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1) Baker, Monya. "1,500 scientists lift the lid on reproducibility." Nature, no. 533 (May 26, 2016): 452-54. doi:10.1038/533452a.

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STEM CELLS TRANSLATIONAL MEDICINE®

HUMAN CLINICAL ARTICLE

Results from Phase I Clinical Trial with Intraspinal Injection of Neural Stem Cells in Amyotrophic Lateral Sclerosis: A Long-Term Outcome

LETIZIA MAZZINI,[a,*] MAURIZIO GELATI,[b,c,*] DANIELA CELESTE PROFICO,[c] GIANNI SORARÙ,[d] DANIELA FERRARI,[e] MASSIMILIANO COPETTI,[f] GIANMARCO MUZI,[b] CLAUDIA RICCIOLINI,[b] SANDRO CARLETTI,[g] CESARE GIORGI,[g] CRISTINA SPERA,[g] DOMENICO FRONDIZI,[g] STEFANO MASIERO,[d] ALESSANDRO STECCO,[h] CARLO CISARI,[i] ENRICA BERSANO,[a] FABIOLA DE MARCHI,[a] MARIA FRANCESCA SARNELLI,[a] GIORGIA QUERIN,[d] ROBERTO CANTELLO,[a] FRANCESCO PETRUZZELLI,[j] ANNAMARIA MAGLIONE,[j] CRISTINA ZALFA,[e] ELENA BINDA,[k] ALBERTO VISIOLI,[l] DOMENICO TROMBETTA,[m] BARBARA TORRES,[n] LAURA BERNARDINI,[n] ALESSANDRA GAIANI,[d] MAURILIO MASSARA,[a] SILVIA PAOLUCCI,[a] NICHOLAS M. BOULIS,[o] ANGELO L. VESCOVI,[b,c,e] ON BEHALF OF THE ALS-NSCS TRIAL STUDY GROUP

Key Words. Adult stem cells • Cellular therapy • Clinical trials • Fetal stem cells

ABSTRACT

The main objective of this phase I trial was to assess the feasibility and safety of microtransplanting human neural stem cell (hNSC) lines into the spinal cord of patients with amyotrophic lateral sclerosis (ALS). Eighteen patients with a definite diagnosis of ALS received microinjections of hNSCs into the gray matter tracts of the lumbar or cervical spinal cord. Patients were monitored before and after transplantation by clinical, psychological, neuroradiological, and neurophysiological assessment. For up to 60 months after surgery, none of the patients manifested severe adverse effects or increased disease progression because of the treatment. Eleven patients died, and two underwent tracheotomy as a result of the natural history of the disease. We detected a transitory decrease in progression of ALS Functional Rating Scale Revised, starting within the first month after surgery and up to 4 months after transplantation. Our results show that transplantation of hNSC is a safe procedure that causes no major deleterious effects over the short or long term. This study is the first example of medical transplantation of a highly standardized cell drug product, which can be reproducibly and stably expanded ex vivo, comprising hNSC that are not immortalized, and are derived from the forebrain of the same two donors throughout this entire study as well as across future trials. Our experimental design provides benefits in terms of enhancing both intra- and interstudy reproducibility and homogeneity. Given the potential therapeutic effects of the hNSCs, our observations support undertaking future phase II clinical studies in which increased cell dosages are studied in larger cohorts of patients. STEM CELLS TRANSLATIONAL MEDICINE *2019;8:887–897*

LESSONS LEARNED

- Nonimmortalized human neural stem cells derived from spontaneously miscarried donor fetuses offer a highly reproducible and standardized method of Good Manufacturing Practice production.
- This approach provides a feasible and safe therapy for neurodegenerative disorders.

SIGNIFICANCE STATEMENT

This study is the first example of a procedure in which nonimmortalized clinical grade human neural stem cells lines are isolated from fetal brain biopsies following natural death in utero, are reproducibly and stably expanded ex vivo, and are medically transplanted. This approach enables the injection of bona fide stem cells, as demonstrated in the authors' previous paper on the first six patients, and not only facilitates use of the same donor cells in all patients in this investigation but also warrants that these same cells can be used in numerous upcoming trials. As such, this will permit implementation of more homogenous future phase I, II, and III studies and will overcome many of the current issues of standardization and reproducibility that afflict this field.

[a]Eastern Piedmont University, "Maggiore della Carità" Hospital, Dipartimento di Neurologia, Novara; [b]Laboratorio Cellule Staminali, Cell Factory e Biobanca, Terni Hospital, Italy; [c]Fondazione IRCCS Casa Sollievo della Sofferenza, Advanced Therapies Production Unit, San Giovanni Rotondo, Foggia, Italy; [d]Department of Neuroscience, University of Padua, Padua, Italy; [e]Biotechnology and Bioscience Department Bicocca University, Milan, Italy; [f]Fondazione IRCCS Casa Sollievo della Sofferenza, Biostatistic Unit, San Giovanni Rotondo, Foggia, Italy; [g]Department of Neurosurgery and Neuroscience, "Santa Maria" Hospital, Terni, Italy; [h]Department of Diagnostic and Interventional Radiology, "Eastern Piedmont" University, "Maggiore della Carità" Hospital, Novara; [i]Department of Physical Therapy, "Eastern Piedmont" University, "Maggiore della Carità" Hospital, Novara; [j]Fondazione IRCCS Casa Sollievo della Sofferenza, Obstetrics and Gynaecology Department, San Giovanni Rotondo, Foggia, Italy; [k]Fondazione IRCCS Casa Sollievo della Sofferenza, Cancer Stem Cells Unit, San Giovanni Rotondo, Foggia, Italy; [l]StemGen SpA, Milan, Italy; [m]Fondazione IRCCS Casa Sollievo della Sofferenza, Department of Oncology, San Giovanni Rotondo, Foggia, Italy; [n]Fondazione IRCCS Casa Sollievo della Sofferenza, Cytogenetics Unit, San Giovanni Rotondo, Foggia, Italy; [o]Department of Neurosurgery, Emory University, Atlanta, USA

*Contributed equally

Correspondence: Angelo L. Vescovi, Ph.D., Fondazione IRCCS Casa Sollievo della Sofferenza, viale dei Cappuccini, 71013 San Giovanni Rotondo, Foggia, Italy. Telephone: 0882 416350; e-mail: vescovia@gmail.com; or Letizia Mazzini, M.D., Department of Neurology, Maggiore della Carità Hospital, Eastern Piedmont University, 18 - 28100 Novara, Italy. Telephone: 0321 3733834; e-mail: mazzini.l@libero.it

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Introduction

Amyotrophic lateral sclerosis (ALS) is incurable [1]: Riluzole and edaravone are the only drugs approved for ALS, but they have modest benefits [2, 3]. Stem cells hold great promise as novel therapeutic agents for untreatable neurological disorders, particularly ALS. Previous studies by us and other groups have documented that human neural stem cells (hNSCs) have the capacity to integrate with brain tissue, and show therapeutic effects in rodent models of neurological diseases [4–7]: These studies demonstrate that, upon transplantation, the progeny of the hNSCs effectively integrate into and survive in the parenchyma of the host brain, likely eliciting beneficial actions through bystander effects that include the release of growth factors and immunomodulation.

Here we report on results of a phase I clinical trial in which multipotent hNSCs were isolated from tissues derived from miscarried human fetuses, were reproducibly expanded, and were stereotactically implanted into 18 patients with ALS, according to published procedures by Glass et al., who performed the first study of this kind [8]. Initial results from the first group of six patients, in whom the hNSCs were transplanted into the lumbar spinal cord, have already been reported [9]. We also show that the cell injection and transplantation surgery led to no serious adverse effects and thus resulted in approval from the Italian Institute of Health (ISS) to continue the trial with more complex injections into the cervical spinal cord. Finally, we present findings from the long-term follow-up of the 12 patients who received implants of hNSCs in the cervical cord, as well as an extended follow-up of the first six lumbar transplants.

Materials and Methods

Trial Design and Participants

This was a prospective, open, pilot study, in which 18 patients (aged 20 through 75 years) with a definitive diagnosis of ALS with spinal onset (according to El Escorial-Revised criteria [10]) were enrolled between 2012 and 2015. Electro diagnostic assessment was used to confirm lower motor neuron degeneration in at least three regions of the cord (cervical, thoracic, and lumbosacral). Patients were eligible for enrollment if they had exhibited clinical signs of progressive weakness attributed to ALS over the last 6 months, had a forced vital capacity (FVC) that was ≥60% of their predicted value, and were not using invasive respiratory support. The cutoff of FVC > 60% was used because these patients have fewer complications, with minor care procedures [11].

Patients were excluded if they presented with psychiatric diseases or neurological diseases other than ALS; presented with mental deterioration or cognitive disturbances; were unable to understand the informed consent form and study aims; showed evidence of concurrent illness; or were receiving corticosteroids, immunoglobulin, or immunosuppressive treatment (Table 1). The trial was approved and monitored by the relevant Italian authorities and registered within the European Clinical Trials Database (EudraCT) identification number 2009-014484-39 as well as ClinicalTrials.gov, NCT 01640067. All patients provided written, informed consent, and all patient data recorded throughout this study were registered in the "*Database for Clinical Studies with Gene and Somatic Therapy*" of the ISS and was communicated to an independent Safety Monitoring Board of multidisciplinary experts who monitored adherence to the protocol criteria and possible adverse effects (AEs).

Study Settings

Enrollment occurred sequentially, in three cohorts of six patients each. Given the hazard of iatrogenic damage, a risk-escalation paradigm was adopted [12] for the number of injections and the amount of potential damage leading to neurological side effects. The first group comprised patients with severe lower limb disability (not-ambulatory), that is, subjects with a lower risk of exacerbated weakness caused by the procedure: Given that the patients in this group showed no signs of iatrogenic damage following the transplant [8], the relevant regulatory bodies approved the recruitment of patients that displayed fair functional autonomy (i.e., ambulatory patients) into the second and third groups. A psychologist assessed the fitness of each patient soon after recruitment. A clinical interview and Minnesota multiphasic personality inventory 2 tests were used to ensure that the participants fully understood that this was a safety trial and that they were aware of all risks associated with the overall procedure.

Interventions

The current Good Manufacturing Practice hNSC primary lines, the surgical procedure, and the immunosuppressive regimen used here have been described [5, 9].

After enrollment—and prior to surgery—each patient was subjected to a 3-month observation period, to evaluate inherent clinical progression [13, 14]. After surgery, patients underwent monthly routine control visits for 1 year, and then every 3 months until death.

Follow-Up

Clinical assessment and rate of disease progression were evaluated by the ALS-functional rating scale revised (ALS-FRS-R), Ashworth Spasticity Scale, and the Medical Research Council (MRC) scale of 34 muscle groups in the upper and lower limbs and FVC. At each examination, a clinical psychologist evaluated the patients, assessing their mood. The Quality of Life Profile of Mood State [15] and the Schedule for Evaluation of the Individual Quality of Life-Direct Weighting (SEIQoL-DW) [16] questionnaires were provided to patients. One year after surgery, the patients came in for trimestral control visits; the exceptions were magnetic resonance imaging (MRI) and bladder ultrasound, which were performed every 6 months. Clinical follow-up was prolonged until the patient died. A telephone follow-up with the registration of the ALS-FRS-R score was planned for patients who were no longer able to visit the hospital.

MRIs of the brain and spinal cord were obtained using a 1.5-T imaging system (Achieva Intera; Philips, Netherlands). Spinal cord MRIs were performed preoperatively, and 21 days, plus 3, 6, 9, and 12 months after surgery, whereas brain MRIs were performed as patients entered the study, and 12 months after surgery. In addition to the full conventional MRIs, the spinal cord was also imaged by a diffusion tensor imaging pulse sequence in the axial plane, with 64 directions, to identify and quantitatively characterize tissues. Using the fiber-tracking

Table 1. Treatment groups and inclusion/exclusion criteria

Group	Site of injection	Dose[a]	Injection	Patient ID	Inclusion criteria[b]
A	Lumbar [T8/11]	2.25×10^6	N°3, monolateral	740, 746, 753	Maximum score of 1 on walking item of ALS-FRS Forced vital capacity ≥60
		4.5×10^6	N°6, bilateral	766, 767, 779	
B	Cervical [C3/5]	2.25×10^6	N°3, monolateral	799, 804, 807	Forced vital capacity ≥60 Ambulation difficulties (maximum score of 2 on walking item of ALS-FRS)
		4.5×10^6	N°6, bilateral	831, 833, 842	
C	Cervical [C3/5]	4.5×10^6	N°6, bilateral	864, 862, 873, 897, 919, 942	Independent ambulation (score of 4 on ALS-FRS) Forced vital capacity ≥70

[a]The concentration of human neural stem cells for each infusion was 50,000 cells/μl, with a total of 15 μl being delivered per injection site, for a total of 750,000 cells/injection.

[b]Common inclusion criteria: diagnosis of definite or possible ALS according to revised EL Escorial criteria; age: 20–75 years; documented progression of disease during the last 6 months; absence of concomitant disease; adequate assurances of adherence to protocol; the patient must be able to communicate verbally or with the use of a nonverbal communication system. Exclusion criteria: psychiatric diseases or other neurological diseases other than ALS; mental deterioration or cognitive sphere disturbances; unable to understand informed consent form and study aims; evidence of any concurrent illness; they were receiving corticosteroids, immunoglobulin, or immunosuppressive treatment. Women with childbearing potential for the duration of the study or who were pregnant were excluded.

Abbreviation: ALS-FRS, amyotrophic lateral sclerosis-functional rating scale.

algorithm, we calculated the values for fractional anisotropy (FA) and apparent diffusion coefficient (ADC). The detailed MRI protocol has been described [9].

Routine laboratory blood tests (for renal function, liver function, glucose) and hepatitis B or C, HIV, and tuberculosis analyses were performed at the time of study entry and 7 days before surgery. Routine and hematological (full blood count and CD3+; CD3+/CD8+ CD4+/8+ count) tests, and assessments for tacrolimus dosage, were performed weekly during the first postoperative month and then monthly over the 6 months of immunosuppression. Bladder ultrasound was used to measure the postvoid residual volume at the time of study entry, on the 2nd and 21st days after surgery, and then every 3 months, for 1 year.

Cerebrospinal Fluid Analysis

Cerebrospinal fluid (CSF) of patients was collected at the time of surgery, for standard biochemical analysis. No abnormalities were detected in any subject (data not shown).

CSF hNSCs Interaction

Neurospheres from the cell line used to treat all patients were mechanically dissociated; cells were resuspended in the same culture medium that was used for routine culturing, but without Epidermal Growth Factor (EGF) and in the presence of only basic Fibroblast Growth Factor (bFGF), or of bFGF plus 5% CSF from patients with ALS, bFGF plus 5% CSF from healthy controls, or bFGF plus 5% saline. Cells were then seeded on a cultrex layer (Cultrex; Trevigen, Maryland, USA) and incubated in 5% O_2, 5% CO_2, and at 37°C for 3 days. Culture medium was replaced with Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 2% FBS, without growth factors, or in 2% FBS plus 5% CSF from patients with ALS, 2% FBS plus 5% CSF from healthy controls, or 2% FBS plus 5% saline. Cells were incubated for an additional 4 days, received another change of medium, and were then incubated for an additional 3 days, following which they were fixed in 4% paraformaldehyde and immunostained using a standardized method.

Supernates of all cell cultures were collected at each time point, and frozen at −80°C, to be used in the enzyme-linked immunosorbent assay (ELISA) tests as follows:

T1: 3 days in culture in the absence of EGF and presence of only bFGF, or with bFGF and 5% of CSF from patients with ALS/healthy controls, or with bFGF plus 5% saline.

T2: After T1, 4 days of additional culture with DMEM/F12 supplemented with 2% FBS, without growth factors, or with 2% FBS and 5% CSF from patients with ALS/healthy controls, or 2% bFGF plus 5% saline.

T3: After T2, 3 days of additional culture with DMEM/F12 supplemented with 2% FBS, without growth factors, with 2% FBS plus 5% of CSF from patients with ALS/healthy controls, or with 2% bFGF plus 5% saline.

ELISA Tests

Conditioned medium was thawed and used to quantify the presence of brain-derived neurotrophic factor (BDNF), osteopontin (OPN), interleukin 10 (IL-10), interleukin 1β (IL-1), and vascular endothelial growth factor (VEGF), using commercially available ELISA kits, according to manufacturer instructions (R&D Systems, Minnesota, USA).

Outcomes

The main objective of this trial was to assess the safety and tolerability of transplanting hNSCs into the spinal cord of patients with ALS. Patients were closely monitored for immediate AEs, including allergic reactions (tachycardia, fever), respiratory failure, local complications (intraparenchymal hematoma, local infection at the site of surgery), systemic complications (systemic infections), and paralysis or sensory loss below the level of the injection site. The following potential delayed AEs were also monitored: intraspinal tumor formation, aberrant connections (spinal myoclonus), and persistent sensory loss or paralysis unrelated to disease progression. Key secondary endpoints included changes in functional outcomes measured by the ALS-FRS-R scale and FVC in the post-treatment period relative to pretreatment values, variation in quality of life, and behavioral scales.

Every AE was mandatorily reported during the study, was classified as "expected" or "unexpected," and was then categorized for apparatus, type, and severity, according to the World Health Organization (WHO) classification. All AEs were evaluated for their relationship with the hNSC treatment.

Table 2. Clinical characteristics and outcomes of patients

Patient (code)	Group	Age, years	Sex	ALS-FRS-R score	FVC, %	Time from symptoms onset to inclusion in the trial, months	Time from diagnosis to inclusion in the trial, months	Time from symptoms onset to death or tracheostomy, months	Clinical form	Follow-up duration, months from surgery	NIV, months from surgery	PEG, months from surgery
740	A1	30	M	25	74	60	50	110	Classical	51[a]	22	23
746	A1	57	M	28	64	68	52	76	LMN-prevalent	8[b]	Refused	Refused
753	A1	54	F	29	83	16	8	24	Classical	8[b]	4	Refused
766	A2	35	M	30	82	72	26	107	Classical	35[b]	10	—
767	A2	67	F	35	88	36	18	44	LMN-prevalent	8[b]	7	—
779	A2	38	M	24	82	24	10	38	UMN-prevalent	14[b]	10	6
799	B1	46	M	43	100	24	12	67	LMN-prevalent	43[a]	37	—
804	B1	60	F	28	94	17	23	61	LMN-prevalent	44[b]	22	—
807	B1	50	F	28	100	16	12	43	Classical	27[b]	10	14
842	B2	49	M	40	69	10	4	35	Classical	25[b]	8	17
831	B2	64	M	28	70	22	15	32	Classical	10[a]; 14[b]	5	—
833	B2	25	F	36	94	17	14	37[c]	UMN-prevalent	20	—	—
864	C1	48	M	41	75	24	18	67[c]	LMN-prevalent	43	8	—
873	C1	65	M	45	93	12	9	19	Classical	7[b]	6	—
897	C1	39	M	36	71	10	3	34[c]	UMN-prevalent	24	20	18
862	C1	48	M	36	70	17	14	45	Classical	28[a]	1	28
919	C1	49	M	43	110	10	3	64	Classical	21[b]	—	17
942	C1	47	M	43	110	9	3	39[c]	Classical	30	27	—

[a]Months from surgery to tracheostomy.
[b]Months from surgery to death.
[c]Alive without tracheostomy.
Abbreviations: —, not applicable; PEG, percutaneous endoscopic gastrostomy; ALS-FRS-R, amyotrophic lateral sclerosis-functional rating scale revised; F, female; FVC, forced vital capacity; LMN, lower motor neuron; M, male; NIV, noninvasive ventilatory; UMN, upper motor neuron.

Statistical Analysis

Enrollment was completed when all 18 patients were recruited. No formal calculation of sample size was made. For the purposes of ad hoc or post hoc exploratory analysis, we considered the entire sample in all patients who had received treatment and had a follow-up of at least 12 months after treatment. Generalized longitudinal linear models were used to assess the temporal trend of continuous outcomes. Progression rates were estimated and compared with the 3-month period prior to treatment, including the "pre-post" covariate into the same models. A p value less than .05 was considered statistically significant. All analyses were performed using SAS Statistical Package Release 9.4 (SAS Institute, Cary, NC).

Results

A total of 1,020 patients applied to participate in this study, but most were ineligible, as they did not meet the inclusion criteria at the time of application. The most frequent reasons for exclusion were as follows: poor spirometry results, MRI contraindications (claustrophobia, need of assisted ventilation), walking subscore at ALS-FRS-R, and underlying medical conditions (cardiovascular pathologies, autoimmune and oncologic diseases, positivity for infectious diseases). The final cohort of patients comprised 18 patients with ALS (5 females and 13 males). Median age was 48 years (range: 25–67). Median follow-up after implantation was 24 months (range: 7–51); the last recruited patient had been followed for 30 months. The principal characteristics and outcomes of the recruited patients are described in Table 2.

The treatment led to no serious AEs. All patients were extubated without problems in the operating room and displayed no immediate postoperative respiratory difficulties. One patient developed transitory reversible acute respiratory failure on day 1 after surgery, which required treatment with noninvasive ventilatory support. The most common and expected AE was acute postsurgical pain. This was confined to the surgery site and to the corresponding dermatomes: Only one patient experienced transient, painful spasms in the lower limbs. Pain was mild (classified as II–III on the WHO scale) and was controlled by narcotic and non-narcotic analgesics; most importantly, the pain disappeared over an average of 18 days after surgery (range: 1–60). There was no correlation between the pain and the number of cell injections; in fact, in every case, the pain was related to the surgical procedures that involved a laminectomy. Table 3 reports the registered AEs. One patient developed iatrogenic diabetes 7 days after surgery and was placed on long-term insulin treatment. Two patients developed pneumonia—270 and 120 days after surgery; in one case, antibiotic treatment sufficed to resolve the infection, whereas in the second case, a tracheotomy was required.

Tacrolimus was generally well tolerated and did not require premature withdrawal, except in one patient who developed postural tremor; this disappeared when the drug was discontinued. All patients displayed tacrolimus blood levels within the

Table 3. Adverse events

Adverse event	Patient	Time after surgery, days	Duration, days	WHO grade	Treatment
Pain	740	1	5	II	
	753	1	6	III	
	766	1	3	III	
	767	1	30	I	Analgesics
	897	1	120	II	
	753	1	6	III	
	799	1	4	I	
	942	1	15	II	
Spasms in the lower limbs	753	1	2	0	Narcotic analgesic
Deep vein thrombosis	779	90	30	III	Anticoagulant
Hematoma at the site of surgical scare	779	100	15	III	Drainage
Acute respiratory failure	862	0	1	III	NIV
Iatrogenic diabetes	799	7	Persistent	II	Insulin
Tremor	799	30	180	I	Remission after discontinuation of tacrolimus
Tingling sensation in the first and second finger of both hands	864	1	15	1	—
Tingling sensation in the left lower limb	799	6	25	I	—
Pneumonia	753	210	10	IV	Antibiotics
	831	270	—	IV	Tracheotomy
	897	120	10	IV	Antibiotics
	746	165	12	II	Antibiotics

Abbreviations: —, not applicable; NIV, noninvasive ventilatory; WHO, World Health Organization.

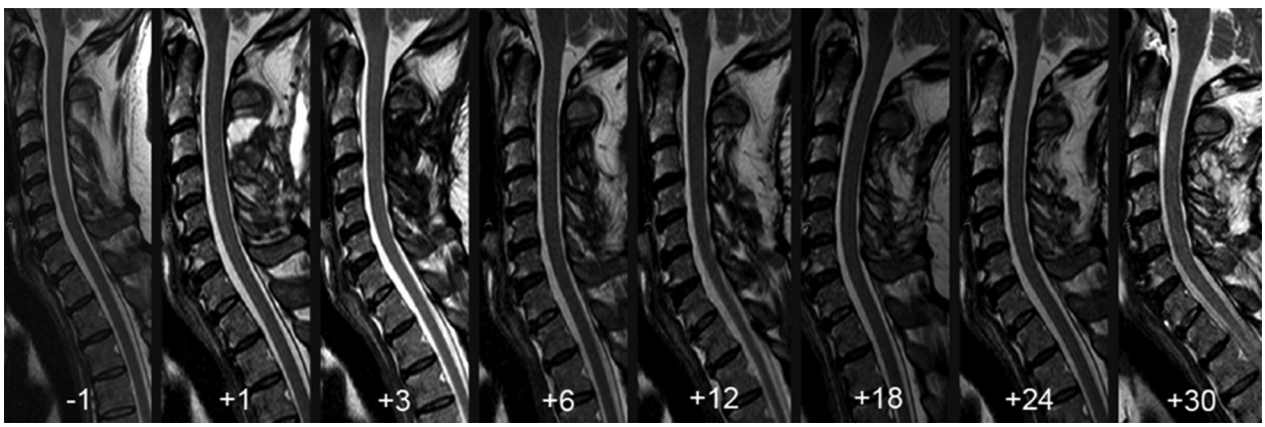

Figure 1. Magnetic resonance imaging follow-up in patient ISS-799. Turbo Spine Echo T2-weighted sequences acquired on sagittal plane before and 1, 3, 6, 12, 18, 24, and 30 months after cervical stem cell implant showing no consequences on spinal cord of cervical laminectomy.

therapeutic target range. Renal and liver function as well as blood and $CD3^+$; $CD3^+/CD8^+$ $CD4^+/8^+$ cell counts remained within the normal range.

At the time of analysis, median duration of post-treatment follow-up was 24 months (range: 7–51). Eleven of the treated patients died, and two patients underwent tracheotomy because of progressive respiratory failure caused by the natural course of the disease. For the three autopsies performed, the cause of death was confirmed and revealed only the presence of adhesions with meningeal structures.

No changes were detected in Sensory Evoked Potentials and Motor Evoked Potentials regarding the sensory and motor nerve conduction times, upon and following surgery. Bladder ultrasound showed no abnormal postvoid residual volumes. MRI revealed no postprocedural complications. In all patients, postsurgical MRI scans revealed an expected extradural fluid collection at the site of surgery, which resolved spontaneously within 3 to 6 months (Fig. 1). Repeated controls revealed no structural changes (including tumor or syrinx formation) within the brain and the spinal cord up to 3 years after transplantation, relative to the baseline. A 12-month follow-up analysis yielded no significant long-lasting changes in the ADC and FA (Fig. 2).

Clinical assessments after transplantation showed that the treatment did not accelerate the course of the disease. No

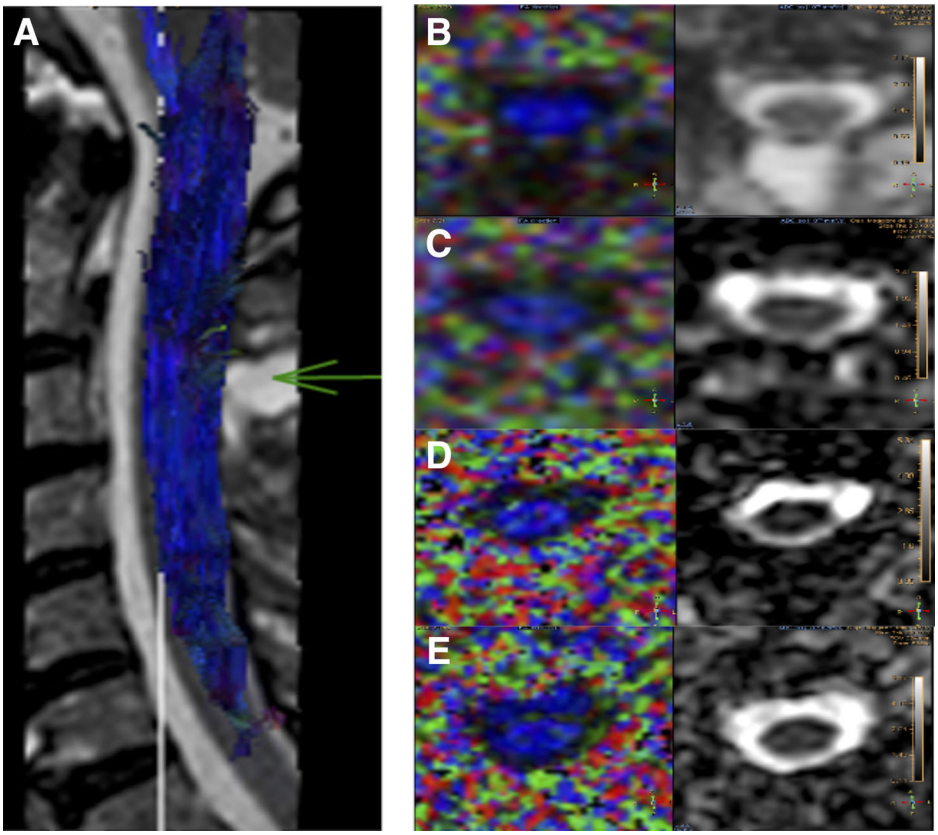

Figure 2. Spinal cord fibre Tracking **(A):** Whole cervical spinal cord fiber tracking reconstruction; the arrow indicates the laminectomy and implant site. **(B)** Magnetic resonance imaging (MRI) 15 days after stem cell graft. Directional color-coded fractional anisotropy map at the implant site level, obtained from diffusion tensor imaging acquisition, with evidence of apparent diffusion coefficient map. **(C–E):** Follow-up MRI with directional fractional anisotropy map and apparent diffusion coefficient map, respectively, at 12 months, 24 months, and 30 months.

significant acceleration in the decline of ALS-FRS-R and FVC (%) (Figs. 3, 4) were observed between the pre- and post-transplantation phases, in all groups of patients. The quality of life main value (SEIQoL) remained high (mean 73%, range: 69%–77%) at all clinical visits and did not decrease with time and disease progression. The psychological profile was not problematic for any of the recruited patients: Most did not develop clinical depressive symptoms or symptoms of anxiety. The one exception was a subject who manifested mood changes, with depressive symptoms in the postsurgery period, because of poor support and assistance provided by family.

Although our study was not primarily designed to determine the efficacy of the treatment, this was one of the secondary endpoints. Thus, during the entire follow-up period after cells transplantation, the rate of change of the ALS-FRS-R score and the FVC (percentage predicted) was compared with that of the 3-month run-in period. The treatment did not worsen the progression of the disease in any patient (Figs. 3, 4). Statistical analysis to compare the pre- and post-treatment rates of progression of the clinical scores in the three groups of patients revealed a transitory decrease in the ALS-FRS-R immediately after transplantation, beginning within the first month after surgery and lasting up to 4 months following transplantation (p = .0136). No statistically significant differences were found in the FVC rate of progression before and after treatment. No effects on survival were observed.

Notably, 5 out of 18 patients (patients 740, 779, 833, 842, and 897) reported specific, temporary subjective clinical improvement of the ambulation score following the surgery (typically lasting 2 to 6 months). Also, in 4 out of 18 patients (patients 799, 807, 842, and 919), the upper limbs (UL) score on the ALS-FRS-R scale improved by one point (cutting food and handling utensils, handwriting, dressing, and hygiene). Patients 740 and 897 demonstrated an objective improvement in the MRC score in the proximal muscles of the lower limb (LL; hip abductors, hip adductors, iliopsoas, biceps femoris, quadriceps femoris) beginning within the first month after surgery, and lasting up to 6 months: Both subjects had a juvenile phenotype, but patient 897 had shown a rapid progression of the disease before transplantation that attenuated after surgery, and the patient maintained a stable ALS-FRS-R score for up to 6 months. Patient 833 manifested a decreased stiffness in both the UL and LL for 3 months, as measured with the Ashworth scale, whereas patient 779 showed a lesser decline in the ALS-FRS-R score following surgery. Patient 833 had a juvenile phenotype with a slowly progressive form of ALS and manifested an improved ALS-FRS-R score after surgery that lasted for up to 12 months. Patients 807 showed a clear postsurgery improvement of the MRC score in the proximal muscles of the UL (deltoid, triceps brachii, biceps brachii). Both patients showed a rapid decline of ALS-FRS-score before transplantation that attenuated after surgery, for up to 3 and 6 months, respectively (Fig. 3).

Analysis of CSF Culture

As shown in Table 4, we detected no differences in differentiation pattern between cells treated with CSF derived from the three different groups of patients with ALS and cells treated

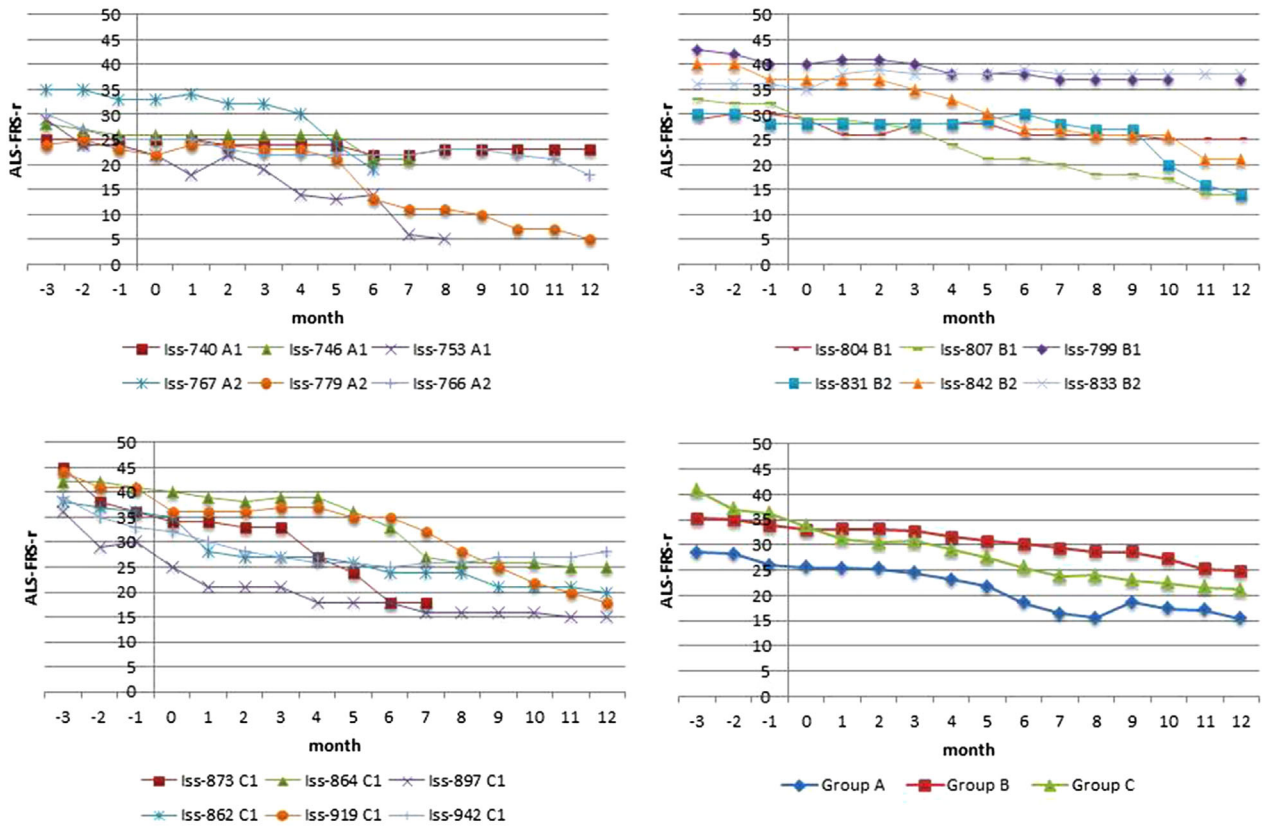

Figure 3. Longitudinal progression of ALS-FRS-R score in the 3-month period of natural history observation and 12 months after transplantation in the three groups of patients. Group A (upper left panel) group B (upper right panel), and group C (lower left panel). Mean values (lower right panel). Abbreviation: ALS-FRS-R, amyotrophic lateral sclerosis-functional rating scale revised.

with saline or CSF derived from healthy volunteers. Nonetheless, there was a slight increase in the differentiation of GalC-positive cells induced by CSF.

ELISA Tests

Quantification of BDNF, IL-10, IL-1, OPN, and VEGF in conditioned media derived from cultured hNSCs and collected during differentiation at T1, T2, and T3 revealed that stem cells can produce relevant amounts of VEGF (1261.6 $\pm$ 358.7 pg/ml) during routine culture and during the differentiation modulated by CSF or saline (Fig. 5A), and produced OPN only during differentiation (Fig. 5B).

Neurosphere and differentiated cells did not produce BDNF, IL-10, and IL-1β. VEGF was produced abundantly by hNSCs and was reduced during cell differentiation; also, CSF from patients with ALS or from healthy controls induced VEGF production in differentiating cells. Of note, OPN was not produced by hNSCs, and only CSF was able to induce production in differentiating cells. CSF from patients with ALS induced twice as much production of OPN as did CSF from healthy controls.

Discussion

A recent Cochrane analysis of safety and efficacy data from phase I/II clinical stem cell-based transplantation studies of neurological disorders [17] revealed that the numbers of trials are still too low to warrant guidance for reliable clinical practice. This issue is of relevance when developing experimental therapies for diseases that are both lethal and incurable, such as ALS/motor neuron disease, for which standardization and reproducibility of treatments are emerging as critical but thorny issues. There is an urgent need to undertake major efforts in this field to turn pleiotropic biological entities such as stem cells into the standardized drug products that are vital for undertaking reproducible clinical trials and for developing consistent therapeutic applications to treat these profoundly debilitating disorders. The present study describes some positive results in the area of clinical trials for brain stem cell-based therapy for patients with ALS. We also report on a donor cell system that resolves the outstanding critical issues that afflict the process of standardizing neural stem cell-based drug development for clinical applications.

Our study is the first to use medical transplantation of stable, clinical-grade hNSC lines that are isolated from brain biopsies from fetuses that are miscarried, and that can be reproducibly and stably expanded ex vivo. This approach enabled us to use the same two donor cells in all the patients in this investigation, and warrants further development of these cells in numerous upcoming human trials. As such, this will permit the implementation of more homogenous future phase I, II, and III clinical studies and should resolve many of the issues with standardization and reproducibility that currently constrain this field.

Primary Endpoint: Our study reached the primary endpoint, and our results underscore that micro-transplantation of cells into the human anterior spinal cord is a safe procedure, even when performed in subjects as fragile as patients with ALS; also, unlike previous studies, this procedure can be accomplished without causing serious short- or long-term AEs. We observed

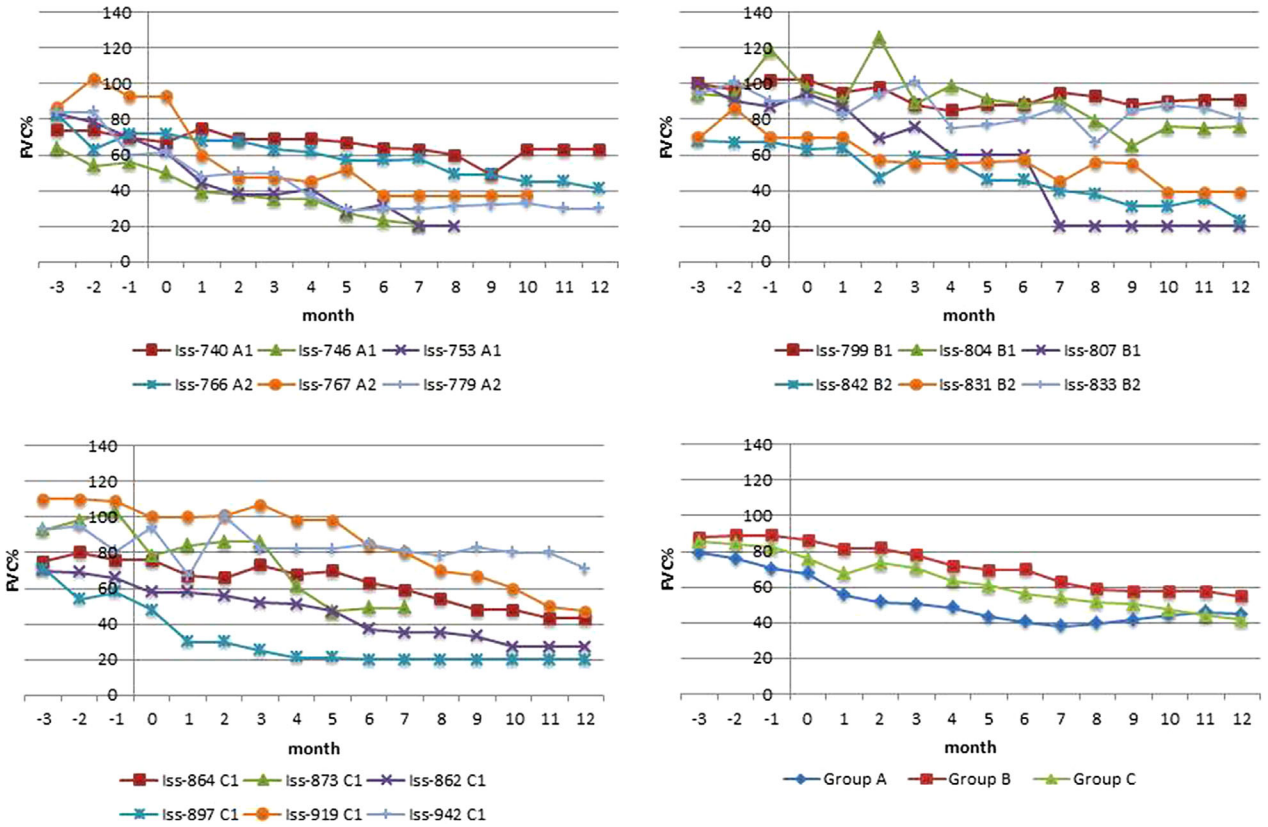

Figure 4. Longitudinal progression of FVC (%) score in the 3-month period of natural history observation and 12 months after transplantation in the three groups of patients. Group A (upper left panel) group B (upper right panel), and group C (lower left panel). Mean values (lower right panel). Abbreviation: FVC, forced vital capacity.

Table 4. Differentiation percentages in human neural stem cells treated with saline or 5% cerebrospinal fluid from either patients with amyotrophic lateral sclerosis or healthy volunteers

Marker	Standard method	Saline	Healthy donors	Group 1	Group 2	Group 3
β-tubulin III	9.3	10.1	10 ± 3.2	10.3 ± 1.6	11.8 ± 2.6	11.4 ± 3.5
GFAP	59.2	54.6	53.3 ± 11.4	58 ± 6.7	53.4 ± 5.2	53 ± 8.5
GalC	16.9	20.5	27.6 ± 11	23.2 ± 9.4	25.2 ± 6.8	22.8 ± 8.5

Abbreviation: GFAP, glial fibrillary acidic; GalC, galactocerebroside protein.

no AEs from the cell injection—in fact, all AEs that occurred in our patients could be referred to surgery or to the progression of the disease. By the low expression profile of HLA determinants in hNSCs, and the limited immunological reaction that they appear to elicit (thus only requiring temporary immunosuppression after transplant) [18], we adopted a transient immunosuppressive regimen in our trial. This may explain why, unlike in previous reports [9], our patients suffered no significant side effects caused by the tacrolimus treatment, which also had no negative influence on the psychological profile and quality of life of our patients.

Secondary Endpoint: Our patient cohort is too small to draw final conclusions approximating putative neurological or functional effects elicited by our treatment in patients with ALS. Notwithstanding this, however, we obtained some interesting results that highlight a significant transitory decline of the progression of the ALS-FRS-R score within the first month after transplantation that continues for up to 4 months after transplantation; 50% of the patients also reported a transitory functional improvement within this same window of time after surgery. Whether these improvements were the consequence of the cell implantation or were elicited by other factors (such as engagement in the trial assessments and increased generic medical input) remains to be determined, but the latter possibility is unlikely, particularly because the transitory improvement in patients with ALS is generally not believed to be accomplished through these factors. Our findings are also consistent with the report for the NCT 01348451 trial that a transient functional improvement was also observed within 6 months after transplantation, with similar effects emerging when cells are reinjected in the same subject 15 months later [19–21]. Although the transient improvement of function is not an important functional outcome for the overall course of the disease, it is nevertheless relevant for the design of future efficacy trials.

The above findings are reinforced by their concordance with the results of preclinical transplantation studies done in ALS

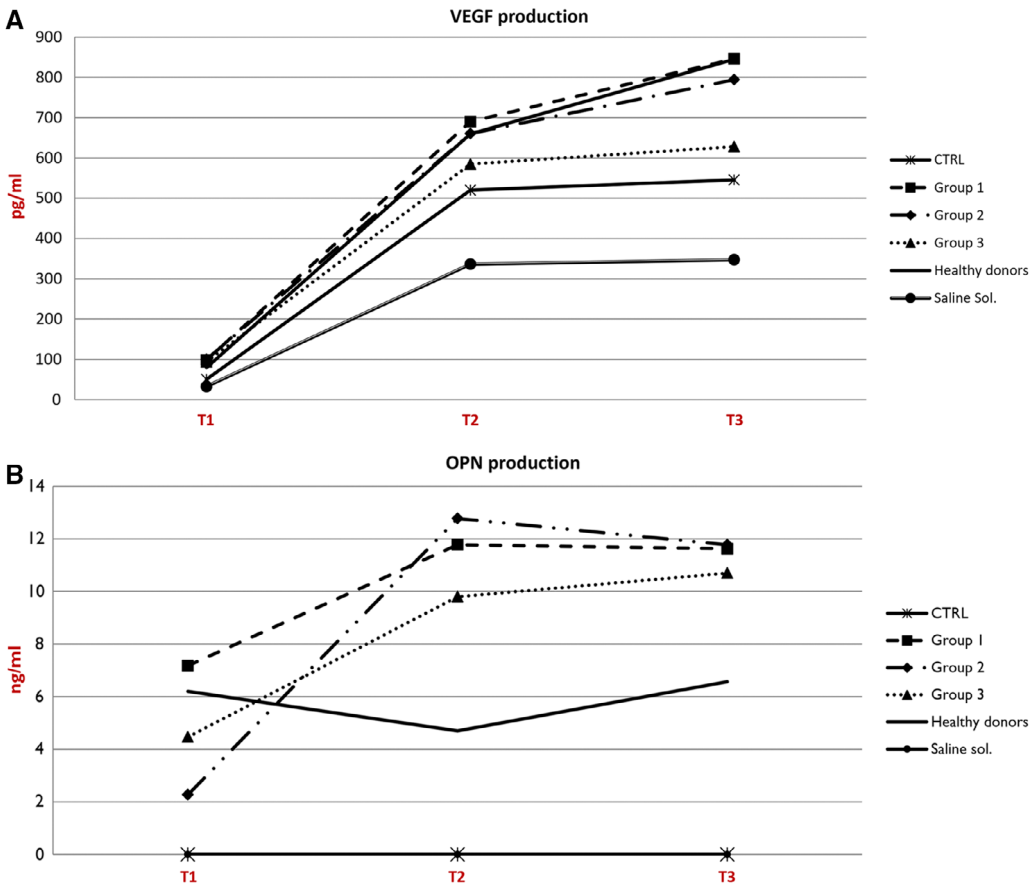

Figure 5. Cytokines production evaluation **(A):** VEGF quantification in conditioned media during differentiation process modulated by saline or CSF derived from patients with amyotrophic lateral sclerosis (ALS) or healthy volunteers. **(B):** OPN quantification in conditioned media during differentiation process modulated by saline or cerebrospinal fluid derived from patients with ALS or healthy volunteers. Abbreviations: OPN, osteopontin; VEGF, vascular endothelial growth factor.

animal models. Thus, hNSCs delivery into the spinal cord of SOD1G93A rodents delays the onset and progression of the disease and prolongs animal survival [22]. Together with the reduction in local astro- and micro-gliosis caused by hNSCs, a major noncell autonomous pathogenic mechanism that drives ALS progression [23], some of putative therapeutic actions could also derive from the release of neurotrophic factors and cytokines (BDNF, Glial cell line-derived neurotrophic factor, and VEGF) by these cells [23]. In our preclinical evaluation for this trial, the same type of hNSCs that were administered to our patients were also injected into the lumbar spinal cord of SOD1G93A rats at early symptomatic stages: This procedure ameliorated disease progression, delayed the deterioration of motor function, and significantly extended the animal's survival (manuscript submitted). The improvements were associated with (a) a better expression profile of histopathological markers of ALS; (b) preservation of motor neurons in the transplanted areas; and (c) reduction of reactive astrogliosis and microglial activation. And similar to the results of our phase I trial, the beneficial effects of hNSCS in SOD1G93A rats were more prominent at early times following the transplant. Collectively, these findings indicate that effects of hNSCs on ALS progression in humans can be extrapolated from rodent models when the same cells are used at the preclinical as well as clinical stage.

The cGMP hNSC stable cell lines: A second set of findings concerns the donor hNSCs we have used in this study. Even though the number of cells from a single donor largely exceeded our needs, we nevertheless used two donors, to increase the safety of the entire patient cohort, considering that this was the first human trial undertaken with this system. The same cells will now suffice for the upcoming phase II ALS trial (similar to the ongoing phase I trial on secondary progressive multiple sclerosis [EudraCt 2015-004855-37]) and for additional future trials. Thus, these stable hNSC lines provide a reliable and consistent cell system that is plentiful and is also reproducible, such that it can be used to complete homogeneous clinical trials using a standardized cell drug product. The hNSCs that we used to transplant into our patients with ALS are based on results of 25 years of preclinical work from our group [24–26] that defined the parameters for establishing our system of stably expanding hNSC lines, which retain their properties of self-renewal, growth rate, and differentiation potential over extensive serial passages in culture. Our system provides a standard amplification process that enables us to produce identical hNSCs lots from a single tissue specimen, in large enough quantities such that they can be transplanted into hundreds of patients. This, along with their single-donor origin, stable dependence on growth factors, karyotype stability, and lack of tumorigenicity, yields a functional and safety profile that warrants the GMP certification of hNSCs. The safety profile of these hNSCs has been further validated over a period of up to 4 years after transplantation by results of the present study in patients with ALS: We saw no changes in the MRIs and did not detect negative changes in neurological outcomes suggestive of

aberrant cell proliferation or of the development of proliferative lesions. We believe a longer-term follow-up will reinforce this conclusion, and as such, we intend to surveil our transplanted patients over their lifetimes.

We have also analyzed possible interactions between the CSF of patients with ALS and hNSCs: CSF from patients with ALS does not significantly influence the differentiation of hNSCs. This is in contrast with the results of the study by Cristofanilli et al. [27], which show how CSF from patients with multiple sclerosis significantly increases the numbers of neural precursor cells-derived neurons and oligodendrocytes in vitro. The slight increase that we detect in the differentiation of GalC-positive cells induced by CSF is probably due to low amounts of BDNF (produced by the choroid plexus) that are present in the CSF under physiological conditions. These differences can be attributed to the use of a commercially available immortalized cell line by the Cristofanilli group, but may also be due to differences in CSF composition between patients with ALS and healthy controls, as recently reported by Collins [28].

VEGF administration slows disease progression and prolongs survival in animal models of ALS [29–31]. Levels of VEGF also elevated in the serum and CSF of patients with ALS in North India who have significantly higher survival rates (~10 years following diagnosis), compared with patients in the U.S. or Europe (3–5 years after diagnosis) [32]. However, the source of the increased VEGF production has not been identified. VEGF is also a potent neurotrophic factor that protects motor neurons from oxidative stress in vitro [33]. In our experiments, hNSCs in vitro produce VEGF when in neurosphere, and CSF from patients with ALS and from healthy controls induces VEGF production in differentiating cells. This observation could indicate a possible neuroprotective mechanism in our cell line and may account at least in part for the transient amelioration of clinical symptoms in treated patients with ALS.

OPN is a matricellular protein with a wide spectrum of biological activities, and it is highly expressed in the healthy CNS, mostly by neurons [34], consistent with our in vitro observations on the supernates of differentiating hNSCs. A recent study shows significant upregulation of the OPN receptor CD44 in microglia and astrocytes in SOD1G93A mice, suggesting that this protein plays a yet undetermined role in ALS [35]. Increased OPN levels are also found in the CNS during Parkinson's and Alzheimer's diseases, stroke, and spinal cord injury; however, depending on the specific pathology, the effects of OPN can also be the opposite of those described above. For example, OPN is neuroprotective in spinal injury, whereas it is detrimental in Parkinson's disease [36, 37]. Kalluri [38] showed that OPN affects survival, proliferation, migration, and neuronal differentiation of NSCs derived from rats, in presence of bFGF, and that these effects are mediated, at least in part, via IGF-1 (affects proliferation) and CXCR4 (affects migration). In addition, results from Kalluri's group suggest that OPN has potential for use in the targeted activation of NSCs in future experimental therapies for neurodegenerative disorders.

Thus, our findings underscore the safe use of stable hNSC lines in the context of stem cell experimental therapy in humans and suggest a mechanism of action whereby the hNSCs mediate this effect in ALS treatment. We also describe a safe and standardized brain cell drug product that is plentiful and can be used for larger and more homogenous trials; the availability of these cells may even enable implementation of international, multicentric clinical studies, in which each center delivers the same treatment to patients. This obviates the limitations of currently available systems that hinder standardization and reproducibility in multicenter trials and lead to heterogeneous results (due to implantation of acutely isolated and/or transiently expanded neural cells that have been derived from multiple donors) not just across different studies but also even within the same trial.

The above considerations are important in the context of the low numbers of clinical trials that are approved to test use of hNSC: The major reason for this is the difficulty in obtaining and handling donor cells. This has led to "stem cell tourism," or the application of unapproved, costly, and dangerous treatments to desperate patients [38], with a disregard of proper safety standards, including of the risks associated with the inherent proliferation potential of stem cells and of changes and aberrations that might occur in the cells as they are being passaged in vitro. The profound dangers of this issue are emphasized by recent reports of hyperplastic and tumor lesions that follow transplantation of highly uncharacterized so-called "brain stem cells" in cases of uncontrolled "stem-cells tourism" [39–41]. The hNSCs we have developed and described here should enable approval of greater numbers of comprehensive, authorized, and controlled clinical trials and also help to implement the mandatory use of GMP-certified brain cells that are required to abide by strict, predefined, and broadly accepted standardization criteria.

Conclusion

Our results support the use of GMP-grade fetal hNSCs derived from in utero spontaneous death in future efficacy-seeking clinical trials for treatment of ALS. Substantial challenges remain to be addressed and resolved in upcoming phase IIa/IIb trials, including determination of the optimal number of cells to be injected, how long the cells remain active in humans, the criteria for patient selection, biomarkers for monitoring the disease course, and efficacy of the hNSCs. These issues have been discussed in a previous paper [42], and we expect to address them in our next phase II trial, currently in preparation.

Author Contributions

L.M.: conception and design, manuscript writing, data collection, data analysis and interpretation, final approval of manuscript, provision of patients; M.G., D.C.P.: conception and design, manuscript writing, provision of study material, administrative support; G.S., G.Q., E.B., F.D.M.: provision of patients, data analysis and interpretation; R.C.: manuscript writing; A.S.: data collection and interpretation; C.S., D.F., C.C., M.M., M.F.S., S.P., S.M., A.G.: data collection; S.C., C.G.: performed surgical procedures; D.F., C.Z., E.B., A.V., D.T., B.T., L.B.: contributed to clinical and nonclinical characterization of the cells; M.C.: data analysis and interpretation, manuscript writing; G.M., C.R.: contributed to cells production and characterization; N.M.B.: study design and data interpretation; A.M., F.P.: provided study materials; A.L.V.: financial support, final approval of manuscript, manuscript writing, conception and design.

Disclosure of Potential Conflicts of Interest

N.B. is a consultant for NeuralStem and is the patent owner for a device used to transplant the cells (Cleveland Clinic Licensed to NeuralStem). The other authors indicated no potential conflicts of interest.

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