Cytotherapy 24 (2022) 1259–1267

Contents lists available at ScienceDirect

CYTOTHERAPY

journal homepage: www.isct-cytotherapy.org

FULL-LENGTH ARTICLE
Manufacturing

Marrow changes and reduced proliferative capacity of mesenchymal stromal cells from patients with "no-option" critical limb ischemia; observations on feasibility of the autologous approach from a clinical trial

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ARTICLE INFO

Article History:
Received 30 March 2022
Accepted 7 July 2022

Key Words:
Abnormal karyotype
Autologous approach
Clinical trial
Critical limb ischemia
Mesenchymal stromal/stem cells
Proliferative capacity

ABSTRACT

Background aims: Approximately 1 in 3 patients with critical limb ischemia (CLI) are not suitable for surgical or endovascular revascularization. Those "no-option" patients are at high risk of amputation and death. Autologous bone marrow mesenchymal stromal cells (MSCs) may provide a limb salvage option. In this study, bone marrow characteristics and expansion potentials of CLI-derived MSCs produced during a phase 1b clinical trial were compared with young healthy donor MSCs to determine the feasibility of an autologous approach. Cells were produced under Good Manufacturing Practice conditions and underwent appropriate release testing.
Methods: Five bone marrow aspirates derived from patients with CLI were compared with six young healthy donor marrows in terms of number of colony-forming units–fibroblast (CFUF) and mononuclear cells. The mean population doubling times and final cell yields were used to evaluate expansion potential. The effect of increasing the volume of marrow on the CFUF count and final cell yield was evaluated by comparing 5 CLI-derived MSCs batches produced from a targeted 30 mL of marrow aspirate to five batches produced from a targeted 100 mL of marrow.
Results: CLI-derived marrow aspirate showed significantly lower numbers of mononuclear cells with no difference in the number of CFUFs when compared with healthy donors' marrow aspirate. CLI-derived MSCs showed a significantly longer population doubling time and reduced final cell yield compared with young healthy donors' MSCs. The poor growth kinetics of CLI MSCs were not mitigated by increasing the bone marrow aspirate from 30 to 100 mL.
Conclusions: In addition to the previously reported karyotype abnormalities in MSCs isolated from patients with CLI, but not in cells from healthy donors, the feasibility of autologous transplantation of bone marrow MSCs for patients with no-option CLI is further limited by the increased expansion time and the reduced cell yield.
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Introduction

Critical limb ischemia (CLI) is the most advanced clinical manifestation of peripheral vascular disease, with a prevalence of 500 to 1000 per million individuals in Western countries [1]. It recently has been referred to as chronic limb-threatening ischemia, a term reflecting the continuum nature of the underlying pathology [2]. Clinically, patients present with pain at rest and/or tissue loss. Revascularization is the preferred therapy; however, this is unachievable in 25–40% of patients due to comorbidities or complex anatomical distribution of the disease [1]. "No-option" CLI is associated with high risk of amputation and mortality and represents a significant unmet medical need [3,4].

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https://doi.org/10.1016/j.jcyt.2022.07.002
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Mesenchymal stromal cells/stem cells (MSCs) are the subject of a great deal of interest, as they represent a potentially useful cell type for cell therapy applications. The safety and efficacy of MSCs are being evaluated in hundreds of clinical trials [5–10], and they have already been approved for some indications. In 2012, Osiris Therapeutics was given approval to use an allogeneic bone marrow (BM)-derived MSCs to treat children with graft-versus-host disease in Canada, New Zealand and Japan [6]. In 2018, the European Medicines Agency granted marketing authorization to Tigenix for an allogenic adipose–derived MSC product for the treatment of complex perianal fistulas in patients with Crohn's disease [11,12].

MSCs are multipotent, non-hematopoietic, fibroblast-like cells that can be isolated from various tissues, mainly BM, adipose tissue, placenta and umbilical cord [13]. 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 [14,15]. MSCs have been shown to be effective in multiple reports in pre-clinical models of CLI [16–19]. In addition, there is a substantial amount of evidence on the safety of MSC administration to humans [20].

Several human studies have used MSCs based on their proangiogenic effect [21–26]. They also mediate anti-inflammatory, immunomodulatory, antifibrotic, antiapoptotic, mitogenic and wound-healing effects [27,28]. When compared with BM–derived mononuclear cells (MNCs), MSCs resulted in better lower-limb perfusion and ulcer healing in patients with diabetes mellitus (DM) [15].

"CLI stromal cells" was a phase 1b, non-randomized, uncontrolled, open-label, dose-escalation study that examined the safety and feasibility of intramuscular transplantation of autologous BM MSCs as a novel therapy to promote angiogenesis in patients with no-option CLI (EudraCT: 2013-003447-37, NCT: 03455335) [29]. The trial aimed to treat nine patients, three at each dose of 20, 40 or 80 million cells, delivered as a single dose via multiple intramuscular injections. Nine eligible patients proceeded to BM harvest. One patient underwent a second marrow aspiration after the MSCs isolated from the marrow harvest failed to meet the required Good Manufacturing Practice (GMP) release criteria. This resulted in 10 MSC batches being produced by the GMP manufacturing facility, Centre for Cell Manufacturing Ireland (CCMI), based at NUI Galway [29].

Six MSC cell batches were successfully released by the manufacturer and were approved for clinical administration by the primary investigator. Investigational Medicinal Product (IMP) was administered to four patients; three received the low dose (20×10^6 cells per patient) and one patient received the mid-dose (40×10^6 cells per patient). Two patients were withdrawn from the study after IMP release due to ineligibility detected during rescreening before cell transplantation [29].

Four of the produced MSC batches were not approved for clinical administration, two because they failed to meet the karyotype release criteria [30] due to abnormal karyotype analysis, one batch was not released due to recall of the dimethyl sulfoxide batch used in the MSC cryopreservation, and the fourth batch passed the manufacturer release criteria but was not used by the primary investigator for clinical administration due to an unusual/atypical karyotype analysis result. Thus, three of the 10 manufactured autologous MSC batches had abnormal karyotypes. It was concluded that this therapy, while apparently safe, may not be feasible with such a high batch failure rate. It should be noted that the 3 BM-MSC batches from healthy young volunteers that were prepared to validate the GMP manufacturing process at CCMI did not exhibit any karyotypic abnormalities.

In this study, we compared the characteristics of the BM derived from healthy donors and from the patients with CLI enrolled in the "CLI stromal cells" phase 1 clinical trial. The properties of the MSCs from both sources following a GMP manufacture process also were compared.

Methods

Patient recruitment

The trial was conducted according to the International Conference for Harmonization Good Clinical Practice guidelines 2001/20/EC, along with full ethical approval from the Health Product Regulatory Authority (approval reference 2163688) and Research Ethics Committee at Galway University Hospital (approval reference 15/12). Written informed consent was obtained from all patients [29].

Patients with CLI who were considered potential candidates for the trial were referred for screening by the primary treating surgeon. Eligible candidates were enrolled in the trial and proceeded to BM harvest.

BM harvest

BM from patients with CLI was harvested as described previously [29]. To summarize, eligible patients had BM aspirated by a consultant hematologist in the operating theater. For patients enrolled in the low-dose cohort of the trial, 30 mL of bone was aspirated under local anesthesia from the posterior iliac crest. Detailed review of the BM characteristics and growth kinetics of the low-dose cohort cell batches raised concerns around the ability to obtain enough cells for the targeted prescribed mid and high doses. Therefore, a substantial amendment to the study protocol was proposed to the Health Product Regulatory Authority and the hospital ethics committee to obtain up to 100 mL of BM under conscious sedation via a single skin puncture and needle redirection within the unilateral iliac crest. This amendment was approved by both regulatory authorities. Therefore, five CLI-derived MSCs batches were produced from 30 mL and five batches from 100 mL of BM aspirate.

BM from healthy donors was obtained following approval from the Research Ethics Committee at Galway University Hospital. The marrow was obtained for validation of the GMP manufacturing process as well as for screening to determine the optimal fetal bovine serum batch used for the cell culture media.

MSC manufacturing

The harvested marrow was processed under GMP conditions and protocols at CCMI facility at NUI Galway. MSCs were isolated by direct plating of marrow at a seeding density of $40–50 \times 10^6$ MNC per T175 Nunc flask. Cells were cultured in MSC growth medium (alpha-medium essential media; Life Technologies, Carlsbad, CA, USA) supplemented with 10% selected fetal bovine serum (HyClone, Logan, UT, USA) with no antibiotics. Cells were incubated until large non-overlapping colonies of cells with spindle-like morphology formed. At this point, they were passaged using Trypsin (Life Technologies). Cells were replated at a seeding density of 2.4–3 million cells per 500-cm^2 triple flask. MSCs were passaged at 80–90% confluence and the cells were harvested and passaged at the same seeding density to P2. At the end of P2, cells were harvested and cryopreserved using 4.5% human serum albumin and 10% dimethyl sulfoxide. Cells were frozen using a controlled rate freezer at 1°C per minute until they were at −80°C. Cells were stored in a −150°C freezer.

Patient-derived MSC batches underwent release testing (as detailed in the approved IMP Dossier, specifically Sterility, Mycoplasma, Endotoxin, Immunophenotype, Viability and Karyotype) in order to be released by the Qualified Person for clinical administration. The same release tests were performed for the GMP manufactured cells from healthy donor marrows.

Colony forming unit-fibroblastic (CFUF) assays

Undiluted BM or BM diluted 10-fold or 100-fold using MSC growth medium was plated in T-75 flasks (100 μL per flask; Nunc, Roskilde, Denmark). Three flasks were seeded for each dilution and cells were cultured in MSC growth medium. Cells were washed with phosphate-buffered saline 5 days after plating then were incubated with MSC growth medium. The medium was replaced on day 8 and day 11. Cells were cultured for between 10 and 14 days until distinct colonies were visible with at least 20 cells per colony. Colonies were fixed with methanol, stained with crystal violet solution, washed then counted. The number of colonies per flask were used to calculate the number of CFUF per milliliter of undiluted BM.

Calculation of population doublings, doubling time and theoretical maximum cell yield

The number of population doublings in each passage was calculated according to the following equation, $n = \log_2 (N_f / N_i)$, where n = number of population doublings, N_f = Number of cells harvested and N_i = number of cells plated. The doubling time for each passage was calculated as (days in culture)/ (number of population doublings).

The number of doublings in the P0 passage after initial plating was calculated using the aforementioned formula using the value for the total number of CFUF corresponding to the volume of BM plated as the initial number of cells plated.

During the GMP MSC manufacturing process, some cells were removed from culture at passaging for in process testing, quality control testing, characterization of cells or to be retained for future experiments. To account for this, the theoretical maximum cell yield for each cell batch was calculated based on the number of MSCs seeded at P0 (determined from the CFUF data) and the total number of doublings which occurred before harvest. This represents the cell harvest if all cells had been maintained in culture.

Karyotype analysis

A karyotype analysis was performed on each batch of MSCs at harvest as part of the suite of GMP release assays. This testing was performed as described previously [29]. To summarize in brief, G banding of metaphase spreads was performed at TDL Genetics, London, United Kingdom. A cell batch passed this assay when 20 observable metaphase cells spreads were analyzed and 10 or more cells have a normal karyotype and no karyotypic instability is found. A batch failed this assay if three or more cells exhibit the same chromosome loss (monosomy), two or more cells exhibit the same chromosome gain (trisomy), or two or more cells exhibit the same structural rearrangement such as a deletion, inversion, or translocation [30].

Statistical analysis

The demographic characteristics are presented as descriptive occurrence of events. Continuous data were tested for normality of distribution using Kolmogorov–Smirnov test. Normally distributed variables were expressed as mean ± standard deviation and analyzed using parametric tests (unpaired t test and one-way ANOVA test). Skewed data were expressed as medians with 95% confidence interval and analyzed using non-parametric tests (Mann–Whitney *U* test and Kruskal–Wallis test).

Results

A total of nine patients with CLI and six healthy donors were included in the study. The CLI group included predominantly males (n = 6) with a mean age of 67.3 years (range: 52–84 years). The majority of healthy donors were female (n = 4), and the group mean age was 21.3 years (range: 20–24 years). Ten CLI-derived cell batches were produced, as one patient underwent two BM aspirates due the first batch failing to meet the release test criteria. Five CLI-derived MSCs batches were produced from a targeted BM harvest volume of 30 mL (mean: 32.88 mL, range: 31.9–33.5 mL) and five batches from a targeted BM harvest volume of 100 mL (mean: 106.72 mL, range: 90.7–117.0 mL). The six healthy donor-derived MSC batches were all produced from an intended BM volume of 30 mL (mean: 30.5 mL, range: 26.0–39.0 mL). These MSC batches from healthy donors were produced during the validation of the manufacturing process. All cell batches were produced under GMP conditions. Donor demographic data and volume of marrow aspirate are shown in Table 1.

The marrow characteristics of patient-derived marrow were compared with healthy donor marrow at the end of the recruitment phase for the low-dose cohort. This compared five batches of MSCs derived from patients with CLI with six healthy-donor cell batches; all were produced from an intended volume of 30 mL of BM aspirate. This analysis indicated a significantly lower number of MNCs per milliliter of BM derived from patients with CLI ($11.3 \times 10^6 \pm 8.1 \times 10^6$) compared with healthy-donor marrow ($29.3 \times 10^6 \pm 1.7 \times 10^6$) (Figure 1A; $P = 0.0173$). The number of CFUFs per milliliter and CFUFs per 100 000 MNCs was also lower in the BM derived from patients with CLI compared with healthy donor marrow, but this difference did not reach statistical significance (Figure 1B, $P = 0.083$ and Figure 1C, $P = 0.331$).

The GMP manufacturing process for the clinical trial involved harvesting cultured cells at the end of P2; this represented a range of 9.9–13.7 population doublings. The mean population doubling time for MSCs isolated from patents with CLI was not significantly different from that of MSCs from healthy donors when analyzed from plating to harvest at P2 (Figure 2A). The mean population doubling at P0 did not show any significant difference between the two groups (Figure 2B). However, the mean population doubling time from the end of the P0 passage to harvest at P2 (the "post P0" culture) showed that CLI-derived MSCs had a significantly longer doubling time (4.11 days) compared with healthy donor MSCs (2.99 days) ($P = 0.0151$) (Figure 2C). The theoretical maximum cell yield (defined as the final cell yield at P2 if all cells had been kept in culture) was also significantly lower for the CLI donors (121.5×10^6) compared with healthy donors (286.9×10^6) ($P = 0.0458$) (Figure 2D). The growth curves for the CLI patients and the healthy donors are presented in Figure 2E. All cells derived from patients with CLI were confirmed to meet the International Society for Cellular Therapy immunophenotype criteria for MSC [31] (Table 2).

Table 1
Demographics of patients with CLI and healthy donors.

Group	Age, y	Sex	BM volume (mL)
Patients with CLI			
CLI001	52	M	33.5
CLI002	63	M	31.9
CLI004	83	M	33.0
CLI006	68	M	33.5
CLI007	72	M	32.5
CLI009	80	F	94.7
CLI010	84	M	114.3
CLI011	39	F	117.0
CLI012	65	F	First batch: 116.9 Second batch: 90.7
Healthy donors			
Healthy donor 1	20	F	31.5
Healthy donor 2	21	M	29.0
Healthy donor 3	21	F	30.0
Healthy donor 4	21	F	27.5
Healthy donor 5	21	M	26.0
Healthy donor 6	24	F	39.0

BM, bone marrow; CLI, critical limb ischemia; F, female; M, male.

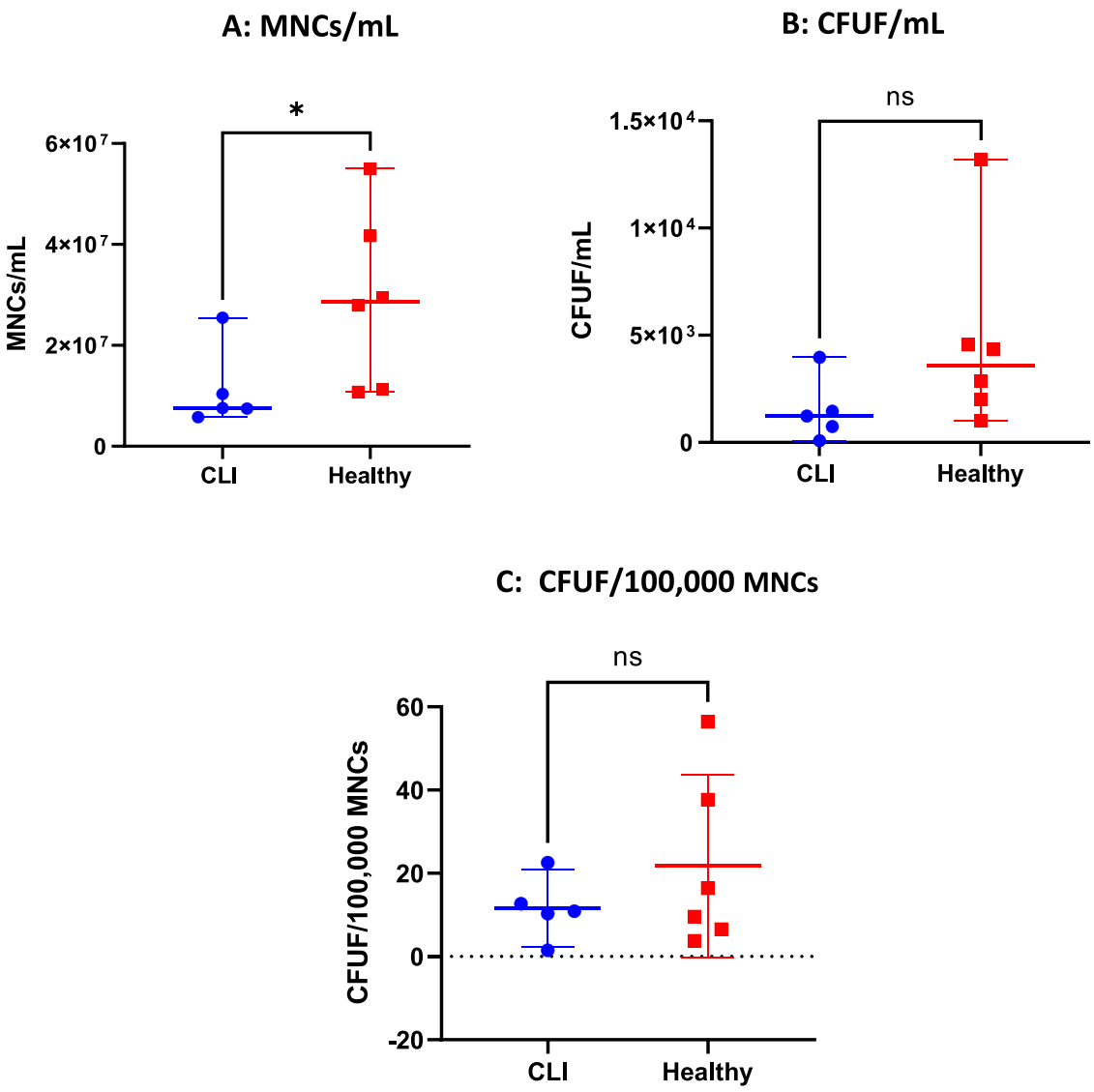

Figure 1. Comparison of BM characteristics from patients with CLI and healthy donors. For an intended volume of BM aspirate of 30 mL, marrow derived from patients with CLI (n = 5) showed significantly lower numbers of total MNCs per milliliter ($P = 0.0173$) than healthy donor-derived marrow (n = 6) (A) and no statistically significant difference in CFUFs per milliliter (B) and CFUF/100 000 MNCs (C); $P = 0.0823$ and 0.3315, respectively. Data in (A) and (B) were analyzed with the Mann–Whitney *U* test and expressed as median with 95% confidence interval. Data in (C) were analyzed with an unpaired Student's *t*-test and expressed as mean with 95% confidence interval. ns, not significant. (Color version of figure is available online).

The results of the analysis of cell yield led to concerns regarding the ability to obtain enough autologous MSCs for the mid- and high cell dose cohorts, considering that cells are also needed for release testing and for the required "retain" samples. Therefore, the study protocol was amended to increase the volume of BM aspirate from 30 to 100 mL in an attempt to ensure enough cells were produced for the targeted treatment dose and all associated quality control testing. However, although increasing the volume of the BM aspirate led to a significant increase in the number of MNCs harvested, it did not result in a significant increase in the number of CFUFs isolated or the maximum final MSC yield (Figure 3). CFUFs represent the population of MSCs and MSC precursors; therefore, this lack of increase in CFUF is of clinical significance and contributes to the lack of increase in the final cell yield. There was no difference in the mean population doubling time (from plating until the end of p2) for MSCs isolated from a 30-mL BM harvest and those isolated from a 100-mL BM harvest (1.61 ± 0.25 days versus 1.59 ± 0.17 days, $P = 0.875$).

We previously reported that karyotype abnormalities present a major limitation to autologous transplantation of CLI patient–derived MSCs. In that study, three CLI-derived batches showed abnormal karyotypes, with two batches demonstrating aneuploidy in the format of trisomy 5 (6/20 and 12/20 metaphase, respectively), whereas the third batch showed a greater-than-usual incidence of translocation, addition and monosomy [29]. A subgroup analysis was performed to compare the BM characteristics and growth kinetics of MSC batches with normal (n = 7) and abnormal (n = 3) karyotype. This comparison revealed no statistically significant difference between the two groups in terms of MNCs, CFUF, population doubling time or maximum cell yield (Figure 4 A-G).

Discussion

"CLI stromal cells" was the first in-human stromal cell clinical trial in Ireland to examine the safety and feasibility of intramuscular transplantation of autologous BM MSCs for patients with no-option CLI. This clinical trial indicated that the treatment was safe but not feasible due to concerns about abnormal karyotype associated with cells manufactured from the BM of patients with CLI.

In the study described here, the BM and MSC characteristics of patients with CLI enrolled in this clinical trial were compared with those of the young healthy donors used to validate the GMP manufacturing process at CCMI. Although the healthy donors were

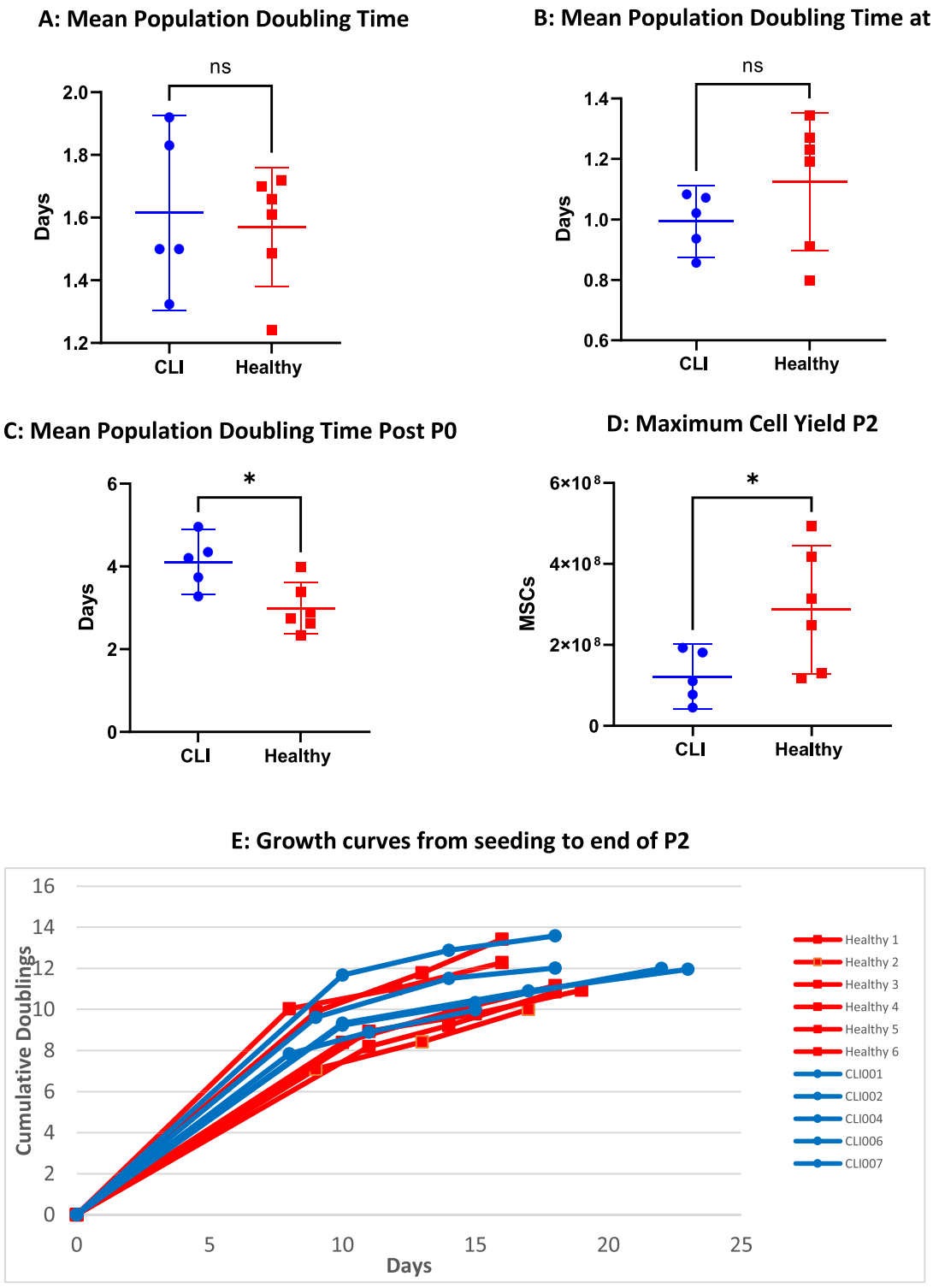

Figure 2. Comparison of growth kinetics of CLI-derived MSCs and healthy donor-derived MSCs. The overall mean population doubling time from plating until the end of P2 was not significantly different for MSCs isolated from the BM of patients with CLI compared with those from healthy donors, $P = 0.7361$ (A), nor was the mean doubling time from seeding to end of P0, $P = 0.2481$ (B). MSCs derived from patients with CLI had significantly greater mean population doubling time post P0 (from end of P0 to harvest at P2), $P = 0.0151$ (C) with significantly lower theoretical maximum cell yield at P2, $P = 0.0458$ (D) compared with healthy donor-derived MSCs. The growth curves from seeding to the end of P2 are presented in (E). Data are expressed as mean with 95% confidence interval and analyzed using the parametric Student's *t*-test. (Color version of figure is available online).

not age-matched to patients with CLI, the donor's ages are representative of the age of donors used in recent allogenic studies [23,32,33]. Both sets of marrow and cells were processed according to GMP and subjected to regulatory quality control testing and release in accordance with GMP and the regulatory approved specifications. BM from patients with CLI had statistically fewer MNCs per milliliter than that of young healthy donors. Following MSC isolation under GMP conditions, there was no difference in the CFUF per milliliter or the CFUF/100 000 MNCs, which suggests that the proportion of MSCs in the marrow was not altered by CLI and/or age. However, after the initial colony formation, the proliferation rate of MSCs from patients with CLI was significantly slower than that of young healthy donors.

Table 2
Immunophenotype quality testing (flow cytometry).

Patients with CLI	Positive markers (≥90%)			Negative markers (≤5%)
	CD90	CD105	CD73	CD45, CD34, CD11b, CD19, HLA-DR
CLI001	98.9	96.6	98.9	1.2
CLI002	99.6	99.7	100	0.7
CLI004	100	99.7	100	3.23
CLI006	99.4	99.9	99.9	0.1
CLI007	100	100	100	0.4
CLI009	96.4	97.0	98.1	0.26
CLI010	99.9	99.9	100	0.4
CLI011	100	99.9	99.9	0.7
CLI012(1)[a]	–	–	–	–
CLI012(2)	99.7	91	99.7	1.1

[a] Flow cytometry analysis not completed for this cell batch, as it failed release due to recall of dimethyl sulfoxide.

This has clear implications for the time taken to reach the desired number of cells required for a clinical dose of MSCs. For a condition such as CLI, which may progress rapidly, this delay could have an impact on patient well-being and thus may impact the feasibility of an autologous approach. For example, one patient's condition in the clinical trial progressed from Rutherford class 4 (rest pain) to Rutherford class 5 (tissue loss) during the nine-week interval between marrow harvest and clinical application of the IMP [29]. Although a visual check indicated that cells were sufficiently confluent to meet the GMP criteria for cell harvest, the cell yield from patient-derived marrow was lower than that of healthy donor marrow. One possible reason is that the patient derived MSCs were

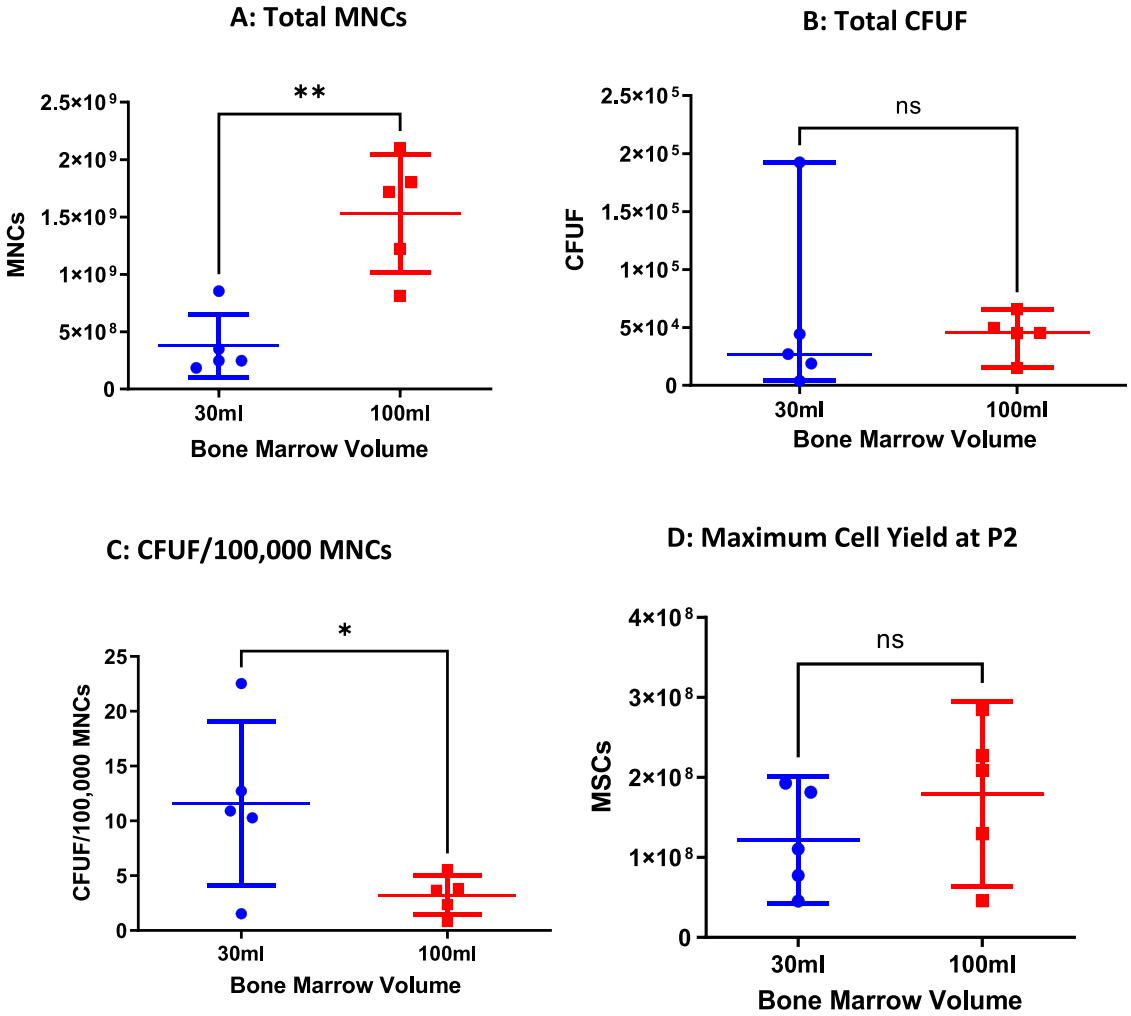

Figure 3. Increasing the BM aspirate volume to 100 mL increased MNC harvest but not the total CFUF or the final MSC yield. Comparison of two cohorts (30 mL [n = 5] versus 100 mL [n = 5]) of BM derived from patients with CLI and MSC batches revealed no statistically significant increase in the total CFUF ($P = 0.4206$) and the theoretical maximum cell yield at the end of P2 when a BM aspiration of approximately 100 mL was collected versus 30 mL ($P = 0.2222$). However, the larger marrow volume contained a significantly greater number of MNCs ($P = 0.0021$) and significantly lower CFUF per 100 000 MNCs ($P = 0.0417$). Data in (A) and (C) were analyzed using the non-parametric Mann–Whitney *U* test and expressed as median with 95% confidence interval . Data in (B) and (D) were analyzed with parametric Student's *t*-test and expressed as mean with 95% confidence interval. (Color version of figure is available online).

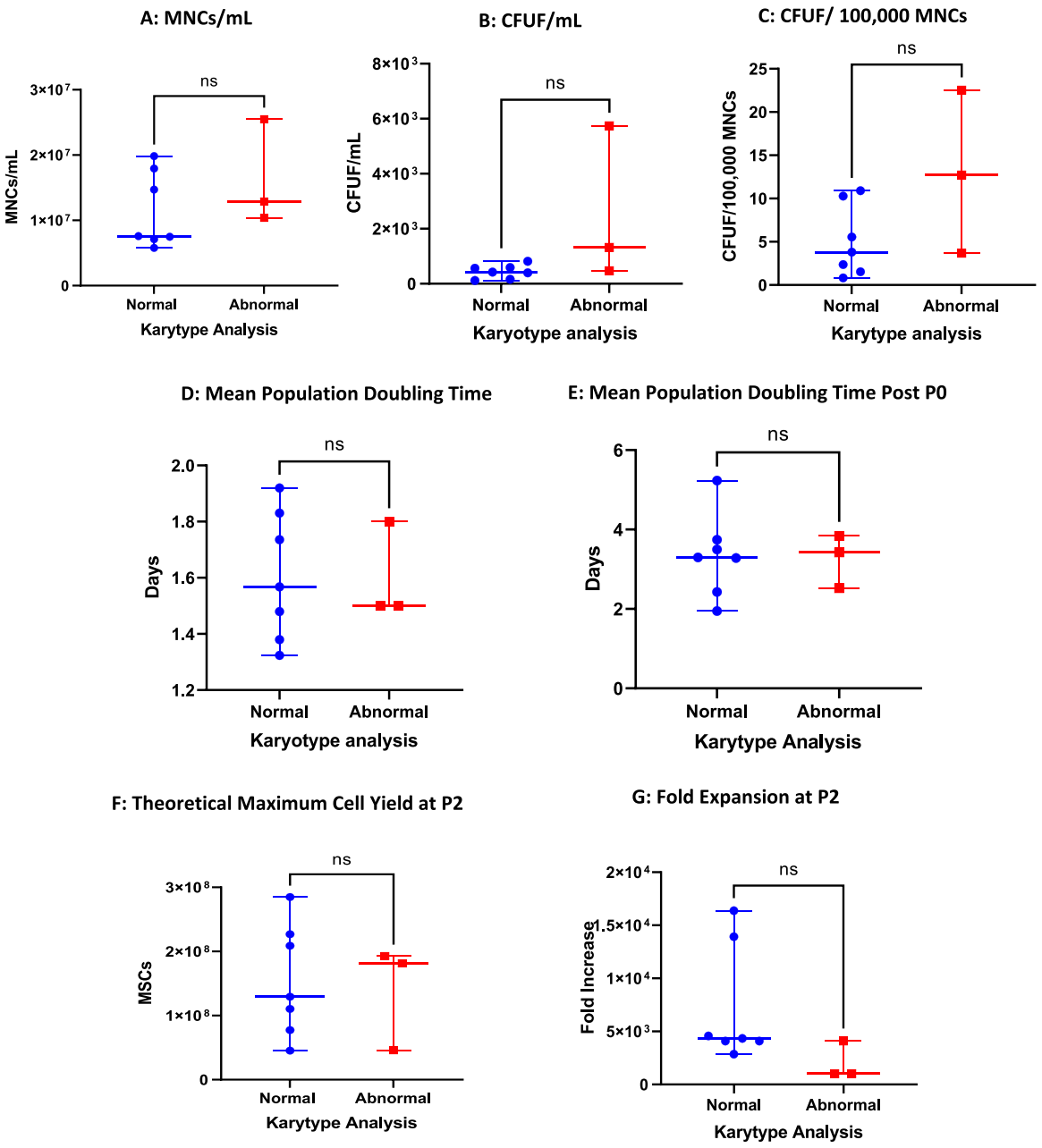

Figure 4. Comparison of the BM characteristics and growth kinetics between CLI-derived MSCs with normal and abnormal karyotype analysis. Comparison of CLI-derived MSCs batches that showed normal karyotype (n = 7) with batches with abnormal karyotype (n = 3) revealed no significant difference in terms of BM characteristics and growth kinetics or the total MSC yield. (A) MNCs per milliliter, $P = 0.3833$. (B) CFUF per milliliter, $P = 0.1167$. (C) CFUF/100 000 MNCs $P = 0.1833$. (D) Mean population doubling time $P = 0.9667$. (E) Mean population doubling time post P0 $P = 0.8333$. (F) Theoretical maximum cell yield at P2 $P = 0.8333$. (G) Fold expansion at P2 $P = 0.0833$. Data are expressed as median with 95% confidence interval and analyzed with the non-parametric Mann–Whitney *U* test. (Color version of figure is available online).

larger on the tissue culture plastic than the health donor cells, but this was not quantified.

In this study, we did not determine whether the differences in cell proliferation were associated with disease state or the increased age of the CLI patients compared with the healthy donors. The association between low number and expansion potential of BM MSCs and increased age has been widely reported in literature [34,35]. A study by Gremmels *et al* [36] reported a significantly greater rate of senescence in CLI-derived MSCs when compared with young healthy donors, measured by β- galactosidase activity and spontaneous DNA break damage foci. While this senescence was found to affect the differentiation potential of MSCs, it reportedly did not affect the proangiogenic potential of the CLI-derived MSCs.

GMP-manufactured MSCs from patients with CLI for the clinical trial proliferated more slowly than GMP-manufactured cells from young healthy donors. In this study, we did not perform any studies to compare the functions of MSCs isolated from these populations. Other researchers have previously reported that MSCs from patients with CLI had similar capacity for adipogenesis and osteogenesis to those from age-matched controls but a decreased potential for chondrogenesis [36]. Cells from both donor cohorts showed comparable *in vitro* and *in vivo* functional angiogenic potentials. MSCs from patients with CLI and from healthy donors had the same ability to differentiate and to secrete factors such as vascular endothelial growth factor, fibroblast growth factor and hepatocyte growth factor, which are implicated in MSC stimulation of angiogenesis [37]. It should be

noted that the cells assessed in these functional comparisons were not manufactured to GMP process and scale of cell production, nor did they compare the initial BM characteristics.

As atherosclerosis is the most common underlying cause of CLI, the reported poor expansion potential of CLI-derived MSCs may be linked to reported impaired BM function in patients with atherosclerotic CLI, indicated by the decreased number and efficacy of the circulating EPCs [38,39].

As CLI is often associated with complex medical comorbidities, the reported poor growth kinetics of CLI-derived MSCs could also be related to coexisting diseases. For example, increased apoptosis, autophagy and mitochondrial deterioration in MSCs isolated from patients with DM was reported [40]. Impaired cellular differentiation and decreased proliferation of MCSs isolated from type I and type II DM also has been reported in literature [41]. A comparison of MSCs isolated from the amputated limbs of patients with severe CLI or CLI and DM showed that these cells proliferated more slowly than those from young heathy donors when cultured in fetal bovine serum or human platelet lysate. This became significant at higher passages, resulting in a decreased cell yield [37]. DM is considered to be a risk factor for CLI. Reports suggest that the proliferation of MSCs isolated from the BM of patients with DM was not different from those from age-matched individuals without DM when cultured in the presence of 1 ng/mL basic fibroblast growth factor [42], although others have noted that a number of BM characteristics and cell properties are adversely impacted in individuals with DM [43]. However, MSCs from patients with end-stage renal disease have been reported to have equivalent phenotypic and functional capabilities when compared with MSCs from healthy donors [44]. Therefore, the impact of disease on the characteristics of BM MSCs is controversial in the existing literature.

The slower proliferation of MSCs from patients with CLI led to concerns around the time required to reach the number of cells required for each patient dose as well as cells required for release testing and the required regulatory "retain" samples. In an attempt to circumvent this problem, the clinical trial was granted permission to obtain a larger volume of BM from patients enrolled in the higher-dose cohorts. This required optional conscious sedation and involved repositioning the marrow draw needle. However, in this study, it is apparent that although increasing the marrow harvest volume led to an increase in the number of MNCs, it did not lead to an increase in CFUFs or to a significantly increased number of MSCs at the end of P2. It has been known for some time that harvested BM is diluted with peripheral blood if volumes greater than 10 mL are aspirated in one draw. Thus, the apparent CFUFs per milliliter is lower for a larger volume blood aspiration [45]. This can be mitigated by multiple draw sites versus a single site and repositioning the needle.

A normal karyotype is one of the criteria suggested in the International Society for Cell Therapy guidelines for defining MSCs, although it is not one that is recommended for their routine identification [31]. A normal karyotype (by G-banding and cytology) was one of the release criteria for the MSCs manufactured for this clinical trial. We have previously reported karyotypic abnormalities (aneuploidy) in a proportion of the MSCs in three of nine batches from patients with CLI, whereas no cells with abnormal karyotype were observed in 6 GMP MSC batches from young healthy donors [29]. Others have also reported abnormalities in MSC batches [46,47]. (MSCs with an abnormal karyotype, trisomy 7, have been used in clinical studies with no reported adverse effects [48]). The concern about karyotype abnormalities centers around the possibility that cells with genetic alterations may be transformed and thus more likely to cause tumors via rapid proliferation and anchorage independent growth [49]. Previously, we reported that there was no evidence of anchorage-independent growth for the aneuploid MSCs [29]). Here, we report that MSC batches containing cells with an abnormal karyotype did not have a significantly shorter population doubling time than cells with a normal karyotype. Although this was only measured until the cell harvest at the end of passage 2, it suggests that the karyotypic abnormalities observed were not ones that led to enhanced MSC proliferation. To study this further, cells would need to be passaged for longer and the proportion of cells with abnormal karyotypes should be measured to determine whether they increase in proportion (suggesting a growth advantage) or decrease in proportion (suggesting proliferation deficits such as senescence).

Taken together, the experiences in this study suggest that using autologous MSCs from patients with CLI may not be feasible. CLI MSCs cultured under GMP conditions had a greater rate of abnormal karyotype, slower proliferation and reduced cell yield compared with those from healthy young donors. An attempt to circumvent the time and yield problem by starting the MSC culture with a larger volume of BM was not successful. The long time between consenting to treatment and receiving treatment, coupled with the need for two medical procedures (the BM harvest and the cell administration) suggest that autologous treatment may not be feasible for CLI. In the CLI stromal cell trial, there was a wait of up to 10 weeks between the marrow harvest and when patients received MSCs.

The feasibility issues associated with autologous MSCs suggest that allogenic MSCs may be preferable for conditions such as CLI. Allogenic MSCs have been administered without adverse effects in many clinical studies [23,32,50]. They can be isolated from young healthy donors and they can be cryopreserved for immediate use as and when required. There are also economies of scale associated with manufacturing enough MSCs for many patients from one donor, which makes this approach more viable commercially.

Conclusions

In addition to the previously reported greater rate of karyotype abnormalities, the feasibility of autologous transplantation of BM MSCs is limited by the reported slower proliferation and reduced cell yield, which was not mitigated by increasing the volume of BM. Allogenic transplantation should be considered in future studies as alternative approach.

Funding

This study 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 Health Research Board Clinical Research Facility Galway (CRFG).

Author Contributions

Conception and design of the study: SAM, AD, JK, AH, SN, AF, MH, AL, MT, SRW, TO and LH. Provision of study material: AD, JK, SN, AF, MH, MT and SRW. Acquisition of data: SAM, AD, VM, JK, SRW and TO. Clinical trial management and study coordination: VM. Analysis and interpretation of data: SAM, AD, TO and LH. Drafting or revising the manuscript: SAM, AD, TO and LH. Approval of the final article: TO and LH.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 patient's confidentiality will be maintained and will not be made publicly available to the extent permitted by the applicable laws and regulations.

Declaration of Competing Interest

TO is a Director and Equity Holder in Orbsen Therapeutics (a stem cell company). All other authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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