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# Vitamin D confers protection to motoneurons and is a prognostic factor of amyotrophic lateral sclerosis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is an incurable paralytic disorder primarily typified by the selective and progressive degeneration of motoneurons in the brain and spinal cord. ALS causes muscle wasting and atrophy, resulting eventually in respiratory failure and death within 3–5 years of diagnosis. Vitamin D is a potent secosteroid hormone with diverse biological functions that include protection against neuronal damage. The detrimental consequences of vitamin D dietary deficiency have been documented in other neurodegenerative diseases. However, the protective effect of vitamin D on motoneuron and the influence of its levels on disease course remains elusive. Here we found that the biologically active form of vitamin D significantly potentiated the effect of neurotrophic factors and prevented motoneurons from a Fas-induced death, while electrophysiological properties of motoneurons were not affected. In ALS patients, we report that a severe vitamin D deficiency accelerates by 4 times the rate of decline and were associated with a marked shorter life expectancy. Our findings support a neuroprotective function of vitamin D on motoneurons and propose vitamin D as a reliable prognostic factor of ALS.



## 1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disorder characterized by the progressive degeneration of upper and lower motoneurons. Motoneuron loss causes muscle weakness and atrophy, leading to death in a median time of 3 years (Hardiman et al., 2011). Except for rare inherited cases, ALS etiology remains largely unknown. ALS prognosis is highly variable, ranging from few months to more than 30 years. Some ALS-related conditions have been associated with an increased severity such as age of onset, weight loss, or vital capacity (Chio et al., 2009). The influence of other factors such as depression, smoking, dyslipidemia, or statin treatment remains controversial and, to date, no biological marker, detectable before onset, has been unequivocally linked to ALS onset or progression (Alonso et al., 2010; Armon, 2009; Bowser et al., 2011; Dupuis et al., 2008; Paganoni et al., 2011).

Vitamin D is a steroid hormone, which in its active form forms 1α, 25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), is involved in a wide variety of biological processes including calcium ($Ca^{2+}$) and bone metabolism, regulation of the immune response, or cancer-related metabolic pathways (Abrams et al., 2013). Interestingly, Vitamin D has been proposed to act as a neuroprotective factor in several neurologic disorders or conditions including Parkinson's disease, multiple sclerosis, cognitive troubles, and neurovascular disorders. Moreover, low plasma levels of vitamin D have been associated with an overall worse prognosis (Balion et al., 2012; Kojima et al., 2012; Mowry et al., 2012; Suzuki et al., 2012). These data are consistent with a neuroprotective activity of vitamin D against neuronal damage, thus prompting the recent proposal of vitamin D as a potential treatment option for ALS (Karam and Scelsa, 2011). However, recent works gave differing results in mice expressing ALS-linked mutated superoxide dismutase-1 (SOD1) mice, a key animal model closely resembling the human pathology. Indeed, while vitamin D intake increased the strength of ALS mice, the lifespan of treated mutant mice was unchanged (Gianforcaro and Hamadeh, 2012; Gianforcaro et al., 2013). In another study, a vitamin $D_3$-deficient diet delayed disease onset but decreased motor performance of SOD1 mutant mice

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(Solomon et al., 2011). However, recently, vitamin D supplementation in a small cohort of ALS patient suggested beneficial effects on the revised ALS functional rating scale (ALSFRS-R) scores (Karam et al., 2013). This evidence led us to further explore the potential role of vitamin D on motoneurons in vitro and in ALS patients.

Here, we show that vitamin D has significant effect on motoneuron survival by potentiating neurotrophic factor activity and by protecting motoneurons from Fas-induced death. Moreover, when we analyzed the plasma levels of vitamin D in ALS patients, we found that low levels of vitamin D were associated with a worse outcome, defined by the rate of decline (severity score) and survival, compared with patients with normal levels.

## 2. Methods

### 2.1. Culture reagents

Glial-derived neurotrophic factor (GDNF) was purchased from Sigma-Aldrich (St. Louis, MO, USA), brain-derived neurotrophic factor (BDNF) from ImmunoTools (Friesoythe, Germany) and ciliary neurotrophic factor (CNTF) from R&D Systems (Minneapolis, MN, USA). Rat monoclonal antibody against vitamin $D_3$ receptor (9A7 clone), soluble human recombinant FasL set, vitamin $D_2$, vitamin $D_3$, and $1,25(OH)_2D_3$ were purchased from Enzo Life Sciences (Farmingdale, NY, USA).

### 2.2. Motoneuron culture

All animal experiments were done in compliance with the European Community and National directives for the care and use of laboratory animals. *Hb9::GFP* mice (T.M. Jessell's laboratory, New York, NY, USA) were maintained on a C57BL/6 background (Charles Rivers laboratories, Wilmington, MA, USA) (Wichterle et al., 2002). For *Hb9::GFP* motoneuron cultures, transgenic embryos at embryonic day 12.5 were sorted under a fluorescence microscope before dissection of the spinal cord. Motoneurons from spinal cord embryos were isolated as described (Arce et al., 1999) modified by (Aebischer et al., 2011), using iodixanol density gradient centrifugation. Motoneurons are plated on poly-ornithine/laminin-treated wells in the presence (or not when mentioned) of a cocktail of neurotrophic factors (NTFs) (0.1 ng/ml GDNF, 1 ng/ml BDNF, and 10 ng/ml CNTF in supplemented neurobasal medium [Invitrogen, Carlsbad, CA, USA]). Supplemented neurobasal contains 2% (vol/vol) horse serum, 25 mM L-glutamate, 25 mM β-mercaptoethanol, 0.5 mM L-glutamine, and 2% (vol/vol) B-27 supplement (Invitrogen).

### 2.3. Immunocytochemistry

*Hb9::GFP* motoneurons were seeded at the density of 5000 cells in polyornithine/laminin–coated 12-mm diameter glass coverslip in the presence of NTFs and maintained at 37 °C, 7.5% $CO_2$ for 24 hours before being treated with vitamin $D_2$, vitamin $D_3$, or $1,25(OH)_2D_3$ (100 nM each) for 8 hours. Neurons were fixed on ice first for 15 minutes with 2% formaldehyde in phosphate-buffered saline (PBS)-neurobasal medium (1:1), then for 15 minutes with 3.7% formaldehyde in PBS. Cells were washed 3 times with PBS and incubated for 1 hour at room temperature in PBS containing 4% BSA, 4% donkey serum, and 0.1% triton-X100. The 9A7 primary antibody was diluted in PBS containing 4% BSA, 4% donkey serum, and 0.1% triton-X100 at concentrations of 5 μg/mL. Immunocomplexes were detected using fluorochrome-conjugated secondary antibodies (AlexaFluor555, Invitrogen).

### 2.4. Electrophysiology

Electrophysiological recordings in motoneurons were done at 20 °C–22 °C after 6 days in vitro. As we previously reported (Hilaire et al., 2005), for action potential recordings, the bathing solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and the pH was adjusted to 7.4 with NaOH. Recording pipettes were filled with the following solutions: 140 mM KCl, 10 mM HEPES, 2 mM Mg-ATP, 0.5 mM $Na_2$-GTP, 0.1 mM EGTA, pH 7.35, adjusted with KOH. For $Ca^{2+}$ current recordings, external NaCl and KCl were replaced with TEA-Cl, pH adjusted with CsOH; internal KCl was replaced with CsCl.

Potentials or currents were recorded with an Axopatch 200B amplifier (Molecular devices, Dipsi Industrie, Chatillon, France). The experimental parameters were controlled with a computer equipped with a Digidata 1300 analogue interface (Molecular devices). We used pClamp software (Clampex 8.02; Molecular devices) for data acquisition and analysis. Signals were filtered at 2 or 5 kHz and sampled at 5 or 10 kHz, respectively. Glass electrodes (3–4 MΩ) were made from capillary glass, using a Narishige puller, and coated with paraffin wax to minimize pipette capacitance. For each experiment, to ensure recordings of motoneurons, green fluorescent *Hb9::GFP* neurons with a large somatic diameter (>25 μm) were selected. Vitamin $D_3$ and $1,25(OH)_2D_3$ (100 nM each) were added to culture 24 hours before electrophysiological recordings.

### 2.5. Neurite outgrowth and survival assay

For the analysis of neurite outgrowth, motoneurons were cultured for 24 hours before being treated or not with 100 nM of vitamin $D_2$, vitamin $D_3$, and $1,25(OH)_2D_3$. Twenty-four hours later, *Hb9::GFP* neurons were immunostained using primary antibodies directed against the green fluorescent protein (GFP) (TP401; Torrey Pines Biolabs, East Orange, NJ, USA; 1:500). Process outgrowth was analyzed by measuring the length of the longest neurite for GFP-positive motoneuron using the ImageJ software and NeuronJ plugin V10.2 (National Institute of Health, Bethesda, Maryland, USA). In each independent experiment, a minimum number of 40 motoneurons per experimental condition were considered for process analysis. Values were expressed relative to the values in the absence of treatment (none, taken as 100%). For trophic factor deprivation and potentiation, motoneurons were plated at the density of 1200 cells per $cm^2$ in the presence or not of a cocktail of NTFs, vitamin $D_2$, vitamin $D_3$, and $1,25(OH)_2D_3$ at the indicated concentration. After 24 hours of culture surviving neurons were directly counted under fluorescence microscopy. sFasL (100 ng/mL in the presence of 1 μg/mL enhancer antibody), vitamin $D_2$, vitamin $D_3$, and $1,25(OH)_2D_3$ (100 nM each) were added to motoneurons that were previously grown for 24 hours in the presence of GDNF, BDNF, and CNTF. Forty-eight hours later, neuron survival was determined by directly counting under a fluorescence microscope.

### 2.6. Patients

All ALS patients followed in our ALS Center that underwent a blood measure for 25-hydroxy vitamin D levels (i.e., vitamin $D_2$ + vitamin $D_3$) between January 2010 and December 2011, were considered for inclusion in the retrospective clinical study. For each of them, the diagnosis of ALS fulfilled the diagnostic criteria (Airlie House/El Escorial revised criteria) of either probable or definite ALS (Traynor et al., 2000). The following clinical data for ALS were available for all the patients: the ALSFRS-R score at the time of vitamin D levels measurement (Kollewe et al., 2008); gender, age of birth, age at vitamin D levels measurement, age at ALS onset, date of ALS onset, site of onset, date of death. ALS onset was defined as the

first time the patient reported the initial muscle weakness. Site of onset was defined as the level at which muscle weakness first appeared: bulbar, upper limb, lower limb, or respiratory onset. Two different criteria for ALS outcome were obtained as: (1) patient survival, defined as the time between ALS onset and either death or the time of database lock (April 2012); (2) rate of decline, defined by the severity score that corresponded to the number of ALSFRS-R points lost each month, between onset and the time of vitamin D levels measurement. The ALSFRS-R scale gives a score of 48 points through 12 items rated 0–4 and exploring functional aspects of ALS, evaluating bulbar, upper limb, lower limb, and respiratory involvement. An individual without deficit is thus rated 48/48. The severity score was then = (48−ALSFRS-R score/number of months between onset and vitamin D levels measurement) (Kimura et al., 2006). ALS duration was defined as the time, in months, between ALS onset and vitamin D dosage. For subgroup analysis, 3 patient groups were considered: normal vitamin D (NVD) levels (NVD > 75 nmol/L), vitamin D deficiency (VDD ≤75 and >25), and severe vitamin D deficiency (SVDD ≤25) (Soni et al., 2012).

### 2.7. Statistical analysis

For in vitro experiments comparisons were done by a 1-way analysis of variance with a Bonferroni post hoc test or an unpaired 2-tailed *t* test. Analyses were performed using the GraphPad Prism 5 software.

In ALS patients, severity score was compared according to patient characteristics as a quantitative and categorical variable. Spearman correlation coefficient was used for testing the association between quantitative variables, which are not normally distributed. To compare the distribution of severity score between patient characteristics (gender, site of onset…), Wilcoxon Mann-Whitney test (2 groups), or Kruskal-Wallis test (more than 2 groups) were used. In the case of a significant difference for the Kruskal-Wallis test, an adapted Newman-Keuls test was performed. Severity score was then considered as a categorical variable (using the median as a cut-off point) for the implementation of a multivariate logistic regression, which aimed to find the risk factors of a high severity score (above the median). The survival of ALS patients was analyzed with the Kaplan-Meier nonparametric estimator and compared between groups using the log-rank test. After verification of proportional hazard assumption a multivariate Cox model was performed with a stepwise variable selection. Fifteen percentages were taken as a decision threshold for the risk factors selection to perform the multivariate logistic regression and the Cox proportional hazards model. The decision threshold was set at 5% for the rest of the statistical analyses in ALS patients. Analyses were performed using SAS software 9.3, SAS Institute Inc, Cary, NC, USA.

## 3. Results

### 3.1. Electrophysiological properties of motoneurons are not modified by 1,25(OH)$_2$D$_3$

To facilitate motoneuron identification, neurons were isolated from the spinal cord of transgenic mouse embryos that express GFP under the control of the motoneuron-selective promoter *Hb9 (Hb9::GFP)*. We first observed by immunocytochemistry that all *Hb9::GFP* neurons expressed the vitamin D receptor (VDR) on which 1,25(OH)$_2$D$_3$ binds to exert its biological function. Addition of 1,25(OH)$_2$D$_3$ in the culture medium increased the intensity of nuclear staining of VDR, while addition of vitamin D$_2$ and vitamin D$_3$, 2 biologically inactive vitamin D metabolites (Ross et al., 1994), did not differ from the basal level of nuclear VDR of motoneurons cultured in the presence of NTFs alone (not shown and Fig. 1A–C). This suggests that 1,25(OH)$_2$D$_3$ efficiently binds and promotes nuclear translocation of VDR in cultured motoneurons, thus stimulating an appropriate and functional biological response.

It was demonstrated that the neuroprotection conferred by 1,25(OH)$_2$D$_3$ against N-methyl-D-aspartic acid or glutamate insult in hippocampal neurons was associated to decrease the activity of L-type voltage-sensitive $Ca^{2+}$ channel (Brewer et al., 2001). Impaired $Ca^{2+}$ homeostasis and a hyperexcitability have been proposed to contribute to motoneuron vulnerability in ALS (Guatteo et al., 2007). We first asked whether 1,25(OH)$_2$D$_3$ could influence intrinsic electric properties and $Ca^{2+}$ current in motoneurons. Embryonic motoneurons present an electrophysiological maturation when kept several days in culture (Alessandri-Haber et al., 1999). Thus, our experiments were carried out on electrically mature motoneurons, that is, at 6 days after the initial isolation of the cells. We first investigated electrical properties of motoneurons in the presence or absence of 1,25(OH)$_2$D$_3$ (n = 12) or vitamin D$_3$ (n = 4) and show that the properties were similar to untreated neurons (n = 6). More precisely, the resting membrane potential was the same for the 3 conditions and varies between −56 and −60 mV (Fig. 1D). Another essential feature of the electrical activity is the threshold required for eliciting a $Na^+$ spike. Results suggest that both voltage and current thresholds are unchanged in the 3 experimental conditions (Fig. 1E and data not shown). Finally, the maximum amplitude of action potentials is not modified by 1,25(OH)$_2$D$_3$ or vitamin D$_3$ (Fig. 1F). Altogether these results suggest that these molecules do not interfere in motoneuron electrical properties.

Besides electrical activity, voltage-gated $Ca^{2+}$ currents play fundamental roles in neuronal cells physiology, such as neurotransmission and gene regulation (Belardetti and Zamponi, 2008), which led us to determine whether 1,25(OH)$_2$D$_3$ could alter the global voltage-gated $Ca^{2+}$ current amplitude and biophysical properties. Using a voltage ramp from −100 mV to 20 mV for 500 ms, we observed typical $Ca^{2+}$ current with similar maximal amplitude in control cells (n = 21), and cells treated with 1,25(OH)$_2$D$_3$ (n = 21), or vitamin D$_3$ (n = 14) (Fig. 1G). Furthermore, voltage steps from a holding potential of −100 mV were performed to determine the $Ca^{2+}$ current density-voltage relationships (Fig. 1H). Recordings on untreated mature motoneurons (n = 11) show the threshold potentials for activation were around −50 mV and potentials for maximal activation were close to −10 mV, under the 3 experimental conditions. Importantly, neither 1,25(OH)$_2$D$_3$ (n = 13) or vitamin D$_3$ (n = 11) have an effect on the current density, amounting roughly −11 pA/pF at −10 mV. Thus, our results suggest that 1,25(OH)$_2$D$_3$ does not induce changes in voltage-gated $Ca^{2+}$ current properties. 1,25(OH)$_2$D$_3$ has also been shown to promote neurite outgrowth in embryonic hippocampal neurons (Brown et al., 2003). We next determined whether 1,25(OH)$_2$D$_3$ could elicit neurite outgrowth in embryonic motoneurons. Quantification of neurite length indicated that neither vitamin D$_2$, vitamin D$_3$ or 1,25(OH)$_2$D$_3$ promoted neurite elongation in motoneurons (none, 100 ± 10.6%; vitamin D$_2$, 95.8 ± 12.5%; vitamin D$_3$, 91.5 ± 13, and 1,25(OH)$_2$D$_3$, 97.5 ± 12.5; means ± standard error of the mean , n = 3, nonsignificant, analysis of variance with Bonferroni post hoc test).

### 3.2. 1,25(OH)$_2$D$_3$ potentiate neurotrophic activity in motoneurons

1,25(OH)$_2$D$_3$ had been previously shown to confer neuroprotective effects against death induced by serum deprivation in hippocampal neurons (Brewer et al., 2001). We first investigated whether 1,25(OH)$_2$D$_3$ may confer neurotrophic benefits to motoneurons. An optimal survival of purified embryonic motoneurons is obtained when a combination of neurotrophic factors (GDNF, BDNF,

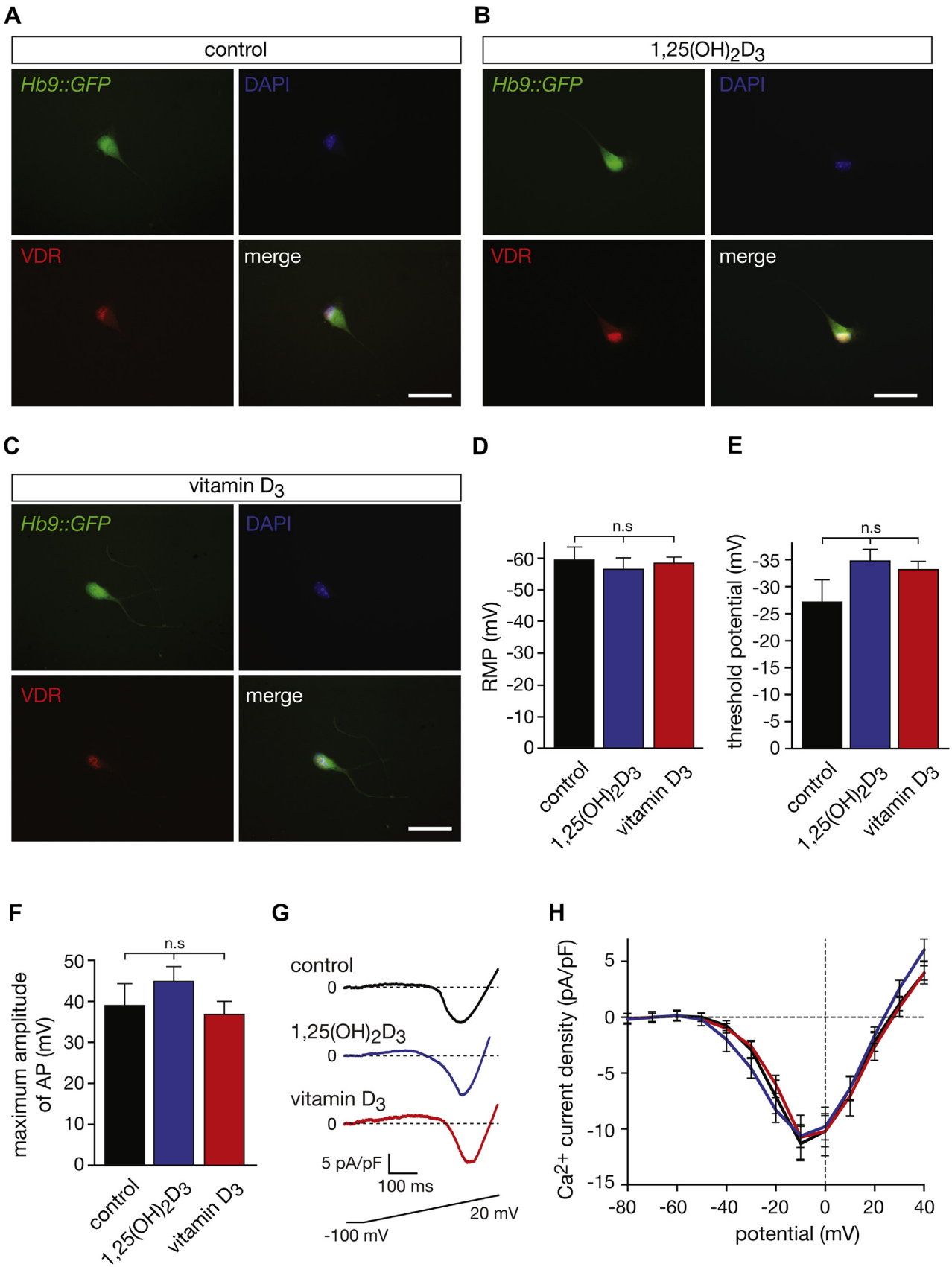


**Fig. 1.** Impact of $1,25(OH)_2D_3$ on electrophysiological features of motoneurons. (A–C) Embryonic motoneurons isolated from *Hb9::GFP* (green) mice were cultured in the presence of neurotrophic factors (NTFs) for 24 hours before being incubated or not (control) with 100 nM of $1,25(OH)_2D_3$ (B), and vitamin $D_3$ (C) for 8 hours. Cells were then immunolabeled for vitamin D receptor (VDR, red). Nuclei were stained with DAPI (blue). Controls using secondary antibody alone gave no staining (data not shown). Scale bar, 50 μm. (D–F) Electrical

and CNTF, referred to as NTFs) is added to culture media. However, in the absence of NTFs more than half of the initially seeded motoneurons are eliminated during the first 24 hours of culture (Arce et al., 1999). Motoneuron survival was studied following the addition of increased concentrations of vitamin $D_2$, vitamin $D_3$, and $1,25(OH)_2D_3$ in the culture medium of motoneurons plated in the absence of NTFs. As expected, the absence of GDNF, BDNF, and CNTF led to a loss of about 70% of motoneurons after 24 hours. The dose-response analysis of $1,25(OH)_2D_3$, vitamin $D_2$ and vitamin $D_3$ did not show any neurotrophic activity in primary cultures (Fig. 2A). Higher concentration of $1,25(OH)_2D_3$, vitamin $D_2$, and vitamin $D_3$ (200 nM) increased motoneuron death (data not shown).

We then investigated whether $1,25(OH)_2D_3$ could potentiate the trophic effect of GNDF, BDNF, and CNTF. Motoneurons were seeded in the presence of NTFs and in the presence of increasing concentrations of $1,25(OH)_2D_3$. Vitamin $D_2$ and vitamin $D_3$ were added at the maximum concentration motoneurons could tolerate. After 24 hours, cell counting showed that $1,25(OH)_2D_3$, but not vitamin $D_2$ or vitamin $D_3$, significantly improved motoneuron survival (Fig. 2B). This suggests that $1,25(OH)_2D_3$ acts by potentiating trophic activity of NTFs in motoneurons.

### 3.3. $1,25(OH)_2D_3$ rescues motoneurons from Fas killing effect

Accumulating evidence suggests that the motoneuron-restricted Fas death pathway is involved in the selective degeneration of motoneuron in ALS mice and may also contribute to the degenerative process in sporadic ALS patients (Aebischer et al., 2013; Bernard-Marissal et al., 2012; Raoul et al., 2006). In addition, we have previously demonstrated that Fas death signaling can be inhibited by the prolonged presence of neurotrophic factors (Raoul et al., 1999). Following the potentiating effect of $1,25(OH)_2D_3$ on NTFs activity in motoneurons, we investigated whether $1,25(OH)_2D_3$ could protect these cells from Fas killing effect. *Hb9::GFP* motoneurons were cultured for 24 hours in the presence of NTFs before being treated for 48 hours with soluble FasL in combination of either vitamin $D_2$, vitamin $D_3$, or $1,25(OH)_2D_3$. After this delay, $1,25(OH)_2D_3$ completely rescued motoneurons from Fas-induced death, while neither vitamin $D_2$ nor vitamin $D_3$ influenced cell survival (Fig. 2C).

### 3.4. Vitamin D in ALS patients

To evaluate whether vitamin D could be a valid treatment option for ALS, we investigated if plasma vitamin D levels influenced the clinical course of ALS. A total of 94 ALS patients had a plasma determination of total vitamin D level in the considered period. Twenty of them were excluded as one of them had prior vitamin D supplementation; potentially modifying outcome and 19 others were not able to walk without assistance. We considered this handicap as a potential bias as a significant lower limb handicap lead a patient to become homebound and sedentary, thus artificially lowering vitamin D levels because of reduced sun exposure. This resulted in a final group of 74 ALS patients with a male to female ratio of 1.47 (30 women and 44 men), and a mean age at ALS onset of 64.3 years (Table 1). Predominant sites of onset were lower limb (44.6%) and bulbar (31%, Table 2). Of these 74 ALS patients, 28 (37.8%) had already died at the time of database lock. At the time of determination of vitamin D levels, mean duration of ALS was 29.8 months and mean severity score was 0.92 (points of ALSFRS-R lost each month). Mean vitamin D levels were low (52.9 nmol/L, normal value >75 nmol/L) with 12 patients in the NVD group, 52 for VDD, and 10 ALS patients in the SVDD group.

Men had a lower severity score compared with women, 0.78 and 1.12, respectively, but this difference was not significant ($p = 0.84$, Table 2). The severity score was negatively correlated with ALS duration ($r = -0.62$, $p < 0.001$) and ALSFRS-R ($-0.57$, $p < 0.001$). Conversely no correlation between severity score and age of onset of ALS could be noted. Patients with upper limb onset had the highest severity score (1.04) and those with lower limb onset had the lowest severity score (0.82), but this was not statistically meaningful. Although already deceased patients had higher average severity score, comparison with living patients did not demonstrate a significant difference.

Severity score and vitamin D levels were studied both quantitatively and qualitatively. Both approaches gave significant results as, first, severity score and vitamin D levels were negatively correlated ($-0.27$, $p = 0.019$) and, secondly, NVD patients had the lowest severity score, SVDD the highest, and VDD was intermediate (0.36, 1.42, 0.95, respectively, $p = 0.00013$). Newman-Keuls post hoc showed that severity score in the NVD group was significantly lower than in both the SVDD and VDD groups. This showed that ALS patients with normal vitamin D levels had the lowest rate of decline.

In univariate models, lower vitamin D levels and shorter ALS duration were associated with higher severity scores ($p = 0.015$ and $p = 0.003$, respectively), while for gender, age of onset, and site of onset, no differences could be noted. The multivariate results were similar with higher risk of high severity score in patients with shorter ALS duration (odds ratio, 0.13; 95% confidence interval, 0.043–0.42; $p = 0.0006$) and lower vitamin D levels in ALS patients with a high severity score (odds ratio, 63; 95% confidence interval, 4–988; $p = 0.0057$).

Median survival of the 74 patients was 43 months (95% CI, 32–51). In the NVD group, 9 patients (75%) were alive and a median survival was 52.8 months. For VDD patients, 35 patients (67%) were alive with a median survival of 47.9 months, while only 2 patients from the SVDD group (20%) were alive, corresponding to a median survival of 29.5 months. Kaplan-Meier estimates of survival between these groups confirmed the influence of vitamin D levels on survival (log-rank, 0.002, Fig. 3). Aside with vitamin D levels, age at onset of ALS, and the ALSFRS-R score were the only clinical criteria found to influence survival in this univariate analysis (log-rank, $p = 0.02$ and 0.049, respectively). When these 3 variables were included in a Cox proportional hazards analysis, an older age of onset was associated with a higher risk of death (hazard ratio, 2.337; 95% CI, 1.003–5.444; $p = 0.049$). After adjustment for age at ALS onset, the risk of death was significantly increased in SVDD patients compared with NVD ones (hazard ratio, 5.9; 95% CI, 1.4–24.3; $p = 0.01$).

## 4. Discussion

Here, we show that vitamin D promotes survival of motoneurons by potentiating the trophic activity of neurotrophic factors and completely blocked death receptor-induced death. Moreover, we show that vitamin D deficiency is associated with both a more rapid

properties of motoneurons, including resting membrane potential (RMP) (D), threshold potential (E) and maximum amplitude of action potential (AP) (F) were recorded at 6 days in vitro (DIV) following the treatment or not (control; n = 6) with 100 nM of $1,25(OH)_2D_3$ (n = 12) or vitamin $D_3$ (n = 4) for 24 hours. (G) Representative traces of $Ca^{2+}$ current recorded at 6 DIV in untreated motoneurons (black line) or neurons incubated with 100 nM of $1,25(OH)_2D_3$ (red line) or vitamin $D_3$ (blue line) for 24 hours using a voltage ramp shown on bottom. Dashed line represents zero current. (H) Current density-voltage relationship recorded at 6 DIV in untreated cells (black line) or in treated cells with 100 nM of $1,25(OH)_2D_3$ (red line) or vitamin $D_3$ (blue line) for 24 hours. Values are represented as mean ± standard error of the mean (SEM). Abbreviations: AP, action potential; DIV, days in vitro; NTFs, neurotrophic factors; RMP, resting membrane potential; SEM, standard error of the mean; VDR, vitamin D receptor.

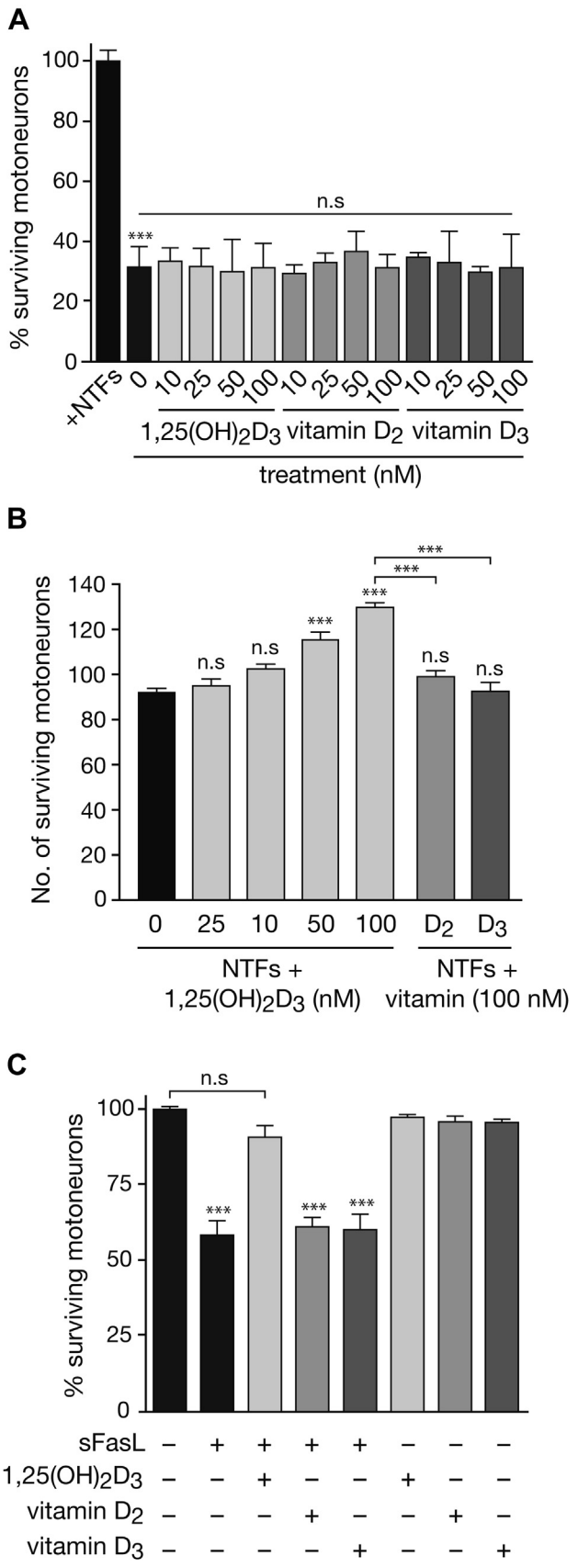


**Fig. 2.** 1,25$(OH)_2D_3$ potentiates neurotrophic factor activity and saves motoneurons from Fas killing effect. (A) Isolated *Hb9::GFP* motoneurons were seeded in the presence or the absence of a cocktail of neurotrophic factors (NTFs) including glial-derived neurotrophic factor (GNDF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF). Increasing concentrations (nM) of 1,25$(OH)_2D_3$, vitamin $D_2$, and vitamin $D_3$ were added in the absence of NTFs. Motoneuron survival was determined 24 hours of culture and expressed relative to the survival of neurons in the presence of NTFs alone. (B) Motoneurons were plated in the presence of NTFs and increasing concentration of 1,25$(OH)_2D_3$ and 100 nM of vitamin $D_2$ or vitamin $D_3$. Neuron survival was determined by direct counting of *Hb9::GFP* cells under fluorescence microscope 24 hours after seeding. (C) Motoneurons were cultured for 24 hours in the presence of NTFs and then treated (or not) with indicated reagent. sFasL, 100 ng/mL (in the presence of 1 μg/mL enhancer antibody used to cross-link sFasL); 1,25$(OH)_2D_3$, vitamin $D_2$ or vitamin $D_3$ 100 nM each. Motoneuron survival was determined 48 hours later and expressed relative to nontreated neurons. Values are means ± standard error of the mean (SEM), *** $p < 0.001$, analysis of variance (ANOVA) with Bonferroni post hoc test. Graphs are representative of 3 independent experiments performed in triplicate. Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; GNDF, glial-derived neurotrophic factor; NTFs, neurotrophic factors; SEM, standard error of the mean.

rate of decline, patients evolving almost 4 times more rapidly, and a significantly shorter survival time.

Vitamin D has already been demonstrated to be neuroprotective in vitro. However, studies are scarce and have, to date, never focused on primary motoneurons. In hippocampal neurons, 1,25$(OH)_2D_3$ has a protective effect against excitotoxic insults which has been associated to a decrease in L-type voltage-sensitive $Ca^{2+}$ channel messenger RNA levels and activity (Brewer et al., 2001, 2006). In motoneurons, we consistently found that, at the same concentration of 1,25$(OH)_2D_3$ that was neuroprotective against N-methyl-D-aspartic acid-induced excitotoxicity in hippocampal neurons, 1,25$(OH)_2D_3$ exerts a neuroprotective effect against the motoneuron-restricted Fas death pathway. We also observed that at higher concentrations, 1,25$(OH)_2D_3$ was neurotoxic in motoneurons, as similar to what was reported in hippocampal neurons (Brewer et al., 2001). However, we did not observe any effect on $Ca^{2+}$ current and electrical activity in motoneurons, suggesting that molecular mechanisms of 1,25$(OH)_2D_3$ action might be cell-type dependent.

Another intriguing finding relates to the potentiation of NTF activity by 1,25$(OH)_2D_3$ in motoneurons (Fig. 2A and B). While we did not observe that 1,25$(OH)_2D_3$ protects against death induced by NTF deprivation, suggesting that it cannot substitute for motoneuron survival factor; we found that when added at the time of seeding, when the dependence of motoneurons on exogenous trophic factors is high, 1,25$(OH)_2D_3$ significantly potentiates the survival effect of NTFs. Additionally, we found that 1,25$(OH)_2D_3$ saved motoneurons from Fas-induced death (Fig. 2C). This result is consistent with an effect of 1,25$(OH)_2D_3$ on NTF signaling. Indeed, it was previously demonstrated that NTFs negatively regulates susceptibility of motoneurons to Fas killing effect (Raoul et al., 1999). Interestingly, the activation of Fas leads to the decreased expression of calreticulin, a $Ca^{2+}$-binding protein implicated in the homeostatic control of endoplasmic reticulum $Ca^{2+}$ levels and endoplasmic reticulum stress response, selectively in motoneurons expressing ALS-linked mutated SOD1 (Bernard-Marissal et al., 2012). The forced expression of calreticulin protected SOD1 mutant motoneurons against Fas-induced death, and reduced levels of calreticulin were documented in the vulnerable population of motoneurons in ALS mice (Bernard-Marissal et al., 2012; Saxena et al., 2013). Hence, an impaired homeostatic regulation of $Ca^{2+}$ was proposed to underlie the motoneuron susceptibility to neurodegeneration (Ho et al., 1996; Saxena et al., 2013). To our concern, mice with a targeted deletion of the VDR gene showed a severe motor impairment, which was associated with an altered $Ca^{2+}$ homeostasis (Kalueff et al., 2004). This is consistent with the evidence showing that vitamin D upregulates the expression of 2 $Ca^{2+}$-binding proteins, calbindin and parvalbumin (Alexianu et al., 1998; de Viragh et al., 1989; Zheng et al., 2004). Further studies are

**Table 1**
Characteristics of 74 patients with ALS

| Charateristics | Mean ± SD | Min | Max | Median | First quartile | Third quartile |
|---|---|---|---|---|---|---|
| Age at onset (y) | 64.3 ± 10.2 | 35.7 | 89.5 | 64.3 | 58.5 | 71.3 |
| Duration (mo) | 29.8 ± 13.5 | 6.4 | 64.5 | 28 | 20.1 | 38.4 |
| ALSFRS-R (/48) | 38.4 ± 6.6 | 20 | 47 | 40.5 | 36 | 43 |
| Severity score | 0.92 ± 1.13 | 0.07 | 8 | 0.58 | 0.33 | 1.11 |
| Vitamin D (nmol/L) | 52.9 ± 24.2 | 0 | 135.1 | 51 | 35.8 | 69 |

Key: ALS, amyotrophic lateral sclerosis, ALSFRS-R, revised amyotrophic lateral sclerosis functional rating scale; max, maximum value; min, minimum value; SD, standard deviation.

therefore warranted to explore the effect of vitamin D on the homeostatic regulation of $Ca^{2+}$ in motoneurons.

NTFs, such as GDNF, BDNF, insulin-like growth factor or vascular endothelial-derived growth factor, have been regarded as promising therapeutic candidates because of their beneficial effects in ALS experimental models (Gould and Oppenheim, 2011). Unfortunately, the clinical outcome of systemically administrated NTFs in ALS patients has been to date either ineffective or resulted in harmful side effects (Gould and Oppenheim, 2011). A more tissue-targeted and long-sustained delivery of NTFs using cell-or gene therapy-based approaches therefore need to be considered. However, another appealing approach targets the potentiation of endogenous NTFs signaling. Indeed, it has been demonstrated that the amplification of the endogenously-produced hepatocyte growth factor by an enhanced Met signaling in motoneurons, significantly ameliorated motor performance and increased lifespan of SOD1 mutant mice (Genestine et al., 2011). Thus, it is possible that vitamin D acts by potentiating endogenous NTF signaling in ALS patients, therefore offering a potential therapeutic alternative.

In the present study, low plasma levels of vitamin D were found to be associated with a higher rate of decline of ALS patients, as defined by the severity score, and also with shorter survival. The disease in patients with the lowest vitamin D levels (SVDD) evolved almost 4 times more rapidly than those with normal levels (severity score, 1.42 vs. 0.36), severity score from VDD patients was intermediate and also significantly increased compared with NVD patients. Similarly, median survival of SVDD patients was reduced to 29.5 months compared with 52.8 months for the group with NVD, and SVDD patients had a 6 times higher risk of death (Cox model). The population studied has classical characteristics of an ALS

**Table 2**
Severity score and characteristics of 74 patients with ALS

| Characteristics | n | Mean ± SD | Median | p |
|---|---|---|---|---|
| Gender | | | | 0.8385[a] |
| Female | 30 | 1.12 ± 1.59 | 0.56 | |
| Male | 44 | 0.78 ± 0.63 | 0.59 | |
| Site of onset | | | | 0.73[b] |
| Bulbar | 23 | 0.97 ± 0.98 | 0.62 | |
| Upper limb | 17 | 1.04 ± 1.85 | 0.46 | |
| Lower limb | 33 | 0.82 ± 0.71 | 0.57 | |
| Respiratory | 1 | 1 | 1 | |
| Outcome | | | | 0.117[a] |
| Alive | 46 | 0.8 ± 0.87 | 0.46 | |
| Dead | 28 | 1.11 ± 1.45 | 0.85 | |
| Vitamin D | | | | 0.00013[b] |
| Normal level | 12 | 0.36 ± 0.47 | 0.25 | |
| Deficiency | 52 | 0.95 ± 1.23 | 0.6 | |
| Severe deficiency | 10 | 1.42 ± 0.84 | 1.52 | |

Key: SD, standard deviation.
[a] Wilcoxon test.
[b] Kruskall-Wallis test.

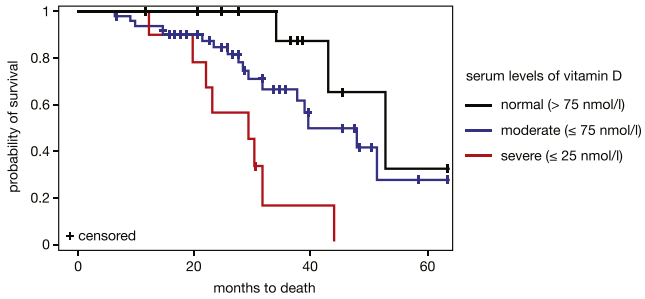


**Fig. 3.** Kaplan-Meier survival estimate of ALS patients according to vitamin D levels. Abbreviation: ALS, amyotrophic lateral sclerosis.

population with similar sex ratio (male predominance, men and women ratio of 1.47) and mean age at onset (64.3 years) (Hardiman et al., 2011). The impact of vitamin D levels on ALS phenotype was studied using both the severity score and overall survival. ALS severity score is based on the ALSFRS-R scale which is the commonly used scale to follow patients' progression, evaluating the 4 main domains of disability in ALS, for example bulbar involvement, upper and lower limb deficits, and respiratory involvement. It has been shown that the ALSFRS-R score was 1 parameter closely correlated to patients' survival (Kollewe et al., 2008). ALS severity score is a validated measure of ALS rate of decline and, in our hands, the correlative influence of vitamin D levels on median survival, may confirm its robustness (Kimura et al., 2006). These results seem clinically important as, to date, no biological marker has been be strongly linked to ALS prognosis (Dorst et al., 2011; Dupuis et al., 2008).

As our study in ALS patients is retrospective, a selection bias may have occurred. To minimize such a bias, we selected a period of time for analysis of patient's files and considered all the patients followed in our ALS Center during this period. At the time of vitamin D dosage, in SVDD patients, mean ALSFRS-R score was the lowest as did ALS duration, and this was consistent with the higher severity of this group. Those patients with SVDD also represent the smallest group and account for 13.5% of the population which is, in proportion, comparable to what is usually described in the normal population and in other studies examining the impact of vitamin D on clinical phenotype of diseases (Berry et al., 2011; Thomas et al., 2012). For these reasons, a selection bias seems unlikely. Another caveat regarding this cohort is lower limb handicap that could indirectly lower vitamin D levels because of reduced sun exposure. To try to minimize such a bias, patients that no longer walked were excluded. In an attempt to ensure that such exclusion, on the contrary, did not impact our results, we also did a post hoc analysis including these primarily excluded patients. Statistical difference between SVDD and NVD was indeed increased when including these patients, confirming that the initial exclusion of these patients was a good and conservative option (data not shown).

Low vitamin D levels have been associated with an increased severity of various neurologic disorders and aging (Balion et al., 2012; Bischoff-Ferrari et al., 2004; Kojima et al., 2012; Mowry et al., 2012; Suzuki et al., 2012). Consequently, the impact of vitamin D deficiency on disease progression we observed is not likely to be specific to ALS. While our study show that vitamin D has a strong neuroprotective effect on the main cellular target of ALS, the motoneurons, and that vitamin D levels may impact ALS prognosis, we do not provide evidence that vitamin D supplementation would be beneficial to individuals with ALS. However, it has been recently suggested that vitamin D supplementation might provide therapeutic benefits in ALS patients (Karam et al., 2013). All together, this evidence may pave the way towards a randomized trial of vitamin D supplementation in patients with this devastative disorder.

## Disclosure statement

Authors declare that they have no conflicts of interest.

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