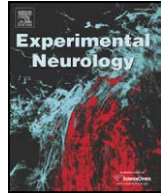




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## Mesenchymal stem cell transplantation in amyotrophic lateral sclerosis: A Phase I clinical trial

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### ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a devastating incurable disease. Stem-cell-based therapies represent a new possible strategy for ALS clinical research. The objectives of this Phase 1 clinical study were to assess the feasibility and toxicity of mesenchymal stem cell transplantation and to test the impact of a cell therapy in ALS patients. The trial was approved and monitored by the National Institute of Health and by the Ethics Committees of all participating Institutions. Autologous MSCs were isolated from bone marrow, expanded in vitro and analyzed according to GMP conditions. Expanded MSCs were suspended in the autologous cerebrospinal fluid (CSF) and directly transplanted into the spinal cord at a high thoracic level with a surgical procedure. Ten ALS patients were enrolled and regularly monitored before and after transplantation by clinical, psychological, neuroradiological and neurophysiological assessments. There was no immediate or delayed transplant-related toxicity. Clinical, laboratory, and radiographic evaluations of the patients showed no serious transplant-related adverse events. Magnetic resonance images (MRI) showed no structural changes (including tumor formation) in either the brain or the spinal cord. However the lack of post mortem material prevents any definitive conclusion about the vitality of the MSCs after transplantation. In conclusion, this study confirms that MSC transplantation into the spinal cord of ALS patients is safe and that MSCs might have a clinical use for future ALS cell based clinical trials.

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### Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating incurable disease that targets motor neurons (Rowland and Shneider, 2001). Despite the relative selectivity of motor neuron cell death, animal and tissue culture models of familial ALS suggest that non-neuronal cells contribute significantly to neuronal dysfunction and death (Boillée et al., 2006; Yamanaka et al., 2008). Marked microglial activation, IgG deposits, and lymphocytic infiltration have been demonstrated in the affected areas of tissue from ALS patients (Henkel et al., 2004). A cell-based therapy may have the advantage of exerting multiple therapeutic effects (Svendsen and Langston, 2004; Lepore et al., 2008; Suzuki and Svendsen, 2008) at various sites and times within the

lesion, as the cells respond to a particular pathological microenvironment (Liu et al., 2006) by protecting existing motor neurons from ongoing degeneration.

Mesenchymal stem cells (MSCs) are multipotent stem cells that are very attractive in view of a possible cell therapy approach in ALS because of their great plasticity (Chen et al., 2008) and their ability to provide the host tissue with growth factors or modulate the host immune system (Garbuzova-Davis et al., 2006). Their protective effects might function in concert with immunosuppressive and anti-inflammatory activities (Uccelli et al., 2007). MSCs promote "by-stander" immunomodulation (Uccelli et al., 2008) as they can release soluble molecules such as cytokines and chemokines and express immuno-relevant receptors such as chemokine receptors and cell-adhesion molecules (CAMs). These can have anti-inflammatory and anti-proliferative effects on microglial cells and astrocytes, resulting in the induction of a neuroprotective microenvironment (Uccelli et al., 2008). Moreover, bone-marrow-derived MSCs are widely used

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clinically and few adverse effects have been attributed to their administration. They can be safely cultured *in vitro* with no risk of malignant transformation (Bernardo et al., 2007).

MSCs isolated from the bone marrow (BM) of ALS patients maintain all their peculiar characteristics and their extensive *in vitro* expansion does not involve any functional modification including chromosomal alterations or cellular senescence (Ferrero et al., 2008). Moreover, they acquire, under specific conditions, new morphological characteristics and neural markers which are suggestive of neural differentiation as in healthy donors (Ferrero et al., 2008). Whole BM transplantation has been shown to delay the onset of disease, to increase lifespan in SOD1G93A mice and participate in striated muscle regeneration (Corti et al., 2004). Moreover, expanded MSCs can survive and migrate after transplantation in the lumbar spinal cord of SOD1G93A mice, where they prevent astrogliosis and microglial activation and delay ALS-related decrease in the number of motoneurons, resulting in an amelioration of motor performance (Vercelli et al., 2008). Early clinical investigations indicated that transplantation of autologous MSCs into the spinal cord is feasible in ALS patients (Mazzini et al., 2003, 2006, 2008) however as that study was limited to 9 patients, the safety of intraspinal transfer of MSCs for ALS leaves some questions relative to the results open (Badayan and Cudkovic, 2008). We therefore performed a Phase I trial for a further assessment of the feasibility and toxicity of the procedure and to test the impact of a cell therapy approach in patients.

## Methods

The trial (Registration number 16454-pre21-823) was approved and monitored by the National Institute of Health and by the Ethics Committees of the Piedmont Region, the “Maggiore della Carità” and “San Giovanni Bosco” Hospitals before starting the study. All patients provided written informed consent. All the recorded data of the recruited patients during the entire follow-up period were registered with the database for clinical studies with gene and somatic therapy of the National Institute of Health and communicated to the Ethics Committee of the recruitment centre which strictly controlled adherence to the protocol and monitored adverse events.

## Patients

Patients between 20 and 65 years old were eligible if they had definite or probable sporadic ALS according to the El Escorial Revised Criteria (Brooks et al., 2000), spinal onset, duration of the disease less than 3 years, evidence of progression disease in the last 6 months, mild to moderate disability documented by satisfactory bulbar and spinal function (minimal score of 3 on ALS-FRS (The ACTS Phase I-II Study Group, 1996) for swallowing, and 2 for cutting food and handling utensils, and walking), FVC greater than or equal to 50% of that predicted, normal polysomnography, good acceptance and understanding of the informed consent. Patients over 65 were excluded as we have previously demonstrated that cell growth of expanded *in vitro* MSCs is strictly related to the donor's age (Mareschi, 2006).

Patients were excluded if they had familial ALS, evidence of any concurrent illness (diabetes, cardiovascular disorders, arterial hypertension, kidney and liver disorders, dysthyroidism, autoimmune disorders, neoplasms or any other diseases reducing life expectancy) or were receiving any medications which might affect BM. A case-report of eligible patients were sent to the National Institute of Health whose experts strictly controlled adherence to the protocol and authorized the treatment. After approval, the patients were informed of recruitment in the study. The patients were closely examined by a psychologist before and immediately after recruitment. A clinical interview and an MMPI-2 test were used to ensure that the participants fully comprehended that this was a safety trial and the risks associated with the procedure.

Informed consent was structured as an interview which clearly stated the experimental and preliminary nature of the clinical study and the risks associated with the procedure. Each question was discussed by the neurologist with the patients and their relatives. Subjects were made aware that their participation was entirely voluntary and that participation or non-participation would not interfere with their ongoing clinical care. Before signing, patients and close relatives were offered the possibility of meeting separately with their family physician, the neurosurgeon, and a consultant neurologist, who was not the neurologist in charge, to discuss all pending issues.

## Assessment

To estimate the disease progression rate the patients were assessed every 3 months. A 9-month period of natural history observation, after enrollment, was performed before transplantation.

The clinical progression of the disease was assessed using the ALS-FRS and the Medical Research Council (MRC) scale. Manual muscle testing of 34 muscle groups of the upper and lower limbs was independently performed by a neurologist and a physiotherapist and the mean value of the scores obtained by the two assessors were considered in the analysis. Whole respiratory assessment included pneumological examination, functional respiratory tests and nocturnal respiratory monitoring.

All patients were also evaluated at each examination by a clinical psychologist. Profile of Mood State (POMS) (McNair et al., 1992) and SEIQoL-DW (Clarke et al., 2001) questionnaires were provided to assess the mood state and the quality of life.

Electromyographic assessment was performed at the time of recruitment and every 6 months by statistical MUNE (Shefner et al., 2004), which was evaluated in recordings from the bilateral abductor digiti minimi (ADM) muscle, and neurophysiological index (de Carvalho et al., 2003). Averaged MUNE of both sides were considered in the final analysis for each patient.

Somatosensory evoked potentials (SEPs) following tibial nerve stimulation were recorded before and after surgery.

Brain and spinal cord (SC) MRIs were obtained using a 1.5-T imaging system (Achieva Intera, Philips). See Table 1 for our imaging protocol. MRIs were performed 15 days before and at 15 days, 3, 6, 9, 12, 18 and 24 months after surgery. MRI scans were obtained every 6 months thereafter. At each MRI session the spinal cord diameter at the level of engraftment was also quantitatively measured in the axial

**Table 1**  
Sequences and parameters used for brain and spine MR scan.

Sequence	Plane	TR (ms)	TE (ms)	NEX	THK (mm)	MATRIX	IT (ms)
<i>Sequences and parameters used for spine MR scan</i>							
TSE T2w	sagittal	3500	120	4	3	576 × 427	
TSE T2w	axial	3438	120	8	4	224 × 174	
TSE T1w	sagittal	400	6	4	3	576 × 432	
STIR T2w	sagittal	2500	60	4	3	488 × 357	170
Contrast medium (c.m.) bolus intravenous injection (0.1 mmol/kg, double molarity gadolinium-based c.m.)							
TSE T1w	sagittal	400	6	4	3	576 × 432	
TSE T1w	axial	shortest	9	4	4	224 × 174	
<i>Sequences and parameters used for brain MR scan (scans obtained after c.m. administration)</i>							
SE T1w	axial	shortest	15	1	5	256 × 163	
TSE dual	axial	shortest	20/120	2	5	328 × 205	
FLAIR	coronal	11000	140	2	5	256 × 154	2800

TR = time of repetition; ms = milliseconds; TE = time of echo; NEX = number of excitations; THK = slice thickness; mm = millimeters; IT = inversion time; TSE = Turbo Spin Echo; T = relaxation time; w = weighted; min = minimum; SPIR = spectral pre-saturation inversion recovery; SE = spin echo; FLAIR = fluid attenuation inversion recovery; PD = proton density.

**Table 2**  
Summary of MSC characteristics before implantation.

Patients	Number of implanted cells ( $\times 10^6$ )	Sterility			Viability (%)	Karyotype	Telomere shortening (kb)
		Bacteriology	Mycoplasma	Endotoxin level (EU/ml)			
1	80	NC	NC	<0.4	96	46XX	NS
2	90	NC	NC	<0.5	98	46XX	NS
3	109	NC	NC	<0.4	96	46XY	0.4
4	70	NC	NC	<0.4	98	46XY	0.9
5	45	NC	NC	<0.4	100	46XX	NS
6	95	NC	NC	<0.4	99	46XY	0.4
7	75	NC	NC	<0.4	99	46XY	0.6
8	120	NC	NC	<0.4	97	46XY	0.7
9	11.4	NC	NC	<0.5	99	46XY	1.4
10	51	NC	NC	<0.4	99	46XY	NS

The expanded cells had been characterized, before implantation, according to the GMP conditions to evaluate sterility, viability, telomere length and karyotype. After *in vitro* expansion MSCs showed no bacterial, fungal, mycoplasma or endotoxin contamination. No telomere shortening was noted after three passages in MSCs of all patients. No chromosomal alteration was evidenced in any samples. NC = no contamination; EU = endotoxin units; NS = no shortening.

and sagittal planes by analyzing the antero-posterior (AP) and right-left (RF) width.

In addition to the full conventional MRI, we also acquired, when technically feasible, a diffusion tensor imaging (DTI) pulse sequence in the coronal plane, with 16 directions in order to identify and quantitatively characterize tissues. On the fiber tracking algorithm we calculated the fractional anisotropy (FA) and apparent diffusion coefficient (ADC) values. We used FiberTrack Software (Philips) and, by placing single point ROI, we measured fractional anisotropy (FA) and ADC at the implant site (T4–T5, T5–T6) and in the appearing normal near cranial segments of the spinal cord. Measurements were acquired placing 4 Regions of Interest (ROI) drawn in each site, and the mean of the 4 measures was considered in the analysis.

#### Bone marrow collection

To evaluate the proliferate potential and characteristics of MSCs from each patient, about 10 ml of BM was aspirated, in local anesthesia, from the posterior iliac crest of recruited patients. MSCs were isolated, expanded for 4 passages and analyzed as described elsewhere (Mazzini et al., 2003, 2006, 2008). Patients whose cultures did not produce the established number of  $110 \times 10^6$  were eliminated.

In the enrolled patients, BM was then collected from the iliac crest in epidural anesthesia, according to standard procedures (Bacigalupo et al., 1992).

#### Isolation and expansion of MSCs

MSCs were isolated, expanded and analyzed as previously described in detail (Mazzini et al., 2003, 2006, 2008), according to the GMP conditions (European Medicines Agency, 1999).

#### Surgical procedure

The details of the transplant protocol and the rationale for choosing transplant variables have been previously described in detail (Mazzini et al., 2003, 2006, 2008). In brief, before implantation, the cells were maintained for at least 3 h in basal MSC Medium (Lonza Group Ltd, Basel, Switzerland) without serum, detached and washed 3 times with PBS  $1 \times$  containing 1% human albumin and once with autologous cerebrospinal fluid (CSF). The cells were suspended in about 1 ml of autologous CSF in all patients. The syringe had an 18-gauge cannula needle mounted on a table fixed arm with a micrometric system which permitted a constant flow of cellular suspension. The cannula was pre-modified so as to inject upwards and downwards. The cells were then, using an operating microscope, injected into the most central part of the spinal cord toward the anterior horns at the high thoracic level (T4–T5; T5–T6), in a pattern of three rows 3 mm apart. At the same level of myelotomy the number of injection sites varied from 2 to 5. All surgical procedures were performed by the same neurosurgeon in general anesthesia using short-acting drugs.

#### Safety end-points

We assessed the safety of MSC transplantation by the development of immediate or delayed adverse events. Immediate reactions included allergic reactions (tachycardia, fever), respiratory failure, local complications (intraparenchymal hematoma, local infection at the site of surgery), systemic complications (systemic infections), paralysis or sensory loss below the level of the injection site. Delayed reactions included intraspinal tumor formation or syringomyelia, persistent sensory loss or paralysis not due to the progression of the disease.

**Table 3**  
Summary of MSC characteristics before implantation.

Patients	CD45+ (%)	CD14+ (%)	CD90+ (%)	CD106+ (%)	CD29+ (%)	CD44+ (%)	CD166+ (%)	CD105+ (%)
1	3.02	1.81	94.00	3.59	96.00	93.00	30.90	89.00
2	2.60	6.20	92.00	22.00	83.00	78.00	10.40	78.00
3	5.00	4.50	72.00	38.00	76.00	82.00	13.60	70.00
4	4.80	11.50	87.70	47.10	83.72	72.10	60.97	76.61
5	0.00	0.00	71.00	40.00	80.00	80.50	26.80	68.00
6	0.40	0.50	92.00	13.90	90.60	97.80	90.80	91.70
7	0.10	0.10	96.90	71.70	98.50	99.10	8.60	98.70
8	0.40	0.00	92.00	3.20	93.30	96.00	79.00	93.30
9	1.50	1.20	98.10	55.30	97.00	91.00	0.00	95.50
10	2.20	1.60	97.00	79.60	97.60	94.20	99.60	99.00

Median percentage expression of MSC-specific surface markers at the last passage, before implantation. MSCs expressed low levels of hematopoietic antigens CD45 and CD14, high levels of CD90, CD29, CD44 and CD105 and lower levels of CD106 and CD166.

**Table 4**  
Averse events.

Adverse event	No of patients	OMS grade	Mean duration (days)
Pain	7 (70%)	II	8 ± 3.12 (range: 3–10)
Sensory light-touch impairment in one leg	4 (40%)	I–II	51.75 ± 85.6 (range: 6–180)
Tingling sensation in one leg	6 (60%)	I–II	43.3 ± 38.7 (Range range 3–90)
Sensory light-touch impairment in sacral region	1 (10%)	I	20

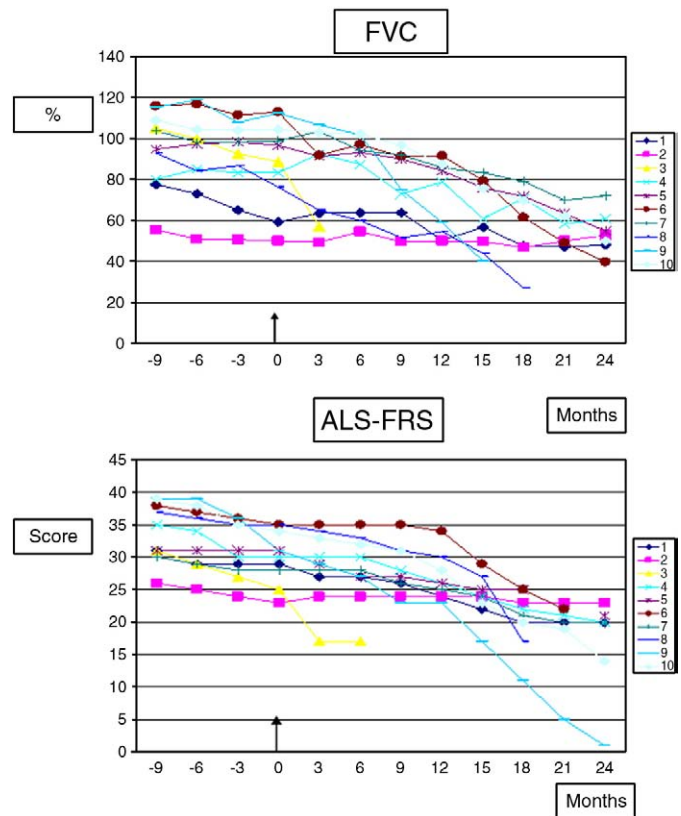
*Discontinuation of the study*

The study may be stopped at any time by the project monitors at the Italian Institute of Health, and/or the investigator(s) and/or the ethics committees on the basis of any medical event of clinical significance experienced by the patient that might occur after the treatment. Severity was graded according to the modified WHO criteria.

**Results**

*Patients*

Twenty of 270 clinically definite and probable ALS patients who had been consecutively visited in our tertiary ALS Center between September, 2003, and June, 2006 were selected. To analyze the proliferative potential of MSCs, a median number of 12 ml (range: 5–22.5) of BM was aspirated from the posterior iliac crest of 16 of the 20 patients recruited for the study (the remaining 4 patients refused the protocol). A median number of  $206.4 \times 10^6$  (range:  $37.8$ – $398.2$ ) total cells were obtained. The analysis of MSC expansion after 4 culture passages, in terms of growth rate, immunophenotype, telomere length and karyotype analysis, revealed that 2 patients were unsuitable for MSC transplantation due to the low proliferative potential. After performing the BM aspirate, 2 patients refused treatment, 1 patient showed positivity to hepatitis B serology and 1 patient developed a severe respiratory failure during the 6-month period of natural history observation which represented a contraindication to the general anesthesia. Therefore, the final cohort included 10 patients (3F and 7M). The median age was 41 years (range: 20–61). The median duration of the disease from diagnosis was 8 months (range: 3–30). The median ALS-FRS score at entry was 33 (range: 26–39) while the median FVC was 99% (range: 51–117). All patients received ordinary medical treatment. The mean



**Fig. 1.** Clinical follow-up. Changes of the forced vital capacity (upper panel) and of the ALS-FRS score (lower panel) in the 9-month period of natural history observation and after transplantation. The arrow indicates the time of MSCs transplantation.

time from symptom onset to BM cell harvest was 22 months (range: 10–40). A median number of 525 ml (range: 60–886) of BM was harvested from enrolled patients during epidural anesthesia; a median number of  $124.9 \times 10^8$  (range: 15.2–232.5) total cells were obtained. There were no local or general complications after BM collection.

*MSCs characteristics*

*In vitro* expanded MSCs showed no bacterial, fungal, mycoplasma or endotoxin contamination. MSCs were expanded within 2 passages for two patients, 3 passages for 5 patients and 4 passages for 3 patients.

**Table 5**  
Clinical characteristics of patients at entry and outcomes.

Patient	Age	Sex	ALS-FRS score	FVC (%)	Disease duration at entry (months from diagnosis)	PEG (months from surgery)	NNIV (months from surgery)	Follow-up duration (months from surgery to death (†) or to the last visit)
1	35	F	31	73	19	33	No	40
2	20	F	26	51	30	No	36	44
3	37	M	31	105	13	No	9	12†
4	29	M	35	80	3	33	27	36
5	50	F	31	95	18	29	30T	40
6	61	M	37	117	3	23	25T	28†
7	40	M	30	103	24	No	No	30
8	46	M	37	93	3	9	17	22†
9	57	M	39	115	3	15	19T	26
10	43	M	33	104	3	No	19	24

FVC = forced vital capacity  
PEG = percutaneous endoscopic gastrostomy  
NNIV = nocturnal non-invasive ventilation  
T = tracheotomy

## SEIQoL-DW

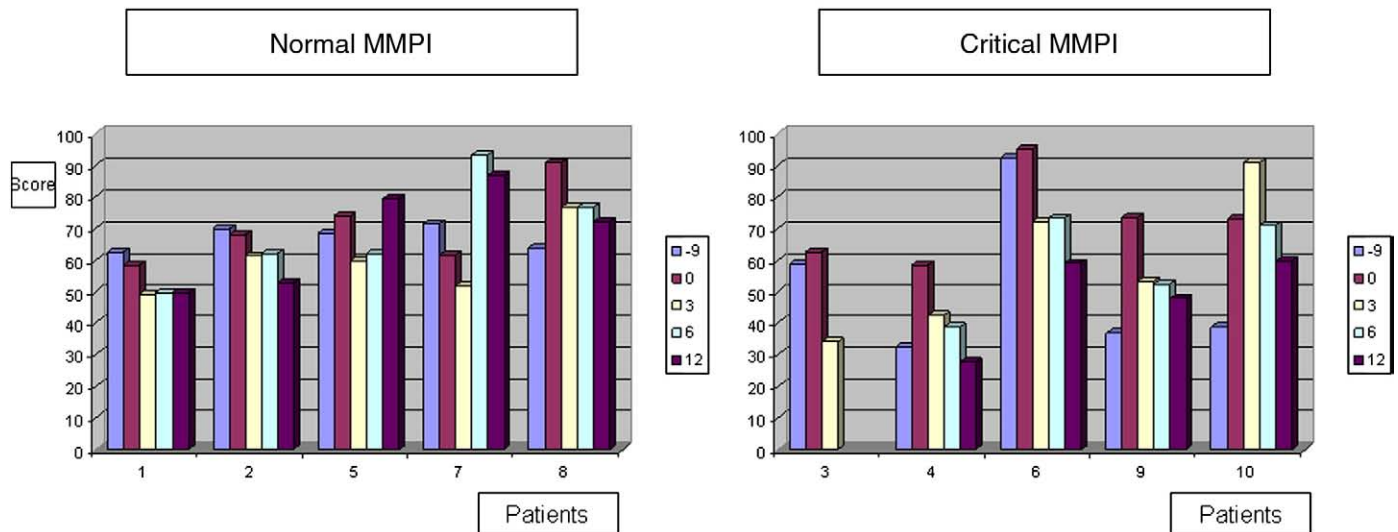


Fig. 2. Psychological follow-up. Changes of the SEIQoL-DW score in the 9-month period of natural history observation and after transplantation.

A median of  $75 \times 10^6$  cells (range:  $11\text{--}120 \times 10^6$ ) was obtained for transplantation. During the expansion time, the cells were analyzed at each passage for the expression of CD45 and CD14, CD90, CD29, CD44, CD105, CD166 and CD106. At the last passage, before implantation, they expressed low levels of hematopoietic antigens CD45 (median: 2.32%, range: 0.10–5.00) and CD14 (median: 3.28%, range: 0.00–11.50), high levels of CD90 (median: 90.45%, range: 72.00–97.00), CD29 (median: 89.84%, range: 76.00–98.50), CD44 (median: 89.03%, range: 72.10–99.10) and CD105 (median: 87.04%, range: 70.00–99.00) and lower levels of CD106 (median: 34.89%, range: 3.59%–79.60%) and CD166 (median: 49.23%, range: 8.60%–99.60%). No telomere shortening was noted after three passages in all the patients' MSCs. No chromosomal aberrations were evidenced in any samples. See Tables 2 and 3 for MSC characteristics before implantation.

#### Adverse events

Clinical, laboratory, and radiographic evaluations of the patients showed no deaths or serious treatment-related adverse events. There was no immediate or delayed toxicity related to MSCs transplantation. SEPs showed no changes of the sensory conduction time after surgery. Table 4 reports the most common adverse events. All symptoms were noted immediately after surgery. Pain was referred to the injection

sites and to the corresponding thoracic dermatomes. No correlation was found between the severity and duration of the side effects and the cell doses or the number of injection sites.

#### Follow-up

At the time of writing, all patients have had at least a 24-month follow-up period after transplantation. Table 5 reports the clinical characteristics of patients at entry and the time of principal outcomes during the follow-up. Five patients needed PEG placement due to a worsening of bulbar symptoms. Nocturnal Non Invasive Ventilation (NNIV) was needed for evidence of sleep apnea syndrome at the polysomnography follow-up in five patients. One patient required tracheotomy 25 months after transplantation and died 3 months later for cardiovascular complications. One patient died 1 year after the surgery of a hemorrhagic stroke as was hypothesized by his general practitioner. One patient died 22 months after surgery of a pulmonary embolism. Despite the informed consent stating, in the event of death, the request for a partial or complete autopsy, the authorizations were not obtained by the relatives.

No significant modifications of the decline of all clinical and instrumental measures including MUNE and NI were observed between the pre- and post-treatment periods (Fig. 1).

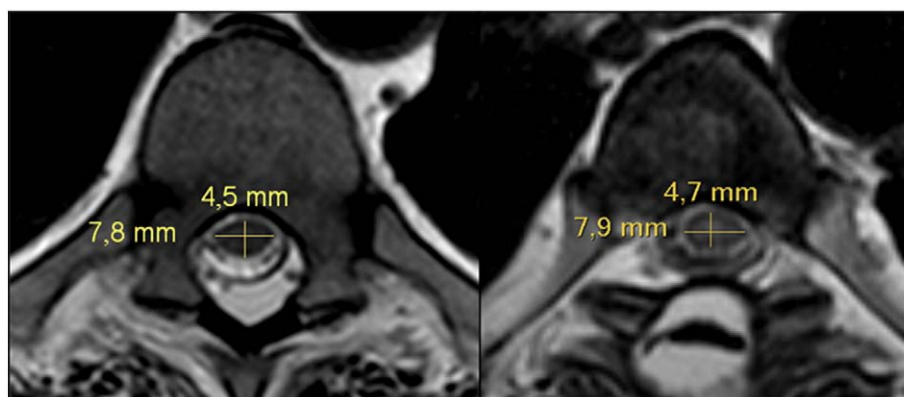


Fig. 3. SC diameter calculation at the site of implant. TSE T2 weighted images acquired before (left image) and 14 days after surgery (right image). SC diameters show a slight increase at the site of the graft after stem cell implant.

**Table 6**

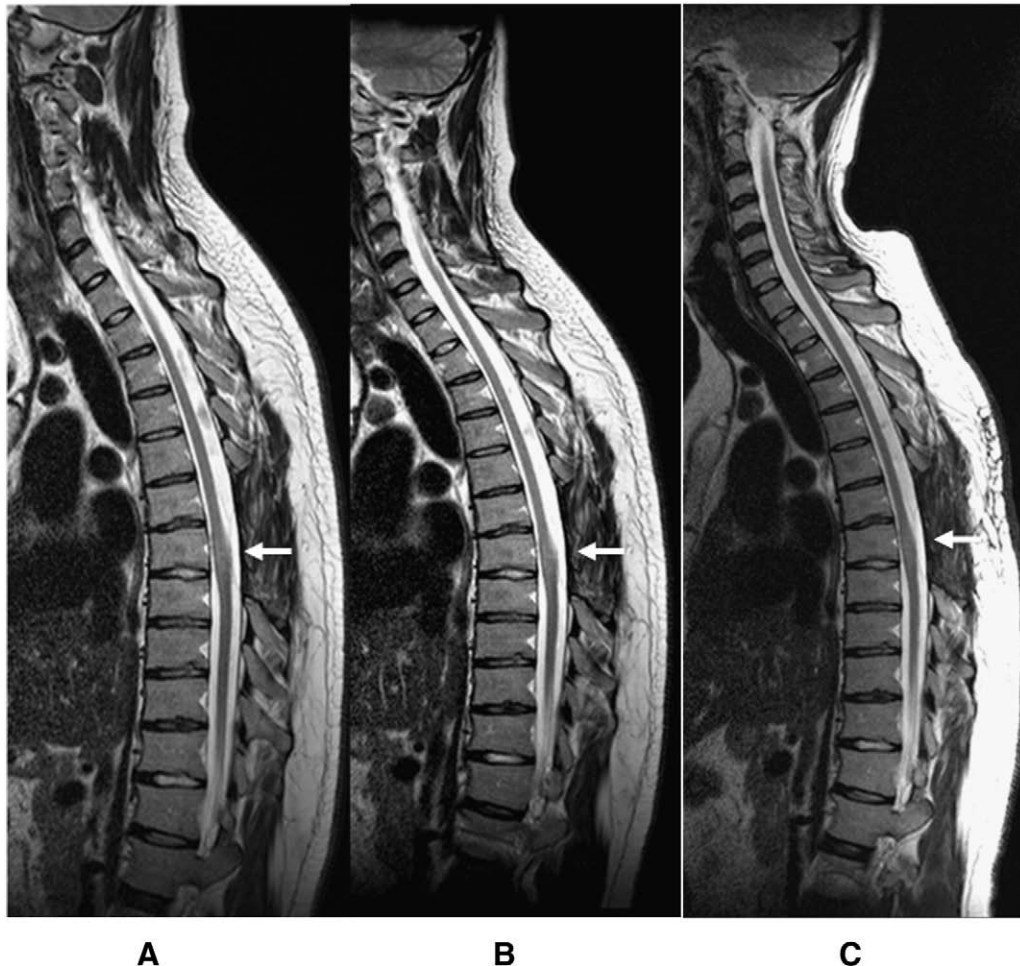
Patient	Basal	15 days	3 months	6 months	9 months	12 months	18 months
<i>(a) Spinal cord diameter (axial plane) at the level of engraftment antero-posterior width (mm)</i>							
1	5.00	5.10	7.15	7.09	7.04	6.95	6.98
2	7.81	8.51	9.81	9.73	9.76	9.75	9.78
3	4.50	4.70	9.08	9.79	10.21	10.58	10.61
4	5.11	7.01					
5	5.70	6.15	7.91	7.19	7.11	5.96	6.01
6	6.00	6.25	9.00	9.23	9.43	10.15	10.01
7	5.20	6.01	5.57	6.15	6.05	5.85	6.01
8	5.65	5.79	5.83	6.20	6.15	6.03	6.19
9	5.25	5.42	5.56	6.15			
10	5.12	6.01	5.98	6.66	6.31	6.92	6.35
<i>(b) Spinal cord diameter (axial plane) at the level of engraftment width (mm)</i>							
1	5.20	5.22	7.04	7.12	7.10	7.17	7.13
2	8.10	7.91	6.01	6.01	6.07	6.09	6.25
3	7.80	7.90	7.95	6.12	6.08	6.00	6.30
4	8.50	9.05					
5	7.01	8.12	9.10	6.85	7.09	7.11	7.01
6	9.00	8.41	7.75	7.80	7.54	6.97	6.30
7	9.08	7.22	7.15	7.61	7.71	7.95	8.05
8	8.10	10.37	7.75	8.91	8.15	7.33	7.85
9	7.09	8.52	8.93	9.12			
10	7.15	9.11	6.01	6.16	6.11	5.82	6.01

SEIQoL-DW scores improved after recruitment throughout the 6-month lead-in phase in all patients and they declined linearly during the whole post-treatment follow-up period in 5 patients while in the

other 5 patients a trend toward stabilization after transplantation was performed (Fig. 2). The patients did not differ as regards clinical variables but their personality profiles at the basal MMPI-2 showed some critical aspects in the patients with a progressive decline of SEIQoL-DW scores. The same patients manifested higher levels of mood disturbance during the follow-up period as measured by POMS particularly in depression and tension scores.

#### MRI results

There were no post-procedural complications. In particular, post-surgical MR scans revealed no pathologic intradural fluid collection, SC or brain post-implant neoformations, or pathological intramedullary contrast enhancement at the site of the graft. Serial MRI showed no structural changes (including tumor or syrinx formation) within the brain and the spinal cord after MSC transplantation relative to the baseline. A slight segmental increase of the spinal cord volume with no contrast enhancement after gadolinium injection at the injection site of the cells (Fig. 3) was observed in all patients after surgery. Table 6 reports the axial measures of AP and RL diameter. Measurements of the AP diameter of the spinal cord of all cases were increased by up to two and a half-fold above the baseline measurement at all post-operative time points. Measurements of the RL width of the spinal cord of 7/10 cases were increased at 15 days after surgery and at later post-operative time points changes from baseline measurements were more variable. MRI confirmed the existence of an area of hyperintensity on T2-weighted images at the graft site in all patients which was



**Fig. 4.** TSE T2-weighted sequences acquired on sagittal plane 3 (image A), 6 (image B) and 12 (image C) months after stem cell implant. Intradural signal hyperintensity at the site of the graft is shown (white arrow). Long-term findings show a slight reduction of SC hyperintensity (white arrow).

probably due to the MSC suspension. In the long term (3, 6, 9, 12 months) T2 relaxivity relative values measured in ROIs showed a progressive reduction of SC hyperintensity (Fig. 4). In 4 of the cases the spinal cord was tethered anteriorly and posteriorly by post-operative scarring, producing marked distortion of the cord due to traction (Fig. 5). In the other 6 cases the cord retained a normal shape (Fig. 6).

A trend toward a decrease of FA and a parallel increase of ADC was evident in the early–mid period of the follow-up after transplantation which rose back to pre-treatment values in the late period. Fig. 7 shows the data recorded in a patient with long-term follow-up.

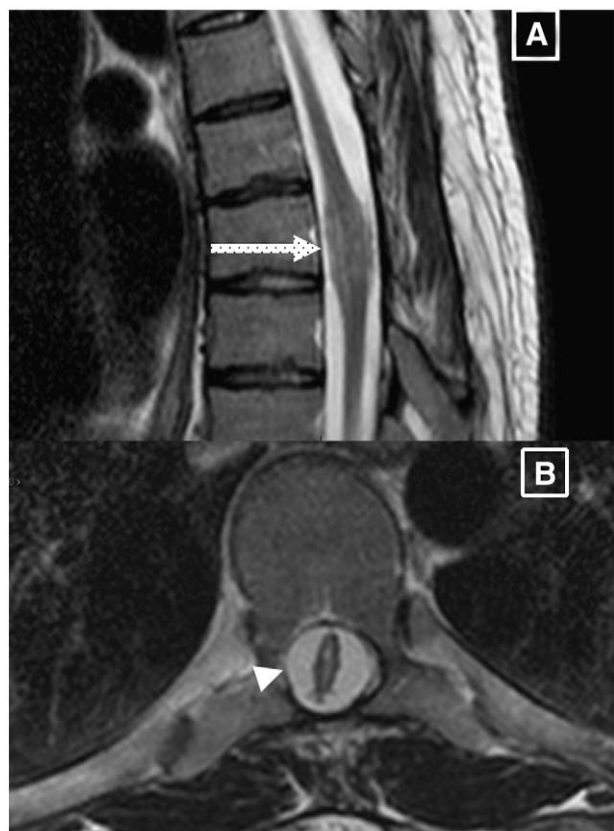
## Discussion

Our study represents the first demonstration of the safety of MSC use after focal transplantation in the central nervous system. The use of stem cells for therapy requires that they can easily access the target tissue to exert their therapeutic effect as the cells respond to a particular pathological microenvironment (Liu et al., 2006). *In situ* administration can directly achieve this goal. Most of the successful clinical outcomes with both neuronal and non-neuronal stem cells in ALS animal models have been achieved by direct intraspinal implantation (Garbuzova-Davis et al., 2001, 2002; Hemendinger et al., 2005; Deshpande et al., 2006; Corti et al., 2007). Therefore the results of our study represent an opening point for future studies in neurodegenerative diseases.

Extensive *in vitro* expansion of ALS patients' MSCs does not involve any functional modification of the cells, including chromosomal



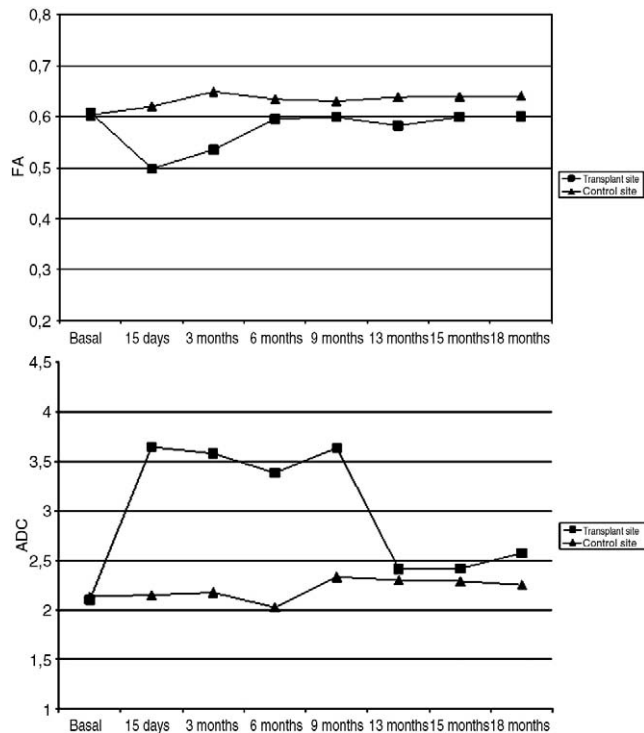
**Fig. 6.** (A) T2-weighted acquisition in the sagittal plane, showing a regular shape and no tumefactive aspect at 12 months control (white arrow); (B) T2-weighted acquisition in the axial plane (same patient and same time of exam), showing the spinal cord morphology at the site of the implant (see posterior surgical breach), without evidence of distortion (white arrowhead).



**Fig. 5.** (A) T2-weighted acquisition in the sagittal plane, showing a pseudo-tumefactive shape (white arrow) of the spinal cord at 12 months control; (B) T2-weighted acquisition in the axial plane (same patient and same time of exam), shows the band-like morphology (white arrowhead) distortion responsible of the sagittal appearance without evidence of spinal cord tumefaction or swelling at the site of the implant (see posterior surgical breach).

aberrations or cellular senescence (Ferrero et al., 2008). Analyzing MSC expansion potential before performing BM collection allowed us to optimize the *in vitro* manipulation protocol and to obtain a high number of viable, safe and functional MSCs. After *in vitro* expansion MSCs showed no bacterial, fungal, mycoplasma or endotoxin contamination and showed an elongated, fibroblastic shape and the expression of specific surface markers, as defined by the minimal criteria of human MSCs (Dominici et al., 2006). Though the possibility that MSCs undergo malignant transformation after *in vitro* expansion has been raised by some authors (Rubio et al., 2005; Miura et al., 2006; Houghton et al., 2004; Tolar et al., 2007), no telomere shortening or chromosomal alteration was noted in our patients' expanded MSCs. In a previous report we found that ALS does not affect MSCs, since the expansion and differentiating potential are the same as the donors' (Ferrero, 2008). This consideration tallies with Bernardo et al. (2007) reporting no chromosomal abnormalities and telomerase activity in human BM-MSCs after *in vitro* expansion up to the 15th passage. The susceptibility to the previously described malignant transformation might be due to the different origin of the cells or to the different MSC expansion conditions.

No structural changes of the parenchyma, tumor formation or development of syringomyelia have been detected by MRI in either the short or the long term. Axial measurements showed an increase in AP diameter but a decrease of the right-left diameter, maintaining, on the whole, the same volume. The opposite behavior of AP and RL diameters particularly evident in 4 patients may be explained by a tent-like triangular shape or "ribbon" acquisition of morphology. This modification was not evident immediately after surgery but 3 months later and it was ascribed to posterior spinal canal surgical fibrotic changes. The lack of spinal cord damage evidence in the morphologic



**Fig. 7.** DTI with FiberTracking. Measurements in a patient with long term follow-up (transplanted vs. non transplanted-control site). The temporal profile of FA (upper panel) decreases after transplantation and progressively rise back to pre-treatment value in the late period. ADC (lower panel) parallel increases in the early–mid period of the follow-up after transplantation, to rise back to pre-treatment value in the late period.

Spin Echo T1 and T2-weighted sagittal and axial images and the transient decrease of FA and increase of ADC seem to suggest an “edema-like” state that does not behave as damage, that goes back to “normal” values.

Although no severe morbidity was associated with the surgical procedure, the technique can be improved to reduce the side effects and to facilitate MSC transplantation in the critical regions of the spinal cord. To maximize the safety of direct parenchymal injection, a microinjection platform has recently been standardized and the targeting of the ventral horn was undertaken with the aid of microelectrode recording (Riley et al., 2008).

Although the informed consent requested, in the event of death, a partial or complete autopsy be carried out, the authorizations were not obtained by the relatives. This is a very delicate issue. We are conscious of the importance of having access to postmortem material to obtain more information about the morphological modifications in the spinal cord and the vitality of the cells after transplantation. However, we must consider the cultural and familial sensitivities that greatly influence this choice.

Stem cell trials represent a new scenario in ALS clinical research. Our study is the first clinical trial with MSCs transplantation and the first surgical trial in ALS.

We have demonstrated that the concerns regarding possible additional lesions of the spinal cord, at least at the thoracic level, that might be caused by the surgical procedure or the severe complications after general anesthesia are, apparently, not legitimate.

Given the novelty and unpredictability of our clinical research and the great hopes of patients, we placed great importance on minimizing the risks for all possible adverse events associated with MSCs transplantation and on analyzing the psychological status and the behavioral changes in addition to the usual follow-up. Institutional supervision by the scientific committee of the National Institute of Health and Ethic Committees ensured the study was carried out

with the highest possible rigor and integrity. All patients manifested their satisfaction at having participated in the study because the feeling anticipated possible benefits for their own health and that it might help other patients in the future.

In most patients we detected no significant changes of the progression of the disease in the post-transplantation period. Only in patients 1 and 2 did we find a trend toward a slower deterioration of FVC and upper limb MRC in the 12 months after transplant rather than in the 9 month pre-treatment period. However, these data are due to the younger age of these patients (Beghi et al., 2006). These results are apparently different from those obtained in our previous study (Mazzini et al., 2003, 2006, 2008) showing a trend toward a slowing down of the progression of the disease after transplantation and prolonged survival in 5/9 patients. Although inclusion criteria were slightly different, no procedural variations distinguished the 2 studies. In the first study patients already showed a severe disability at the time of recruitment. Moreover, clinical and neurophysiological assessment in the long-term follow-up shows a predominant lower motor neuron involvement in 3/5 patients with better prognosis. Considering the location, severity, and clinical form of the disease, the selection of ALS patients who should undergo transplantation still represents the main open question. Patients with a spinal form of the disease with predominant lower motor neuron involvement or patients with focal MND variants might benefit most from MSC transplantation.

The lack of a clear advantage in the clinical course of the disease in this study can also be explained by the site of MSC transplantation. Because of the low iatrogenic risk of surgery, we chose to inject stem cells at the thoracic levels, even though these segments of the spinal cord are not involved in crucial functions such as the respiratory capacity or limb functions. Moreover, quantitative measures to monitor the progression of the disease at this level are not reliable.

Because we can hypothesize a mainly focal activity of the cells in the transplanted segments and as this study demonstrates good safety and feasibility, new protocols involving MSCs transplantation in the cervical segments of the spinal cord should be planned. Recently, autologous BM-derived hematopoietic stem cell transplantation in C1–C2 level has been shown to be safe and probably effective for ALS patients (Deda et al., 2009).

Another main point that needs to be addressed in future studies concerns the optimal number of cells that must be injected. At the beginning of the trial, patients were selected on the base of the proliferative potential of MSCs isolated from the marrow aspirate and the optimal number of  $110 \times 10^6$  cells were established. However during the treatment phase after bone marrow collection different numbers of cells were obtained in each subject. This variability has been described also in normal subjects and on samples obtained at different times from the same donor (Phinney et al., 1999). Despite there being differences according to the patients' ages (Mareschi, 2006), only one patient received fewer than  $15 \times 10^6$  cells. We choose to inject different amounts of cells because it was quite unreliable to determine the optimal dose in animals and transpose it to humans. Moreover, a single dose might miss adverse events that might emerge in later trials or large effects. We have found no correlation between the number of cells and the incidence and severity of the side effects or the outcome.

It is very important for the application and development of stem cell therapeutic approaches to develop non-invasive methods to monitor the modifications of cerebral and spinal cord tissues. Methods for labeling cells with ferromagnetic particles while being useful for MRI evaluation, are not currently approved for clinical use. We hope to obtain a better understanding of the mechanisms of the action of MSCs from ongoing neuro-imaging studies (diffusion tensor imaging).

In conclusion, the results of this study confirm, as previously reported (Mazzini et al., 2003, 2006, 2008, 2009), that MSC transplantation

into ALS patients' thoracic spinal cord is safe and that MSCs represent a good source of stem cells for future ALS cell-based clinical trials. Moreover, the active participation of the Italian institute of Health and ethics committees in the study design guaranteed adherence to the protocol and the strict control of the patient's status also in the long term.

Questions related to the optimal number of cells to engraft, the location and the selection of patients are still open. However, we can hypothesize that transplantation in critical regions of the spinal cord involved in crucial functions such as the respiratory capacity or controlling limb movements might represent the best target for future Phase II clinical trials.

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### References

- Bacigalupo, A., Tong, J., Podesta, M., Piaggio, G., Figari, O., Colombo, P., et al., 1992. Bone marrow harvest for marrow transplantation: effect of multiple small (2 ml) or large (20 ml) aspirates. *Bone Marrow Transplant.* 9 (6), 467–470 (Jun).
- Badayan, I., Cudkovic, M.E., 2008. Is it too soon for mesenchymal stem cell trials in people with ALS? *Amyotroph. Lateral Scler.* 9 (6), 321–322 (Review).
- Beghi, E., Logroscino, G., Chiò, A., Hardiman, O., Mitchell, D., Swinger, R., et al., 2006. The epidemiology of ALS and the role of population-based registries. *Biochim. Biophys. Acta* 1762 (11–12), 1150–1157.
- Bernardo, M.E., Zaffaroni, N., Novara, F., Cometa, A.M., Avanzini, M.A., Moretta, A., et al., 2007. Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term *in vitro* culture and do not exhibit telomere maintenance mechanisms. *Cancer Res.* 1:67 (19), 9142–9149.
- Boillée, S., Yamanaka, K., Lobsiger, C.S., Copeland, N.G., Jenkins, N.A., Kassiotis, G., et al., 2006. Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* 2:312 (5778), 1389–1392.
- Brooks, B.R., Miller, R.G., Swash, M., Munsat, T.L., 2000. World Federation of Neurology Research Group on Motor Neuron Diseases. El Escorial revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph. Lateral Scler. Other Mot. Neuron. Disord.* 1, 293–299.
- Chen, Y., Shao, J.Z., Xiang, L.X., Dong, X.J., Zhang, G.R., 2008. Mesenchymal stem cells: a promising candidate in regenerative medicine. *Int. J. Biochem. Cell Biol.* 40 (5), 815–820.
- Clarke, S., Hickey, A., O'Boyle, C., Hardiman, O., 2001. Assessing individual quality of life in amyotrophic lateral sclerosis. *Qual. Life Res.* 10, 149–158.
- Corti, S., Locatelli, F., Donadoni, C., Guglieri, M., Papadimitriou, D., Strazzer, S., et al., 2004. Wild-type bone marrow cells ameliorate the phenotype of SOD1-G93A ALS mice and contribute to CNS, heart and skeletal muscle tissues. *Brain* 127, 2518–2532.
- Corti, S., Locatelli, F., Papadimitriou, D., Del Bo, R., Nizzardo, M., Nardini, M., et al., 2007. Neural stem cells LewisX+ CXCR4+ modify disease progression in an amyotrophic lateral sclerosis model. *Brain* 130, 1289–1305.
- de Carvalho, M., Scotto, M., Lopes, A., Swash, M., 2003. Clinical and neurophysiological evaluation of progression in amyotrophic lateral sclerosis. *Muscle Nerve* 28 (5), 630–633.
- Deda, H., Inci, M.C., Kurekci, A.E., Sav, A., Kayihan, K., Ozgün, E., 2009. Treatment of amyotrophic lateral sclerosis patients by autologous bone marrow-derived hematopoietic stem cell transplantation: a 1-year follow-up. *Cytotherapy* 11 (1), 18–25.
- Deshpande, D.M., Kim, Y.S., Martinez, T., Carmen, J., Dike, S., Shats, I., et al., 2006. Recovery from paralysis in adult rats using embryonic stem cells. *Ann. Neurol.* 60 (1), 32–44.
- Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., et al., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8, 315–317.
- European Medicines Agency, 1999. The rules governing medical products in European Union. *Good Manufacturing Practices* 4.
- Ferrero, I., Mazzini, L., Rustichelli, D., Gunetti, M., Mareschi, K., Testa, L., et al., 2008. Mesenchymal stem cells from healthy donors and sporadic amyotrophic lateral sclerosis patients. *Cell Transplant.* 17, 255–266.
- Garbuzova-Davis, S., Willing, A.E., Milliken, M., Saporta, S., Sowerby, B., Cahill, D.W., et al., 2001. Intraspinal implantation of hNT neurons into SOD1 mice with apparent motor deficit. *Amyotroph. Lateral Scler. Other Mot. Neuron. Disord.* 2 (4), 175–180.
- Garbuzova-Davis, S., Willing, A.E., Milliken, M., Saporta, S., Zigova, T., Cahill, D.W., et al., 2002. Positive effect of transplantation of hNT neurons (NTERA 2/D1 cell-line) in a model of familial amyotrophic lateral sclerosis. *Exp. Neurol.* 174 (2), 169–180.
- Garbuzova-Davis, S., Willing, A.E., Saporta, S., Bickford, P.C., Gemma, C., Chen, N., et al., 2006. Novel cell therapy approaches for brain repair. *Prog. Brain Res.* 157, 207–222. *J. Cell Physiol.* 211 (1), 27–35.
- Hemendinger, R., Wang, J., Malik, S., Persinski, R., Copeland, J., Emerich, D., et al., 2005. Sertoli cells improve survival of motor neurons in SOD1 transgenic mice, a model of amyotrophic lateral sclerosis. *Exp. Neurol.* 196 (2), 235–243.
- Henkel, J.S., Engelhardt, J.L., Siklós, L., Simpson, K., Houkin, K., Kim, S.H., Pan, T., et al., 2004. Presence of dendritic cells, MCP-1, and activated microglia/macrophages in amyotrophic lateral sclerosis spinal cord tissue. *Ann. Neurol.* 55 (2), 221–235.
- Houghton, J., Stoicov, C., Nomura, S., Rogers, A.B., Carlson, J., Li, H., et al., 2004. Gastric cancer originating from bone marrow-derived cells. *Science* 26:306 (5701), 1568–1571.
- Lepore, A.C., Rauck, B., Dejea, C., Pardo, A.C., Rao, M.S., Rothstein, J.D., et al., 2008. Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. *Nat. Neurosci.* 11 (11), 1294–1301.
- Liu, H., Honmou, O., Harada, K., Nakamura, K., Houkin, K., Hamada, H., et al., 2006. Neuroprotection by PlGF gene-modified human mesenchymal stem cells after cerebral ischaemia. *Brain* 29 (Pt. 10), 2734–2745.
- Mazzini, L., Fagioli, F., Boccaletti, R., Mareschi, K., Oliveri, G., Olivieri, C., et al., 2003. Stem cell therapy in amyotrophic lateral sclerosis: a methodological approach in humans. *Amyotroph. Lateral Scler. Other Mot. Neuron. Disord.* 4 (3), 158–161.
- Mazzini, L., Mareschi, K., Ferrero, I., Vassallo, E., Oliveri, G., Boccaletti, R., et al., 2006. Autologous mesenchymal stem cells: clinical applications in amyotrophic lateral sclerosis. *Neurol. Res.* 28 (5), 523–526.
- Mazzini, L., Mareschi, K., Ferrero, I., Vassallo, E., Oliveri, G., Nasuelli, N., et al., 2008. Stem cell treatment in amyotrophic lateral sclerosis. *J. Neurol. Sci.* 15:265 (1–2), 78–83.
- Mazzini, L., Vercelli, A., Mareschi, K., Ferrero, I., Testa, L., Fagioli, F., 2009. Mesenchymal stem cells for ALS patients. *Amyotroph. Lateral Scler.* 10 (2), 123–124.
- McNair, D.M., Lorr, M., Droppleman, L.F., 1992. Profile of mood state manual. Educational and Industrial Testing Service, San Diego.
- Miura, Y., Gao, Z., Miura, M., Seo, B.M., Sonoyama, W., Chen, W., et al., 2006. Mesenchymal stem cell-organized bone marrow elements: an alternative hematopoietic progenitor resource. *Stem Cells* 24 (11), 2428–2436.
- Phinney, D.G., Kopen, G., Righter, W., Webster, S., Tremain, N., Prockop, D.J., 1999. Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. *J. Cell. Biochem.* 75, 424–436.
- Riley, J., Butler, J., Baker, K.B., McClelland III, S., Teng, Q., Yang, J., et al., 2008. Targeted spinal cord therapeutics delivery: stabilized platform and microelectrode recording guidance validation. *Stereotact. Funct. Neurosurg.* 86, 67–74.
- Rowland, L.P., Shneider, N.A., 2001. Amyotrophic lateral sclerosis. *N. Engl. J. Med.* 31:344 (22), 1688–1700.
- Rubio, D., Garcia-Castro, J., Martín, M.C., de la Fuente, R., Cigudosa, J.C., Lloyd, A.C., et al., 2005. Spontaneous human adult stem cell transformation. *Cancer Res.* 15:65 (8), 3035–3039.
- Shefner, J.M., Cudkovic, M.E., Zhang, H., Schoenfeld, D., Jilapalli, D., 2004. Northeast ALS Consortium. The use of statistical MUNE in a multicenter clinical trial. *Muscle Nerve* 30 (4), 463–469.
- Suzuki, M., Svendsen, C.N., 2008. Combining growth factor and stem cell therapy for amyotrophic lateral sclerosis. *Trends Neurosci.* 31, 192–198.
- Svendsen, C.N., Langston, J.W., 2004. Stem cells for Parkinson disease and ALS: replacement or protection? *Nat. Med.* 1, 224–225.
- The ALS-CNTF treatment Study (ACTS) Phase I-II study group, 1996. The amyotrophic lateral sclerosis functional rating scale: assessment of activities of daily living in patients with amyotrophic lateral sclerosis. *Arch. Neurol.* 53, 141–147.
- Tolar, J., Nauta, A.J., Osborn, M.J., Panoskaltis Mortari, A., McElmurry, R.T., Bell, S., et al., 2007. Sarcoma derived from cultured mesenchymal stem cells. *Stem Cells* 25, 371–379.
- Uccelli, A., Pistoia, V., Moretta, L., 2007. Mesenchymal stem cells: a new strategy for immunosuppression? *Trends Immunol.* 28 (5), 219–226.
- Uccelli, A., Moretta, L., Pistoia, V., 2008. Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.* 8, 726–736.
- Vercelli, A., Mereuta, O.M., Garbossa, D., Muraca, G., Mareschi, K., Rustichelli, D., et al., 2008. Human mesenchymal stem cell transplantation extends survival, improves motor performance and decreases neuroinflammation in mouse model of amyotrophic lateral sclerosis. *Neurobiol. Dis.* 31 (3), 395–405.
- Yamanaka, K., Chun, S.J., Boillee, S., Fujimori-Tonou, N., Yamashita, H., Gutmann, D.H., et al., 2008. Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat. Neurosci.* 11 (3), 251–253.