

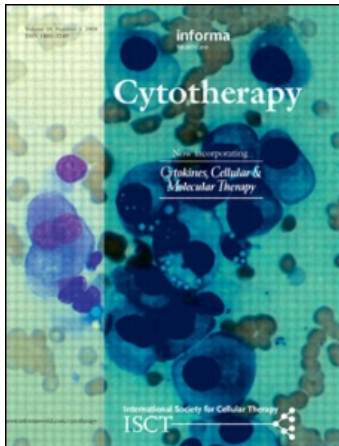
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Stem-cell transplantation into the frontal motor cortex in amyotrophic lateral sclerosis patients

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Background aims

Amyotrophic lateral sclerosis (ALS) is characterized by the selective death of motor neurons. CD133⁺ stem cells are known to have the capacity to differentiate into neural lineages. Stem cells may provide an alternative treatment for ALS and other neurodegenerative diseases.

Methods

Five men and five women (aged 38–62 years) with confirmed ALS were included in this study. Our institutional ethics and research committees approved the protocol. After informed consent was obtained, patients underwent Hydrogen-Magnetic Resonance Imaging (H-MRI) spectroscopy and were given scores according to an ALS functional rating scale, Medical Research Council power muscle scale and daily living activities. Bone marrow was stimulated with 300 µg filgrastim subcutaneously daily for 3 days. Peripheral blood mononuclear cells were obtained after admission by leukapheresis. The cell suspension was conjugated with anti-human CD133 superparamagnetic microbeads, and linked cells were isolated in a magnetic field. The isolated cells (2.5–7.5 × 10⁵) were resuspended in 300 µL of the patient's

cerebrospinal fluid, and implanted in motor cortexes using a Hamilton syringe. Ten patients with confirmed ALS without transplantation were used as a control group. Patients were followed up for a period of 1 year.

Results

The autologous transplantation of CD133⁺ stem cells into the frontal motor cortex is a safe and well-tolerated procedure in ALS patients. The survival of treated patients was statistically higher (P= 0.01) than untreated control patients.

Conclusions

Stem-cell transplantation in the motor cortex delays ALS progression and improves quality of life.

Keywords

Amyotrophic lateral sclerosis, autologous transplant, CD133, neurodegenerative disorders, stem cells.

Introduction

Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative disorder characterized by rapid deterioration and selective death of motor neurons in the cerebral cortex, brain stem and spinal cord [1–3]. A wide variety of clinical features are present early in the course of ALS [4]. There is no specific diagnostic test, although a clinical diagnosis of ALS is probably correct in more than 95% of cases [5]. Sometimes it is difficult to separate ALS from other motor neuron diseases, such as Kennedy's disease, monomelic

amyotrophy, X-linked spinobulbar muscular atrophy and cervical spondylotic myelopathy [4,5]. Formal criteria are used for clinical trials but these may be too restrictive, as some patients die of ALS without qualifying for a therapeutic trial. The median survival of ALS patients is usually 29.1 months after presentation [6] and, despite exciting advances in understanding the molecular basis of ALS, the etiology of sporadic cases remains unexplained [4].

There is only one medication approved by the FDA to treat ALS. However, this drug is expensive and only delays

disease progression modestly [4]. Stem-cell therapy is considered to be an alternative method for treating ALS and other neurodegenerative disorders [7–10]. Stem-cell transplantation is a potential therapeutic strategy not only via cell replacement but also by modification of the extracellular motor neuronal environment, through a trophic and neuroprotective effect [11]. A variety of cell sources have been considered for cell therapy, including neural stem cells, embryonic stem cell-derived neurons and somatic stem cells, such as hematopoietic stem cells [12–15]. In SOD1 transgenic mice, an experimental model that resembles ALS, the transplantation of hematopoietic stem cells from murine bone marrow (BM) or from human cord blood cells increased the animals' lifespan [13]. However, at present, no animal model reproduces all of the salient features of ALS, particularly the involvement of corticospinal and corticobulbar tracts [13–15]. CD133 is a stem-cell glycoprotein antigen selectively expressed on progenitor cells [16,17]. These cells have been isolated from different sources, such as BM, peripheral blood and umbilical cord, and are known to have the capacity to differentiate into several lineages, including cardiac, endothelial, hepatic and neural lineages [12–19]. It has been demonstrated by several groups that stem cells isolated from human BM can be induced to differentiate into neurons. Reports indicate that these differentiated cells express nestin, neuron-specific enolase, neuron-specific nuclear protein, glial fibrillary acidic protein, neurofilaments, Tau, Nurr1 and neuron-specific tubulin-1 [20–24]. An additional report has described these cells displaying synaptic transmission capabilities [25]. Finally, *in vivo* models in rodents have demonstrated that systemic transplantation of human BM-derived CD133⁺ cells displays migration, engraftment and improvement of neurologic deficits [26,27].

Clinical studies using stem cells in humans have been described for Huntington's disease, Parkinson's disease, complete spinal cord injury, stroke patients and Batten's disease [28–32]. Recently, a method of intraspinal cord implantation of autologous hematopoietic stem cells in patients with confirmed ALS was described, and the authors reported this method as safe [10]. Another study has assessed the safety and therapeutic efficacy of autologous human BM stromal cell transplantation in 35 complete spinal cord injury patients [30–32]. To assess both safety and efficacy, we have performed an uncontrolled, open-label non-randomized clinical trial using

autologous CD133⁺ stem-cell transplantation into the frontal motor cortex of confirmed ALS patients. The scientific rationale for our clinical research was that CD133⁺ stem cells obtained from peripheral blood are capable of differentiating into neurons, and their transplantation into the frontal motor cortex was aimed at improving upper motor neuron function.

Methods

Study subjects

All patients were recruited and evaluated for eligibility at the neurology service of the Hospital Universitario UANL, Monterrey, Mexico, and the Neurosciences Center of the Hospital San Jose Tec de Monterrey, Monterrey, Mexico, from June 2005 to December 2006. The ethics and research committees of the Hospital San Jose Tec de Monterrey and the Tecnológico de Monterrey School of Medicine, Monterrey, Mexico, approved the protocol, and all the participating patients signed an informed consent form. A trained neurologist conducted examinations for the diagnosis of ALS, which was made using the well-established El Escorial criteria [33,34]. Patients who had signed the consent form and who had had a definite diagnosis of ALS for more than 1 year and less than 4 years were enrolled in the study. Patients with a current, or past, history of neurologic disease other than ALS, and those enrolled in other clinical trials, were excluded. The approval by ethics and research committees was set strictly at 10 patients initially, and they suggested close monitoring of the safety of the procedure. The follow-up period in the study for ALS cases and controls ended in February 2008.

The inclusion criteria for patients were: (a) confirmed ALS according to the El Escorial clinical and neurophysiologic criteria; (b) cervical and cranial magnetic resonance imaging (MRI) excluding structural damage to the brain and spinal cord; (c) a functional respiratory test showing the occurrence of forced vital capacity (FVC) values; and (d) an appropriate nutritional state (above 20%). Exclusion criteria were: (a) severe bulbar involvement; (b) an inadequate nutritional state; (c) tracheostomy or gastrostomy; (d) the presence of systemic disorders, such as malignant neoplasm, cardiovascular disease, previous stroke or coagulation abnormalities; and (e) evidence of cervical spondylotic myelopathy or structural abnormalities by MRI.

Confirmed ALS patients complying with the inclusion criteria who did not accept treatment, or who applied after

the study number had been met, served as control ALS subjects. These patients signed an informed consent form, remained with their current medical treatment (riluzole and antioxidants) and were followed up by direct examination, clinical information obtained from their neurologist, or by telephone conversation with themselves or their caregiver. For these control ALS subjects, detailed information concerning their progression, medication and treatment, and percutaneous endoscopic gastrostomy and tracheostomy were collected. The date and cause of death were registered where appropriate. All the patients included were examined at baseline and in follow-up visits by a neurologist and a clinical evaluator who had experience with both ALS patients and neuromuscular disorders. Each examination consisted of testing for muscle stretch reflexes, pathologic reflexes, muscle tone, full manual muscle testing in four limbs, as well as the Medical Research Council (MRC) scale for grading muscle power and strength [35]. The ALS Functional Rating Scale Revised (ALSFRS-R) [36], which is the most widely used and extensively validated global scale for assessing motor function in ALS, and the Spitzer quality of life (QOL) scale were used by a clinical evaluator at each visit. The entire clinical evaluation lasted 30–40 min and was performed at baseline and at 1, 3, 6, 9 and 12 months after surgery. All control and ALS patients with stem-cell transplants were assessed six times, and the treatment group patients remained in follow-up for more than 1 year.

Stem-cell preparation

After informed consent was obtained, patients in the treatment group received a subcutaneous daily dose of 300 µg filgrastim (Neupogen, Basel, Switzerland) for a period of 3 days. This drug, a human granulocyte colony-stimulating factor (G-CSF) obtained by recombinant DNA technology, acts on hematopoietic cells by binding to specific cell surface receptors, stimulating proliferation, differentiation and some end-cell functional activation. Absolute monocyte and lymphocyte counts have been reported to increase in both patients and normal subjects receiving G-CSF [37,38]. The day following the final G-CSF dose, patients were admitted to hospital and a white blood cell count was obtained. Peripheral blood mononuclear cells were isolated by leukapheresis (Baxter CS3000+, Deerfield, IL, USA; or Haemonetics MCS, Braintree, MA, USA). A 2-mL sample of cerebrospinal fluid (CSF) was also obtained by lumbar puncture after the

apheresis procedure. The cells obtained were washed three times with phosphate-buffered saline (PBS). The CD133 immunoreactive cells in the cell suspension were conjugated with anti-human CD133 superparamagnetic microbeads and isolated in a magnetic field over a MiniMACS separation column (Miltenyi Biotech, Gladbach, Germany). Enrichment of CD133⁺ cells on a patient's samples was confirmed by fluorescence-activated cell sorting (FACS). The cells were counted in a Beckman Z2 Coulter Counter (Fullerton, CA, USA) and $2.5\text{--}7.5 \times 10^5$ cells were suspended in 0.3 mL autologous CSF and dispensed in sterile tubes.

Surgery

To avoid respiratory complications, the procedure was performed while patients were awake, under mild sedation and local anesthesia. The stem cells were transplanted bilaterally by stereotaxy or neuronavigation guidance into the frontal motor cortex. Based on MRI or computed tomography (CT) scan, the motor cortex strip was identified and the target was defined 3–4 cm from the mid-line. Setting of stereotactic co-ordinates with a Leksell G frame (Elekta AB, Stockholm, Sweden) or identification of the area on the navigation system (VectorVision 2, BrainLab AG, Munich, Germany) determined the place where bur holes were to be made. After dura incision, the suspension of $2.5\text{--}7.5 \times 10^5$ CD133⁺ stem cells in CSF was injected 7 mm in depth into the cortex using a Hamilton syringe, held by a mechanical arm to maintain stability during the procedure. Vascular structures and subarachnoid spaces were avoided. The patients were discharged the following day. Compliance and adverse effects were monitored throughout the study period

Data analysis

All statistical analyzes were performed using the SPSS 16.0 software package (SPSS, Chicago, IL, USA). Univariate comparison of demographic and clinical variables used either the *t*-test or Mann–Whitney *U*-test. Survival curves were estimated using the Kaplan–Meier method, and the differences in survival were measured by the log rank test. Patients who underwent tracheostomy or gastrostomy were coded as deceased on the date of the procedure. Multivariate analyzes of the risk for death associated with selected independent variables were performed using the Cox proportional hazards model. The following variables were considered in the analysis: age, gender, time interval

from diagnosis to baseline and diagnosis to death and, in the treatment group, the number of stem cells transplanted. Survival time was measured from time of diagnosis because the classification of patients based on the El Escorial criteria was performed on the date of the diagnosis [33,34]. The primary endpoint of this study was the survival rate. The secondary outcome measured changes in global function, as scored by the ALSFRS-R (normal score = 48). The Friedman test was performed in the QOL evaluation of transplanted patients after diagnosis and during the follow-up period. Differences among groups (for example age and ALSFRS-R score at several time intervals) were analyzed using the Mann–Whitney *U*-test and the Wilcoxon signed rank test. Significance was tested at the 5% level. The χ^2 method was used to compare cell number against ALSFRS-R score in the treatment group.

Results

Thirty-two ALS patients were evaluated during the 19-month surveillance period (17 men and 15 women). Fifty-five per cent of patients attended the Neurology ALS Clinic at the Hospital Universitario and the Neuroscience Center at the Hospital San Jose Tec de Monterrey. Neurologists from abroad referred the remaining 45% of patients. No familial ALS cases were present. Twenty-three ALS patients fulfilled the inclusion criteria. Ten cases with confirmed ALS were included in the treatment group, and 13 patients with confirmed ALS continued their current treatment with riluzole, vitamins, antioxidants and physical therapy and served as the control group. The control and transplanted ALS patients were followed for a period of 1 year. Three control ALS patients did not complete the follow-up.

The demographic and clinical characteristics of the Hispanic ALS patients are shown in Table I. The median age at diagnosis was 48.8 years (range 31–70 years) in both groups. There were no differences in mean age and sex between the groups ($P = 0.35$). The time from diagnosis to baseline was longer in the treatment group (30.1 months) compared with the control ALS patients (14.3 months). Sixty per cent of the included ALS cases were of spinal onset, while 40% were of bulbar onset. For both groups, and at baseline, the upper motor neurons (UMN) were more affected than the lower motor neurons (LMN) in 50% of the cases. In the remaining 50% of cases, the LMN were more affected than the UMN (40%), with only 10% of these patients affected equally.

In the group receiving transplanted cells, two patients died during the 1-year follow-up period. One patient died 10 days after surgery because of myocardial infarction, and the other died 6 months later because of respiratory insufficiency. No tracheostomy was performed in any patient of this group. However, one patient underwent gastrostomy 12 months after transplantation. In the control group, three patients died during the 1-year follow-up period. The death of these patients was because of respiratory insufficiency at 3, 5 and 6 months, respectively, after baseline (mean 4.6). Tracheostomy and gastrostomy were performed in two of these patients before death. These procedures were also conducted in three additional control patients.

The median survival time from diagnosis to the end of follow-up was 19 months [range 14–32 months, 95% confidence interval (CI) 11.77–26.23 months] and 66 months (range 24–71 months, 95% CI 17.53–114.47 months) for the control and transplanted groups, respectively. Significant differences among the groups (log rank = 6.45, $P = 0.0111$) were determined (Figure 1).

The ALSFRS-R scores in the treatment group showed a statistically significant improvement at 1 and 2 months by Friedman test ($P = 0.017$) compared with baseline scores. The ALSFRS-R scores at 6 months and 1 year after stem-cell transplantation also revealed a significant improvement compared with baseline scores (Figure 2). The baseline ALSFRS-R scores were higher in the control group (mean 31.4) than in the treatment group (mean 24.6). When this score was compared among groups at 6 and 12 months after baseline, the treatment group showed a stable mean score (27.9 and 24, respectively) whereas the control group showed a decline in the mean score at 6 months (25.1) ($P < 0.001$) and 12 months (15.7) ($P < 0.001$). The control group also showed a significant decrease in ALSFRS-R score at 1-year follow-up compared with baseline values (Friedman test, d.f. = 3, $P = 0.007$).

ALS patients in the treatment group remained under surveillance in the outpatient clinic until February 2008, when this trial ended. Two other patients died during this extended surveillance period. The deaths occurred at 18 and 22 months after transplantation. All other patients were still alive at February 2008. The Spitzer QOL scale in the treated ALS patients did not reveal any significant differences during follow-up compared with the baseline scores.

Table I. Demographic, objective clinical variables and outcomes in the ALS control and treatment groups.

Patient/gender*/age (years)	Duration		ALSFRS-R		Outcome baseline to 12 months			Survival time** (months)	FVC (%)	
	Diagnosis to baseline (months)	Baseline	6 M†	12 M	Tracheostomy	Gastrostomy	Death		Baseline	12 M
Control group										
1/M/70	13	30	14	7	Yes	Yes	No	14‡	20	VS***
2/F/52	12	26	19	4	Yes	Yes	No	19‡	87.60	VS
3/M/54	13	35	32	8	Yes	Yes	No	15‡	22	VS
4/M/65	29	30	27	NP	Yes	Yes	Yes	32‡	36	NA
5/M/34	14	40	39	32	No	No	No	25	64	64
6/M/46	12	20	18	4	Yes	Yes	Yes	18‡	20	NA
7/F/31	13	20	16	NP	No	No	Yes	18‡	36	NA
8/F41	12	43	40	37	No	No	No	24	60	60
9/F/55	13	36	20	20	No	No	No	25	68	65
10/F/65	12	34	26	14	No	No	No	24	80	75
Treatment group										
1/M/52	42	19	23	19	No	Yes	No	53‡	28	20
2/F/38	45	16	22	17	No	No	No	57	46	21
3/M/35	45	19	22	21	No	No	No	57	36	25
4/F/62	32	18	NP	NP	No	No	Yes	32‡	25	NA
5/M/38	30	19	29	25	No	No	No	42	NA	NA
6/M/40	22	34	35	33	No	No	No	34	49	49
7/F/47	16	39	35	20	No	No	No	28	48	28
8/F/50	27	20	32	23	No	No	No	39	NA	NA
9/F/37	24	29	42	34	No	No	No	36	70	58
10/M/51	18	33	39	NP	No	No	Yes	39‡	58	NA

*M, male; F, female. †M, month. **Survival time at the end of the study or time of death, tracheostomy or gastrostomy. ‡Months at the primary endpoint (mortality) or at the 1-year follow-up point. ***VS, ventilatory support; NP, not performed because of patient death; NA, not available because patients were either on ventilatory support or had poor respiratory effort.

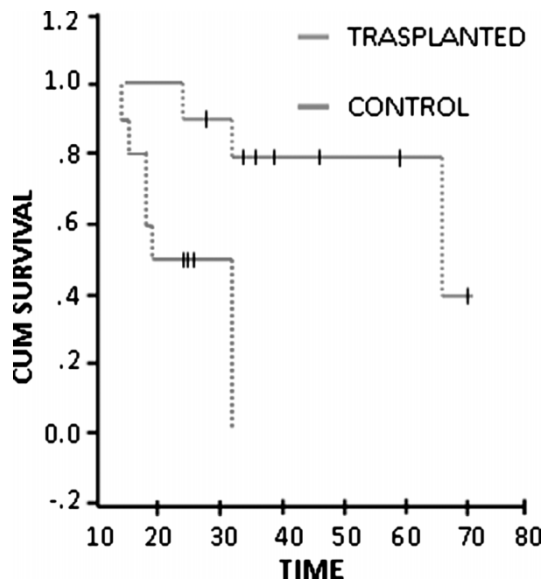


Figure 1. Mean survival time from diagnosis estimated using the Kaplan–Meier method, showing differences between the treatment and control groups (log rank = 6.45, $P = 0.01$).

There were no differences in FVC between the control and transplant-treated groups. The FVC values ranged from 25% to 70% (mean 44.6%) in the treatment group, and from 20% to 80% (mean 49.3%) in the control group. Statistical comparison of the FVC values could not be performed at the 1-year point. Three patients in the control group were on ventilatory support and three had

died. FVC remained stable at the 1-year follow-up point in the treatment group, and no tracheostomy was performed in these patients. However, two patients in the treatment group were unable to complete the functional respiratory test satisfactorily. Five ALS patients in the control group showed a significant decrease in FVC and, therefore, underwent a tracheostomy during this time.

G-CSF produced an increase in the number of absolute monocytes, as well as the lymphocyte count. Before G-CSF injection in the treatment group, the numbers of white blood cells ranged from 3800 to 7560 (mean 6979). After G-CSF, the white blood cells increased in numbers from 14 400 to 52 000 (mean 33 260). Mononuclear cells isolated by leukapheresis produced a significant number of CD133⁺ stem cells after being conjugated with anti-human CD133 superparamagnetic microbeads (range 500 000–5 360 000). Among the patients in the treatment group, a trend in clinical improvement was observed with the ALSFRS-R score when the number of transplanted CD133⁺ stem cells was greater than 500 000 stem cells ($\chi^2 = 5.77$, d.f. = 1, $P = 0.0162$; Figure 3).

Discussion

ALS is a devastating neurodegenerative disorder with no effective treatment that usually leads to death within 3–5 years of diagnosis [39–42]. Mortality in these patients is associated with respiratory insufficiency, myocardial infarction and cardiac arrhythmia [1]. In this study, one patient died because of myocardial infarction in the treatment group early after stem-cell transplantation, and three control ALS patients died because of respiratory insufficiency at a mean of 4.6 months after baseline. Control patients remained on their current treatment with riluzole and antioxidants during the follow-up period. The mortality of ALS patients is expected because of these

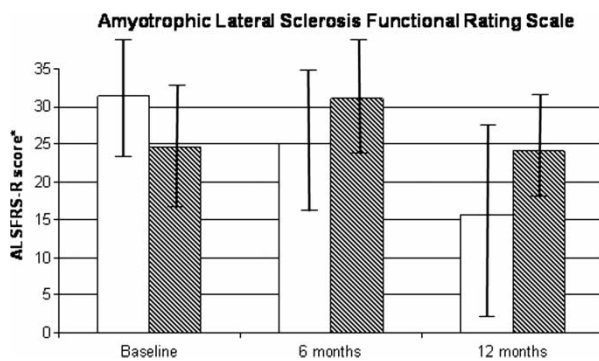


Figure 2. ALSFRS-R score of the control group (CG, open bars) (normal score = 48) and the treatment group (TG, hatched bars). There were significant differences in the score of the treatment group at 6 months ($P = 0.013$) and 12 months ($P = 0.01$) compared with their baseline score. The baseline ALSFRS-R score was higher in the control group than in the treatment group ($P = 0.00$). The control group showed a decline in the mean score and, compared with the treatment group, we observed significant differences at 6 months ($P = 0.00$) and 12 months ($P = 0.00$).

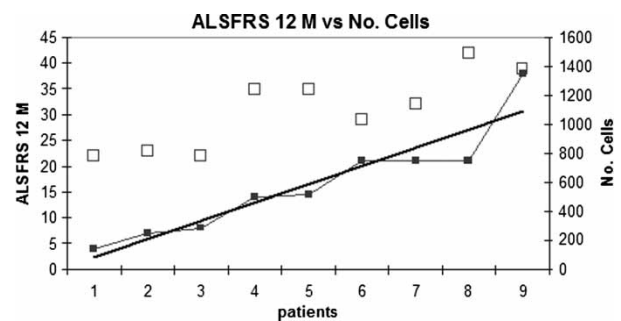


Figure 3. Improvement in ALSFRS-R score at 12 months (open squares) versus the number of transplanted cells (closed squares).

complications no matter what type of treatment is administered. Treated patients only received antioxidants after stem-cell transplantation.

There has been great variability in the described clinical presentation of ALS cases, as well as in the time when mortality presents, which is usually between 3 and 5 years after diagnosis. However, recent reports describe the median survival time of ALS patients as around 29.1 months after presentation. In the present series, the median survival time from diagnosis to the end of follow-up was 19 months in the control group (95% CI 11.77–26.23) and 66 months in the treatment group (95% CI 17.53–114.47) (log rank = 6.45, $P=0.0111$). Moreover, survival at the end of the 1-year follow-up showed a significant difference between the control and treated ALS patient groups ($F=11.096$, $P=0.004$).

The burgeoning field of stem-cell biology has attracted considerable interest over the last decade. Stem-cell therapy is considered to be an alternative treatment for ALS, as well as for cell-replacement therapy in other neurodegenerative disorders [10,11,14,28,29]. Stem-cell transplantation is a potential therapeutic strategy in ALS patients that may operate via cell replacement or by modifying the extracellular motor neuronal environment [11]. Adult stem cells isolated *in vivo* from BM or peripheral blood may give rise to neural cells and, therefore, these stem cells represent an alternative to embryonic cells for therapeutic transplantation [11,12].

CD133⁺ stem cells isolated from peripheral blood are capable of differentiating into neurons [13,14]. Clinical studies using stem cells in humans have been described for several neurodegenerative disorders. However, we are still learning about the possible benefits of cell replacement therapy in such neurologic disorders. ALS is a neurodegenerative disorder that produces selective death of motor neurons in the cerebral cortex, brain stem and spinal cord. The rationale in the present study was to improve the upper motor neuron function through stem-cell transplantation.

Our study indicates that autologous CD133⁺ stem-cell transplantation into the frontal motor cortex is a safe and well-tolerated procedure in ALS patients. Stem-cell transplantation in ALS patients delays disease progression and mortality. The median survival time from diagnosis to the end of follow-up was higher in the treatment group compared with the control ALS patient group. Survival at

the end of the 1-year follow-up also showed significant differences between the groups ($F=11.096$, $P=0.004$). The Spitzer QOL score for the treatment group was not modified at the end of the study compared with baseline values. Absence of a decrease in the Spitzer score suggests that the patients remained stable regarding their QOL, with no decrease, and that they showed a good acceptance of the procedure.

The ALSFRS-R score in the treatment group improved after stem-cell transplantation in comparison with the baseline score. The baseline score was higher in the control group in comparison with the treatment group (mean 31.4 versus 24.6). However, at 6 and 12 months the score remained stable in the treatment group (27.9 and 24, respectively) whereas the control group showed a decline (25.1 and 15.7, respectively) ($P=0.007$). This finding suggests that there was a delay in disease progression in the stem-cell transplant-treated ALS patients. We observed a preservation of pulmonary function in ALS patients in the treatment group. During the 1-year follow-up, no transplant-treated patient required tracheostomy or respiratory assistance. In contrast, the disease progressed markedly in the control group patients, and five out of 10 patients required tracheostomy and ventilatory support.

G-CSF induces BM-derived cell mobilization. In the present series, G-CSF treatment was feasible, safe and well tolerated, with a few, reversible adverse effects in ALS patients. G-CSF produces BM-derived cell mobilization in ALS patients, as is also observed in a healthy population. In our patients, we observed an increase in the number of white blood cells and, consequently, a significant number of CD133⁺ stem cells were obtained (range 500 000–5 360 000). Among the patients in the treatment group, we observed that the number of transplanted stem cells was related to a higher clinical score obtained with the ALSFRS-R. This trend in clinical improvement was observed when the number of transplanted CD133⁺ stem cells was greater than 500 000 ($P=0.0162$). It is believed that G-CSF has a direct neuroprotective effect on motor neurons. The differences observed in favor of patients with a higher number of transplanted stem cells suggests that the stabilization or improvement is related to the stem cells and not in fact to direct G-CSF neuroprotection.

The autologous transplantation of CD133⁺ stem cells into the frontal motor cortex is a safe and well-tolerated

procedure in ALS patients. Adequate numbers of CD133⁺ stem cells from peripheral blood may be obtained by stimulation with G-CSF. Although our study indicates that stem-cell transplantation into motor cortex delays ALS progression in human patients, further studies with a greater number of patients are necessary to define the usefulness of stem-cell therapy in patients with confirmed ALS.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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