



Randomized Clinical Trial of the Safety and Efficacy of Autologous Bone Marrow Aspirate Concentrate for No-Option Critical Limb Ischemia in Type 2 Diabetes Mellitus Patients (DIALEG)

František Jalůvka^{1,2}, Jan Roman^{1,2}, Katerina Vítková³, Jaromír Gumulec^{2,4}, Adéla Vrtková⁵, Lubomír Pavliska³, Kusinová Pavlína⁶ and Václav Procházka^{6,7*}

¹Department of Surgery, University Hospital Ostrava, Czech Republic

²Department of Surgical Studies, Faculty of Medicine, Ostrava, Czech Republic

³Department of Science and Research Deputy, University Hospital Ostrava, Czech Republic

⁴Department of Hemato-Oncology, University Hospital Ostrava, Czech Republic

⁵Department of Applied Mathematics, Faculty of Electrical Engineering and Computer Science, VSB – Technical University of Ostrava, Czech Republic

⁶Department of Radiology, University Hospital Ostrava, Czech Republic

⁷Department of Imaging Methods, Faculty of Medicine, Ostrava, Czech Republic

Abstract

Critical limb ischemia belongs among the major vascular complications of long-lasting type 2 diabetes mellitus. Administration of autologous Bone Marrow Aspiration Concentrate (BMAC) is a potential treatment to improve the outcomes of patients who have no other available treatment option for critical limb ischemia. In the present study, we aimed to evaluate the safety, tolerability, and efficacy of different routes of administration of Bone Marrow Mononuclear Cells (BM-MNC) in patients with otherwise No-Option Critical Limb Ischemia (NO-CLI) in the setting of type 2 diabetes mellitus. We conducted a single-center parallel randomized controlled open-label trial, with computer-generated tables used to allocate treatment methods. The analysis included 55 patients with a history of type 2 diabetes mellitus and critical limb ischemia with depleted standard treatment. Patients in interventional groups received a single dose of bone marrow concentrate administered intramuscularly, intraarterially, or intravenously. Patients in all groups were followed for two years. BM-MNC administration statistically significantly prolonged the overall survival of patients, regardless of the route of application, and improved quality of life. BM-MNC administration did not significantly reduce the frequency of limb amputations. ClinicalTrials.gov, Identifier: NCT01818310.

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*Correspondence:

Václav Procházka, Department of Radiology, University Hospital Ostrava, 17. Listopadu 1790/5, 708 52 Ostrava, Czech Republic,

E-mail: vaclav.prochazka@fno.cz

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Introduction

Peripheral Arterial Disease (PAD), particularly of the lower limbs, is among the most common manifestations of a prolonged course of diabetes mellitus, associated with a 2- to 4-fold increased risk of PAD development [1]. Diabetes mellitus duration and disease severity significantly correlate with complications, including PAD [2]. Clinical manifestations predominantly include exertion and claudication during walking and, in more advanced stages, limb rest pain, local hair loss, changes in skin color, sensitivity, wounds with impaired mobility. Paraclinical manifestations include the absence of peripheral arterial pulsations, abnormal Ankle-Brachial Index (ABI) [3], and Doppler signs of slowing blood flow with changes in the physiological pulse curve. Advanced tissue and skin changes accompanied by rest pain terminate in Critical Limb Ischemia (CLI), which has significant adverse effects on patients' quality of life. Standard therapeutic options include pharmacological, surgical, or interventional revascularization to restore diminished blood flow. These interventions are impossible in no-option CLI (NO-CLI) cases, and amputation is the last treatment option [4]. The experimental treatment method for NO-CLI is the local or systemic application of autologous

stem cells [5]. The bone marrow mononuclear cells secrete several angiogenic cytokines for angiogenesis improvement. Progenitor cells, together with cytokines, develop a stabilized capillary network. This angiogenesis can lead to peripheral limb perfusion improvement. An autologous stem cell concentrate is prepared by concentrating Bone Marrow Mononuclear Cells (BM-MNC) or mobilizing Peripheral Blood Stem Cells (m-PBSC). BMAC is administered intramuscularly, intra arterially, or intravenously. However, there is currently insufficient evidence regarding the efficacy and safety of the different administration methods [6]. The present randomized clinical trial was to verify the safety and effectiveness of different routes of administration of BM-MNC autologous concentrate in patients with type 2 diabetes mellitus and NO-CLI.

Materials and Methods

The DIALEG study is the parallel open-label clinical interventional study of the safety and efficacy of different routes of administration of BM-MNC autologous concentrate in patients with type 2 diabetes mellitus and NO-CLI. This study was registered at ClinicalTrials.gov under the identifier NCT01818310 and conducted at the University Hospital Ostrava. The local Ethics Committee approved the study before it commenced. The study had to be prematurely terminated, with agreement from the Ethics Committee, due to the unequivocally positive effect of the intervention based on examination on the clinical condition of the treated limb.

Participant selection and randomization

The main inclusion criteria for the randomized study included type 2 diabetes mellitus, critical limb ischemia with exhausted interventional or surgical therapy, and a chronic non-healing wound of the index limb. Other inclusion criteria were ankle-brachial index ABI<50 mmHg or its ratio <0.4, Toe-Brachial Index (TBI) <40 mmHg or its ratio <0.4, and transcutaneous oxygen pressure ($T_c PO_2$) <20 mmHg in supination. Participants had to be over 18 years of age and had to sign an informed consent. Exclusion criteria were noncompliance with all inclusion criteria; expected survival of <6 months; history of bone marrow disease, renal failure, or dialysis addiction; known malignant disease; inability to undergo general anesthesia or sedation; life-threatening coronary arterial disease; extensive necrosis of the limb under study; active infection or antibiotic treatment; immunosuppression; pregnancy; and breastfeeding. The study planned for randomization of 80 subjects; however, after the interim data analysis, only 55 participants were randomized, and the study stopped. MedCalc software for Windows, version 19.4 (Ostend, Belgium), was used for the computer-generated randomization table, allocating patients into four groups (A, B, C, and control group D). The utilized intervention was known only to the doctor performing the application but not to the follow-up team. The patient received information about the possible routes of bone marrow concentrate administration and the informed consent signature.

Clinical intervention

The BM-MNC concentrate was prepared according to the standard operation protocol. Bone marrow aspiration procedure is provided under TIVA - total intravenous analgesia using propofol (Propofol, B Braun, Melsungen, Germany) or under local anesthesia with trimecaine (Zentiva, Prague, Czech Republic). According to the standardized protocol, the heparinized aspiration Yamshidi needle was used for dorsal iliac crest bone puncture and marrow aspiration. A total of 240 mL of bone marrow aspirated. The sampling needle position

is changed after aspiration of every 10 mL with needle rotation to ensure sufficient marrow collection yield. The bone marrow aspirate is collected in the collection bag containing 30 mL Anticoagulant Citrate Dextrose Solution A (ACD-A). The bag content is then visually checked for clots or large clusters. A 5-mL aliquot of the aspirate was analyzed for cell count and cytokines levels. The remaining 240 mL of the heparinized bone marrow aspirate was then directly transferred with four 60 mL syringes to the centrifuge machine. The gradient density centrifugation for 14 min is applied, and 40 mL of BMAC is finally obtained. A 5 mL of BMAC volume was analyzed with a cell counter for the number of individual cells, and the remaining 35 mL was used for the patient treatment. Patients in the intervention groups received a 35-mL total injection volume of BM-MNC. BM-MNC was administered into the muscles in group A by injecting the area around the affected tissues or occluded parts of arteries. In group B, BM-MNC was administered intra arterially on the side of the affected limb through a common femoral artery with a catheter introduced into the area under the knee or higher depending on the occlusion height. In group C, the BM-MNC concentrate was intravenously administered by joint injection into the peripheral vein of the upper extremities. Antibiotic application of 1 g cefazolin was issued before the procedure. In cases of cefazolin intolerance, alternatives included 1 g vancomycin or 600 mg clindamycin. Patients were monitored for possible adverse reactions throughout the collection and application. Further monitoring included clinical control visits at predetermined dates, 3 to 7 days after BM-MNC application, and then at 1, 3, 6, 12, and 24 months after application.

Study end-points

The primary study end-points were to evaluate the efficacy and safety of the application of BM-MNC via different administration routes. We assessed the amputation-free survival and mortality of patients in the other groups. The secondary end-points were to evaluate the paraclinical parameters of BM-MNC application to the affected limb. We analyzed the quality of the bone marrow used for capillary network formation. All participants were repeatedly assessed by laser Doppler to measurement in the treated limb objectively. Quality of life was evaluated using the standardized medical outcomes Study Short Form 36 (SF-36) and European Quality of Life Questionnaire (EQ-50) combined with a Visual Analog pain Scale (VAS) to measure subjective pain.

Statistics

Statistical analysis was performed using R software (v. 4.0.3, www.r-project.org). The significance level was set to 0.05. Numerical variables were expressed as the median and range (minimum and maximum) or interquartile range (lower and upper quartiles). Categorical variables were presented as absolute frequency and relative frequencies in percentages. Defined groups were compared using the Kruskal-Wallis test or Fisher's exact test. The significance of changes over time in the selected parameters was tested using the paired Wilcoxon test. Finally, Kaplan-Meier curves and the Log-rank test were used to analyze amputation-free and overall survival.

Results

Before the early termination of the study, a total of 55 patients were enrolled, including eight women (15%) and 47 men (85%). Of these patients, 14 were randomized to group A, 12 to group B, 15 to group C, and 14 to group D. Table 1 summarizes the demographic data, medical history, and the input risk parameters in the different

groups.

Among all patients, 16 required amputation (29%), while the limb was preserved in the remaining patients. Five amputations (42%) were performed in group B, four each in groups C and D (27% and 29%, respectively), and 3 (21%) in group A. However, the amputation rate did not significantly differ between the groups. This study enrolled patients with clinical PAD in stages 3 to 6 according to the Rutherford classification and staged IIb-IV of the Fontaine classification (Table 2). Following the University of Texas Wound Classification (Table 3), the highest percentage of patients were characterized as stage IIIC and IIID (i.e., patients having a more ischemic severe defect with verified bacterial colonization). Following the Wagner photographic classification, most patients were classified in stages III and IV, and no patient had extensive gangrenous affection putting the limb at risk of amputation. The overview suggests that patients were compromised by limb ischemia with numerous severe defects before study enrolment.

Table S1 presents the monitored laboratory parameters. Patients in group B (intra arterial BM-MNC administration) achieved higher glycemic values (at entry and after six months) and higher glycated hemoglobin concentration (after six months). The groups did not significantly differ in C-peptide concentration, C-reactive protein, or thrombophilic parameters, such as von Willebrand factor activity or fibrinogen concentration. Group A (intramuscular administration) exhibited significant decreases in platelets and PAI-1 antigen levels six months after administration. Group C (intravenous administration) showed a significantly increased glycated hemoglobin concentration and substantial reductions of C-reactive protein, fibrinogen concentration, and D-dimers at six months after BM-MNC administration. Laser Doppler revealed almost no statistically significant differences in individual groups over time and between the intervention and control groups. The only

Table 1: Demography data, medical history, occurrence of risk factors, amputations, and death count.

	Median (range) or n (%) ^a					
	Group A (i.m.)	Group B (i.a.)	Group C (i.v.)	Group D (w/o)	Total	<i>p</i> ^b
	(n = 14)	(n = 12)	(n = 15)	(n = 14)	(n = 55)	
Sex, male	11 (79)	10 (83)	14 (93)	12 (86)	47 (85)	0.726
Age, years	66 (33; 82)	64 (50; 75)	60 (38; 77)	68 (54; 82)	64 (33; 82)	0.052
Weight, kg	86 (67; 125)	98 (74; 130)	84 (60; 104)	86 (58; 125)	87 (58; 130)	0.351
Height, cm	173 (154; 186)	175 (150; 187)	174 (162; 186)	175 (160; 188)	174 (150; 188)	0.97
Body mass index	30.1 (23.1; 37.3)	31.0 (25.4; 39.7)	27.5 (22.9; 32.1)	29.5 (19.4; 37.3)	29.9 (19.4; 39.7)	0.101
Systolic blood pressure, mmHg	130 (120; 140)	133 (110; 150)	130 (120; 150)	130 (120; 150)	130 (110; 150)	0.512
Diastolic blood pressure, mmHg	80 (70; 90)	80 (60; 85)	80 (70; 85)	80 (60; 85)	80 (60; 90)	0.96
Swelling	2 (14)	3 (25)	4 (27)	5 (36)	14 (25)	0.647
Neuropathy	3 (21)	5 (42)	2 (13)	1 (7)	11 (20)	0.186
Diabetes mellitus	10 (71)	11 (92)	7 (47)	10 (71)	38 (69)	0.098
Hyperlipidemia	9 (64)	8 (67)	5 (33)	7 (50)	29 (53)	0.285
Renal insufficiency	4 (29)	0 (0)	2 (13)	2 (14)	8 (15)	0.293
Smoking	1 (7)	2 (17)	2 (13)	1 (7)	6 (11)	0.838
Alcohol	1 (7)	0 (0)	1 (7)	1 (7)	3 (5)	>0.999
Amputation	3 (21)	5 (42)	4 (27)	4 (29)	16 (29)	0.715
Death count	2 (14)	1 (8)	0 (0)	4 (29)	7 (13)	0.103

^aData are shown as the median and range (minimum and maximum) or the absolute and relative frequency in percentages

^bThe *p*-value from the Kruskal-Wallis test or Fisher's exact test

i.m.: intramuscular; i.a.: intra-arterial; i.v.: intravenous; w/o: control group

significant difference was that groups B and C exhibited a higher $TcpCO_2$ at six months after BM-MNC administration compared to the other groups. Table S2 presents a comparison of the parameters. Bone marrow quality analysis reveals individual angioproliferative and a mesenchymal Cluster of Differentiation (CD) marker (Figure 1). Our results showed statistically significant differences in $CD3^+$, $CD4^+$, $CD13^+$, and $CD49^+$, significantly more common in group B (intra-arterial administration), and in $CD90^+$, which was substantially more common in group A (intramuscular administration). Table S3 presents a detailed comparison.

Quality of life

The central aspect evaluated was pain, assessed by VAS at multiple control intervals regarding the quality of life. One year after BM-MNC application, the intervention groups showed a significantly more frequent improvement of VAS score when compared to the control group (Fisher's exact test, *p*=0.044). Figure 2 roughly illustrates the distribution of pain over time for all groups. Based on the SF-36 questionnaire, we found a statistically significant improvement of quality of life one year after BM-MNC application, with the median growth of the SF-36 index being 10.9 (paired Wilcoxon test, *p*=0.003). Patients exhibited improved overall physical health, reduced physical pain, reduced physical activity restrictions, and improvements in several other psychological parameters. Table 4 presents these data and also includes a comparison with the European Standard of the Group Oxford Healthy Life Survey.

Survival

We monitored amputation-free survival and overall survival rates in all study groups and compared the difference in overall survival between patients with or without necessary amputation. The amputation-free survival rate did not significantly differ between the intervention and control groups (Log-rank test, *p*=0.660). Group

Table 2: Distribution of patients according to the Fontaine and Rutherford classifications.

	University of Texas Wound Classification							
	n (%) ^a							
	Fontaine classification			Rutherford classification				
Group	IIb	III	IV	3	4	5	6	
A (i.m.)	1 (7)	5 (36)	8 (57)	---	6 (43)	5 (36)	3 (21)	
B (i.a.)	---	6 (50)	6 (50)	---	4 (33)	7 (59)	1 (8)	
C (i.v.)	1 (7)	2 (13)	12 (80)	---	4 (27)	8 (53)	3 (20)	
D (w/o)	2 (14)	2 (14)	10 (72)	1 (7)	4 (29)	5 (35)	4 (29)	
Total	4 (7)	15 (27)	36 (66)	1 (2)	18 (33)	25 (45)	11 (20)	
p ^b	0.257			0.821				

^aThe absolute and relative frequencies in percentages^bThe p-value from Fisher's exact test

i.m.: intramuscular; i.a.: intra-arterial; i.v.: intravenous; w/o: control group

Table 3: According to the University of Texas Wound Classification (Fisher's exact test, p=0.695).

Group	I-A	I-B	I-C	I-D	II-A	II-B	II-C	II-D	III-A	III-B	III-C	III-D
A (i.m.)	3 (21)	---	4 (29)	1 (7)	---	---	1 (7)	---	---	---	---	5 (36)
B (i.a.)	1 (8)	---	3 (26)	1 (8)	---	1 (8)	---	1 (8)	---	1 (8)	2 (17)	2 (17)
C (i.v.)	1 (7)	---	4 (27)	---	---	---	2 (13)	---	1 (7)	1 (7)	4 (27)	2 (13)
D (w/o)	3 (22)	---	1 (7)	---	1 (7)	2 (14)	1 (7)	---	---	1 (7)	2 (14)	3 (22)
Total	8 (15)	---	12 (21)	2 (4)	1 (2)	3 (6)	4 (7)	1 (2)	1 (2)	3 (6)	8 (15)	12 (21)

^aThe absolute and relative frequencies in percentages

i.m.: intramuscular; i.a.: intra-arterial; i.v.: intravenous; w/o: control group

Table 4: The physical and mental health of patients before administration and 12 months after the administration of cell therapy.

	Median(IQR) ^a				
	Before BM-MNC application	After BM-MNC application	Difference	p ^b	European standard (Oxford)
Physical function	37.5 (15.6; 56.3)	50.0 (27.8; 66.7)	4.2 (-7.6; 16.0)	0.085	88.4
Restriction of physical activities	0.0 (0.0; 25.0)	25.0 (0.0; 75.0)	25.0 (0.0; 50.0)	0.003	85.82
Physical pain	36.9 (26.2; 49.4)	56.7 (34.4; 82.2)	9.0 (7.0; 21.5)	0.004	81.49
General health	36.6 (27.4; 48.8)	39.0 (26.0; 45.5)	-0.3 (-17.1; 13.0)	0.604	73.52
General physical health	27.8 (20.5; 43.2)	44.2 (32.9; 51.9)	5.6 (-2.5; 25.2)	0.003	
Vitality	43.8 (31.3; 59.4)	50.0 (43.8; 62.5)	6.3 (-6.3; 18.8)	0.103	61.13
Social function	37.5 (18.8; 56.3)	50.0 (37.5; 75.0)	12.5 (0.0; 25.0)	0.011	88.01
Restriction of emotional role	33.3 (0.0; 83.3)	66.7 (33.3; 100.0)	0.0 (0.0; 66.7)	0.083	82.93
Mental health	61.9 (40.5; 73.8)	66.7 (44.4; 88.9)	1.6 (-9.5; 21.4)	0.322	73.77
General mental health	43.6 (29.9; 61.9)	58.2 (47.9; 73.4)	9.9 (-5.6; 18.9)	0.028	

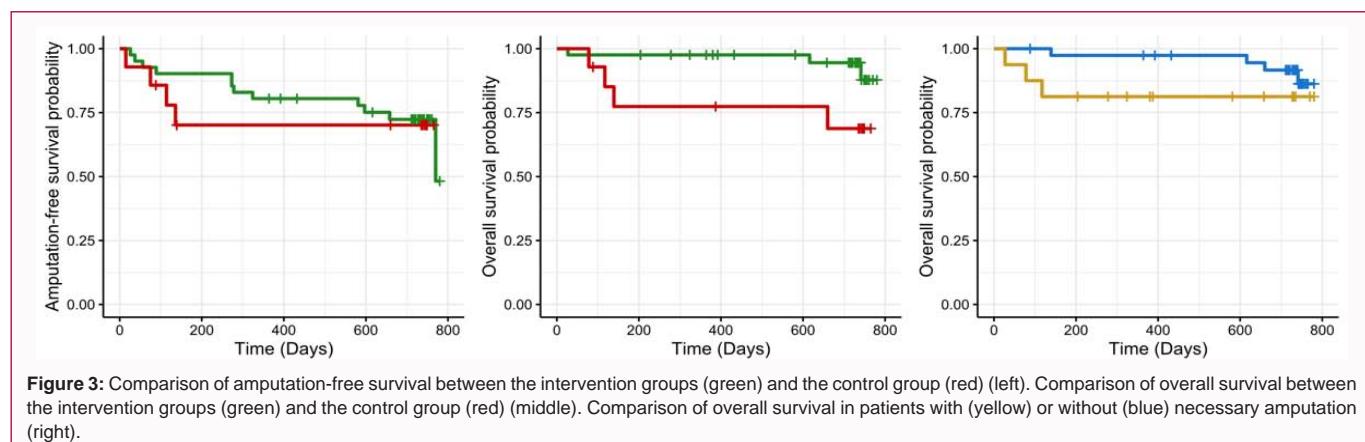
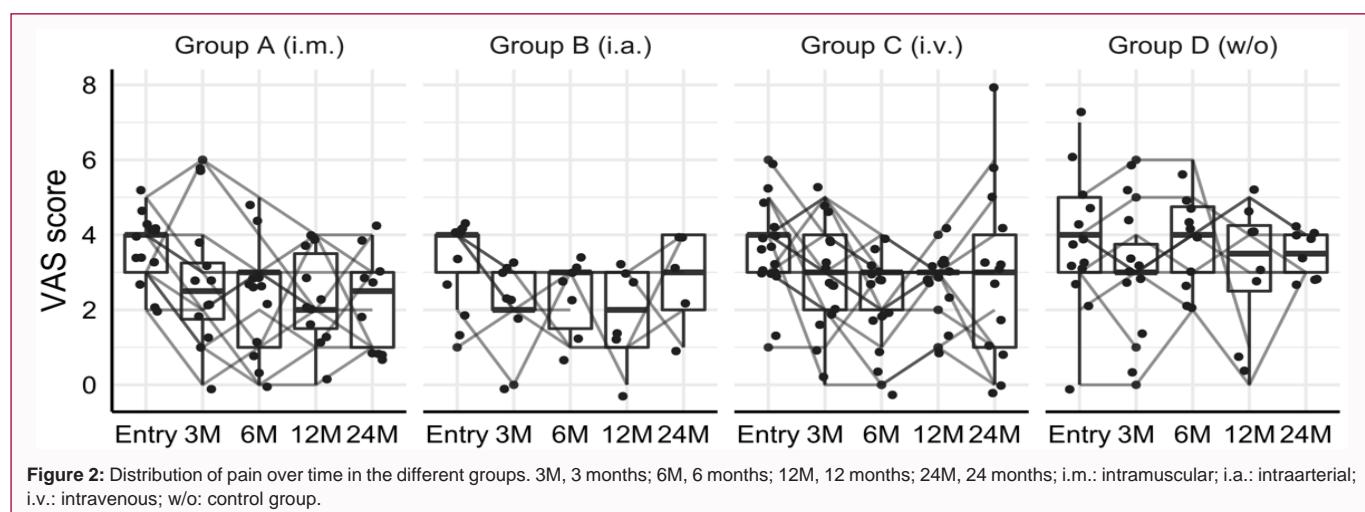
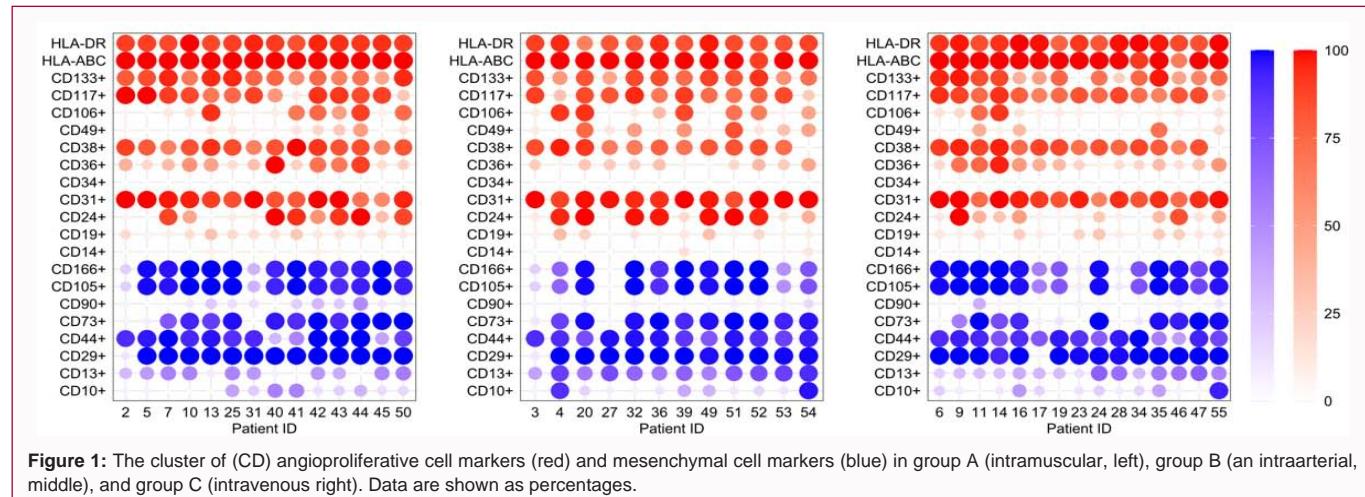
^aThe median and the interquartile range^bThe p-value of the paired Wilcoxon test

D (control) included the highest number of patients who died, constituting 29% of the patients in this group and 57% of all deaths in the study. Due to the higher death rate in the control group and the early achievement of the primary end-point, the study was terminated early. The Ethics Committee agreed with this early termination. The results indicate significantly better overall survival of patients in the intervention groups (Log-rank test, $p=0.039$) than the control group. However, we found no significant difference in the overall survival between patients with or without necessary amputation (Log-rank test, $p=0.170$). Figure 3 provides a graphical illustration of the Kaplan-Meier curves. The death occurred in a total of 7 cases. All deaths were due to cardiovascular failure in these a priori compromised patients, with two instances of failure occurring after an amputation procedure. No death appeared to be connected with the administered

treatment, and the progression of the underlying disease caused all amputations. Throughout the study, no metabolic, allergic, or inflammatory responses to BM-MNC administration were observed.

Discussion

Critical Limb Ischemia (CLI) is the most advanced stage of Peripheral Artery Atherosclerotic Disease (PAD) and is among the most severe complications of diabetes mellitus. Other risk factors include smoking, arterial hypertension, and hypercholesterolemia. PAD currently affects about 200 million people worldwide, representing a substantial burden in terms of morbidity and mortality and the cost of care for disabled patients [7-9], with about 10% of these patients having CLI manifestations [10]. PAD treatment of the affected limbs comprises pharmacotherapy, long-term mitigation



of risk factors, interventional and surgical revascularization. The results of different revascularization modalities do not significantly differ [11], and, so far, there is no clear consensus regarding the optimal treatment approach. However, this should be changed by the upcoming randomized BEST-CLI (Best Surgical Therapy in Patients with CLI) trial, which is currently in its final phase [12,13]. Notably, due to the irreversible progression of atherosclerotic changes with increasing age, many patients run out of conventional therapeutic methods. In such cases, patients are typically treated by amputation

of the affected limb, which significantly deteriorates the quality of life and numerous postoperative complications. Influencing the prognosis of the affected limb with CLI by therapeutic angiogenesis is an intensively researched method. The first controlled trials involved local or overall administration of growth factors or stem cells, but the results were controversial. It appears to be preferable to apply bone marrow cells taken directly from the marrow or peripheral blood. This method has been successfully tested in several smaller randomized experiments, but high-quality evidence of the efficacy of this therapy

is lacking [5,14,15]. The basic principle of cell therapy using the BMAC is the regenerative action of cells and the angiogenic factors. They can improve local angiogenesis and thus increase oxygen levels in the index limb. Compared to treatment using a single cell type (e.g., CD34⁺ cells) or a specific growth factor, therapy with an aspiration concentrate seems to be more beneficial, likely due to the expected range of reciprocal relationships among the cells themselves and with the microenvironment. These interrelations are synergistic in the angiogenesis process. The desired effect appears to require the presence of platelets, which are necessary for adequate homing, adhesion, and differentiation of bone marrow mononuclear cells [16,17]. This highly complex interaction results in accelerated angiogenesis in the area of ischemic tissue affected by PAD, which yields better perfusion of the periphery and subsequent pain reduction, improvement of healing of peripheral defects, and thus also in improvement of the overall quality of life. Our present results unequivocally confirmed the positive effect of BM-MNC on the overall survival and quality of life of treated patients. The main findings included a reduction in bodily pain, physical activity restrictions, and an overall improvement in the patients' physical and mental health perceptions. We did not observe statistically significant differences among the different interventional groups, except in several parameters (SF-36 index, glycemia, glycated hemoglobin concentration, and differences among several CD markers). From a clinical perspective, it can be concluded that BM-MNC administration significantly improves patients' quality of life, irrespective of the type of administration. The overall survival of treated patients was significantly better compared to traditional treatment methods. Two patients enrolled in this study died after significant limb amputation while hospitalized. In our research, BM-MNC administration did not decrease the need for limb amputation. Notably, undergoing an amputation was not associated with lower overall survival in our patients. In contrast, several other studies have reported substantially increased mortality after amputation. The 30-day mortality rate after significant limb amputation ranges from 4% to 22% [18]. The 5-year mortality rate of patients after significant amputations is reported to reach 30% to 70%, showing association with the level of amputation, with above-knee amputation having the worst outcomes [19]. Diabetes mellitus significantly worsens the prognosis, reducing the median survival from 79 to 27 months, with mortality exceeding some malignant tumors [20].

Therefore, one goal of PAD and CLI treatment should be to reduce necessary amputations, especially in diabetic patients. Several studies [21-23], including a small-scale randomized trial [24], have shown positive effects of BM-MNC administration in these patients; however, there remains a need for a large randomized study. The meta-analysis by Wang et al. shows improved ABI values, increased T_{CO_2} , and reduced Visual Analog Scale (VAS) levels [25]. More importantly, this meta-analysis revealed significantly improved Amputation-Free Survival (AFS) at one year (OR 8.05, $p<0.00001$) and at three years (OR: 22.33, $p=0.0003$). Moreover, BM-MNC administration (irrespective of the administration method) has been associated with few to no adverse effects in several studies. Most reported side effects are usually connected to the primary disease (cardiac arrest, myocardial infarction, sepsis, etc.) and not to the administration of BM-MNC itself [26], in concordance with our present data. However, Jonsson et al. [27] reported two deaths following BM-MNC administration, and the study was prematurely terminated. There are currently several written techniques for BM-MNC preparation, including the Ficoll or Harvest BMAC system techniques [28]. BM-MNC can also be prepared in a point-of-care

setting using the RES-Q60 BMC system [29]. The optimal dose of injected BM-MNC has not yet been established. The present clinical randomized interventional study has several limitations. The primary limitation is the relatively small number of enrolled patients due to its early termination. Since our results indicate a clear benefit of BM-MNC application for patients, this limitation can be eliminated in a subsequent study by only comparing different administration methods and not including a control group of patients. Another possible limitation is the heterogeneity within other intervention groups. These would likely be eliminated with an increased number of patients, or the negative impact of the difference between populations could be reduced by using specific statistical methods (e.g., paired analysis, propensity score matching, etc.).

Supplementary Materials

The following are available online at www.mdpi.com/xxx/s1: Table S1. Biochemistry analysis and hematology analysis, Table S2. Laser Doppler analysis, and Table S3. Bone marrow quality analysis.

Author Contributions

Conceptualization, F.J., V.P., and J.G.; methodology, F.J., J.R., K.V., software, K.V., P.K., A.V., L.P., and V.P.; validation, J.G., P.K., and V.P.; formal analysis, K.V., V.P., A.V., L.P., and J.R.; investigation, F.J., V.P., and J.G.; resources, V.P. and F.J.; data curation, F.J., V.P., A.V., L.P., and J.G.; writing—original draft preparation, J.R., and F.J.; writing—review and editing, V.P., A.V., and J.G.; visualization, A.V., L.P.; supervision, V.P.; project administration, V.P., F.J., and K.V.; funding acquisition, V.P. and F.J. All authors have read and agreed to the published version of the manuscript.

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