Circ J 2018; **82:** 2165–2174
doi:10.1253/circj.CJ-17-1220

ORIGINAL ARTICLE
Peripheral Vascular Disease

Outcome From a Randomized Controlled Clinical Trial
— Improvement of Peripheral Arterial Disease by Granulocyte Colony-Stimulating Factor-Mobilized Autologous Peripheral-Blood-Mononuclear Cell Transplantation (IMPACT) —

Takashi Horie, MD, PhD; Seiji Yamazaki, MD; Sayaka Hanada, MD; Shuzo Kobayashi, MD, PhD; Tatsuo Tsukamoto, MD, PhD; Tetsuya Haruna, MD, PhD; Katsuhiko Sakaguchi, MD, PhD; Ken Sakai, MD, PhD; Hideaki Obara, MD, PhD; Kiyofumi Morishita, MD, PhD; Kenichi Saigo, MD, PhD; Yoshiaki Shintani, MD; Kohmei Kubo, MD, PhD; Junichi Hoshino, MD, PhD; Teiji Oda, MD, PhD; Eiji Kaneko, MD, PhD; Masaharu Nishikido, MD, PhD; Tetsuya Ioji; Hideaki Kaneda, MD, PhD; Masanori Fukushima, MD, PhD
for the Japan Study Group of Peripheral Vascular Regeneration Cell Therapy (JPRCT)

Background: The clinical usefulness of peripheral blood (PB) mononuclear cell (MNC) transplantation in patients with peripheral arterial disease (PAD), especially in those with mild-to-moderate severity, has not been fully clarified.

Methods and Results: A randomized clinical trial was conducted to evaluate the efficacy and safety of granulocyte colony-stimulating factor (G-CSF)-mobilized PBMNC transplantation in patients with PAD (Fontaine stage II–IV and Rutherford category 1–5) caused by arteriosclerosis obliterans or Buerger's disease. The primary endpoint was progression-free survival (PFS). In total, 107 subjects were enrolled. At baseline, Fontaine stage was II/III in 82 patients and IV in 21, and 54 patients were on hemodialysis. A total of 50 patients had intramuscular transplantation of PBMNC combined with standard of care (SOC) (cell therapy group), and 53 received SOC only (control group). PFS tended to be improved in the cell therapy group than in the control group (P=0.07). PFS in Fontaine stage II/III subgroup was significantly better in the cell therapy group than in the control group. Cell therapy-related adverse events were transient and not serious.

Conclusions: In this first randomized, large-scale clinical trial of G-CSF-mobilized PBMNC transplantation, the cell therapy was tolerated by a variety of PAD patients. The PBMNC therapy was significantly effective for inhibiting disease progression in mild-to-moderate PAD.

Key Words: Granulocyte colony stimulating factor; Peripheral arterial disease; Peripheral blood mononuclear cells; Progression-free survival

Regenerative medicine is being actively studied, and in particular, a number of investigations, including clinical trials of cell therapy in cardiovascular diseases, indicate the safety and effectiveness of therapeutic neovascularization. Asahara et al demonstrated that endothelial-cell progenitors are present as $CD34^+$ hematopoietic stem cells in human peripheral blood (PB) and that transplantation of $CD34^+$ cells promotes angiogenesis in animal models of lower-limb ischemia,[1] which drew attention to hematopoietic cells as a new therapeutic modality in ischemic diseases. Inaba et al,[2] Kawamoto et al,[3] Losordo et al[4] and Dong et al[5] have reported clinical

Received November 17, 2017; revised manuscript received April 2, 2018; accepted April 25, 2018; released online June 7, 2018
Time for primary review: 24 days
Department of Surgery, Sapporo Hokuyu Hospital, Sapporo (T.H.); Cardiovascular Center, Sapporo Higashi Tokushukai Hospital, Sapporo (S.Y.); Department of Vascular Surgery, Tobu Hospital, Gotenba (S.H.); Department of Kidney Disease and Transplant Center, Shonan Kamakura General Hospital, Kamakura (S.K.); Nephrology and Dialysis (T.T.), Cardiovascular Center (T.H.), The Tazuke Kofukai Medical Research Institute, Kitano Hospital, Osaka; Department of Nephrology and Hypertension, Sumitomo Hospital, Osaka (K. Sakaguchi); Department of Nephrology, Toho University Faculty of Medicine, Tokyo (K. Sakai); Department of Surgery, Keio University School of Medicine, Tokyo (H.O.); Department of Cardiovascular Surgery, Hakodate Municipal Hospital, Hakodate (K.M.); Division of Surgery, NHO Chiba-East National Hospital, Chiba (K. Saigo); Department of Cardiology, Shin-Koga Hospital, Kurume (Y.S.); Department of Hematology, Aomori Prefectural Central Hospital, Aomori (K.K.); Nephrology Center, Toranomon Hospital, Tokyo (J.H.); Department of Thoracic and Cardiovascular Surgery, Shimane University Faculty of Medicine, Izumo (T.O.); Institute of Education, Tokyo Medical and Dental University, Tokyo (E.K.);
(Footnote continued the next page.)

trials regarding transplantation of $CD34^+$ cells purified from PB mononuclear cells (PBMNCs) in patients with critical lower-limb ischemia (CLI). These early-phase clinical trials indicated the safety and potential effectiveness of $CD34^+$ cell therapy in CLI.

On the other hand, Kawamura et al attempted transplantation of granulocyte colony-stimulating factor (G-CSF)-mobilized MNCs derived from PB instead of isolated/purified $CD34^+$ cells in patients with chronic CLI and reported clinical effectiveness without severe adverse events (AEs).[6] However, Kawamura et al reported limited therapeutic effects of PBMNC transplantation in advanced cases.[7] Huang et al,[8] Ishida et al,[9] and Hoshino et al[10] similarly reported usefulness of transplantation of G-CSF-mobilized PNMNCs in small numbers of patients with CLI.

As just described, the clinical benefit of PBMNC transplantation was expected in lower-limb peripheral arterial disease (PAD). However, the efficacy and safety of this treatment have not been clearly demonstrated because a well-designed, large-scale, randomized clinical trial has not been conducted to date. It is also unclear which severity stage of PAD is responsive to PBMNC transplantation. To assess this issue, the risk and benefit of PBMNC administration should be investigated in not only CLI but also in the mild-to-moderate stage of PAD.

In this study, we included mild-to-moderate cases (Rutherford category 1–3)[11] as well as CLI patients (Rutherford category 4–5), and excluded the most advanced cases unlikely to benefit from PBMNC transplantation (Rutherford category 6), in either limb of chronic PAD caused by arteriosclerosis obliterans (ASO) or Buerger's disease (BD). We randomly assigned patients to either the group receiving standard of care (SOC) based on the Trans-Atlantic Inter-Society Consensus Document on Management of Peripheral Arterial Disease (TASC) II[12] or the group receiving SOC combined with transplantation of autologous and G-CSF-mobilized PBMNCs in order to verify the superior efficacy and equivalent safety of the combination therapy relative to SOC only.

Methods

Trial Design

This was a multicenter, randomized (1:1), open-label, parallel group study. The trial was approved by the institutional review boards, and written informed consent was given by the participants. The trial was conducted in accordance with the Declaration of Helsinki, the Ethical Guideline on Clinical Studies, and the Guidelines for Clinical Study Using Human Stem Cells by the MHLW of Japan. This trial was registered at http://www.umin.ac.jp/ctr/index.htm (identifier: UMIN000002280).

Participants

Inclusion criteria required participants to have angiographically documented obstructive PAD, Fontaine classification stage II–IV, Rutherford classification category 1–5[11] in one or both lower extremities despite an appropriate and stable medication regimen. The classification of Shionoya was used for diagnosis of BD: (1) smoking history; (2) onset before the age of 50 years; (3) infrapopliteal arterial occlusions; (4) either upper limb involvement or phlebitis migrans; and (5) absence of atherosclerotic risk factors other than smoking.[13] For assessing the Rutherford classification, a treadmill test (5 min at 2 km/h on 12% incline) was used if necessary.[14] Participants in whom the medical condition was not indicated for angioplasty or bypass surgery to the lower-extremity arteries (diffuse stenotic region or stenosis in peripheral arterioles that are technically challenging for bypass surgery or angioplasty), or participants with poorly controlled symptoms despite a history of these conventional revascularization treatments. The participants had to have quit smoking within the past month. Exclusion criteria included progressive symptoms (Fontaine stage or Rutherford category had increased within the past 1 month); proliferative diabetic retinopathy; active infection; lower-extremity revascularization or low-density lipoprotein apheresis within the past 1 month; a history of acute coronary or cerebrovascular syndrome within the past 6 months; and a history of cancer within the past 36 months.

Initially, only patients with Rutherford category 3–5 were enrolled in this study, but because of insufficient accrual of patients, patients with Rutherford category 1–2 were also included after protocol amendment in March 2010. The enrollment period was also extended from 2 to 5 years.

Randomization

Patients were enrolled and randomly assigned to either the SOC for PAD plus G-CSF-mobilized PBMNC therapy group (hereafter termed the "cell therapy group") or the SOC group (hereafter termed the "control group") via a web-based registration system and a follow-up system provided by an independent data center. Minimization was applied to randomization and assignment adjustment factors were (1) Fontaine stage (II and III vs. IV) and (2) hemodialysis (HD) status (performed vs. not performed).

Interventions

SOC for PAD In both groups, the SOC was conducted according to TASC II.[12]

Collection and Transplantation of Autologous PBMNCs In the cell therapy group, G-CSF (filgrastim, GRAN®, Kyowa Hakko Kirin, Japan) was administered subcutaneously at a single dose of 200 μg/m^2 per day for 4 days.

Leukapheresis was performed on the 4th or 5th day for collection of PBMNCs. COBE Spectra (Gambro BCT, USA), COM.TEC, AS-104, or AS.TEC204 (Fresenius, Germany) was used with the PB stem cell collection program or PBMNC collection program. Processed blood volume was 100–200 mL/kg. Using a hemocyte counter and flow cytometry, we calculated the total nuclear-cell count, cell fraction, and total $CD34^+$ cell count.

The PBMNC transplantation was performed using the entire volume of the cells collected under general or local anesthesia. Ischemic muscle including the gastrocnemius,

Blood Purification, Nagasaki University Hospital, Nagasaki (M.N.); and Translational Research Informatics Center, Foundation for Biomedical Research and Innovation, Kobe (T.I., H.K., M.F.), Japan

JPRCT Co-investigators listed in **Appendix**.

Mailing address: Takashi Horie, MD, PhD, Department of Surgery, Sapporo Hokuyu Hospital, 6-6-5-1 Higashisapporo, Shiroishi-ku, Sapporo 003-0006, Japan. E-mail: saiseichiryo@hokuyu-aoth.org

ISSN-1346-9843 All rights are reserved to the Japanese Circulation Society. For permissions, please e-mail: cj@j-circ.or.jp

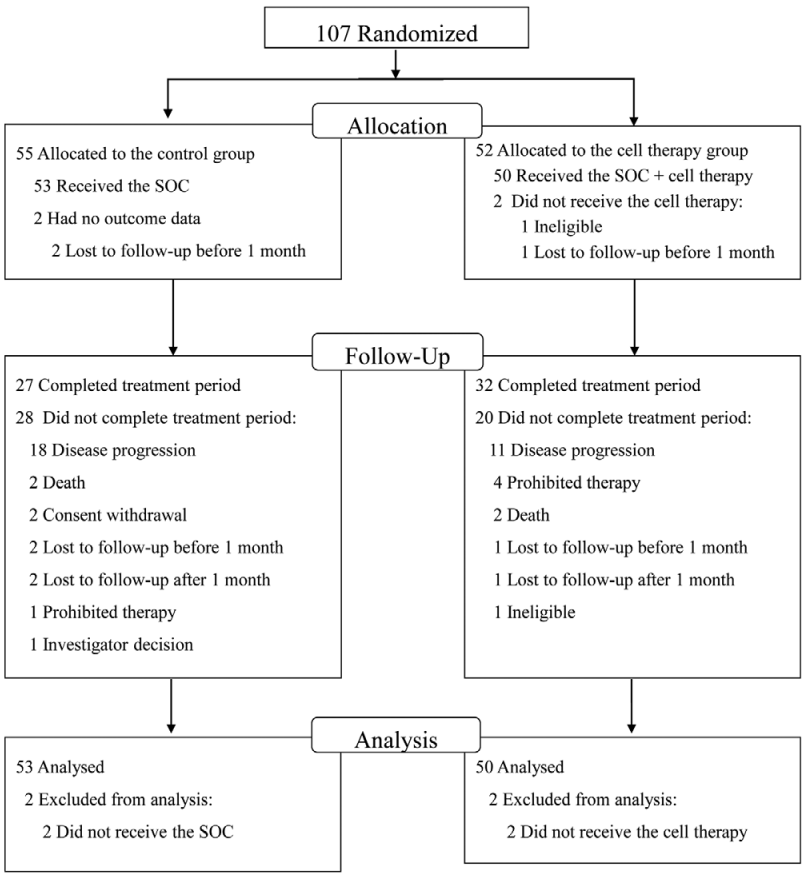

Figure 1. CONSORT flow diagram showing that of the 107 randomized patients, 4 (2 in each group) were excluded from analysis. SOC, standard of care.

anterior tibial, plantar or toe muscle was chosen as the transplantation site. Assuming a 0.5-mL injection volume per site, the number of injection sites was estimated on the basis of the collected fluid volume (≈70–150 sites). If ischemic lesions were located in both lower limbs, transplantation was performed in both limbs. After disinfection, the cells were injected intramuscularly into each planned site using 23–27-G needles.

Prohibited Therapies During the protocol treatment period (1 year from registration), prohibited medications were as follows: (1) G-CSF other than the protocol treatment, (2) sympathetic nerve blockers other than nonsteroidal anti-inflammatory drugs and narcotics, (3) basic fibroblast growth factor (fiblast® spray, Kaken Pharmaceutical, Japan), and (4) other investigational agents. Prohibited therapies were as follows: (1) angiogenic therapy other than the protocol treatment, (2) surgical revascularization, (3) endovascular treatment, (4) sympathectomy, and (5) low-density lipoprotein apheresis. After completion or premature discontinuation of the protocol treatment period, any treatment, including G-CSF-mobilized PBMNC transplantation for the control group, was permitted. Revascularization was performed at the discretion of the physicians. Follow-up was continued until 1 year after registration in patients with premature discontinuation except for withdrawal of consent.

Endpoints

The primary endpoint was progression-free survival (PFS) within 1 year after registration. Disease progression was defined as: (1) worsening of Rutherford category, (2) an increase in skin ulcer size (>50% increase in sum of the longest diameter), (3) gangrene extension, (4) a new ulcer or gangrene, or 5) major limb amputation (above the ankle). Disease progression was evaluated in both limbs of all participants. Progression was adjudicated by the Event Evaluation Committee.

Secondary endpoints included: (1) all-cause death, (2) frequency of major limb amputation, (3) major limb amputation-free survival, (4) changes from baseline in Fontaine stage or Rutherford category, (5) ulcer size, (6) rest pain scales (Wong-Baker FACES Pain Rating Scale [hereafter termed the "FACES" scale]; rest pain scale), (7) ankle-brachial index (ABI) and toe-brachial index (TBI) in patients in whom these parameters were measurable, and (8) intermittent claudication distance (ICD) and absolute claudication distance (ACD). Death and major-limb-amputation data were collected until 1 year after the last patient registration, and other outcomes were assessed at 1, 6, and 12 months after registration. AEs, vital signs, and laboratory data were analyzed during the treatment and follow-up periods. The Common Terminology Criteria for Adverse Events (CTCAE) grading score was used for AE grading.[15]

Statistical Analysis

Based on previous clinical studies,[12,16–18] the 1-year PFS was anticipated to be 65% in the cell therapy group and

Table 1. Baseline Characteristics of the PAD Patients

	Control (n=53)	Cell therapy (n=50)
Age (years)	62.0±12.0	62.6±10.9
Male sex	33 (62.3)	37 (74.0)
Body mass index (kg/m^2)	22.9±3.8	21.7±3.2
Buerger's disease	8 (15.1)	9 (18.0)
Hypertension	31 (58.5)	31 (62.0)
Hyperlipidemia	14 (26.4)	16 (32.0)
Diabetes	29 (54.7)	27 (54.0)
Hemodialysis	27 (50.9)	27 (54.0)
History		
Ischemic heart disease	16 (30.2)	19 (38.0)
Stroke	10 (18.9)	8 (16.0)
Major or minor amputation	9 (17.0)	7 (14.0)
Angioplasty	14 (26.4)	11 (22.0)
Bypass graft	10 (18.9)	4 (8.0)
Laboratory data		
LDL-cholesterol (mg/dL)	90.0±37.5	93.7±29.8
HDL-cholesterol (mg/dL)	53.3±17.1	54.4±17.1
Triglyceride (mg/dL)	132.3±95.3	127.1±58.6
Hemoglobin A1c (%)	5.7±1.0	5.6±0.8
TASC II-Recommended SOC		
Lipid-lowering drugs	15 (28.3)	21 (42.0)
Blood-pressure lowering drugs	33 (62.3)	35 (70.0)
Antidiabetic drugs	21 (39.6)	21 (42.0)
Antithrombotic drugs	48 (90.6)	45 (90.0)
Exercise therapy	17 (32.1)	18 (36.0)
Prostanoids	25 (47.2)	20 (40.0)
Vascular measures		
Fontaine stage		
II	33 (62.3)	36 (72.0)
III	8 (15.1)	5 (10.0)
IV	12 (22.6)	9 (18.0)
Rutherford category*		
0	0 (0.0)	0 (0.0)
1	9 (17.0)	13 (26.0)
2	15 (28.3)	10 (20.0)
3	9 (17.0)	13 (26.0)
4	8 (15.1)	5 (10.0)
5	12 (22.6)	9 (18.0)
Gangrene	7 (13.2)	7 (14.0)

Data are mean±standard deviation for continuous variables and numbers and percentages for discrete variables. *Severity in the more severe limb of each patient. HDL, high-density lipoprotein; LDL, low-density lipoprotein; PAD, peripheral artery disease; SOC, standard of care; TASC, Trans-Atlantic Inter-Society Consensus Document on Management of Peripheral Arterial Disease.

40% in the control group. Sample size was calculated as 64 for each group (two-tailed 5% significance level, power level of 80%).[19] Assuming that 10% of patients would be lost during follow-up, we planned to enroll 144 patients.

Kaplan-Meier survival estimate curves for time-to-event variables were generated for each group and compared by log-rank test. A stratified log-rank test using Fontaine stage (II/III vs. IV) and HD status (+ vs. −) was also performed for analysis of PFS. We chose Fontaine stage and HD status as the assignment adjustment factors, because a significant difference was found between Fontaine II/III and IV, and between groups with and without HD on amputation-free survival in a retrospective study of G-CSF-mobilized PBMNC implantation.[18] For subgroup analysis of the protocol treatment effect on PFS, a Cox proportional-hazards model was used to calculate the hazard ratios (HRs) and corresponding 95% confidence intervals. The Chi-square test was used for changes in Fontaine stage, Rutherford category, and the rest pain scale. A mixed model for repeated measures was used for changes in ulcer size, ABI, TBI, ICD, and ACD. Frequencies of AEs and serious AEs were compared between groups. The significance level was 0.05 for a two-sided test. Data are expressed as mean±standard deviation for continuous variables and as

numbers and percentages for discrete variables, unless specifically mentioned. All analyses were conducted using SAS version 9.3 (Cary, NC, USA).

Interim Analysis

Prespecified interim analyses for PFS and safety were performed when study data were available until the 6-month follow-up in 35 patients in each group. In June 2013, the Independent Data Monitoring Committee recommended the investigators continue the study in consideration of the results of the interim analysis.

Results

Subject Disposition and Baseline Characteristics

From January 1, 2009 to December 31, 2013, 107 PAD patients (74% of the target number) in 17 centers across Japan were randomly assigned to the cell therapy group (n=52) or the control group (n=55); 2 patients in the cell therapy group who did not undergo cell therapy and 2 patients in the control group who had no outcome data were excluded from the analysis (**Figure 1**). Baseline characteristics are shown in **Table 1**. The study population included 70 men and 33 women with a mean age of 62 years; 54 patients (52.4%) were on chronic HD and 17 patients (16.5%) had BD. Fontaine stage was IV in 21 patients (20.4%). Rutherford category was 4 in 25 limbs (12.1%) and 5 in other 25 limbs (12.1%). A total of 16 patients (15.5%) had undergone major or minor amputation, and 33 patients (32.0%) had undergone lower-extremity bypass surgery or percutaneous intravascular intervention. There were no statistically significant differences in the baseline demographics, medical history, and disease characteristics of the 2 groups.

Treatments

The cell transplantation procedure was performed in 49 left limbs and 43 right limbs. The transplanted PBMNC count per patient was a median 1.41 (range 0.55–36.00×10^{10}), including a median 1.64 (range 0.16–26.80×10^{7}) $CD34^{+}$ cells (in 69.1±21.5 mL). After disease progression or completion of 1-year follow-up, 17 patients in the control group underwent the cell therapy. Furthermore, 21 patients underwent revascularization after completion of the protocol treatment period or after discontinuation of the study (12 in the cell therapy group, 9 in the control group; 18 patients underwent percutaneous transluminal angioplasty, 2 had bypass surgery, and 1 had thrombectomy).

Efficacy

Within 13 months (the outside limit of the 1-year follow-up window), disease progression or death occurred in 15 patients in the cell therapy group (0.40/patient-years) and in 24 patients in the control group (0.73/patient-years). PFS tended to be improved in the cell therapy group compared with the control group (log-rank P=0.07) (**Figure 2**). Early separation of Kaplan-Meier curves was observed (5 events in the cell therapy group vs. 15 in the control group within 3 months) (**Table S1**). Worsening Rutherford category was the most frequent event (6 in the cell therapy group vs. 18 in the control group). An increase in ulcer size, gangrene extension or major limb amputation was rare (**Table 2**). A stratified log-rank test for PFS using Fontaine stage (II/III vs. IV) and HD status (+ vs. −) yielded a similar result (P=0.07).

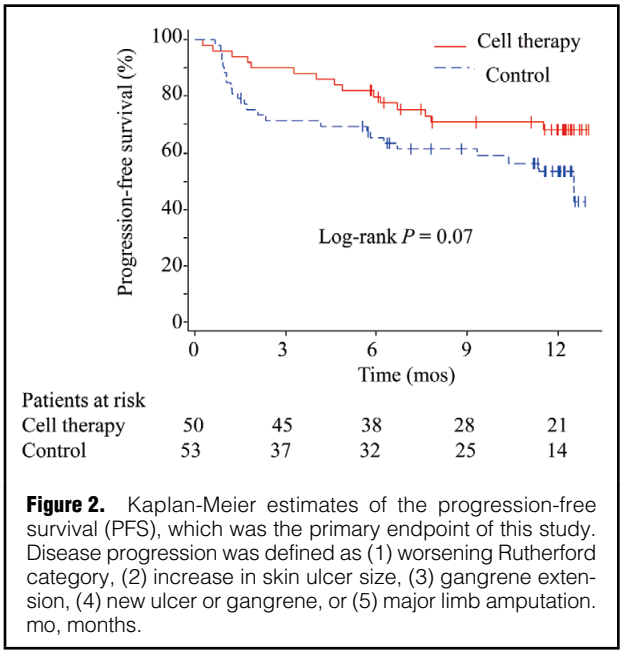

Figure 2. Kaplan-Meier estimates of the progression-free survival (PFS), which was the primary endpoint of this study. Disease progression was defined as (1) worsening Rutherford category, (2) increase in skin ulcer size, (3) gangrene extension, (4) new ulcer or gangrene, or (5) major limb amputation. mo, months.

Subgroup analysis by a forest plot of the HR revealed that PFS in the Fontaine stage II/III subgroup was significantly better in the cell therapy group than in the control group. However, HD status and disease (ASO vs. BD) did not significantly affect the difference of PFS between the 2 treatment groups (**Figure 3**). In a subgroup of non-CLI patients at baseline (n=33 in both groups), there tended to be less progression to CLI in the cell therapy group than in the control group (2 (6%) vs. 7 (21%), P=0.15).

On the other hand, the Cox proportional hazard model utilized for PFS showed a significantly higher HR (P=0.03,

Table 2. Individual Components of the Primary Endpoint

	Control (n=53)	Cell therapy (n=50)	P value*
Worsening Rutherford category	18 (34.0)	6 (12.0)	0.01
Increase in skin ulcer size	3 (5.7)	2 (4.0)	1.00
Gangrene extension	3 (5.7)	5 (10.0)	0.48
New ulcer or gangrene	8 (15.1)	9 (18.0)	0.80
Major limb amputation	3 (5.7)	3 (6.0)	1.00
Death	11 (20.8)	7 (14.0)	0.44

*Fisher's exact test.

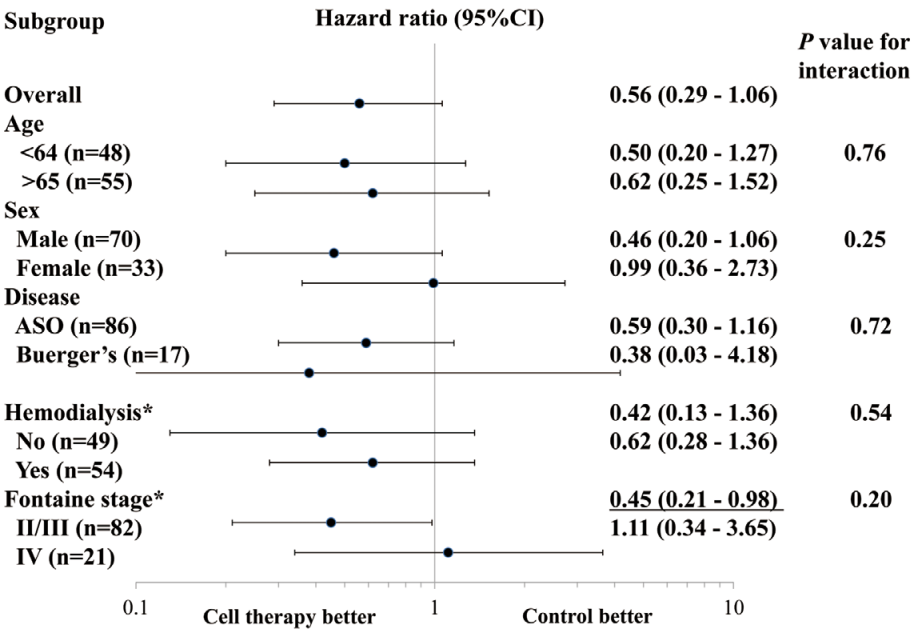

Figure 3. Subgroup analysis of the progression-free survival (PFS), analysis of the interaction and a forest plot of the hazard ratio. In the subgroup of Fontaine stage II/III, the cell therapy was significantly better than the control for PFS improvement. The results of all tests for treatment-by-subgroup interactions were not statistically significant. *Assignment adjustment factors (Fontaine stage and hemodialysis). ASO, arteriosclerosis obliterans; CI, confidence interval.

HR=2.15 [1.10–4.20]) for the subgroup with HD than for the subgroup without. The effect of PBMNC therapy on PFS adjusted by variables (Fontaine stage and HD status) was of a similar magnitude to the unadjusted PFS (**Table 3**).

During a median 870 days of follow-up, 7 patients (6 patients on HD) in the cell therapy group and 11 (9 on HD) in the control group died (log-rank P=0.37) (**Figure S1A**). A stratified log-rank test (ASO vs. BD and HD status) revealed a similar result (P=0.56). The main causes of death included sepsis and cardiovascular diseases in both groups. In both treatment groups, major amputation was rare (3 patients in each group and all patients were on HD) and major amputation-free survival was similar between the 2 groups (P=0.97) (**Figure S1B**).

The cell therapy was associated with a favorable shift in the distribution of Rutherford category at 1, 6, and 12 months from baseline (P=0.04, 0.002, and 0.004, respectively) (**Figure 4A**,**B**) and the FACES scale and the rest pain scale at 1 month (P<0.001 and P=0.004, respectively) (**Figures 4C**,**D**,**S3**).

There were no differences in the changes of ABI, TBI, ICD, or ACD between the 2 groups (**Figure S2**). There were no significant differences in changes of ulcer size or gangrene extension between the 2 groups.

Safety

AEs were reported in 39 patients (78.0%) in the cell therapy group and in 22 patients (41.5%) in the control group (P<0.001). AEs in the cell therapy group were mostly transient fluctuation from the start of G-CSF administration till the date of discharge, and the CTCAE grade of all AEs at this time was 1 or 2. Serious AEs occurred in 10 patients (20.0%) in the cell therapy group and in 6 (11.3%) in the control group (P=0.28) (**Table 4**). Among the AEs that were observed in ≥5% of all the patients, hypertension and laboratory data abnormality (leukopenia, anemia, alkaline phosphatase elevation, hypocalcemia or hyperuricemia) were significantly more frequent in the cell therapy group compared with the control group. Leukopenia, alkaline phosphatase elevation, and hyperuricemia were regarded as AEs of G-CSF administration. Hypocalcemia was regarded as an AE associated with leukapheresis. However, no serious cases were observed among the frequent AEs (**Table S2**).

Discussion

The major importance of this clinical trial can be summarized in the following 3 points. (1) This is the first report of a large-scale, randomized, parallel clinical trial of G-CSF-mobilized PBMNC therapy with 1 year of follow-up in PAD patients. (2) PAD patients with Fontaine stage II–IV and Rutherford category 1–5 were enrolled in this clinical trial. (3) The proportion of PAD patients on HD was as high as 52% of all subjects. The frequency of the HD patients was higher in this study than in any other randomized clinical trials of cell therapy for PAD.[20,21]

In the present study, as many as 103 patients with PAD received the protocol treatment. Even with other types of cell therapy, such as BMMNC or BM mesenchymal stem cell transplantation, a controlled study with more than 100 enrolled cases with PAD and with a follow-up period ≥1 year is rare. A randomized, double-blind, placebo-controlled clinical study (JUVENTAS trial), which enrolled 160 cases with PAD (Rutherford category 3–6)[20] evaluated intra-arterial infusion of autologous BMMNCs for a

Table 3. Univariate and Multivariate Cox Proportional Hazard Models to Assess the Factors Associated With Progression-Free Survival

	Unadjusted HR (95% CI)	P value	Adjusted HR (95% CI)	P value
Treatment		0.08		0.08
Cell therapy (n=50)	0.56 (0.29–1.06)		0.56 (0.29–1.07)	
Control (n=53)	1.00		1.00	
Fontaine stage		0.11		0.12
IV (n=21)	1.78 (0.89–3.58)		1.73 (0.86–3.49)	
II/III (n=82)	1.00		1.00	
Hemodialysis		0.03		0.02
Yes (n=54)	2.15 (1.10–4.20)		2.22 (1.14–4.36)	
No (n=49)	1.00		1.00	
Disease		0.10		
ASO (n=86)	2.68 (0.83–8.71)			
Burger's (n=17)	1.00			
Sex		0.13		
Male (n=70)	0.61 (0.32–1.16)			
Female (n=33)	1.00			
Ischemic heart disease		0.07		
Yes (n=35)	1.79 (0.95–3.38)			
No (n=68)	1.00			
Stroke		0.56		
Yes (n=18)	0.78 (0.32–1.85)			
No (n=85)	1.00			

ASO, arteriosclerosis obliterans; CI, confidence interval; HR, hazard ratio.

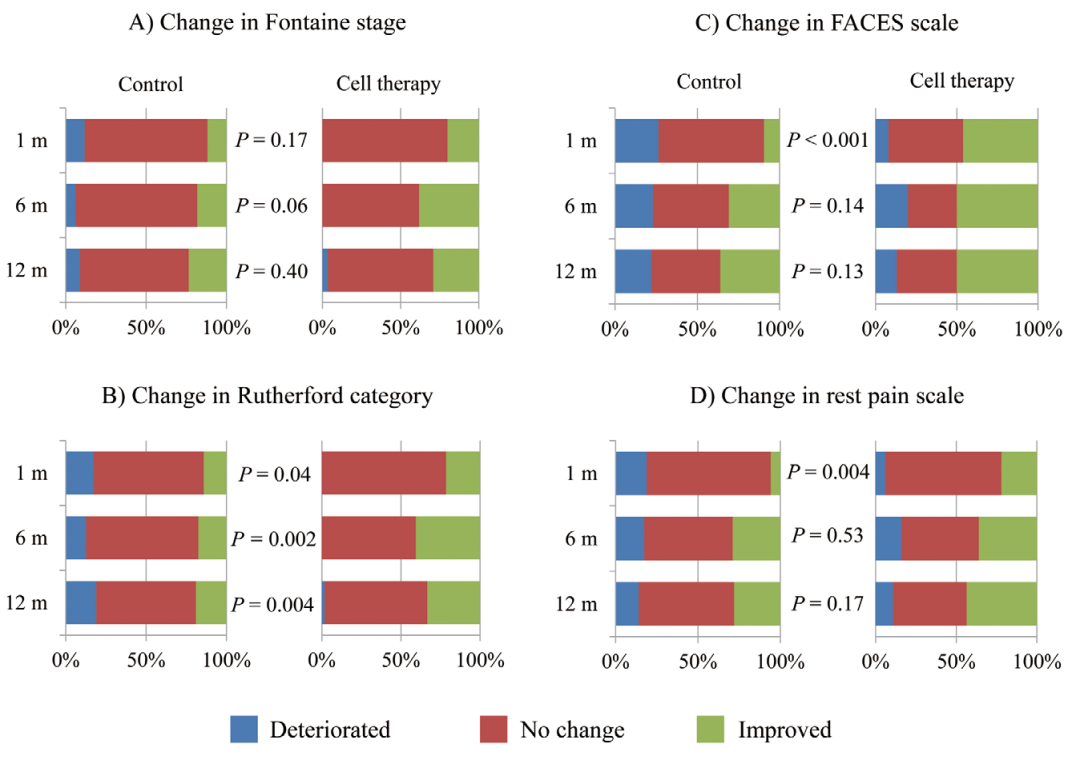

Figure 4. Changes in Fontaine stage (**A**), Rutherford category (**B**), the FACES scale (**C**), and the rest pain scale (**D**). Changes in Fontaine stage and Rutherford category in both limbs and pain scales (FACES scale and rest pain scale) from baseline to 1, 6, and 12 months later were categorized as deteriorated (increased), no change, or improved (decreased). P values are for comparison between the 2 treatment groups.

Table 4. Serious Adverse Events

	Control (n=53)	Cell therapy (n=50)
No. of patients with an event (%)	6 (11.3)	10 (20.0)
Limb edema	0 (0.0)	1 (2.0)
Sepsis	1 (1.9)	1 (2.0)
Lung infection	3 (5.7)	0 (0.0)
Conjunctival chalasis	0 (0.0)	1 (2.0)
Cataract	1 (1.9)	1 (2.0)
Retinopathy	1 (1.9)	0 (0.0)
Rotator cuff tear	1 (1.9)	1 (2.0)
Fracture	0 (0.0)	1 (2.0)
Thromboembolic event	0 (0.0)	1 (2.0)
Peripheral arterial ischemia	0 (0.0)	3 (6.0)
Acute coronary syndrome	1 (1.9)	2 (4.0)
Cerebrovascular ischemia	0 (0.0)	1 (2.0)
Arrhythmia	0 (0.0)	1 (2.0)
Seizure	0 (0.0)	1 (2.0)
Pneumothorax	1 (1.9)	0 (0.0)
Pain in extremity	0 (0.0)	1 (2.0)

6-month follow-up period. Unfortunately, that study revealed no significant difference in the major amputation rate at 6 months between the BMMNC and placebo groups. The current clinical trial of PBMNC therapy is considered to be important for verifying the safety and efficacy of a novel therapeutic modality in PAD patients.

Because most of the previous cell therapy trials[4,20–24] were conducted in PAD patients with severe limb ischemia only, the inclusion/exclusion criteria of this study are unique. In fact, the proportion of Fontaine stage II cases was the greatest, at 67% of all participants, suggesting that mild PAD patients were frequently enrolled in this study. The JUVENTAS trial investigators discussed that their study result may not rule out the potential benefit of BMMNC treatment in patients with milder PAD despite the negative outcomes in severe subjects.[20] Another controlled study[14] was conducted in a small number of PAD patients with Fontaine stage IIb (Rutherford category 2–3) only. In 13 Fontaine IIb cases, autologous BMMNCs were injected intra-arterially into the superficial femoral artery of the ischemic limb and intramuscularly into quadriceps and gastrocnemius at the same time; walking distance, ABI, and other parameters were evaluated before the treatment as well as at 2 and 13 months after the treatment. The same evaluation was performed at the start of the study and 4 months later in another 12 Fontaine IIb cases as the controls. That pilot study revealed that both the short-term (2 months) and the long-term (13 months) effects on ABI and capillary-venous oxygen saturation were attributable to the stem cell transplantation.[14] Because of the small amount of knowledge about the safety and efficacy of cell therapy in cases of milder PAD, the outcome of the present clinical trial is considered to be valuable.

On the other hand, PAD patients on HD were frequently enrolled in this study. Our retrospective study[18] revealed that HD as well as higher Fontaine stage and presence of gangrene were negatively associated with amputation-free survival in PAD patients receiving G-CSF-mobilized PBMNCs. Onodera et al also reported a similar outcome after BMMNC or PBMNC transplantation for CLI.[17] These previous reports suggested that various subjects, including both good responders (lower Fontaine stage) and poor responders (on HD) to cell therapy, would be frequently enrolled in the present clinical trial.

In a study comparing BMMNC and PBMNC transplantations,[23] significant improvements were observed in ABI, skin temperature, rest pain with PBMNC compared with BMMNC. However, there was no significant difference between 2 groups for pain-free walking distance, transcutaneous oxygen pressure, ulcers, and rate of lower-limb amputation. Therefore, further large-scale studies are needed to determine whether PBMNC or BMMNC transplantation is more effective for preventing PAD progression.

The main outcomes of this study were: (1) PFS during the 1-year follow-up tended to be improved in the cell therapy group than the control group, although the difference was not statistically significant; and (2) subgroup analyses revealed that PFS in the Fontaine stage II/III subgroup was significantly better in the cell therapy group than in the control group. In contrast, HD status did not significantly affect the difference in PFS between the cell therapy and control groups.

We selected PFS, not amputation-free survival, as the primary endpoint of this study. The reasons for the endpoint selection were: (1) it is clinically important to prevent PAD progression because PAD is a chronic and progressive disease, and (2) amputation-free survival may not have been appropriate for this study because amputation is generally rare in Fontaine stage II patients. In fact, only 3 patients (5.7%) in each treatment group underwent major amputation in this study.

The main outcomes described suggest that G-CSF-mobilized PBMNC therapy may be effective for preventing PAD progression, especially in patients with mild severity who are ineligible for revascularization. Clinical severity of PAD rather than HD status may be a more significant factor determining the response to the PBMNC therapy. It would be warranted to perform a pivotal, prospective, randomized clinical trial for evaluating the usefulness of

G-CSF-mobilized PBMNC therapy in PAD patients in Fontaine stage II/III in the future.

On the other hand, HD was a significant factor in PAD aggravation, which was consistent with clinical outcomes in previous reports.[17,18,25] The importance of HD status as well as PAD severity should be considered in the design of future studies.

One of the intriguing findings of this study is that disease progression events frequently occurred within 3 months after randomization in the control group compared with the cell therapy group. This result suggested that PBMNC therapy might prevent PAD progression early after the transplantation, although the influence of an unblinded study design should be carefully considered.

It should be noted that the number of cases in Fontaine stage IV was limited to 21 (20.4%) in this study. One of the reasons for this small number was that subjects classified as Rutherford category 6 were excluded because of their extremely poor prognosis in our retrospective study.[18] Also, only patients for which endovascular intervention or bypass surgery was not indicated or those with poor control of the disease following these conventional treatments were enrolled in this study. Because conventional revascularization is the standard therapy for Fontaine stage IV patients and endovascular intervention can be performed repeatedly, the number of Fontaine stage IV patients was smaller than that of milder PAD patients in this study. In the subgroup of Fontaine stage IV, PBMNC therapy was not significantly effective for PFS improvement and a reason of this result may be that there were many difficult-to-treat cases among these no-option or poor-option patients even though they were classified as Rutherford 5 not 6. Considering the current results, it would be worthwhile evaluating the safety and efficacy of combination therapy of PBMNC transplantation and conventional revascularization in Fontaine stage IV patients who are the high-risk population for limb amputation even after successful revascularization.

The point estimate of the HR was numerically lower in the BD group than in the ASO group (0.38 vs. 0.59, **Figure 3**), suggesting more efficacy in the BD group, as previously reported. The small number of patients (n=17) was likely to result in wide confidence interval and no significant difference.

AEs were more frequent in the cell therapy group than in the control group. However, AEs in which a causal relationship with the cell therapy could not be denied were transient and mild to moderate. Cardiovascular AEs, such as thromboembolic events, peripheral arterial ischemia, acute coronary syndrome, and cerebrovascular ischemia, tended to occur more frequently in the cell therapy group (**Table 4**). Based on the temporal relationship, cardiovascular AEs were judged as "unrelated to protocol treatment (including G-CSF)" by the investigators as well as by the Independent Data Monitoring Committee. There was no difference in the frequency of serious AEs between the 2 treatment groups. These results indicated that G-CSF-mobilized PBMNC transplantation may be feasible and tolerable in a variety of PAD patients.

Conclusions

This first randomized, large-scale clinical trial of G-CSF-mobilized autologous PBMNC transplantation revealed that cell therapy was feasible and tolerable in patients with both mild and severe PAD. The PBMNC therapy tended to be effective for preventing PAD progression. PFS in the Fontaine stage II/III subgroup was significantly better in the cell therapy group than in the control group.

Acknowledgments

This work was supported by a grant from the Regional Medicine Research Foundation (Tochigi, Japan).

The authors appreciate the careful review of the manuscript by Shinsuke Kojima, Mikio Yoshidomi and Atsuhiko Kawamoto at the Translational Research Informatics Center, Kobe, Japan.

Disclosures

There is no conflict of interest.

Appendix

JPRCT Co-investigators

Makoto Akamatsu (Itabashi Chuo Hospital); Yukio Ichikawa (Kanagawa Cardiovascular and Respiratory Center); Akaru Ishida (Department of Transfusion Medicine and Cell Therapy, Keio University); Chikara Iwashita (National Hospital Organization, Chiba-East Hospital); Akio Kawamura (Sapporo Hokuyu Hospital); Kazuki Mizuiri (Omori Medical Center, Toho University); Takeyasu Otake (Shonan Kamakura General Hospital); and Akio Sakata (Tokushima Red Cross Hospital).

Name of Grant

Regional Medicine Research Foundation, Tochigi, Japan.

References

1. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275:** 964–967.
2. Inaba S, Egashira K, Komori K. Peripheral-blood or bone-marrow mononuclear cells for therapeutic angiogenesis? *Lancet* 2002; **360:** 2083.
3. Kawamoto A, Katayama M, Handa N, Kinoshita M, Takano H, Horii M, et al. Intramuscular transplantation of G-CSF-mobilized $CD34^+$ cells in patients with critical limb ischemia: A phase I/IIa, multicenter, single-blinded, dose-escalation clinical trial. *Stem Cells* 2009; **27:** 2857–2864.
4. Losordo DW, Kibbe MR, Mendelsohn F, Marston W, Driver VR, Sharafuddin M, et al. A randomized, controlled pilot study of autologous CD34+ cell therapy for critical limb ischemia. *Circ Cardiovasc Interv* 2012; **5:** 821–830.
5. Dong Z, Chen B, Fu W, Wang Y, Guo D, Wei Z, et al. Transplantation of purified $CD34^+$ cells in the treatment of critical limb ischemia. *J Vasc Surg* 2013; **58:** 404–411.
6. Kawamura A, Horie T, Tsuda I, Ikeda A, Egawa H, Imamura E, et al. Prevention of limb amputation in patients with limb ulcers by autologus peripheral blood mononuclear cell implantation. *Ther Apher Dial* 2005; **9:** 59–63.
7. Kawamura A, Horie T, Tsuda I, Abe Y, Yamada M, Egawa H, et al. Clinical study of therapeutic angiogenesis by autologous peripheral blood stem cell (PBSC) transplantation in 92 patients with critically ischemic limbs. *J Artif Organs* 2006; **9:** 226–233.
8. Huang P, Xiao Z, Li S, Yang R, Han M, Han ZC. Autologous transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells improves critical limb ischemia in diabetes. *Diabets Care* 2005; **28:** 2155–2160.
9. Ishida A, Ohya Y, Sakuda H, Ohshiro K, Higashiuesato Y, Nakaema M, et al. Autologous peripheral blood mononuclear cell implantation for patients with peripheral arterial disease improves limb ischemia. *Circ J* 2005; **69:** 1260–1265.
10. Hoshino J, Ubara Y, Hara S, Sogawa Y, Suwabe T, Higa Y, et al. Quality of life improvement and long-term effects of peripheral blood mononuclear cell transplantation for severe arteriosclerosis obliterans in diabetic patients on dialysis. *Circ J* 2007; **71:** 1193–1198.
11. Rutherford RB, Baker JD, Ernst C, Johnston KW, Porter JM, Ahn S, et al. Recommended standards for reports dealing with lower extremity ischemia: Revised version. *J Vasc Surg* 1997; **26:** 517–538.
12. Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG; TASC II Working Group. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *J*

Vasc Surg 2007; **45**(Suppl S)**:** S5–S67.
13. Shionoya S. Diagnostic criteria of Buerger's disease. *Int J Cardiol* 1998; **66**(Suppl 1)**:** S243–S245.
14. Bartsch T, Brehm M, Zeus T, Kogler G, Wernet P, Strauer BE. Transplantation of autologous mononuclear bone marrow stem cells in patients with peripheral arterial disease (the TAM-PAD study). *Clin Res Cardiol* 2007; **96:** 891–899.
15. Cancer Therapy Evaluation Program: Common Terminology Criteria for Adverse Events, Version 3.0. DCTD, NCI, NIH, DHHS, March 31, 2003.
16. London GM, Drueke TB. Atherosclerosis and arteriosclerosis in chronic renal failure. *Kidney Int* 1997; **51:** 1678–1695.
17. Onodera R, Teramukai S, Tanaka S, Kojima S, Horie T, Matoba S, et al. Bone marrow mononuclear cells versus G-CSF-mobilized peripheral blood mononuclear cells for treatment of lower limb ASO: Pooled analysis for long-term prognosis. *Bone Marrow Transplant* 2011; **46:** 278–284.
18. Horie T, Onodera R, Akamastu M, Ichikawa Y, Hoshino J, Kaneko E, et al; Japan Study Group of Peripheral Vascular Regeneration Cell Therapy (JPRCT). Long-term clinical outcomes for patients with lower limb ischemia implanted with G-CSF-mobilized autologous peripheral blood mononuclear cells. *Atherosclerosis* 2010; **208:** 461–466.
19. Freedman LS. Tables of the number of patients required in clinical trials using the logrank test. *Stat Med* 1982; **1:** 121–129.
20. Teraa M, Sprengers RW, Schutgens RE, Slaper-Cortenbach IC, van der Graaf Y, Algra A, et al. Effect of repetitive intra-arterial infusion of bone marrow mononuclear cells in patients with no-option limb ischemia: The randomized, double-blind, placebo-controlled Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-arterial Supplementation (JUVENTAS) trial. *Circulation* 2015; **131:** 851–860.
21. Powell RJ, Marston WA, Berceli SA, Guzman R, Henry TD, Longcore AT, et al. Cellular therapy with Ixmyelocel-T to treat critical limb ischemia: The randomized, double-blind, placebo-controlled RESTORE-CLI trial. *Mol Ther* 2012; **20:** 1280–1286.
22. Wang ZX, Li D, Cao JX, Liu YS, Wang M, Zhang XY, et al. Efficacy of autologous bone marrow mononuclear cell therapy in patients with peripheral arterial disease. *J Atheroscler Thromb* 2014; **21:** 1183–1196.
23. Huang PP, Yang XF, Li SZ, Wen JC, Zhang Y, Han ZC. Randomised comparison of G-CSF-mobilized peripheral blood mononuclear cells versus bone marrow-mononuclear cells for the treatment of patients with lower limb arteriosclerosis obliterans. *Thromb Haemost* 2007; **98:** 1335–1342.
24. Peeters Weem SMO, Teraa M, den Ruijter HM, de Borst GJ, Verhaar MC, Moll FL. Quality of life after treatment with autologous bone marrow derived cells in no option severe limb ischemia. *Eur J Vasc Endovasc Surg* 2016; **51:** 83–89.
25. Idei N, Soga J, Hata T, Fujii Y, Fujimura N, Mikami S, et al. Autologous bone-marrow mononuclear cell implantation reduces long-term major amputation risk in patients with critical limb ischemia: A comparison of atherosclerotic peripheral arterial disease and Buerger disease. *Circ Cardiovasc Interv* 2011; **4:** 15–25.

Supplementary Files

Supplementary File 1

Figure S1. Overall survival (**A**) and major amputation-free survival (**B**).

Figure S2. Changes in ABI (**A**), TBI (**B**), ICD (**C**), and ACD (**D**).

Figure S3. FACES scale (**A**) and the rest pain scale (**B**) before and after the treatment.

Table S1. Cases of aggravated PAD within 3 months of randomization

Table S2. Adverse events reported in ≥5% of patients in either treatment group

Please find supplementary file(s);
http://dx.doi.org/10.1253/circj.CJ-17-1220