsor collected the data and monitored the conduct of the study. After database lock, statistical analyses were conducted by MDS Pharma Services (Lyon, France). The sponsor coordinated writing of the manuscript with a writing committee comprising the first nine academic authors and sponsor authors. The writing committee had full access to study reports and was actively involved in data analysis and interpretation. All the authors approved the final manuscript.

Study design. This was a double-blind, randomized, placebo-controlled, parallel-group, multicenter, multinational phase II study, involving 37 investigational sites in six European countries (Belgium, France, Germany, Italy, Switzerland, and the UK). The protocol was approved by the institutional review board at each center, and the study was conducted in accordance with good clinical practices, the Declaration of Helsinki, and relevant local regulations. All patients provided written informed consent.

A total of 112 patients were to be randomized, 56 into each group, with the aim of verifying the assumption that the success rate for the complete healing of at least one ulcer of the treated leg, evaluated in the MITT population, would be 30% with NV1FGF versus 8% with placebo at week 25. The study would have sufficient power (80%) to detect improvement by 22% (15 NV1FGF successes versus 5 placebo successes) with a total of 50 patients available for evaluation per treatment group.

Patients. The study enrolled men and women patients aged ≥45 years with CLI who presented with nonhealing ulcer(s), and in whom revascularization was not considered a suitable option. CLI was defined according to the TransAtlantic Inter-Society Consensus document.[2] Patients were required to show objective evidence of CLI on the basis of both total arterial occlusion (by angiography or Doppler) and pressure criteria (resting ankle pressure ≤70 mm Hg and/or toe pressure ≤50 mm Hg, and/or $TcPO_2$ ≤20 mm Hg and/or metatarsal pulse volume recording barely pulsatile). Signs of healing of the trophic lesions (reduction in ulcer size or depth) were required to be absent for ≥2 weeks before the first administration of the study drug. All the participants were found unsuitable for revascularization for one or more of the following reasons: (i) there was poor or no autologous graft material; (ii) revascularization would result in incomplete perfusion of the foot (absence of distal runoff); (iii) there was a high risk of failure for technical reasons; (iv) there was a safety risk associated with the revascularization procedure; (v) there was a high risk of amputation on account of conditions such as gangrene.

Randomization and treatment plan. The patients were screened for eligibility 2–8 weeks before the administration of the study drugs. Blocks of study medication for four patients each were allocated to each investigational site. The eligible patients were randomized in the order enrolled, between April 2002 and April 2004, using permuted-block randomization with a block size of four. Patients participated in the trial on an outpatient basis.

On each of days 1, 15, 30, and 45, eight intramuscular injections were administered in a single leg (four in the calf and four in the thigh), each injection containing either 2.5 ml NV1FGF at 0.2 mg/ml (0.5 mg NV1FGF per injection; 4 mg per administration) or placebo saline solution. If CLI affected both of the patient's legs, the leg estimated to benefit most from the treatment, based on lower hemodynamic parameters (including ABI, TBI, and $TcPO_2$) was selected. Because of the diffuse nature of CLI-related atherosclerotic lesions, the sites of injection differed at each administration, and were selected according to available muscle mass while avoiding ulcer locations, and at distance from an artery or main nerve according to the investigator's discretion. Following the 6-week double-blind treatment period, the follow up of patients continued up to week 52, with assessments at week 13 (±5 days), week 25 (±10 days), week 38 (±10 days), and week 52 (±10 days). On clinic visit days during the period of administration of the study drug, patients received their study drug or placebo after all the assessments had been collected.

A panel of experts, blinded as to study group, assigned patients to the MITT population, which included those who (i) had received at least two treatment injections (eight injections each) of a study drug, (ii) had undergone an evaluation for aggregate ulcer size at baseline and had at least one nonhealing ulcer, and (iii) had undergone an evaluation for aggregate ulcer size at or after week 5. All patients who had received at least one treatment injection were included in the safety population.

End points. The prespecified primary end point in this trial was the incidence of complete healing of at least one ulcer in the treated limb at week 25. Ulcer assessments were performed at each visit by clinical assessment (type and characteristic of ulcers) and size assessment. A review panel, blinded as to intergroup allocation of the patients, comprising three investigators and one wound-healing expert, assessed ulcers as "healed" or "not healed". The panel reconciled discordance between the investigator's assessments and the available data. Predetermined secondary end points included major and minor amputation ("rate" and "time to"). Major amputation was defined as through or above the ankle. Minor amputation was defined as below the ankle. Other prespecified secondary assessment variables were time to death, and hemodynamic parameters. At each visit, patients assessed the ischemic rest pain they had experienced during the previous 7 days on a subjective 100-mm visual analog scale: 0 mm = "no pain" to 100 mm = "pain as bad as it could be".

Safety. Safety assessments included adverse event reporting, physical examinations, vital signs, laboratory tests, ophthalmologic examination, chest x-ray, and mammography. An independent Data and Safety Monitoring Board was responsible for the continuous independent safety monitoring of this trial.

Statistical analysis. All efficacy and safety analyses were performed in the MITT and safety populations, respectively. For both, baseline demographic and safety data, comparisons between the NV1FGF and placebo groups were performed, using a Student's *t*-test (the Mann–Whitney rank-sum test for skewed variables) or a χ^2 analysis when appropriate.

The primary end point was compared in the NV1FGF and placebo groups, using a generalized linear mixed model and the last-observation-carried-forward method.[29] The main parameters of the model were treatment agent, baseline aggregate ulcer size and number, diabetes status, smoking status, and country.

Time to event (amputation, death, or combination) was compared by Kaplan–Meier analysis and associated log-rank test with censoring at day 375. HRs were derived using the Cox model. The robustness of the results was assessed by post-hoc sensitivity analysis involving the potential influence of disease prognostic factors in Cox analyses.

Statistical significance was defined as $P < 0.05$.

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SUPPLEMENTARY MATERIAL

Supplementary Data S1. Acknowledgments and additional study material.

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