Effect on walking distance and atherosclerosis progression of a nitric oxide-donating agent in intermittent claudication

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Background: **Peripheral arterial disease (PAD) is almost invariably associated with a generalized atherosclerotic involvement of the arterial tree and endothelial dysfunction. Previous short-term studies showed improvement of vascular reactivity and walking capacity in PAD patients by measures aimed at restoring nitric oxide (NO) production. NO is also known to prevent the progression of atherosclerosis. We wished to assess whether the prolonged administration of an NO-donating agent (NCX 4016) improves the functional capacity of PAD patients and affects the progression of atherosclerosis as assessed by carotid intima-media thickness (IMT).**

Methods: **This prospective, double-blind, placebo-controlled study enrolled 442 patients with stable intermittent claudication who were randomized to NCX 4016 (800 mg, twice daily) or its placebo for 6 months. The primary study outcome was the absolute claudication distance on a constant treadmill test (10% incline, 3 km/h). The main secondary end point was the change of the mean far-wall right common carotid artery IMT.**

Results: **The increase of absolute claudication distance at 6 months compared with baseline was 126 ± 140 meters in the placebo-treated group and 117 ± 137 meters in the NCX 4016-treated group, with no significant differences. Carotid IMT increased in the placebo-treated group (+0.01 ± 0.01 mm; $P = .55$) and decreased in the NCX 4016-treated group (−0.03 ± 0.01 mm; $P = .0306$). Other secondary end points did not differ between the two treatments.**

Conclusions: **Long-term NO donation does not improve the claudication distance but does reduce progression of atherosclerosis in patients with PAD. Further studies aimed at assessing whether long-term NO donation may prevent ischemic cardiovascular events are warranted. (J Vasc Surg 2012;56:1622-8.)**

Peripheral arterial disease (PAD) is a condition of leg ischemia caused by atherosclerosis of the arteries of the lower limbs.[1,2] An early symptom of PAD is intermittent claudication, a disabling pain on walking that can be relieved by rest and that is caused by muscular ischemia, immediately followed by reperfusion during rest. PAD is caused by atherosclerosis, but dysfunction of the endothelium in the lower limbs may also contribute to the development of symptoms by reducing the ability of the arteries to supply muscles with the required quantity of oxygen during exercise. In later stages, progression of atherosclerosis of the lower limb arteries can lead to ischemic pain at rest, ulceration, gangrene, and in some cases, limb amputation.

PAD is almost invariably associated with a generalized atherosclerotic involvement of the arterial tree and is frequently associated with clinical ischemia of the coronary and cerebrovascular circulation. Endothelial dysfunction has been shown to play a role in the initial phases of the atherosclerotic process and in the precipitation of ischemic events[3] and is mainly attributable to the loss of the capacity of the endothelium to produce nitric oxide (NO).

Endothelial dysfunction is the consequence of the noxious effects of several classical and novel risk factors on the

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Financial support for this study was received from NicOx SA. This work was also partly supported by grants to P.G. from Fondazione Cassa di Risparmio di Perugia (Protocol No. 2010.020.161 and 2009.020.0097).

Author conflict of interest: The NCX 4016-X-208 study was funded by NicOx S.A. The sponsor participated in discussions regarding the design and conduct of the study and provided logistical support during the trial. Collection, management, and analysis of the data were performed by the sponsor and the contract research organization under contract with the sponsor. Collection and measurements of carotid intima-media thickness were performed independently from the sponsor by Drs Gresele and Migliacci and by Dr Giuseppe Guglielmini. The manuscript was prepared by Drs Gresele and Migliacci and revised by the members of the Steering Committee of the trial. The sponsor was permitted to review the manuscript; the final approval of contents was exclusively retained by the authors.

*A list of the members of the NCX 4016-X-208 Study Group may be found in Appendix B (online only).

Additional material for this article may be found online at www.jvascsurg.org.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

0741-5214/$36.00

http://dx.doi.org/10.1016/j.jvs.2012.05.064

endothelium.[3,4] However, ischemia-reperfusion damage also contributes to endothelial dysfunction and to the progression of systemic atherosclerotic disease by provoking an inflammatory reaction in the involved tissue and a systemic response with activation of circulating neutrophils, increase of endothelial adhesion molecules, and release of several cytokines.[5-7]

It has thus been hypothesized that therapeutic interventions aimed at restoring NO levels, by favoring endogenous NO biosynthesis or by supplying exogenous NO, may be beneficial to the functional capacity and to the systemic progression of atherosclerotic disease in patients with PAD. The positive effect shown by the long-term administration of some statins, such as simvastatin or atorvastatin,[8-10] has been interpreted as partly due to the ability to restore the production of NO.[11]

Another example of such an approach has been the oral supplementation with large doses of L-arginine, the metabolic precursor of NO. Short-term administration has shown very promising activity on the functional capacity of patients with PAD, with a striking increase in the claudication distance.[12] Moreover, several preclinical studies showed that long-term supplementation with L-arginine inhibited atherosclerosis and myointimal hyperplasia and enhanced angiogenesis.[13] On the basis of these premises, a long-term clinical trial testing long-term L-arginine administration to PAD patients was performed, but with disappointing results: NO synthesis and vascular reactivity were not improved.[14]

An alternative approach is that of directly providing NO using an NO-donating agent. Indeed, treatment with S-nitroso albumin protected skeletal muscle from ischemia-reperfusion injury in rabbits,[15] and some nitrates were shown to prevent endothelial dysfunction induced by ischemia-reperfusion in humans.[16] Moreover, sublingual glyceryl trinitrate improved the maximum walking capacity of patients with intermittent claudication.[17] In addition, transdermal nitroglycerin was shown to decrease inflammatory mediators in patients with PAD,[18] suggesting a possible protective role on atherosclerosis progression.

NCX 4016, an NO-releasing derivative of acetylsalicylic acid able to produce a sustained release of low concentrations of NO in vivo, demonstrated remarkable antiatherosclerotic activity in several preclinical models.[19,20] We previously showed that a 4-week administration of NCX 4016 to patients with intermittent claudication prevented the acute endothelial dysfunction induced by exercise and produced an increase, although not significant, of the initial claudication distance (ICD).[7] Moreover, recent data have shown beneficial effects of the administration of NCX 4016 to patients with type 2 diabetes mellitus, a frequent risk factor in PAD patients, in terms of inhibition of platelet function and metabolic control.[21,22] It thus seemed logical to test whether long-term NO donation with NCX 4016 improves the claudication distance in an appropriately sized clinical trial in patients with intermittent claudication. Another aim of our study was to assess the effects of prolonged NO treatment on atherosclerosis progression as documented by the measurement of intima-media thickness (IMT) at the level of the common carotid artery.[23]

METHODS

Design of the study. This was a prospective, randomized, double-blind, parallel-groups, placebo-controlled study conducted in 43 clinical sites throughout Europe. A total of 442 patients with PAD at Leriche-Fontaine stage II were treated for 6 months with NCX 4016 (800 mg, twice daily) or with an indistinguishable placebo. The twice-daily dosage of 800 mg was selected based on previous clinical studies that demonstrated a good safety profile and a good tolerability in the gastrointestinal tract.[19] Compliance was checked by counting dispensed vs returned study medication. Participants were allocated to treatment according to a centrally generated randomization list, balanced for each participating center. Inclusion criteria are reported in the Supplemental Methods (online only).

All patients received aspirin (100 mg, once daily) for cardiovascular prevention, which was standard for this patient population at the time of study design.[1,2]

The primary end point of the study was the absolute claudication distance (ACD) on a constant treadmill test. Secondary end points were evaluations at 6 months of ICD, the proportion of patients showing a ≥28% or a ≥50% improvement of ACD,[24,25] quality of life as assessed by the Short Form 36 Health Survey,[26] variations of the ankle-brachial index (ABI), and modifications of the IMT of the right common carotid artery. Details on IMT measurement are reported in the Supplemental Methods (online only).

Statistical methods. The primary study objective was to demonstrate a clinically significant difference in the ACD between active treatment and placebo after 6 months of therapy. For this purpose, ACD data were fitted by a generalized linear model with log-link function and assuming γ-distribution of errors. Treatment, centers (dummy covariates), and log-transformed run-in ACD values (continuous covariate) were considered as the independent variables of the model. Results are reported as model-based estimates of treatment differences with associated two-sided 95% confidence limits. A further ad hoc analysis based on ACD data transformed into ranks (nonparametric approach) was used to assess the sensitivity of the analysis to the assumed model (γ-distribution). Sample size calculation and other details on statistical analysis are available in the Supplemental Methods (online only).

The trial was registered at http://ClinicalTrials.gov (NCT01256775).

RESULTS

Demographics and baseline characteristics. A total of 442 patients, who met the inclusion and exclusion criteria, were enrolled in the trial in 43 centers from five European countries and were randomly allocated to either of the two treatment groups; 372 of 442 patients (84%) completed the study (Fig 1). Of the 70 patients who dropped out (44 [19.9%] in the NCX 4016 group and 26 [11.8%] in the placebo group), reasons for discontinuation

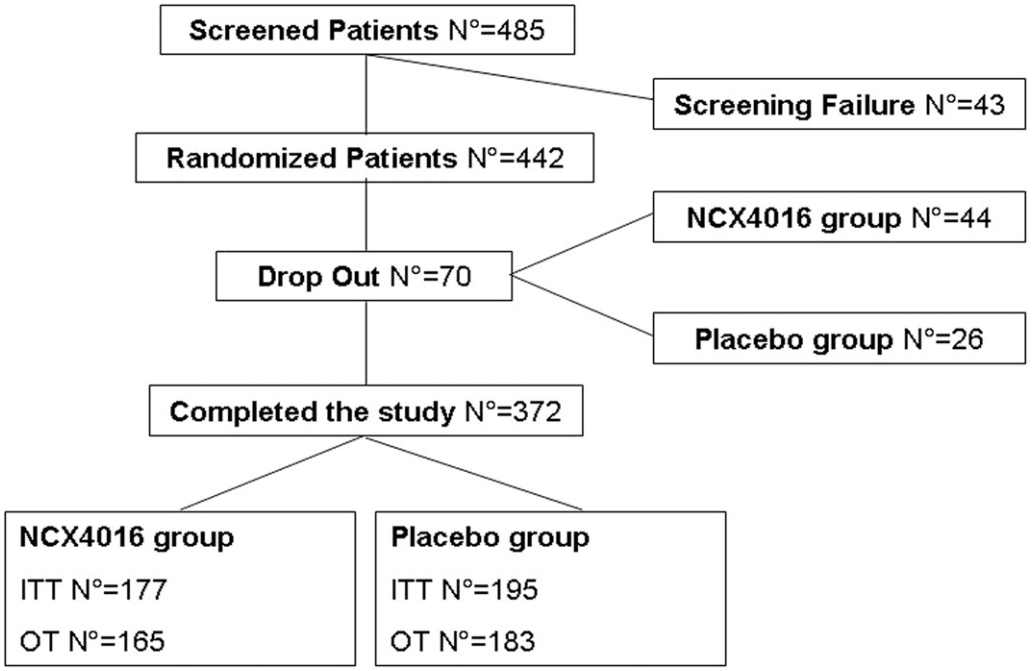

Fig 1. Flow chart of the trial profile. *ITT,* Intention to treat; *OT*, on-treatment.

were adverse events (25 in the NCX 4016 group, nine in the placebo group); protocol violation (three in the NCX 4016 group and five in the placebo group); patient withdrawal of consent (11 in the NCX 4016 group and 10 in the placebo group); and other reasons (five in the NCX 4016 group and two in the placebo group).

No relevant heterogeneity among the two study groups was found in demographic variables (Table I). As expected, most patients were men (80.1% on NCX 4016, 78.3% on placebo), and mean age was 66.7 ± 8.2 (NCX 4016) and 66.4 ± 9.1 years (placebo). In the entire cohort, the most prevalent risk factor was hypertension (69.0%), followed by dyslipidemia (56.3%) and diabetes (32.5%). In the NCX 4016 and placebo groups, respectively, the mean ABI was 0.66 ± 0.15 and 0.64 ±0.15, mean baseline ACD was 210 ± 107 and 214 ±114 meters, and mean baseline ICD was 131.5 ±77 and 131 ±78 meters, respectively. Concerning concomitant medications, 52.2% of patients were taking statins, with a significant imbalance in favor of the NCX 4016 group (57.9% vs 46.6%; $P = .0173$), and 41.4% were taking angiotensin-converting enzyme inhibitors (ACE-Is)/angiotensin receptor blockers (ARBs), with no significant differences between the two groups.

Effects on the walking capacity and health-related quality of life. The ACD increased in both treatment groups from baseline to day 90 and continued to increase from day 90 to day 180 (Fig 2). The increase of ACD at 6 months compared with baseline was +126 ± 140 meters in the placebo group and +117 ± 137 meters in the NCX 4016 group. In the intention-to-treat analysis, the difference in ACD from baseline to day 180 between NCX 4016-treated and placebo-treated patients was not significant (+4.6%; range, −3.5% to +13.4%; $P = .27$). The on-treatment analysis also showed no significant difference in the primary end point between the two study groups (+6.5%; range, −3.1 to 16.9; $P = .1927$). There was no interaction of the primary end point with other background variables, such as hypertension, smoking, dyslipidemia, or

Table I. Demographic characteristics of patients in the two study groups

Variable[a]	*NCX 4016*	*Placebo*	P
Age, years	66.7 ± 8.2	66.4 ± 9.1	.6839
Sex			
Female	44	48	
Male	221	221	.6393
PAD duration, years	5.61 ± 4.87	5.35 ± 4.89	.5906
Body mass index, kg/m^2	26.6 ± 4.3	26.6 ± 4.2	.9512
Hypertension	154 (69.7)	151 (68.3)	.7577
Dyslipidemia	133 (60.2)	116 (52.5)	.1030
Diabetes	77 (34.8)	67 (30.3)	.3102
Current cigarette smoker	91 (41.2)	91 (41.2)	.3243
Medication use			
Statin	128 (57.9)	103 (46.6)	.0173
ACE-I	99 (44.8)	84 (38.0)	.1475
ARB	54 (24.4)	44 (19.9)	.2522
ABI	0.66 ± 0.15	0.64 ± 0.15	.2444
ACD, meters	210 ± 107	217 ± 114	.4896
ICD, meters	131 ± 77	131 ± 78	.9959

ABI, Ankle-brachial index; *ACD,* absolute claudication distance; *ACE-I,* angiotensin-converting enzyme inhibitor; *ARB,* angiotensin receptor blocker; *ICD,* initial claudication distance; *PAD,* peripheral arterial disease.
[a]Continuous data are shown as the mean ± standard deviation and categoric data as number (%).

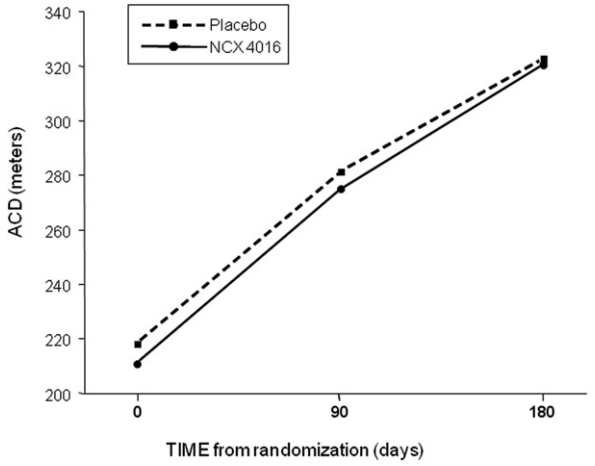

Fig 2. Evolution of the absolute claudication distance (*ACD*) during the trial in patients treated with NCX 4016 or placebo. Data are shown as geometric means ± standard deviation and refer to the intima-media thickness (IMT) analysis.

concomitant drug use. In a post hoc subgroup analysis in 77 diabetic patients in the NCX 4016 group and 67 in the placebo group, the difference in ACD from baseline to 6 months was significantly higher in the NCX 4016 group than in the placebo group (+15.2%; range, 0.9%-31.5%; $P = .0376$). Concerning secondary end points, the ICD increased by 84.5 ± 88.5 meters in the placebo group and by 84.3 ± 100.2 meters in the NCX 4016 group. The difference in ICD at day 180 between NCX 4016 patients and placebo patients was not significant (+5.9%; range, 2.7%-15.1%; $P = .1838$). The proportion of patients with a ≥28% improvement in ACD at 6 months compared with baseline was 44.3% in the NCX 4016 group and 44.8% in the placebo group ($P = .9238$). The proportion of patients with a ≥50% improvement in ACD at 6 months compared with baseline was 34.4% in the NCX 4016 group and 30.8% in the placebo group ($P = .4168$). No statistically significant difference between the two groups was found for any of the Short Form 36-related parameters (Table II).

Effects on the progression of atherosclerosis. The ABI was measured at run-in to verify patient eligibility at baseline and at completion of the 6-month treatment period. The ABI of the most affected limb remained stable throughout the study and did not differ between the placebo group (0.66 ± 0.15) and NCX 4016 group (0.67 ± 0.16) at 6 months. No statistically significant difference between NCX 4016 and placebo treatment was found for changes of ABI between baseline and 6 months.

The common carotid IMT was measured at baseline and at 180 days. A complete set of analyzable IMT data was available from 183 patients (80 NCX 4016 patients and 103 placebo patients). Some of the participating centers did not have the technical means for image acquisition, and several recordings were excluded from the analysis for poor technical quality.

Table II. Walking capacity and health-related quality of life (secondary end points) according to intention to treat analysis[a]

Variable	*NCX 4016*	*Placebo*	P
ACD			
Patients with			
>28% improvement	98 (44.3)	99 (44.6)	.9237
>50% improvement	76 (34.4)	68 (30.8)	.4168
QOL score			
Physical functioning			
6 months-baseline	3.14 ± 14.03	5.66 ± 14.71	.1160
ICD, meters			
Baseline	131.5 ± 77.3	131.5 ± 78	.9959
6 months	203.0 ± 162.6	193.2 ± 138.8	.1838
ABI			
6 months-baseline	0.0 ± 0.08	0.02 ± 0.1	.1376

ABI, Ankle-brachial index; *ACD,* absolute claudication distance; *ICD,* initial claudication distance; *PAD,* peripheral arterial disease; *QOL,* quality of life.

[a]Categoric data shown as number (%); continuous data shown as mean ± standard deviation.

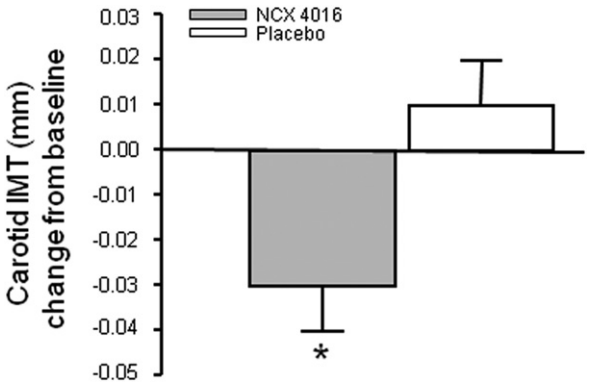

Fig 3. Change from baseline in the mean intima-media thickness (*IMT*) of the right common carotid artery after 6 months of treatment with either NCX 4016 or placebo. Data represent means ± standard deviation. *$P = .0306$ vs baseline.

The subgroup of patients for which IMT measurements were available did not differ from the remaining population for any demographic variable or for ACD. Baseline IMT did not differ significantly between the two treatment groups (Supplemental Table I, online only). Mean IMT decreased from baseline to 6 months in the NCX 4016-treated group (−0.03 ± 0.01; range, 0.06-0 mm; $P = .0306$) but increased in the placebo-treated group (+0.01 ± 0.01; range, −0.01 to 0.01 mm; $P = .55$; Fig 3). The number of patients taking a statin in the two treatment groups was balanced (NCX 4016, 46; placebo, 48; $\chi^2 = 2.141$; $P = .1797$). An analysis of IMT changes at 6 months in the subgroup taking statins confirmed a decrease of IMT in the NCX 4016 group (−0.04 ± 0.18 mm) and not in the placebo group (+0.02 ± 0.16 mm).

Adverse events. Adverse events occurred in 90 patients (40.7%) treated with NCX 4016 and in 69 (31.2%)

treated with placebo ($P = .0374$; Supplemental Table II, online only). The most frequent adverse event described in the NCX 4016 group was diarrhea (11.3%), followed by low back pain (2.3%) and epigastric discomfort (1.8%). A significant difference was found between the two groups with regard to diarrhea (25 patients in the NCX 4016 group and five in the placebo group; $P < .05$). A higher number of patients discontinued the study drug in the NCX 4016 group. Overall, 35 NCX 4016 patients compared with 11 placebo patients interrupted the study drug due to side effects ($P < .0002$); in most cases, drug discontinuation was the consequence of diarrhea.

In the placebo group, the most frequent adverse events reported were diarrhea (2.26%), low back pain and limb pain, and bronchitis and gastroesophageal reflux (for all, 2.26%).

Major cardiovascular events occurred in four patients in the placebo group (one patient each with acute myocardial infarction, angina, worsening of claudication, and sudden death) and in six in the NCX 4016 group (three acute myocardial infarctions, one angina, and two with worsening of claudication; P = NS).

DISCUSSION

The main finding of the present study is that 6 months of administration of an oral NO-donating agent (NCX 4016) does not improve the maximal walking distance in patients with PAD compared with placebo. The study rationale was based on the observation that patients with PAD have endothelial dysfunction with impaired NO production,[4,7] which is partly the consequence of repeated ischemia-reperfusion insults occurring during daily life in these patients; therefore, we hypothesized that an NO-donor, by providing the missing NO, would improve calf blood flow and functional capacity. In agreement with this, a previous study showed that NCX 4016 administered for 1 month to PAD patients prevented the acute endothelial dysfunction produced by maximal leg exercise.[7] Indeed, the administration to healthy volunteers of L-arginine, the metabolic precursor of NO, protects from endothelial dysfunction induced by ischemia-reperfusion.[27] In addition, previous small studies with other NO donors, such as nitroglycerin or with the NO precursor L-arginine, had shown an improvement of the claudication distance in PAD.[12,17]

The lack of an effect of NCX 4016 in the present long-term clinical trial, despite effective NO release in vivo,[7,21] suggests that long-term NO donation is not able to improve the functional capacity of PAD patients. This finding is in agreement with a recent study showing that the long-term supplementation with L-arginine, a precursor of NO synthesis, is of no benefit in patients with intermittent claudication.[14] Development of tolerance to NO might be one possible explanation of the lack of efficacy of NCX 4016 on the claudication distance, although this seems unlikely, because a 4-week administration of this drug in a previous study did not lead to the worsening of endothelial function, a marker of development of tolerance.[7] Therefore, the findings of the present study, together with previous data with L-arginine administration, suggest that the increase of NO to levels not inducing vasodilatory side effects may not be sufficient to significantly improve blood flow to the exercising muscles.

However, despite the lack of effects on the claudication distance, treatment with NCX 4016 inhibited significantly the progression of carotid IMT, an established surrogate marker of cardiovascular events in patients with atherosclerosis.[23,28] In fact, although a slight increase of carotid IMT was observed in the placebo-treated group at 6 months, a clear and significant decrease was evident in the NCX 4016-treated group.

Several previous studies with therapeutic agents of proven efficacy in preventing atherosclerotic cardiovascular events, such as statins, ACE-Is, or ARBs, have shown a limiting effect on the progression of IMT in patients with cardiovascular risk factors.[29-31] Moreover, cilostazol, an antiplatelet agent able to inhibit smooth muscle cell proliferation and prevent restenosis after endovascular treatment of patients with femoropopliteal atherosclerotic lesions,[32] was recently shown to induce regression of carotid atherosclerosis in patients with type 2 diabetes mellitus during a 2-year observation period[33] to an extent similar to that observed in the present study with NCX 4016. The reduction of IMT in the NCX 4016 group in our study was also similar in size to that observed with a 6-month treatment period with pioglitazone in patients with type 2 diabetes mellitus.[34]

The effect of treatment on the progression of IMT was a prespecified end point of our study, although a secondary end point, and although this was determined in a subgroup of the entire study population, this subgroup was rather large (183 patients) and not dissimilar in size to that of previously published clinical trials that had IMT as the principal end point.[30,34] In a subgroup analysis, statin treatment did not affect the result of NCX 4016 treatment on carotid IMT. This finding therefore suggests that NCX 4016 exerts an antiatherosclerotic activity in aspirin-treated patients at cardiovascular risk, even when added to a full set of drugs active in preventing atherosclerosis progression, such as statins, ACE-Is, or ARBs. This finding is interesting in view of the recent report that IMT progression is correlated with the long-term incidence of ischemic cardiovascular events in particular in patients with PAD.[35]

The reduction in the progression of atherosclerosis produced by NCX 4016 in our trial is in accordance with several preclinical studies showing an antiatherosclerotic effect of this NO donor in different models of atherosclerosis.[20,36,37] Moreover, one large, community-based cohort study in young adults showed a significant correlation between the degree of endothelial dysfunction and IMT, confirming that an impaired capacity of the endothelium to produce NO contributes to the progression of atherosclerosis.[38] We previously showed that oral administration of NCX 4016 to diabetic or PAD patients at the doses used in the present trial significantly enhanced plasma levels of NO degradation production.[7,19,21] Altogether, these data suggest that the long-term supply of NO may slow the progression of atherosclerosis. However, that the effect of NCX 4016 on IMT may be partly unrelated to NO cannot be excluded.

Our finding that the ABI did not change significantly in the two treatment groups is not in contrast with this conclusion. In fact, significant ABI changes may be detected only over longer observation periods and for much more extensive progressions of atherosclerosis.

In the subgroup of patients with diabetes (~30% of the study population), a statistically significant improvement of ACD was seen in the NCX 4016 group compared with placebo (+15.2%; $P = .038$). Although this finding is not fully explained, recent data showing that NCX 4016 stimulates glucose transport in adipocytes without negatively affecting insulin sensitivity, thus possibly helping in better controlling glycemia,[22] and that it controls hyperglycemia-induced platelet activation better than aspirin,[21] suggest a preferential effect of NCX 4016 in diabetic individuals. Of course, the hypothesis that the difference in ACD in the diabetic subgroup may be due to the play of chance cannot be excluded.

In terms of safety, NCX 4016 did not provoke hypotension or any other detrimental action to the cardiovascular system. However, a significantly higher incidence of episodic diarrhea was observed in the NCX 4016 group. This side effect has previously been reported for cilostazol, a phosphodiesterase inhibitor, and may be due to an increase of cyclic guanosine monophosphate in the gut with consequent water influx.[39]

Our study has several limitations. The ACD and ICD data showed a large variability, suggesting that the severity of the PAD in the patients enrolled was rather heterogeneous: this may have been partly responsible for the lack of efficacy of NCX 4016 on the claudication distance; however, the claudication distance, as measured by a standardized treadmill test, is an end point with an inherent large variability.

A second limitation may be represented by the prevalence of treatment with statins or ACE-Is, or both, at lower than current recommended standards; however, several large registries of patients with PAD confirm that this patient population is generally undertreated.[40]

Another limitation may be that the population for the IMT substudy was not large; however, many previous studies on the effect of agents active in preventing cardiovascular events on the progression of IMT were of a similar size. There also may have been errors in IMT measurements due to poor reproducibility at the participating centers or to intersonographer differences between scans performed at many different institutions. However, the same expert sonographer examined each patient in our study with an identical ultrasound protocol throughout all the visits. The readings of the recordings were centrally performed in random order by a single expert investigator, unaware of the clinical characteristics of the participants or of the treatment groups, using automated digital edge-detection technology, as indicated by the Mannheim carotid IMT consensus.[41] The striking stability of the values observed at 6 months in the placebo group is a further confirmation of the good reproducibility of IMT measurements.

CONCLUSIONS

Long-term NO donation does not increase functional capacity in PAD patients, in accordance with previous data on the lack of efficacy of 6 months of treatment with L-arginine,[14] but does reduce atherosclerosis progression, as shown by a significant reduction of carotid IMT. NCX 4016 presents several peculiarities compared with previous NO donors, such as slow and sustained NO donation, the ability to prevent the activation of platelets induced by a wide range of agonists, the ability to prevent smooth muscle cell proliferation, and the capacity to induce reparative neoangiogenesis in a mouse model of peripheral ischemia.[19,21,37,42] However, NCX 4016 is poorly absorbed and has formulary limitations that have led to the interruption of its clinical development.[21] Novel NO-donating molecules with a better bioavailability and pharmacokinetic profile may represent ideal candidates for the treatment of patients with atherosclerotic cardiovascular disease. Confirmatory studies on the effect of orally active, slow-release NO donors on carotid IMT progression are warranted.

We gratefully acknowledge the help of Dr Marco Sardina in the planning of the study, Dr Maria Ballabio with help in the analysis of data and with critical discussions (both formerly at NicOx SA, Sophia Antipolis, France), Dr Giuseppe Guglielmini with help in the IMT data handling, and the skilled editorial assistance from Dr Sara Orsini.

AUTHOR CONTRIBUTIONS

Conception and design: PG, RM
Analysis and interpretation: PG, RM
Data collection: PG, RM, EA, EB, PM, FV
Writing the article: PG, RM
Critical revision of the article: PG, RM, EA, EB, PM, FV
Final approval of the article: PG, RM, EA, EB, PM, FV
Statistical analysis: EB
Obtained funding: PG
Overall responsibility: PG

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Submitted Mar 2, 2012; accepted May 8, 2012.

Additional material for this article may be found online at www.jvascsurg.org.

APPENDIX A
Supplemental Methods

Inclusion criteria. Inclusion criteria were: male and female patients between 40 and 80 years with Leriche-Fontaine stage II PAD presenting symptoms of intermittent claudication stable for at least 6 months, an ankle/brachial index <0.9, an absolute claudication distance (ACD) <500 m and an initial claudication distance (ICD) >50 m on a standardized treadmill test (10% incline, 3 km/hr), and clinical stability before inclusion (ie, changes in ACD not exceeding 25% in two standardized treadmill tests during run-in). All patients gave their written informed consent.

Exclusion criteria were: unstable symptoms and/or rapid deterioration of PAD during the previous 3 months; presence of clinically significant renal or hepatic failure, or insulin-dependent type 1 diabetes; uncontrolled type 2 diabetes, arterial hypertension, or dyslipidemia; any clinical condition limiting the patient's exercise ability (angina pectoris, congestive heart failure, respiratory disease, bone and joint disease, neurological disorders); active peptic ulcer during the previous 6 months; any hemorrhagic condition or history of bleeding; acute coronary syndrome or acute cerebrovascular episodes during the previous 6 months; previous revascularization procedures during the last 6 months or indication for vascular surgery; ischemic rest pain; life expectancy <12 months; pregnancy or lactation; participation to other investigational trials within 3 months prior to inclusion; history of hypersensitivity or any form of allergic reaction or contraindications to NSAIDs, aspirin, and NO-donating drugs. The following treatments were not allowed for the period of the study: continuative use (>7 days) of NSAIDs or nitrovasodilating drugs; phosphodiesterase type 5 inhibitors, anticoagulants, heparin, ticlopidine, clopidogrel, indobufen, defibrotide, mesoglycan, picotamide, pentoxyfylline, carnitine, sulodexide. All other concomitant treatments were kept constant as much as possible during the study period.

IMT measurement. Common right carotid artery was examined by B-mode ultrasound in the longitudinal view, 1-1.5 cm proximally to the bifurcation. The measurement of IMT was obtained according to the Mannheim carotid intima-media thickness consensus ([1]) with one modification: instead of acquiring the vascular wall image only with the lateral probe incidence, images were acquired also with the anterior and posterior incidence, in order to obtain a triplicate number of measurements to be used for comparisons. Each position was visualized and recorded for at least 15 sec. with simultaneous taking of the ECG tracing. Images of the far wall of the distal 1 cm of the right common carotid artery were obtained. IMT was calculated from each of the three projections and the final value was calculated from the average of all measurements. The baseline carotid ultrasonographic examinations were used to localize the site of interest at follow-up. Digitized still images from an electrocardiographically defined diastolic frame were analyzed offline. A single observer who was unaware of the treatment assignments and the identities of the patients measured the mean carotid intima media thickness. Focal atherosclerotic plaques were excluded from the measurements. All measurements were performed with the use of an automated border detection system. A number of measurements not inferior to 30 for each of the three image acquisition incidences was carried out in the 1-cm segment of the carotid artery assessed. For each subject the same ultrasound system and transducer and the same operator were used throughout the study. Images were centrally analyzed at the coordinating center by a dedicated, automated computerized edge detection system for the measurement of common carotid far wall intima-media using the software M'ATH 2.0 (Metris Argenteuil, France).

Statistical methods. Analyses were performed both on the full (intention-to-treat) and on the compliant (on-treatment) analysis set. The full analysis set contained all patients having received at least one dose of the randomized treatments; the compliant analysis set was a subset of the full analysis set obtained by excluding patients with less than 80% intake of the investigational drugs and/or a major protocol deviation. Being a superiority trial, the results obtained from the analysis on the intention-to-treat population were considered as primary end point, while the on-treatment population was analyzed with the aim of ensuring that protocol violations/deviations and drop-outs or withdrawals did not affect the results. All computations were carried-out with the SAS version 8.2. Baseline characteristics in the two study groups were compared by Student *t*-test or Mann-Whitney test. Assuming as clinically significant a 28% ACD increase in the active treatment group as compared with the placebo, using the basic methodology for testing hypotheses about the means of two exponential distributions (distance to event data), a total sample size of 420 patients, randomized according to 1:1 allocation ratio, would provide a 80% power to detect a ratio of 1.28 in mean ACD between active treatment and placebo. Therefore, 210 evaluable patients per treatment group were required.

Computations were carried out assuming a 0.05 significance level (alpha) and using a one-sided hypothesis based on the F distribution. The PASS 2002® software was used for sample size calculation.

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APPENDIX B

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Supplemental Table I. Demographic characteristics of the subpopulation of patients for which IMT measurements were available (IMT yes) vs all the remaining study population (IMT no)

Variable	*IMT No*	*IMT Yes*	*All Patients*	P *value*
Age, years				
Mean ± SD (N)	66.91±8.44 (259)	66.01±8.9 (183)	66.53±8.63 (442)	.2797
Median (min-max)	68 (34-81)	68 (39-79)	68 (34-81)	
Weight (kg)				
Mean ± SD (N)	75.92±12.43 (259)	75.07±12 (183)	75.57±12.25 (442)	.4712
Median (min-max)	74 (48-114)	75 (50-110)	75 (48-114)	
Height (cm)				
Mean ± SD (N)	168.32±7.6 (259)	168.47±8.24 (183)	168.38±7.86 (442)	.8402
Median (min-max)	168 (145-188)	170 (140-187)	168 (140-188)	
Alcohol (units/daily)				
Mean ± SD (N)	1.28±1.5 (259)	1.53±1.57 (183)	1.38±1.54 (442)	.0892
Median (min-max)	1 (0-10)	1 (0-10)	1 (0-10)	
Coffee/tea (units/daily)				
Mean ± SD (N)	1.96±1.62 (258)	2.12±2.01 (183)	2.02±1.79 (441)	.3469
Median (min-max)	2 (0-10)	2 (0-20)	2 (0-20)	
Cigarettes (N/daily)				
Mean ± SD (N)	5.8±9.63 (258)	6.61±9.94 (182)	6.14±9.76 (440)	.3932
Median (min-max)	0 (0-60)	0 (0-40)	0 (0-60)	
Basal ACD (minutes)				
Mean ± SD (N)	5.25±0.52 (259)	5.21±0.53 (183)	5.23±0.53 (442)	.4979
Median (min-max)	5.25 (3.96-6.2)	5.19 (4.06-6.19)	5.21 (3.96-6.2)	
Basal ICD (minutes)				
Mean ± SD (N)	4.75±0.53 (259)	4.7±0.55 (183)	4.73±0.53 (442)	.3079
Median (min-max)	4.66 (3.42-6.03)	4.61 (3.91-6.03)	4.65 (3.42-6.03)	
Race				
White/Caucasian	100 (259/259)	100 (183/183)	100 (442/442)	—
Gender (M/F)				
Female	21.2 (55/259)	20.2 (37/183)	20.8 (92/442)	.7953
Male	78.8 (204/259)	79.8 (146/183)	79.2 (350/442)	

ACD, Absolute claudication distance; *ICD,* initial claudication distance; *IMT,* intima-media thickness; *SD,* standard deviation.
No significant differences were detected between the two populations for any of the assessed variables.

Supplemental Table II. Number of patients and % with at least one post-treatment adverse event by type

ICD10 term	*NCX 4016 800 mg bid*	*Placebo 800 mg bid*
Number (%) of patients with at least 1 adverse event	90 (40.72%)	69 (31.22%)
Diarrhea	25 (11.31%)	5 (2.26%)
Low back pain	5 (2.26%)	5 (2.26%)
Fever	4 (1.81%)	1 (0.45%)
Gastralgia	4 (1.81%)	2 (0.9%)
Influenza	3 (1.36%)	1 (0.45%)
Acute myocardial infarction (not specified)	3 (1.36%)	1 (0.45%)
Nausea and vomiting	3 (1.36%)	0 (0%)
Oliguria	3 (1.36%)	0 (0%)
Oral burning	3 (1.36%)	0 (0%)
Sciatic pain	3 (1.36%)	1 (0.45%)
Anxiety	2 (0.9%)	0 (0%)
Arthritis	2 (0.9%)	0 (0%)
Cataract (not specified)	2 (0.9%)	1 (0.45%)
Claudicatio intermittent	2 (0.9%)	2 (0.9%)
Deep venous thrombosis	2 (0.9%)	0 (0%)
Dyspepsia	2 (0.9%)	3 (1.36%)
Hypertension	2 (0.9%)	2 (0.9%)
Itching (not specified)	2 (0.9%)	0 (0%)
Meteorism	2 (0.9%)	0 (0%)
Osteoporosis	2 (0.9%)	1 (0.45%)
Pyrosis	2 (0.9%)	1 (0.45%)
Surgery prophilaxis	2 (0.9%)	0 (0%)
Thrombophlebitis	2 (0.9%)	0 (0%)
Vertigo	2 (0.9%)	0 (0%)
Abscess perianal	1 (0.45%)	0 (0%)
Accidental cut on scalps skin	1 (0.45%)	0 (0%)
Acute pancreatitis	1 (0.45%)	0 (0%)
Acute pharingitis (not specified)	1 (0.45%)	0 (0%)
Acute tendinitis	1 (0.45%)	0 (0%)
Allergy	1 (0.45%)	0 (0%)
Ankle distorsion	1 (0.45%)	0 (0%)
Arthrosis (not specified)	1 (0.45%)	0 (0%)
Ascites	1 (0.45%)	0 (0%)
Asthma	1 (0.45%)	0 (0%)
Atrial fibrillation and atrial flutter	1 (0.45%)	1 (0.45%)
Bronchitis	1 (0.45%)	4 (1.81%)
Bronchospasm	1 (0.45%)	0 (0%)
Cerebral infarction (not specified)	1 (0.45%)	0 (0%)
Cervical trauma due to a car incident	1 (0.45%)	0 (0%)
Cervicoalgia	1 (0.45%)	1 (0.45%)
Chronic sclerosante hepatopathy	1 (0.45%)	0 (0%)
Colon diverticulosis	1 (0.45%)	1 (0.45%)
Constipation	1 (0.45%)	0 (0%)
Cough	1 (0.45%)	0 (0%)
CPK increase	1 (0.45%)	0 (0%)
Critical ischemia on the contralateral leg	1 (0.45%)	0 (0%)
Cystitis	1 (0.45%)	1 (0.45%)
Dental implantation	1 (0.45%)	0 (0%)
Depression	1 (0.45%)	1 (0.45%)
Dermatitis	1 (0.45%)	0 (0%)
Diabetes	1 (0.45%)	1 (0.45%)
Diarrhea and gastroenteritis of suspected infective origin	1 (0.45%)	0 (0%)
Dyslipidemia	1 (0.45%)	0 (0%)
Dyspnea	1 (0.45%)	0 (0%)
Erythema	1 (0.45%)	0 (0%)
Flushing	1 (0.45%)	0 (0%)
Gastritis (not specified)	1 (0.45%)	0 (0%)
Gastrointestinal hemorrhage (not specified)	1 (0.45%)	0 (0%)
Hemorrhoids	1 (0.45%)	0 (0%)
Herpes zoster	1 (0.45%)	1 (0.45%)
High AST/ALT level	1 (0.45%)	0 (0%)
High GGT level - suspected alchol abuse	1 (0.45%)	0 (0%)
Hyperuricemia	1 (0.45%)	4 (1.81%)
Infection wound foot	1 (0.45%)	0 (0%)

Supplemental Table II. Continued.

ICD10 term	*NCX 4016 800 mg bid*	*Placebo 800 mg bid*
Laryngitis	1 (0.45%)	0 (0%)
Limb pain	1 (0.45%)	5 (2.26%)
Liver function test increased	1 (0.45%)	1 (0.45%)
Liver steatosis	1 (0.45%)	0 (0%)
Migratory arthritis	1 (0.45%)	0 (0%)
Periarthritis shoulder	1 (0.45%)	0 (0%)
Pulmonary hypertension	1 (0.45%)	0 (0%)
Renal colic	1 (0.45%)	0 (0%)
Rhinitis vasomotor	1 (0.45%)	0 (0%)
Supraventricolar tachycardia	1 (0.45%)	0 (0%)
Syncope	1 (0.45%)	3 (1.36%)
Teleangectasia	1 (0.45%)	0 (0%)
Thoracic pain (not specified)	1 (0.45%)	1 (0.45%)
Thyroid nodule	1 (0.45%)	0 (0%)
Tooth extraction	1 (0.45%)	1 (0.45%)
Tooth fracture	1 (0.45%)	0 (0%)
Torticollis	1 (0.45%)	1 (0.45%)
Urinary tract infection	1 (0.45%)	0 (0%)
Ustion	1 (0.45%)	0 (0%)
Veins varicose without inflammation or ulcer of lower limbs	1 (0.45%)	0 (0%)
Wound foot	1 (0.45%)	0 (0%)
Acute pulmonary edema	0 (0%)	1 (0.45%)
Aortic murmure	0 (0%)	2 (0.9%)
Atherosclerotic cardiopathy	0 (0%)	1 (0.45%)
Bladder papilloma	0 (0%)	1 (0.45%)
Bradycardia (not specified)	0 (0%)	1 (0.45%)
Cerebral subarachnoid hemorrhage	0 (0%)	1 (0.45%)
Cramps muscle	0 (0%)	1 (0.45%)
Dental abscess	0 (0%)	1 (0.45%)
Epistaxis	0 (0%)	1 (0.45%)
Epithelioma DX back	0 (0%)	1 (0.45%)
Erysipelas	0 (0%)	1 (0.45%)
Foot trauma	0 (0%)	1 (0.45%)
Gastroesophageal reflux disease	0 (0%)	5 (2.26%)
Gastrointestinal problems	0 (0%)	1 (0.45%)
Gouty arthritis	0 (0%)	1 (0.45%)
Hair loss	0 (0%)	1 (0.45%)
Headache	0 (0%)	2 (0.9%)
Hematuria	0 (0%)	1 (0.45%)
Inflammation anal	0 (0%)	1 (0.45%)
Intestinal infection due to Campylobacter J - hypotension - lipothymia - multiple fractures at left hand	0 (0%)	1 (0.45%)
Left iliac murmure	0 (0%)	1 (0.45%)
Lymphedema left leg	0 (0%)	1 (0.45%)
Malleolar edema	0 (0%)	2 (0.9%)
Other wrist and hand fracture (not specified)	0 (0%)	2 (0.9%)
Phlebitis and thrombophlebitis of the lower limbs	0 (0%)	1 (0.45%)
Pink spot at the thorax	0 (0%)	1 (0.45%)
Rib fracture	0 (0%)	1 (0.45%)
Stomach perforation	0 (0%)	1 (0.45%)
Swelling generalized	0 (0%)	1 (0.45%)
Tooth ache	0 (0%)	1 (0.45%)
Transient ischemic attack	0 (0%)	1 (0.45%)
Trophic lesion right foot	0 (0%)	1 (0.45%)
Umbilical hernia	0 (0%)	1 (0.45%)

ALT, Alanine aminotransferase; *AST,* aspartate aminotransferase; *GGT,* gamma-glutamyl transferase.