FULL-LENGTH ARTICLE

Clinical Research

Autologous bone marrow mesenchymal stromal cell therapy for “no-option” critical limb ischemia is limited by karyotype abnormalities

Sara Azhari Mohamed¹, Linda Howard¹, Veronica McInerney², Amjad Hayat³, Janusz Krawczyk^{3,4}, Sean Naughton³, Andrew Finnerty⁴, Miriam Holohan⁴, Aoife Duffy⁴, Tony Moloney⁵, Eamon Kavanagh⁵, Paul Burke⁵, Aaron Liew^{6,7}, Muhammad Tubassam⁸, Stewart Redmond Walsh^{6,8}, Timothy O'Brien^{1,4,6,9,*}

¹ National University of Ireland Galway, Ireland. Regenerative Medicine Institute

² Health Research Board, Clinical Research Facility Galway, Ireland

³ Galway Blood and Tissue Establishment, Ireland

⁴ National University of Ireland Galway, Ireland. Centre for Cell Manufacturing Ireland

⁵ Department of Vascular Surgery, University Hospital Limerick, Ireland

⁶ National University of Ireland Galway, Ireland. School of Medicine

⁷ Portlincula University Hospital, Ballinasloe, Galway, Ireland

⁸ Department of Vascular Surgery, University Hospital Galway, Ireland

⁹ National University of Ireland Galway, Ireland. CURAM, SFI Research Centre for Medical Devices

ARTICLE INFO

Article History:

Received 8 February 2020

Accepted 25 February 2020

Key Words:

abnormal karyotype
 chronic limb-threatening ischemia
 critical limb ischemia
 mesenchymal stromal cells
 phase 1 clinical trial

ABSTRACT

Background: Critical limb ischemia (CLI) is the most severe manifestation of peripheral vascular disease. Revascularization is the preferred therapy, but it is not achievable in 25%–40% of patients due to diffuse anatomic distribution of the disease or medical comorbidities. No-option CLI represents an unmet medical need. Mesenchymal stromal cells (MSCs) may provide salvage therapy through their angiogenic and tissue-trophic properties. This article reports a phase 1b clinical study examining the safety and feasibility of intramuscular transplantation of autologous bone-marrow MSCs for patients with no-option CLI.

Methods: Twelve patients were enrolled in the clinical trial, and nine proceeded to bone marrow aspiration and culture expansion of MSCs.

Results: A high rate of karyotype abnormality (>30%) was detected in the produced cell batches, resulting in failure of release for clinical administration. Four patients were treated with the investigational medicinal product (IMP), three with a low dose of 20×10^6 MSCs and one with a mid-dose of 40×10^6 MSCs. There were no serious adverse events related to trial interventions, including bone marrow aspiration, IMP injection or therapy.

Conclusions: The results of this trial conclude that an autologous cell therapy approach with MSCs for critical limb ischemia is limited by the high rate of karyotype abnormalities.

© 2020 International Society for Cell and Gene Therapy. Published by Elsevier Inc.

This is an open access article under the CC BY license. (<http://creativecommons.org/licenses/by/4.0/>)

Introduction

Peripheral vascular disease (PVD) is a growing health problem in Western societies, affecting up to 3%–10% of the population [1]. Critical limb ischemia (CLI) is the most advanced clinical manifestation of PVD, occurring in 500 to 1000 per million individuals in North America and Europe [2]. The term “chronic limb-threatening ischemia” (CLTI) has

recently been introduced to emphasize the continuum nature of the impaired perfusion [3]. CLI is typically characterized by rest pain and/or tissue loss in the form of ulceration or gangrene. Surgical or endovascular revascularization is the preferred therapy; however, it is unachievable in 25%–40% of patients due to medical comorbidities or anatomically diffuse disease [2]. These “no-option” patients are at high risk for amputation and death, with a 40% risk of amputation [4] and 20% risk of mortality within 6 months [1]. Therefore, no-option CLI is a life-threatening disease representing an unmet medical need.

Cell therapy has been extensively studied as a novel therapy for no-option CLI. The proposed therapeutic advantages of mesenchymal

* Correspondence: Timothy O'Brien, MD, PhD, FRCPI, FRCP (UK), FACP, College of Medicine, Nursing and Health Sciences, National University of Ireland Galway.

E-mail address: timothy.obrien@nuigalway.ie (T. O'Brien).

stromal cell (MSC) transplantation include that these cells are able to reside in hypoxic tissue and thus have enhanced survival properties, and they secrete many factors that have paracrine effects on angiogenesis. Although the use of a variety of cell types has been proposed for vascular regeneration, the current project focused on bone marrow (BM)–derived MSCs.

MSCs are multi-potent non-hematopoietic, fibroblast-like cells that can be isolated from various tissue sources, including BM, adipose tissue, placenta and umbilical cord [5]. They have many characteristics that are advantageous for therapeutic applications. They adhere to tissue culture plastic and proliferate in culture, facilitating *ex vivo* expansion to generate cell doses from relatively small volumes of BM [6,7]. MSCs have been shown to be effective in multiple reports in preclinical models of CLI. In addition, there is substantial evidence on the safety of MSC administration to humans [8].

Several human studies have used MSCs based on their pro-angiogenic effect [9–13]. They also mediate anti-inflammatory, anti-fibrotic, anti-apoptotic, mitogenic and wound-healing properties [14]. Compared with BM-derived mononuclear cells (BM-MNCs), MSCs resulted in better lower limb perfusion and ulcer healing in diabetic patients [7]. Many clinical trials suggested that the difference in therapeutic efficacy of various cell types is probably related to the respective cell potency [15].

“CLI stromal cells” is a phase 1b safety and feasibility study examining the use of intramuscular autologous transplantation of BM-MSCs as a novel therapy to promote angiogenesis in no-option CLI patients (EudraCT number 2013-003447-37).

Methods

Trial overview

This was a nonrandomized, uncontrolled, open-label, dose-escalation phase 1b study conducted with a primary aim of examining the

feasibility and safety of autologous BM-MSCs as a novel therapy for patients with CLI who are not candidates for revascularization. The trial sponsor was the National University of Ireland, Galway. The planned doses were sequentially 20, 40 and 80 million cells delivered as a single dose via multiple intramuscular injections. The targeted sample size was nine patients, three for each dose cohort (Figure 1).

The trial adhered to the declaration of Helsinki and followed the International Conference for Harmonization Good Clinical Practice (GCP) guidelines 2001/20/EC. The trial protocol was approved by the Health Product Regulatory Authority under investigational medicinal products section (approval reference 2163688). The trial was also approved by the Research Ethics Committee at Galway University Hospital (approval reference 15/12). Written informed consent was obtained from all patients. An independent Data Safety Monitoring Board (DSMB) was established to review the safety data at periodic intervals.

Patient recruitment

Patients with CLI attending the outpatient and inpatient vascular surgery service at Galway University Hospital were pre-screened and offered participation in the trial if they were considered suitable candidates by their primary treating vascular surgeon. Patients were also referred from other vascular surgery centers across the Republic of Ireland, including St James's Hospital, Tallaght Hospital and University Hospital Limerick.

BM harvest

Consented patients were screened for eligibility (Table 1). Eligible patients proceeded to have BM aspiration performed by a consultant hematologist in the operating theatre. For patients enrolled in the low-dose cohort, 30 mL of BM was aspirated under local anesthesia from the posterior iliac crest. This volume was increased to 100 mL

Summary of the pathway of the clinical trial procedures

Figure 1. Trial methodology. Summary of the pathway of the clinical trial procedures. ; CCMI, Centre of Cell Manufacturing Ireland; FBS, fetal bovine serum; GUH, Galway University Hospital; HAS, human serum albumin; MEM, minimal essential media; MNCs, mononuclear cells; P2, passage 2; UHL, University Hospital Limerick.

Table 1
Eligibility criteria.

Inclusion criteria
<ul style="list-style-type: none"> • Men or women between the ages of 18 and 85 • Voluntary written informed consent, with the understanding that consent could be withdrawn at any time without prejudice to future medical care • Presented with CLI with rest pain or ulceration with no option for revascularization agreed by an expert panel including an interventional radiologist and vascular surgeon; CLI defined as persistent ischemic rest pain for ≥ 2 weeks and/or ulceration or gangrene of the toe or foot • Estimated life expectancy > 6 months as deemed by patients clinician and/or investigator • Suitable for BM aspiration (deemed by consultant hematologist) • Rutherford class 4 (CCI with rest pain) or Rutherford class 5 (mild to moderate tissue loss) who are not candidates for revascularization • Medically fit to undergo BM harvest and stromal cell intramuscular injection • Have one of the following: ankle systolic pressure < 70 mm Hg or ABI < 0.9 OR toe pressure < 70 mm Hg OR Toe Brachial Index < 0.6 OR TcPO₂ < 60 mm Hg on room air
Exclusion criteria
<ol style="list-style-type: none"> 1. Prior therapy with MSCs 2. Previous amputation of talus or above 3. Failed revascularization within 2 weeks of enrollment 4. Known aorto-iliac disease with $> 50\%$ stenosis 5. Severe comorbidity limiting 6-month survival 6. Significant cognitive impairment (< 22 score on Mini-Mental State Examination) 7. Contraindication to intramuscular procedure, including active infection, wet gangrene or exposed bone or tendon in lower limb with CLI; or, in the opinion of the attending clinician, patient unsuitable for intramuscular procedure 8. Rutherford class 6 CLI 9. Abnormal liver function (aspartate aminotransferase and alanine aminotransferase $> 2.5 \times$ upper normal limit, total bilirubin $1.5 \times$ upper normal limit) 10. Likely inability to comply with the protocol or cooperate fully with the investigator and site personnel 11. Presence of poorly controlled diabetes mellitus with HbA_{1c} $> 10\%$ within previous 3 months 12. Presence of proliferative retinopathy (in participants with diabetes mellitus only) 13. HIV or HBsAg positive 14. Pregnancy 15. Presence of acute coronary syndrome 16. Known active malignancy 17. Patients with known allergy to fetal bovine serum or trypsin 18. Use of other investigational drugs at the time of enrollment or within 28 days of enrollment 19. Significant BM dysfunction, based on assessment by hematologist or an established diagnosis of myelodysplasia, myeloproliferative disorder, etc. 20. Bleeding diathesis, coagulopathy, thrombocytopenia, etc. 21. Patients in whom delay incurred by attempts at limb salvage using MSCs will adversely affect prognosis in the opinion of the responsible attending clinician

under conscious sedation for patients enrolled for the higher-dose cohorts. BM procurement was performed under the Galway Blood and Tissue Establishment quality system.

MSC manufacturing

The marrow was transferred for culture expansion to the Centre for Cell Manufacturing Ireland (CCMI) Good Manufacturing Practice (GMP)-licensed facility. MSCs were produced in accordance with GMP requirements through direct plating at a seeding density of $40\text{--}60 \times 10^6$ MNCs per T175, cultured in alpha-medium essential media (MEM) supplemented with 10% fetal bovine serum with no antibiotics. Cells were harvested at the end of P2 for cryopreservation using 4.5% human serum albumin and 10% dimethyl sulfoxide (DMSO).

MSCs meeting the release criteria as detailed in the approved Investigation Medicinal Product Dossier (Table 2) were released by the Qualified Person (QP) for clinical administration. The release

criteria included assessment of sterility, mycoplasma, endotoxin, immunophenotype [16], karyotype analysis [17] and viability. In the case of failed release for clinical use or insufficient MNCs, patients were offered re-consenting for a second BM harvest.

MSC administration

Once successful release of the cells was confirmed, the clinical team assessed the study participants to reconfirm eligibility. The investigational medicinal product (IMP) injection procedure was performed by a consultant vascular surgeon in the operating theater. The targeted dose was thawed, washed and reconstituted using normal saline into 10 mL, injected into 20 intramuscular sites (0.5 mL each) under ultrasound guidance to avoid inadvertent puncture of a major vascular structure. The injection sites were selected on the basis of the underlying anatomic distribution of the disease, following an anterior or medial linear pattern to the calf and/or distal thigh, replicating a “biologic bypass.” The injection line started at the most distal patent named artery and extended distally to the area of tissue loss or arterial reconstitution with inline flow to the area of tissue loss. The 20 intramuscular injection sites were 1 to 2 cm apart and at least 5 mm away from named arteries. Ulcer beds were not directly injected.

Outcome measures

Participants were reviewed for follow-up at days 7, 30, 90, 180 and 365 after IMP injection. The primary outcome measure of this study was safety determined by the number of serious adverse events (SAEs) probably or definitely attributable to BM aspiration, MSC therapy or MSC injection procedure within 12 months. All captured SAEs were evaluated by an independent adjudicating physician. In addition, after each dose-escalation step, a summary of all the SAE data collected during the 30 days after each participant's treatment was reviewed by the DSMB, which decided whether escalation to the next dose was appropriate. The study protocol defined the safety criteria for stopping the trial as more than two participants within the cohort with two or more SAEs attributable to either MSCs therapy or bone marrow aspiration.

The secondary outcomes included amputation-free survival, ankle brachial index (ABI), transcutaneous oxygen pressure (TcPO₂), ischemic rest pain score, ulcer size and quality of life (measured by EQ-5D-5L assessment) at 12 months.

Table 2
IMP specification.

Test	Method	Specification
Sterility	Direct Inoculation (Ph Eur 2.6.1)	No growth
Mycoplasma	Culture and Indicator Cell Culture (Ph Eur 2.6.7)	No mycoplasma detected
LAL test for Endotoxin	Chromogenic Kinetic Method (Ph Eur 2.6.14)	< 5 EU/mL
Immunophenotype	Flow cytometry	Positive markers CD73 $\geq 90\%$ CD90 $\geq 90\%$ CD105 $\geq 90\%$ Negative marker cocktail CD45, CD34, CD11b, CD19, HLA-DR $\leq 5\%$
Karyotype	20 metaphases analyzed by G banding	No findings indicative of karyotype instability
Viability	Trypan Blue exclusion (Ph Eur 2.7.29)	$\geq 70\%$

LAL, limulus ameobocyte lysate; Ph Eur, European Pharmacopoeia.

Table 3
Clinical and demographic characteristics

Pt. no	Age	Sex	BMI	Rutherford class	Smoking	Diabetes	Hypertension	Hyperlipidemia	IHD	CD	CKD	COPD/asthma
CLI001	52	M	34.0	5	Yes	No	Yes	Yes	No	No	No	No
CLI002	63	M	32.7	4	Ex	Yes	Yes	No	Yes	No	No	No
CLI003	70	M	— ^a	5	Ex	No	Yes	No	No	No	No	Yes
CLI004	83	M	31.0	5	Non	No	Yes	Yes	No	Yes	Yes	No
CLI005	65	M	36.9	5	Ex	Yes	Yes	No	Yes	Yes	Yes	No
CLI006	68	M	28.1	5	Non	No	Yes	Yes	Yes	Yes	No	Yes
CLI007	72	M	23.2	5	Non	Yes	Yes	Yes	Yes	No	Yes ^b	No
CLI008	81	M	37.8	5	Non	No	No	No	No	No	No	No
CLI009	80	F	21.6	5	Yes	Yes	No	No	Yes	No	No	Yes
CLI010	84	M	25.3	5	Yes	No	Yes	Yes	No	Yes	Yes	Yes
CLI011	39	F	22.0	5	Yes	No	No	Yes	No	No	No	No
CLI012	65	F	34.7	5	Non	Yes	Yes	Yes	Yes	No	Yes ^b	Yes

BMI: <18.5 = underweight, 18.5–24.9 = normal, 25.0–29.9 = overweight, 30.0–34.9 = class I obesity, 35.0–39.9 = class II obesity, >40.0 = class III obesity. Rutherford classification: 0 = asymptomatic, 1 = mild claudication, 2 = moderate claudication, 3 = severe claudication, 4 = rest pain, 5 = moderate tissue loss, 6 = severe tissue loss and gangrene. BMI, body mass index; CD, cerebrovascular disease; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; Ex, ex-smoker; F, female; IHD = ischemic heart disease; M, male; Non = nonsmoker.

^a Patient withdrawn before collection of this data.

^b On dialysis.

Soft agar assay

To determine whether MSCs with abnormal karyotypes were transformed, the *in vitro* anchorage independent growth of the cells was tested by growing the cells in soft agar. PC3 prostate cancer cells were used as positive control in this assay. A basal layer of 0.5% or 0.75% agar mixed with an equal volume of 2× medium (alpha-MEM for MSCs and HAM-F12 for PC3 cells) was plated followed by a second layer containing cells in an agar/medium mixture. Using six-well plates, 2500 cells were plated in each well for the positive control and the three CLI-patient derived MSCs batches. To increase the robustness of the testing, higher numbers of MSCs were also tested (7500, 22 500 and 67 500 cells per well). Culture plates were incubated at a temperature of 37°C, 5% carbon dioxide for 30 days. To detect colonies, each well was stained with 200 µL of 1 mg/mL p-iodonitrotetrazolium violet for 24 h at 37°C before imaging.

Statistical analysis

The demographic characteristics, safety data and karyotype analysis results are all presented as descriptive occurrence of events. Efficacy data including ischemic rest pain, ABI and TcPO₂ was expressed as mean ± SD and analyzed using one-way analysis of variance with mixed effect analysis to compensate for missing data.

Results

From March 2015 to January 2019, 263 CLI patients were pre-screened for suitability for inclusion in the study. Of the 93 patients who were not suitable for revascularization “no-option CLI,” 12 were consented and enrolled in the clinical trial (nine men, three women). The demographic characteristics and clinical comorbidities of enrolled patients are outlined in Table 3. Three patients were withdrawn from the study due to ineligibility detected during screening (aorto-iliac stenosis >50%, history of BM dysfunction [polycythemia] or withdrawal of consent). Nine patients proceeded to BM harvest after confirmation of eligibility, with one patient undergoing a second marrow aspiration due to the failed release of the first MSC cell batch. This resulted in 10 cell batches manufactured by CCMi.

Four MSC batches were not approved for clinical administration; two because they had not been released by the manufacturer due to abnormal karyotype analysis, one due to a manufacturing event and recall of the DMSO used in the cryopreservation of the IMP, and the fourth batch passed the manufacturer release criteria but failed release by the primary investigator for clinical administration due to

abnormal karyotypes. Six MSC cell batches were successfully released by the manufacturer. Two patients were withdrawn from the study after IMP release (Figure 2), IMP was administered to four patients; three with low dose of 20×10^6 cells per patient, and one patient treated with the mid-dose 40×10^6 cells per patient. An overview of the trial scheme is summarized in Figure 2.

Safety assessment

Eighteen SAEs were recorded during the clinical trial. After evaluation by an independent adjudicating physician and the DSMB, none of the SAEs were found to be attributable to BM aspiration, IMP therapy or IMP injection procedure. None of the 57 recorded AEs were related to BM aspiration or IMP therapy. Two AEs were related to the injection procedure for one patient (one with pain at one injection site and the other with bruising at one injection site). These AEs were graded as mild in severity and resolved spontaneously without intervention (Table 4). The DSMB safety evaluation concluded that intramuscular autologous transplanatation of BM-MSCs is safe.

Secondary outcome measures

Out of the four treated patients, three had amputation-free survival at the end of the study. One patient underwent above-knee amputation at day 96 post-IMP injection due to the progression of ischemic rest pain and gangrenous tissue loss. This patient had evidence of disease progression between the BM harvest and the time of injection of the expanded product. Two of the treated patients had complete ulcer healing after MSC injection (Figure 3).

Analysis showed clinically significant reduction in mean ischemic rest pain from 7 at baseline to 0 at day 30; this difference did not reach statistical significance. However, in three patients and notwithstanding the non-controlled nature of the trial, the pain relief was life-changing. Changes in ABI and TcPO₂ showed no significant difference from before and after IMP injection (Figure 4). The evaluation of potential therapeutic efficacy of the cell therapy is limited by the fact this trial was not powered or controlled to determine efficacy.

Karyotype analysis

G banding of metaphase spreads was used to test the karyotype of the IMP at the end of P2. A test result was considered a “pass” when 20 observable metaphase cells spreads were analyzed and 10 or more cells have a normal karyotype and no findings of karyotypic

Figure 2. Overview of the trial scheme. Study enrollment diagram for the CLI Stromal Cells clinical trial.

Table 4

Summary of recorded adverse events and abnormal karyotype.

Pt. No.	Total AEs	SAEs	Related to BM aspiration	Abnormal karyotype	Related to IMP	Related to injection procedure
CLI001	0	0	N/A	Yes	N/A	N/A
CLI002	31	7	None	No	None	None
CLI004	5	1	None	No	None	2
CLI006	6	6	None	Yes	N/A	N/A
CLI007	6	1	None	No	None	None
CLI009	2	2	None	Yes	N/A	N/A
CLI010	3	0	None	No	None	None
CLI011	0	0	N/A	No	N/A	N/A
CLI012	4	1	None	No	N/A	N/A
Total	57	18	0	3	0	2

N/A, not applicable

instability are found. A test was considered a “fail” if three or more cells exhibited the same chromosome loss (monosomy), two or more cells exhibited the same chromosome gain (trisomy) or two or more cells exhibited the same structural rearrangement—for example, deletion, inversion or translocation [17].

Three of the 10 MSC batches manufactured by CCMI failed the release criteria for clinical administration due to karyotypic abnormalities. Two of the three batches showed trisomy 5 (Figure 5B) as the predominant karyotypic abnormality (6 of 20 and 12 of 20 metaphase). Although the third batch passed the criteria detailed in the IMPD and was released from the manufacturer, it did not meet the acceptance criteria for clinical use as decided by the primary investigator. This was related to the higher than usual incidence of translocation, addition and monosomy. The details of karyotype analysis for the three batches are summarized in Figure 5A.

Additional karyotype analysis was carried out for the first MSC batch for donor CLI001. A total of 6 additional samples were tested, evaluating 20 metaphase cells in each sample, covering a range of different passages (P2, P3, P4). Five of the six additional samples showed two or more cells with trisomy 5 and would have failed the

criteria for IMP release (Table 5), confirming that the chromosomal abnormality persisted across different passages.

To determine whether MSCs with abnormal karyotype were transformed, the *in vitro* anchorage independent growth of the cells was tested by growing the cells in soft agar. Many colonies of the positive control cells (PC3 prostate cancer cells) were observed, but no colonies were detected for the MSCs at any of the cell numbers tested indicating that despite the karyotypic alteration, they are not transformed cells (Figure 6).

Discussion

Stromal cell therapy has been proposed as a treatment for CLI patients with no option for revascularization. In this study, we examined the safety and feasibility of autologous transplantation of BM-derived MSCs in patients with no-option CLI and report for the first time limitations associated with this approach due to karyotypic abnormalities in the expanded cells.

Many therapeutic strategies have been proposed in an attempt to decrease the morbidity and mortality associated with no-option CLI.

Figure 3. Representation of the treated limbs showing the progression of ischemic ulcer/gangrene for each patient throughout the clinical trial follow-up.

Figure 4. (A) Ischemic rest pain score measured by verbal analogue scale: 0 = no pain, 10 = worst pain in patient's life, $P = 0.1524$. (B) Ankle brachial index of the treated limb, $P = 0.412$. (C) Transcutaneous oxygen pressure in the distal part of the treated limb measured in mm Hg, $P = 0.6194$. Data expressed as mean $\pm$ SD, analyzed using one-way analysis of variance with mixed effect analysis to compensate for missing data

Single angiogenic gene/protein-based therapies have been attempted, with poor clinical outcome [18]. Recombinant growth factor therapy combined with tissue grafting for patients with chronic ulcers showed minimal benefit over standard treatment [19].

Cell-based therapy is currently showing promise in both preclinical and clinical studies. Many cell types have been examined for efficacy in promoting neo-angiogenesis either by direct incorporation into new vessels or by secretion of multiple angiogenic growth factors and cytokines [20].

Of the 263 CLI patients prescreened for inclusion in this clinical trial, 35% had no-option for revascularization. This percentage is consistent with the range reported in the literature (25%–40%) [1]. Of 93 “no-option” CLI patients, only 12 were successfully enrolled in the clinical trial. The most frequently encountered exclusion criteria were advanced disease (including Rutherford class 6 and infected ulcers), age above the upper limit, aorto-iliac stenosis, cognitive impairment, active malignancy and limited life expectancy.

The results of this study confirmed that intramuscular autologous transplantation of MSCs to no-option CLI patients is safe, with no adverse events or SAEs attributable to BM harvest, cell therapy or injection procedure. This result supports the preexisting safety data reported by previous similar clinical trials [7,9,11,21–23]. In this study, two cell doses were examined (20 and 40 million MSCs), with three patients treated with the low-dose and one patient treated

with the higher dose. Both doses were well tolerated by patients with no adverse events.

Autologous cell harvesting was used in this study, and the results indicate limited feasibility with this approach. Despite the advantage of elimination of immunogenicity, there are concerns associated with allogeneic approach, the process of cell manufacturing and quality-controlled testing takes up to 10 weeks; rendering patients with severe or rapidly progressing disease unsuitable for autologous cell therapy. This was apparent from the percentage of patients excluded from the clinical trial due to advanced disease requiring urgent amputation (38%). One of the enrolled patients experienced rapid deterioration from only rest pain (Rutherford class 4) at enrollment to tissue loss and dry gangrene of the toes (Rutherford class 5) at the time of cell injection. This patient experienced transient improvement in ischemic rest pain but eventually required major amputation 96 days after cell therapy. It is acknowledged that there is potential to reduce the time required for some of the release testing. For example, mycoplasma testing was performed using the traditional culture method (Ph Eur 2.6.7) which takes up to 35 days for final report, but rapid microbiological testing using polymerase chain reaction (Ph Eur 5.1.6) could reduce this significantly to approximately 2–3 days.

Although this study was not powered for efficacy outcome measures, we observed clinically significant improvement in ischemic rest pain in all treated patients. Three of the four treated patients had

A: Summary of karyotype abnormality

Donor	Karyotype abnormality
CLI001	Normal male chromosome complement and banding pattern in 13 cells. Anomalous Cell List: <ul style="list-style-type: none"> • Six cells: 47, XY, +5 • One Cell: 45, XY, -22
CLI006	Normal male chromosome complement and banding in 15 cells Anomalous Cell List: <ul style="list-style-type: none"> • One cell: 46, XY, ?t(3;8)(p?21;p?11.2) • One cell: 46, XY, ?t(4;11)(p?34;p?15) • One cell: 46, XY, add(6)(q25) • One cell: 45, X, -Y • One cell: 45, XY, -17
CLI009	Normal Female chromosome complement and banding pattern in only 3 cells Anomalous Cell List: <ul style="list-style-type: none"> • Eleven Cells: 47, XX, +5 • Three cells: 49, XX, +5, +5, +10 • One cell: 48, XX, +2, +5 • One cell: 49, XX, +5, +5, +9, +10, -18 • One cell: 46, XX Add(10)(p13)

B: Karyotype Abnormality

Figure 5. MSCs batches with abnormal karyotype. Three CLI patient-derived MSC batches failed release for clinical use due to abnormal karyotype. (A) Summary of karyotype abnormality. (B) Karyotype abnormality. (1B) Trisomy 5 detected in 6 of 20 metaphase cells for donor CLI001. (2B) Trisomy 5 detected in 11 of 20 metaphase cells for donor CLI009. (3B) An example of chromosomal abnormalities and translocation abnormality detected in one cell batch. Overall, 5 of 20 metaphase cells showed chromosomal abnormality for donor CLI006. This cell passed the IMP karyotype specification but failed release for clinical administration due to abnormally higher rate of karyotype abnormality.

Table 5

Additional testing of CLI patient-derived BM-MSCs cell batch.

Sample	Result
First sample (P4)	Six cells: 47, XY,+5 One cell: 46,XY,-22
Sample 1 (P2)	Two cells: 47,XY,+5 One cell: 47,XY,+6 One cell: 48,XY,+5,+6 One cell: 49,XY,+5,+6,+21 One cell: 45,X
Sample 2 (P2)	Two cells: 47,XY,+5 One cell: 47,XY,+14 One cell: 48,XY,+5,+6 One cell: 49,XY,+5,+6,+14
Sample 1 (P3)	One cell: 46,XY,+5,-16 One cell: 47,XY,+5,+12,+17 One cell: 46,X,t(Y;20)(p11.2;q13.1),add(20)(p13) One cell: 46,XY,add(5)(q34),add(22)(p11.2)
Sample 2 (P3)	Three cells: 47,XY,+5 One cell: 48,XY,+5,+6 One cell: 46,XY,t(14;15)(q31;q15)
Sample 1 (P4)	Four cells: 47,XY,+5 One cell: 48,XY,+5,+7 One cell: 46,XY,t(1;9)(q21;p22) One cell 46,XY,t(12;14)(p11.2;p11.2)
Sample 2 (P4)	No anomalous cells detected

Figure 6. *In vitro* transformation assay. Anchorage independent growth of CLI patient-derived MSCs with abnormal karyotype was examined by growing the cells in soft agar to test *in vitro* transformation potential. (A) Colonies formed by prostate cancer cells (PC₃) as a positive control. (B) No colonies were detected in any of the three CLI-derived MSCs batches (representative image).

complete resolution of the rest pain within 30 days of cell therapy and remained pain free throughout the 1-year follow-up. This improvement in rest pain was also reported by a meta-analysis of 10 autologous cell therapy studies showing significant difference in ischemic rest pain with standardized mean difference of -1.105 [24].

The targeted number for treated patients was nine, with three patients in each dose cohort; however, a decision was made to end

the study after culture expansion of cells from nine patients revealed a high rate of karyotypic abnormalities, suggesting that this study was not feasible. It is still not fully known whether the karyotype abnormalities observed reflect aneuploidy present in patients' cells or are culture-induced. Tarte *et al.* suggested that the abnormalities are not culture-related and represent cells that subsequently became senescent (because they disappeared after the second passage) [25].

The predominant karyotypic abnormality observed was trisomy 5. This chromosomal abnormality was only reported by two other MSC studies [25,26]. In both studies, cells with trisomy 5 were lost to higher passages during cell expansion, suggesting that this mutation confers a growth disadvantage. Our observation is contrary to that report because chromosomal abnormalities were observed in higher passages including passage 4 (Table 5).

Absence of transformation potential both *in vivo* and *in vitro* was confirmed in previous studies by detection of normal expression of transformation-associated genes (p53, p21, c-myc, p16) and no tumor formation when these cells were injected into immune-deficient mice [25]. This is consistent with the result of the soft agar assay reported in this study, indicating the absence of *in vitro* transformation of MSCs with abnormal karyotype.

The detected karyotypic abnormalities limit the feasibility of autologous MSCs therapy under the current IMP specification followed in this study. The European Cell Products Working Party and the Committee for Advance Therapies experts meeting recommended that MSCs with chromosomal aberrations can be used after assessment of the benefit–risk ratio linked to the clinical indication [27]. Further studies are required for characterization of these cells to determine future feasibility of an autologous approach.

Conclusion

This clinical trial was conducted to examine the safety and feasibility of autologous intramuscular transplantation of BM-MSCs as a novel therapy for no-option CLI patients. The results of the study confirm the safety of the use of these cells; however, the feasibility is limited by the high rate of karyotype abnormalities detected in the CLI patient-derived MSCs and the time needed to culture expand cells after patients present with critical limb ischemia. BM-MSCs therapy may also be beneficial in improving rest pain and ischemic ulcer healing.

Funding

This project was funded by Science Foundation Ireland/Health Research Board (SFI/HRB) Translational Research Award (award number: TRA 201115); NUIG, School of Medicine PhD Scholarship; and HRB Clinical Research Facility Galway (CRFG). TOB is a director and equity holder in Orbsen Therapeutics (a stem cell company).

Declaration of Competing Interests

The other authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

Author Contributions

Conception and design: SAM, LH, AH, JK, SN, AF, MH, AD, AL, MT, SRW, TOB. Administrative support: SAM, VM, TOB. Collection and assembly of data: SAM, VM, JK, SN, AF, AL, MT, TOB. Collection of data: SRW. Data analysis and interpretation: SAM, TOB. Manuscript writing: SAM, LH, MH, AL, TOB. Study coordination: SAM, VM. Provision of study material: JK, SN, AF, MH. Provision of study materials or patients: TM, EK, PB, MT, SRW. Final approval of the manuscript: LH, TOB.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material. Raw data are available on request from the corresponding author. The patients' confidentiality will be maintained and will not be made publicly available to the extent permitted by the applicable laws and regulations.

Acknowledgements

The authors thank Anne Marie Dunne (research nurse, University Hospital Limerick, Ireland) for her contribution to recruitment; Margaret Tarpey (Galway Blood & Tissue establishment, Galway, Ireland) for her work obtaining a tissue procurement license and IMP reconstruction protocols; and Adrian O'Callaghan (consultant vascular surgeon, St James's University Hospital, Dublin, Ireland) and Bridget Egan (consultant vascular surgeon, Tallaght Hospital, Dublin, Ireland) for their contributions to patient recruitment.

This clinical trial was registered at European Union Drug Regulatory Authorities Clinical Trials Database (EudraCT number 2013-003447-37).

References

- [1] Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *J Vasc Surg* 2007;45(1):S5–S67.
- [2] Norgren L, Hiatt WR, Dormandy JA, et al. The next 10 years in the management of peripheral artery disease: perspectives from the 'PAD 2009' Conference. *Eur J Vasc Endovasc Surg* 2010;40(3):375–80.
- [3] Conte MS, Bradbury AW, Kolh P, et al. Global vascular guidelines on the management of chronic limb-threatening ischemia. *J Vasc Surg* 2019;69(6s):3S–12S.e40.
- [4] Bradbury AW, Adam DJ, Beard DJ, et al. Bypass versus angioplasty in severe ischaemia of the leg (BASIL): Multicentre, randomised controlled trial. *Lancet* 2005;366(9501):1925–34.
- [5] Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* 2004;36(4):568–84.
- [6] Horwitz EM, Le Blanc K, Dominici M, et al. Clarification of the nomenclature for MSC: the International Society for Cellular Therapy position statement. *Cytotherapy* 2005;7(5):393–5.
- [7] Lu D, Chen B, Liang Z, et al. Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. *Diabet Res Clin Pract* 2011;92(1):26–36.
- [8] Liew A, O'Brien T. Therapeutic potential for mesenchymal stem cell transplantation in critical limb ischemia. *Stem Cell Res Ther* 2012;3(4):28–41.
- [9] Dash NR, Dash SN, Routray P, et al. Targeting nonhealing ulcers of lower extremity in human through autologous bone marrow-derived mesenchymal stem cells. *Rejuven Res* 2009;12(5):359–66.
- [10] Lee HC, An SG, Lee HW, et al. Safety and effect of adipose tissue-derived stem cell implantation in patients with critical limb ischemia—a pilot study. *Circ J* 2012;76(7):1750–60.
- [11] Gupta PK, Chullikana A, Parakh R, et al. A double blind randomized placebo controlled phase I/II study assessing the safety and efficacy of allogeneic bone marrow derived mesenchymal stem cell in critical limb ischemia. *J Transl Med* 2013;11(1):143.
- [12] Lasala GP, Silva JA, Gardner PA, et al. Combination stem cell therapy for the treatment of severe limb ischemia: safety and efficacy analysis. *Angiology* 2010;61(6):551–6.
- [13] Guiducci S, Porta F, Saccardi R, et al. Autologous mesenchymal stem cells foster revascularization of ischemic limbs in systemic sclerosis: a case report. *Ann Int Med* 2010;153(10):650–4.
- [14] Kawamoto A, Katayama M, Handa N, 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;67(5):444–55.
- [15] Liew A, Bhattacharya V, Shaw J, et al. Cell therapy for critical limb ischemia: a meta-analysis of randomized controlled trials. *Angiology* 2016;27(11):2857–64.
- [16] Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;8(4):315–7.
- [17] Schwinger E. An international system for human cytogenetic nomenclature, Lisa G. Shaffer, Marylin L. Slovak, Lynda J. Campbell. Recommendations on the International Committee on Human Cytogenetic Nomenclature. Published in collaboration with Cytogenetic and Genome Research. VI+138pp, 11 fig, 4 tab, soft cover,

- S. Karger, Basel (2009). CHF 43.00/EUR 30.50/USD 43.00, ISBN 978-3-8055-8985-7. *Ann Anat* 2009;191(5):502.
- [18] Liew A, Bhattacharya V, Shaw J, et al. Gene therapy for peripheral arterial disease. *Cochrane Database Syst Rev* 2018;10:CD012058.
 - [19] Kalka C, Asahara T, Krone W, et al. Angiogenesis and vasculogenesis. Therapeutic approaches for stimulation of post-natal neovascularization. *Herz* 2000;25(6):611–22.
 - [20] Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275(5302):964–7.
 - [21] Debin L, Youzhao J, Ziwen L, et al. Autologous transplantation of bone marrow mesenchymal stem cells on diabetic patients with lower limb ischemia. *J Med Coll PLA* 2008;23(2):106–15.
 - [22] Powell RJ, Marston WA, Berceli SA, 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(6):1280–6.
 - [23] Dubský M, Jirkovská A, Bem R, et al. Impact of severe diabetic kidney disease on the clinical outcome of autologous cell therapy in people with diabetes and critical limb ischaemia. *Diabet Med* 2019;36(9):1133–40.
 - [24] Ai M, Yan C-F, Xia F-C, et al. Safety and efficacy of cell-based therapy on critical limb ischemia: a meta-analysis. *Cytotherapy* 2016;18(6):712–24.
 - [25] Tarte K, Gaillard J, Lataillade JJ, et al. Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. *Blood* 2010;115(8):1549–1543.
 - [26] Sensebé L, Tarte K, Galipeau J, et al. Limited acquisition of chromosomal aberrations in human adult mesenchymal stromal cells. *Cell Stem Cell* 2012;10(1):9–10.
 - [27] Barkholt L, Flory E, Jekerle V, et al. Risk of tumorigenicity in mesenchymal stromal cell-based therapies—bridging scientific observations and regulatory viewpoints. *Cytotherapy* 2013;15(7):753–9.