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Could conservative iron chelation lead to neuroprotection in amyotrophic lateral sclerosis?

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ABSTRACT

n accumulation has been observed in mouse models and both sporadic and familial rms of Amyotrophic lateral sclerosis. Iron chelation could reduce iron accumulation and e related excess of oxidative stress in the motor pathways. However, classical iron elation would induce systemic iron depletion. We assess the safety and efficacy of nservative iron chelation (i.e. chelation with low risk of iron depletion) in a murine eclinical model and pilot clinical trial. In *Sod1*^{G86R} mice, deferiprone increased the mean e span as compared with placebo. The safety was good, without anemia after 12 months deferiprone in the 23 ALS patients enrolled in the clinical trial. The decreases in the ALS nctional Rating Scale and the body mass index (BMI) were significantly smaller for the st 3 months of deferiprone treatment (30 mg/kg/day) than for the first treatment-free riod. Iron levels in the cervical spinal cord, medulla oblongata and motor cortex ccording to MRI), as well as cerebrospinal fluid levels of oxidative stress and urofilament light chains were lower after deferiprone treatment. Our observation leads the hypothesis that moderate iron chelation regimen that avoids changes in systemic n levels may constitute a novel therapeutic modality of neuroprotection for ALS.

Amyotrophic lateral sclerosis (ALS) is characterized by rapid progressive upper and lower motor neuron degeneration, leading to paralysis and death. Iron accumulation has been observed in mouse models and both sporadic and familial forms of ALS.(1,3,4,5,6,8,9) Iron accumulation seems to occur at least in microglial cells within motor cortical regions.(6) and has been observed in the motor cortex using MRI.(1,6) In patients with sporadic ALS, cerebrospinal fluid (CSF) levels of iron are elevated,(4) and elevated serum ferritin levels correlate with shorter survival.(9) Importantly, iron chelation has showed therapeutically relevant protective effects in animal models.(3,5) Deferiprone is a unique iron chelator; at low dose levels, it can cross membranes, decrease regional iron accumulation, redeploy the captured iron to extracellular transferrin, and subsequently distribute iron throughout the body (thus avoiding anemia), defining the "conservative" iron chelation.(2)

Dose and sex effect on neuroprotection with deferiprone in the murine model of ALS

A dose- and sex-dependent effect of deferiprone on survival was observed in the $SOD1^{G86R}$ mouse model (**Fig. 1**). The female mice in the 50-mg/kg/day deferiprone groups survived for 13 days longer than those in the vehicle group (**Fig. 1A**). This corresponded to a 56% extension in survival from disease onset (defined as the peak in body weight) (**Fig. 1B**). The dose of 100-mg/kg/day was less effective and the dose of 200-mg/kg/day was not effective (not shown). A significant effect was observed in male mice only with the highest dose (200-mg/kg/day). Deferiprone improved the animals' physical examination, as shown by greater body weight (**Fig.1B**), a lower peak in neurological impairment (i.e. a lower NeuroScore, a quick phenotypic neurological scoring system) (**Fig. 1C**) and return gene expression of *acetylcholine receptor subunit γ (Chrng)* to wild type (WT) levels (marker of muscles denervation) (**Fig. 1D**). Importantly, treated mice had less iron accumulation in the spinal cord (shown by MRI T2* sequence) compared to vehicle-control mice (**Fig. 1E**); demonstrating an ability of deferiprone to hit the biological target. As with treatment in other neurological models,(2) deferiprone did not induce anemia, and serum ferritin were only marginally below normal levels at the highest dose (**Fig. 1F**).

Safety profile of deferiprone in early ALS patients

Twenty-three consecutive sporadic patients were enrolled (22 limb onset and 1 bulbar

onset) (**Table 1**). Four patients dropped out: one patient died after a fall, and 3 withdrew their consent. All were compliant to medication. The non-neurological physical examination remained unchanged. All patients displaying normal hematologic profiles, a slight elevation in urine iron levels and a transient decrease in serum ferritin (in the normal ranges) was observed in the first 3 months of treatment.

Is there a disease modifying effect of deferiprone in early ALS patients?

The decrease in the ALSFRS-R score was significantly smaller for the first 3 months of deferiprone treatment than for the 3-month treatment-free period (p=0.013) (**Table 1**). Likewise, the decrease in the BMI was significantly different, with a decrease during the first 3 months and a small increase during the treatment period (no RIG/PEG feeding) (p=0.047). Then BMI remained unchanged during 9 months. The reduction in MMT scores was lower in patients on deferiprone than in matched patients from the Mitotarget study, although this difference did not reach statistical significant (p=0.09) (**Table 2**).

Iron accumulation and oxidative stress reduction under deferiprone in early ALS patients

A significant decrease in iron concentration (shown by a decrease in R2*) was observed in the cervical spinal cord, the medulla oblongata and the motor cortex but not in areas outside the motor system (i.e. the cerebellum and the occipital cortex) following treatment with deferiprone. Iron levels, oxidative stress marker and neurofilament light chains were lower after deferiprone treatment in the cerebrospinal fluid (**Table 1**).

Conservative iron chelation

The present study is a first to demonstrate the safety of conservative iron chelation in ALS. In both a murine model of familial ALS and sporadic ALS patients, low-dose deferiprone was associated with a decrease in pathologic iron accumulation in the central motor pathways but did not alter iron metabolism in other regions of the brain or in the periphery. This new therapeutic strategy appears to maintain the patient's overall aerobic metabolism and limit excess oxidative stress - as has been observed in Parkinson's disease (2).

Deferiprone significantly increased survival, as previously reported with other iron chelators.(3,5) This was observed despite the treatment initiation at the symptomatic stage in the phenotypically aggressive SOD1^{G86R} model. An interaction between dose and sex was observed; disease severity, iron accumulation and the required dose were higher in males than in females.

Deferiprone had a good safety profile in patients, with adverse events mostly relating to persistent ALS symptoms. We did not detect any of the adverse events occasionally observed in patients with systemic iron overload treated with 100-mg/kg/day deferiprone. Encouragingly for a safety trial with a small number of patients, deferiprone treatment was associated with slower disability progression and weight loss. However, the occurrence of a nocebo effect and then a placebo effect cannot be ruled out, and so a large, multicenter, double-blind, placebo-controlled, randomized clinical trial is underway.

Innovation

The present work provides the first clinical evidence about the neuroprotective potential of a therapeutically safe chelation treatment on early- stage ALS patients and responded significantly to treatment in both brain iron deposits and indicators of disease progression. The novel treatment relied on oral administration of deferiprone that by chelation of labile iron it conferred upon oxidation-stressed animals and improved motor functions, while essentially sparing systemic iron. The paradigmatic modality of chelation with deferiprone in ALS has prompted a multi-center study.

Notes

Material and Methods

SOD1^{86R} transgenic mice

All animal experiments were carried out in accordance with the "Principles of Laboratory Animal Care", the current French and European Union legislative and regulatory framework (APAFIS#4269-2015112317225759) and the European ALS group's preclinical trial guidelines of 2010. A dose-response study was performed in FVB-Tg(Sod1*G86R)M1Jwg/J mice (JAX Laboratories) with 50, 100 or 200 mg/kg p.o. deferiprone or vehicle twice a day (10 in each group). Study treatment was initiated at the

age of 75 days, *i.e.* an age at which these mice are devoid of motor symptoms but already present with weight loss. The investigators were blinded to the study treatment. Magnetic resonance imaging of the cervical spinal cord on a 7-T MR system (Biospec Bruker, Ettlingen, Germany), using a multi-echo T2*-weighted sequence (number of echoes: 12; first echo time: 4 ms; echo spacing: 7 ms; repetition time: 1500 ms; slice thickness: 1 mm; field of view: 200x250mm; matrix: 256x256; number of signal averages: 2)

Clinical trial

A single-center, single-arm, 12-month pilot clinical trial was performed to evaluate the effect of deferiprone in patients with ALS. The patients were followed for a 3-month treatment-free period and then treated for 12 months with the liquid formulation of deferiprone at a dose level of 30 mg/kg/day (morning and evening dose). The patients were recruited between December 2013 and January 2015, and all provided written, informed consent. All patients had been taking riluzole. Treatment compliance (>80%) was assessed by questioning the participants and inspection of the dispensed packs of medication. The primary outcome criterion was disease progression, as measured using the revised ALS Functional Rating Scale (ALSFRS-R). The 3-month treatment-free period was compared with the first 3 months of treatment. The secondary outcomes included manual muscle testing (MMT), body mass index (BMI), slow vital capacity and CSF levels of markers for oxidative stress and neurofilaments. The physical examination was assessed every 3 months together with adverse event reports and reviewed anonymously by an independent safety monitoring board. Weekly blood counts were used to monitor the risk of neutropenia. For exploratory purposes, 19 patients treated with deferiprone for 9 months were compared with 19 matched individuals from amongst the all 512 patients in the Mitotarget trial (negative results with olesoxime; NCT:00868166).(7)

Iron content was quantified by R2* tranverse relaxation rates (=1/T2*) measured in a 3-Tesla MRI system (Achieva, Philips Medical Systems, Best, The Netherlands) using a 2D fast-field echo multi-echo sequence (number of echoes: 15; first echo time: 3.6 ms; echo spacing: 3.3 ms; repetition time: 1803 ms). Two stacks were subsequently acquired in the axial plane; 17 slices for each (slice thickness: 2 mm; isotropic, no gap, field of view: 230x190 mm; matrix: 116x95; number of signal averages: 2) to cover a volume between

the floor of the fourth ventricle and the corpus callosum convexity. Images were processed using a T2* tool on an IDL virtual machine (V2, www.rsinc.com/IDL). A mono-exponential signal decay with the echo time was obtained by voxel-by-voxel nonlinear least-squares fitting of the multi-echo data [$S(t) = S_0.e^{-t.R2^*}$; where t = echo time, S = measured signal, R2* = transverse relaxation rate]. Region of interests were manually drawn on R2* maps by the same operator, who was blinded to the clinical data.

Statistical analysis

Differences in main outcomes between the treatment-free period (Months 0 to 3) and the first three months of deferiprone treatment (Months 3 to 6) were assessed with a paired T test (for normally distributed variables) or Wilcoxon's signed rank test.

In order to take into account the differences in baseline characteristics between the patients on deferiprone and the Mitotarget population, we performed 1:1 matching on three pre-specified factors: age (±5 years), disease duration (±2 months) and sex. Changes in the main outcomes between Month 3 and 15 (9 months of treatment) in the paired groups were compared using linear mixed models with random coefficients. *Group*, *time*, the *group* x *time* interaction and the baseline value were considered as fixed effects, with the participant and block matching considered as random effects. All the statistical tests were two-sided ($p<0.05$), and all data were analyzed using SAS software (version 9.4, SAS Institute Inc., Cary, NC).

Study approval.

All clinical investigations were performed in accordance with the tenets of the Declaration of Helsinki. All patients provided their written, informed consent to participation. A local institutional review board approved the aims and procedures of the main study (national reference number: 2013-001228-21; ClinicalTrials.gov reference: NCT02164253) and a compassionate 12-month extension. The study and the manuscript followed the consort statement.

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AUTHORS CONTRIBUTIONS AND ACCOUNTABILITIES

A: Study concept and design; B: acquisition of data; C: analysis and interpretation; D: critical revision of the manuscript for important intellectual content; E: study supervision

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Maeva Kyheng and Alain Duhamel: C, D & independent and blind statistical analyses

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Luc Defebvre, James Duce, Régis Bordet: A, C, D

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David Devos: A, C, D, E

The indicated authors take responsibility for data collection and analysis and the principal investigator (DD), who had full access to all the study data, takes full responsibility for submitting the final work for publication.

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LIST OF ABBREVIATIONS

ALS: Amyotrophic lateral sclerosis

ALSFRS-R Functional: ALS Rating Scale

BMI: body mass index

CSF: cerebrospinal fluid

DFP: deferiprone

MMT: manual muscle testing

MRI: magnetic resonance imaging

NLF: neurofilaments

SCV: slow vital capacity

SOD: superoxide dismutase

VEH: vehicule

WT: wild type

8-oxdG: DNA adduct of 8-oxo-7,8-dihydro-2'-deoxyguanosine

Table 1: Neurological and systemic parameters in ALS patients at inclusion and with up to 12 months deferiprone treatment. Data are mean ± Standard Deviation (1st line) and median value [Quartile 1-Quartile 3] (2nd line). A revised ALS Functional Rating Scale (ALSFRS-R) score was measured as the primary outcome in the patient cohorts where as body mass index (BMI), muscle strength (measured by manual muscle testing, MMT) and slow vital capacity (SVC) were also evaluated as secondary outcomes (Student's test (*) or Wilcoxon's test (**), depending on the distribution assessed using histograms and the Shapiro-Wilk).

Iron content was quantified by R2* tranverse relaxation rates (=1/T2*) measured in a 3-Tesla MRI system (Analysis of variance for repeat measures; the statistically significant contrast analyses for V0 vs. V3, V3 vs. V6, and V6 vs. V9 are indicated).

Additional secondary biological outcomes were evaluated using previously characterized assays. Units observed in control patients were within the established ranges previously reported. Ferritin in CSF and serum in ng/ml. Iron in urines in µg/24h. 8-oxdG: DNA adduct of 8-oxo-7,8-dihydro-2'-deoxyguanosine in ng/ml (Highly Sensitive 8-OHdG Check, Gentaur France SARL, Paris, France), SOD: superoxide dismutase in U/mL (whole blood, as published elsewhere[8]); NfL: neurofilament light chain in pg/mL (Simoa/Digital ELISA platform) and pNfH: phosphorylated neurofilament heavy chain in pg/mL (Biovendor kit) (Student's test (*) or Wilcoxon's test (**), depending on the distribution assessed using histograms and the Shapiro-Wilk).

	← The SAFE-FAIR-ALS trial (months) →						
	Baseline	**V3** **3 months with no treatment**	**V6** **3 months of DFP**	**p value for V3 vs. V6**	**V9** **6 months of DFP**	**V12** **9 months of DFP**	**V15** **12 months of DFPFP**

Population	Mean ± SD age: 56.2 ± 9.8 years; 4F/13M;						
(n=23)	Mean ± SD disease duration (since first signs): 11.7 ± 5.7 months						
Drop out: **4 patients**: 1 death related to brain hemorrhage after a fall and 3 withdrew their consent							
Adverse events during V3-V18 (n = 23)							
Falls with or without trauma (n=9); respiratory failure with or without bronchitis (n=4); arterial hypertension (n=2); transient chest pain (n=2); transient abdominal pain (n=1); pulmonary embolism (n=1); atrial fibrillation (n=1); arterial thrombosis (n=1); transient nausea (n=1); transient postural tremor (n=1); pruritus (n=1); gastritis (n=1)							
Handicap test (n=19)							
ALSFRS-R	**40.5±4**	**35.6±7**	**33.1±8**	**0.013***	31.3±9	29.6±11	29±11
	41[36-45]	**36[31-42]**	**33[26-41]**		31 [26-41]	31[20-40]	35[20-39]
BMI	**26.3±4**	**25.9±4**	**26.0±4**	**0.047****	26.4±4	26.9±3	25±3
	25[24-28]	**26[23-28]**	**25[24-28]**		26 [24-28]	27[24-28]	24[24-27]
MMT	93.9±7	88.0±11	79.4±13	0.33**	77.0±16	75.2±17	72±14
	92[89-99]	90[81-95]	78[73-90]		75 [68-88]	77[66-84]	72[66-79]
SVC	102.6±18	95.3±20	87.3±25	0.1**	79.8±23	78.8±31	75±35
	102[89-112]	90[79-110]	90[60-96]		83 [67-96]	74[57-96]	76[58-92]
Iron accumulation on MRI: R2* (s^{-1}) (n=19)							
precentral-central	14.9 ±1.3	**15.6 ±1.4**	**14.3 ±1.6**	**0.038**** (V3-V6)	13.3 ±2		

cortex						
medulla oblongata	16.2 ±3	**17.4 ±2**	**15.5 ±1**	**0.047**** (V3-V6)	15.7±2	
cerebellum	16.9 ±1	17.1±1	16.9±1	0.3** (V3-V6)	17.1±1	
cervical spinal cord	**40.2±5.6**	**42.2±5.6**	**39.2±4.8**	**0.002****(V3-V6)	39±4.9	
Ferritin and iron in body fluids (n=19)						
Ferritin	134 [100-175]	**143 [89-193]**	**134 [72-170]**	**0.002 (V3-V6)**	150 [99-206]	160 [101-220]
Ferritin (CSF)	-	**12.3 [10-16]**	-	**0.027 (V3-V9)**	**9.7 [9-14]**	
Iron (urine)	11.4 [4-19]	**12.4 [8-12]**	**98.6 [32-99]**	**0.001 (V3-V6)**	85 [42-122]	72 [21-99]
Oxidative stress marker in CSF (n=19)						
8-OHdG	-	**6 [5.4-6.7]**	-	**0.029 (V3-V9)**	**5.2 [5-6.5]**	-
Superoxide dismutase-1 in CSF (n=19)						
SOD	-	.6 [0.5-0.7]	-	0.3 (V3-V9)	.6 [0.5-0.7]	-
Neurofilaments in CSF (n=19)						
NfL	-	5698 [3332-9599]	-	**0.08 (V3-V9)**	3492 [2807-6288]	
pNfH	-	2578 [1338-3245]	-	0.3 (V3-V9)	2381 [1361-3255]	-

Table 2: Comparisons of 9 months of disease progression in patients on deferiprone vs. a matched population from the Mitotarget trial.

The Revised ALS Functional Rating Scale (ALSFRS-R), body mass index (BMI), muscle strength (measured by manual muscle testing, MMT) and slow vital capacity (SVC) were evaluated.

Model for ALSFRS: $\mathrm{ALSFRS_{ik}} = \beta_0 + \beta_1 t_{6ik} + \beta_2 t_{12ik} + \beta_3 \mathrm{Group_{ik}} + \beta_4 \mathrm{Group_{ik}} * t_{6ik} + \beta_5 \mathrm{Group_{ik}} * t_{12ik} + \beta_6 \mathrm{ALSFRS_{baseline_i}} + \gamma_{0i} + \gamma_{1k} + \varepsilon_{ik}$. ik = for any subject i, for any bloc k, $\gamma_{0i} + \gamma_{1k}$ are random effect.**p-value for the overall interaction between *group* and *time*, adjusted for the outcome level before deferiprone treatment (baseline).

Parameters and populations	**deferiprone treatment**	**3 months of deferiprone treatment**	**9 months of deferiprone treatment**	**p-value***
ALSFRS				
SAFE-FAIR, mean	35.6 (6.7)	33.1 (8.2)	30.6 (11.2)	0.50
(SD)	34.0	33.0	(35.0)	
Median	(30.0 to 43.0)	(26.0 to 41.0)	(20.0 to 41.0)	
[IQR]				
Mitotarget , mean	35.4 (8.4)	32.8 (9.9)	30.6 (10.5)	
(SD)	38.0	35.5	35.5	
Median	(30.0 to 42.0)	(26.5 to 41.0)	(23.0 to 38.0)	
[IQR]				

BMI				
SAFE-FAIR, mean	26.0 (4.5)	26.1 (4.9)	27.4 (2.9)	0.32
(SD)	25.7	25.5	27.4	
Median	(23.6 to 28.4)	(24.0 to 29.0)	(25.2 to 28.9)	
[IQR]				
Mitotarget, mean	23.8 (3.2)	23.2 (3.5)	23.0 (3.6)	
(SD)	23.2	21.9	22.5	
Median	(21.4 to 25.7)	(20.7 to 25.3)	(20.5 to 25.5)	
[IQR]				
MMT				
SAFE-FAIR, mean	88.1 (11.8)	79.4 (13.3)	76.0 (17.9)	**0.090**
(SD)	90.5	78.5	78.5	
Median	(80.0 to 96.0)	(73.0 to 90.0)	(68.5 to 85.5)	
[IQR]				
Mitotarget, mean	118.8 (26.4)	112.8 (29.7)	97.5 (39.0)	
(SD)	127.0	121.5	111.0	
Median	(110.0 to 139.0)	(86.5 to 135.0)	(56.0 to 122.0)	
[IQR]				
SVC				

SAFE-FAIR, mean (SD)	96.3 (20.2) 91.6	89.4 (24.1) 90.4	82.9 (28.3) 80.7	0.97
Median [IQR]	(80.4 to 113.0)	(66.7 to 102.8)	(61.8 to 105.4)	
Mitotarget, mean (SD)	92.1 (20.2) 92.5	84.7 (23.7) 88.0	76.4 (36.4) 75.4	
Median [IQR]	(78.0 to 106.0)	(75.0 to 98.0)	(49.5 to 107.5)	

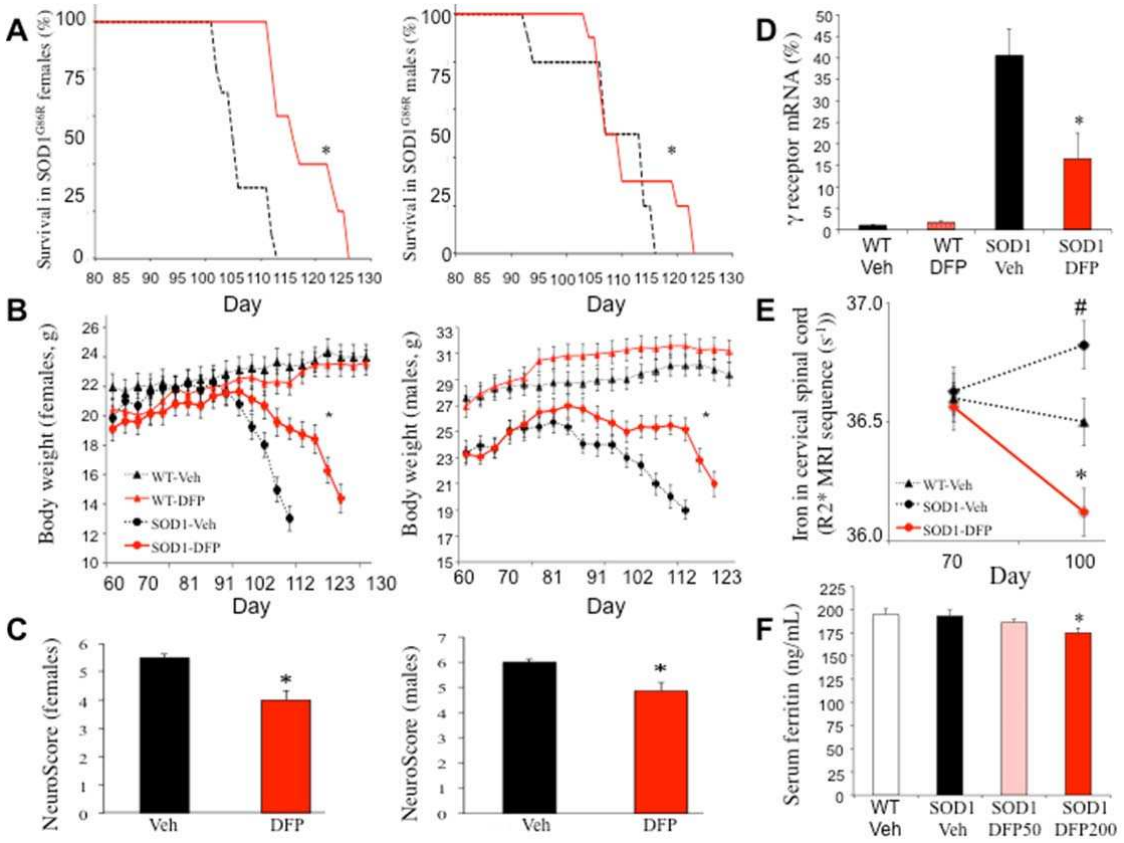

Figure 1: Pharmacological effects of deferiprone in a murine model of ALS.

Data are mean ± SEM in all experiments and doses, n=10 per group for each sex and each dose.

For the females, the best dose was 50-mg/kg/day, the dose of 100-mg/kg/day was less effective and the dose of 200-mg/kg/day was not inefficient. For the males, the only efficient dose was 200-mg/kg/day. All the figures are presented with the dose of 50-mg/kg/day for the females and 200-mg/kg/day for the males. **A.** The survival rates were significantly improved for female (in red) and male (in black) $SOD1^{G86R}$ mice (from disease and treatment onset onwards) in the deferiprone (DFP) group (solid line) and the vehicle group (Veh, dotted line) (Log-rank test; Females: p=0.011; Males: p=0.03)). **B.** Change in body weight in female and male $SOD1^{G86R}$ mice from disease and treatment onset until death, in four groups: wild type (WT)-Veh (black triangles with a dotted line), WT-deferiprone (DFP) (red triangles with a solid line), $SOD1^{G86R}$-Veh (black circles with a dotted line), and $SOD1^{G86R}$-DFP (red circles with a solid line) (ANCOVA adjusted on baseline, * =p<0.05 vs. untreated $SOD1^{G86R}$ mice). **C.** The peak NeuroScore (a quick phenotypic

neurological scoring system for evaluating disease progression in the Mouse Model of ALS: 0: no impairment; 6: greatest possible impairment) in the $SOD1^{G86R}$-Veh group (black bar) and the $SOD1^{G86R}$-DFP group (red bar) (Mann-Whitney test, * =$p<0.05$ vs. untreated $SOD1^{G86R}$ mice). **D.** As measured by qRT-PCR, acetylcholine receptor subunit γ receptor RNA expression as a percentage (%) of the control value in the left gastrocnemius muscle in $SOD1^{G86R}$ female mice treated with 50 mg/kg/day DFP (Mann-Whitney test, * =$p<0.05$ vs. untreated $SOD1^{G86R}$ mice). **E.** Magnetic resonance imaging of the cervical spinal cord at day 70 (i.e. just before the appearance of symptoms and treatment onset) and at day 100 (i.e. with marked motor impairments but before death) in untreated female $SOD1^{G86R}$ mice (triangles with a black solid line), WT female mice (squares with a dotted line) and $SOD1^{G86R}$ female mice treated with 50-mg/kg/day DFP (red line). R2* (= 1/T2*) was obtained in a manually drawn region of interest using a mono-exponential fitting of the signal decay with the echo time. (Kruskal-Wallis test *= $p<0.05$ vs. untreated $SOD1^{G86R}$ mice, ♯ = $p<0.05$ vs. WT non-treated mice) **F**. Serum ferritin levels (ng/mL) from serum obtained at end of treatment: not significantly reduced after 50-mg/kg/day and 100-mg/kg/day (data not shown) but significantly reduced after 200-mg/kg/day. (Kruskal-Wallis test *= $p<0.05$ vs. untreated $SOD1^{G86R}$ mice).