Statistical relationship between levels of autologous bone marrow-derived CD34+ and clinical status of patients with amyotrophic lateral sclerosis.

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal disease characterized by a progressive degeneration of motoneurons in the brain and in the spinal cord. There is **no cure for ALS** and the current available treatments are mostly directed to maintain the quality of life by a multidisciplinary palliative approach. Besides, there are **no consistent explanations of its pathophysiology** and there are also **no reliable biomarkers**. Several trials for stem cell (SC) therapies have proven a beneficial influence in the affected neural tissues promoting motoneuron regeneration(1-14).

OBJECTIVE

To determine the **relationship between Bone marrow-derived CD34+ cells and the clinical status** of patients with ALS.

METHODS

The data was retrospectively collected from an open label pilot study in 85 patients with ALS treated with **Neuron Point-of-care stem cell therapy (N-POCST)** **(Figure 1)** carried-out from August 2011 to October 2013.

Under sedoanalgesia, **bone marrow (BM) was harvested from the posterior superior iliac crest** with the standard procedure of BM aspiration. The amount of milliliters extracted was determined by availability at the moment of aspiration. After harvesting, the BM underwent an on-site cell separation for 30 minutes in a closed system (Sepax II®) that uses density centrifugation technique with a fixed 10% reduction rate that isolates BM-derived mononuclear cells from BM derived-plasma (BMP)(15). **BM before separation sample (BMBSS)** and **bone marrow after separation sample (BMASS)** were sent for laboratory analysis immediately. The BMASS later was the one infused via intrathecal to the patient.

CD34+ cells were determined according to the ISHAGE guidelines with the Stem-kit Beckman Coulter (single platform principle with CD45FITC/CD34 PE detector) (16). The number of CD34+ cells infused in each patient was determined by the product of CD34+ cells/ml. times the number of milliliters infused. The **vitality or viability** of the cells was assessed with 7-Aminoactinomycin D (7-AAD) dye and conventional flow cytometer that reflects the percentage of cells with structural integrity (living cells). The **concentration factor (CF)** of CD34+ was mathematically assessed as the quotient the values in BMASS divided by the values in BMBSS. The **reduction factor (RF)** of vitality was calculated by the subtraction of BMBSS minus the values of BMASS.

Variables of laboratory tests, clinical status before N-PCOST and clinical outcome after treatment were analyzed in different sets of groups and compared to each other using **non-parametric tests (Levene's T test for equality of variances and T test for equality of means, except for the variables without normal distribution in which Wilcoxon-Mann-Whitney test was used.** When divided in variable groups a cut-off point of each variable was arbitrary determined with a value around the mean of the complete samples. Linear and logarithmic regression models were performed between the variables all variables.

Confidence intervals of 95% were used, the null hypothesis was rejected when a significance of p=0,05 was reached and when rejected the test was considered statistically significant. Graphs and analysis were performed using the IBM SPSS® statistics software version 2.0. and SYSTAT13.

The clinical status was determined by the **Amyotrophic Lateral Sclerosis Functional Rating Scale Revised (ALSFRS-R)** in which higher scores mean better clinical status(17).

Figure 1. Neuron Point-of-care stem cell therapy (N-POCST)

RESULTS

The mean ml. of BM taken was 137,4±36,8 ml. (40 to 230). They have statistically significant ***more vitality in BMBSS*** (89,5 vs. 85,3 % [n=65]; p=0,033) and ***higher CF of CD34+ cells*** (12 vs. 4,5 times more CD34+ cells/µl [n=65]; p=0,001) **(Table1).**

Regression models showed ***statistically significant relationship between patients that had more ml. extracted and longer survival*** (logarithmic p=0,045)**(Figure 2).**

Milliliters extracted from BM	<130	>130	p value
Vitality in BMBSS (%) (mean)	85,3	89,4	0,033
CF (times more CD34+/µL) (mean)	4,52	12,02	0,001

Table 1. Milliliters extracted from Bone Marrow

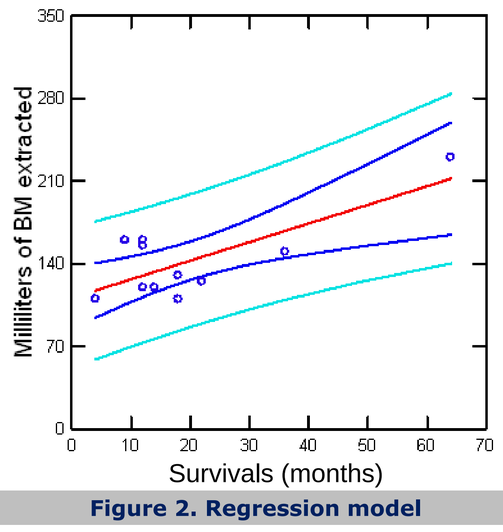

Figure 2. Regression model

Patients with ***more than 100 CD34+ cells/µl in BMBSS had statistically significant more vitality*** (87,2±5,1 vs. 86,4±9,5 % [n=78]; p=0,019), ***lower CF of CD34+ cells*** (6,1±6,9 vs. 11,5±8,4 times [n=73]; p=0,004) and ***more time passed between diagnosis and N-POCST*** (24,9±27 vs. 21,1±16,3 months [n=80]; p=0,039) **(Table 2)**. ***After separation (BMASS) the patients with more than 500 CD34+ cells/µl*** had statistically significant ***higher vitality*** (71,2±10,7 vs. 70,8±16,5 % [n=73]; p=0,036) and ***less time passed since diagnosis*** to N-POCST (21,8 vs. 23,8 months [n=73]; p=0,047) **(Table 3)**.

The regression models were statistically significant between ***high vitality in BMBSS and high ALSFRS-R in the follow-up*** (linear p=0,006 and logarithmic p=0,009)**(Figure 3)**.

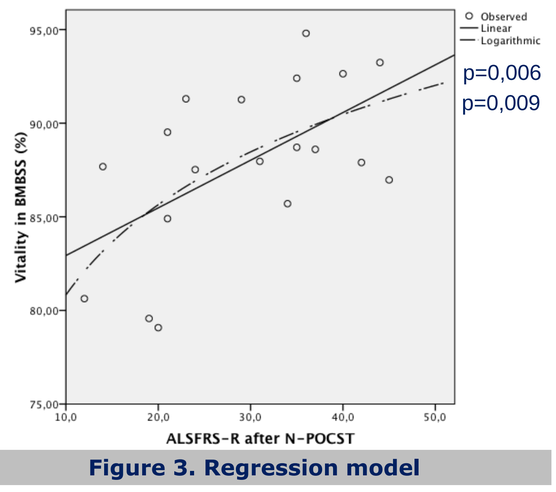

Figure 3. Regression model

Vitality (%) in BMASS	<80	>80	p value
Age (years)	52,7	51,4	0,027
Time from diagnosis to N-POCST (months)	21,9	26,7	0,049
ALSFRS-R before N-POCST (mean)	30,2	28,5	0,035
ALSFRS-R after N-POCST (mean)	28,8	31,6	0,041
How many months after the N-POCST died (mean)	5,6	6,8	0,049

Table 4. Groups of Vitality in BMASS

CD34+/µL in BMBSS	<100	>100	p value
Vitality (%) in BMBSS (mean)	86,4	87,2	0,019
CF of CD34+ (times) (mean)	11,5	6,1	0,004
Time between diagnosis and N-POCST (months)	21,1	24,9	0,039

Table 2. Groups of CD34+/µL in BMBSS

CD34+/µL in BMASS	<500	>500	p value
Vitality (%) in BMASS (mean)	70,8	71,2	0,036
Time between diagnosis and N-POCST (months)	23,8	21,8	0,047

Table 3. Groups of CD34+/µL in BMASS

The group with ***more than 80% of vitality*** in BMASS were younger (51,4±7,6 vs. 52,7±10,8 y/o [n=73]; p=0,027), had ***more time passed between diagnosis and N-POCST*** (26,7±31,4 vs. 21,92±17,3 months [n=72]; p=0,049), ***lower ALSFRS-R before N-POCST*** (28,5±13,8 vs. 30,2±9,5 [n=36]; p=0,035) but ***higher ALSFRS-R after N-POCST*** (31,6±13,2 vs. 28,8±7,5 [n=18]; p=0,041) and ***died later after N-POCST*** (6,8±5,5 vs. 5,6±2,5 months [n=16]; p=0,049)**(Table 4)**.

DISCUSSION AND CONCLUSIONS

High volume of BM available for aspiration correlates with longer survival (Figure 2), more CD34+ concentration per µl and more vitality (Table 1). The patients that had more time passed since diagnosis hence probably more advanced disease and worse clinical status had apparently better BM conformation before the separation process, while they have more CD34+ cells/µl (Table 2), however after the separation the ones that maintain more CD34+ cells/µl were the ones with less time since diagnosis or less progressive disease (Table 3).

A possible explanation is that **the patients with advanced disease had poor quality of SCs.** Therefore, a cornerstone for the pathophysiology could be suggested. Several scientific groups described that normal functioning bone marrow-derived SCs mobilize and migrate into injured tissue where they participate in the process of repair. In parallel to these, a considerable number of recent studies have been associated to the development of several degenerative disease with a reduced number of circulating SCs in peripheral blood. This both previous investigations together provide a possible understanding in which **degenerative diseases do not develop just due to intrinsic cellular loss or external factors but also following an imbalance between cellular loss and tissue renewal**(2,18).

Supporting these descriptions, in our sample the **patients with more advanced disease are probably producing a strong signal from damaged tissue to the BM** (due to an intrinsic or extrinsic factor not yet discovered) **that makes a highly active SCs production measured by high CD34+ cells/µl. However, the lack of migrating capacity or functional deficiency, not measured but assumed by the low resistance to centrifuge, causes an imbalance in tissue renewal.** These assumptions should be further confirm by sampling SCs levels in peripheral blood and BM of the same patient(19). Moreover, with analysis of BM and peripheral blood content we could create prognosis scales.

These findings also support the rationale of N-PCOST, while we infused SCs directly in the affected organs (CNS and muscle) trying to overcome the lack of migration capacity. Even other non-discovered functional deficiencies are not changed by N-POCST the treatment reduces the imbalance of tissue renewal in affected motoneurons and surrounding implicates. This therapies also conferee a kind of system "restart" that may bye preserved after the infused SCs die promoting better tissue renewal.

We found that vitality plays an important role in patients' improvement while high vitality in BMASS correlates with high ALSFRS-R after the N-POCST making vitality an important outcome predictor of N-POCST (Table 4, Figure 3). Furthermore, when we look at patients with more than 80% in BMBSS before N-POCST they had lower ALSFRS-R reversed by N-POCST (Table 4). Also, patients with more than 80% of vitality in BMASS had more survival time after the N-POCST (Table 4). A conclusion arise, **patients with high levels of vitality are the ones who benefit the most from stem cell treatment.** We could determine an initial minimum required vitality of 80% in BMASS to present a clinical improvement.

Further investigations are required to confirm the **tissue renewal imbalance as pathophysiological cornerstone and the use of BM CD34+ cells and their vitality as a biomarker in ALS.**

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