MEDICAL SCIENCES. For the article “Lithium delays progression of amyotrophic lateral sclerosis,” by Francesco Fornai, Patrizia Longone, Luisa Cafaro, Olga Kastsiuchenka, Michela Ferrucci, Maria Laura Manca, Gloria Lazzeri, Alida Spalloni, Natascia Bellio, Paola Lenzi, Nicola Modugno, Gabriele Sililiano, Ciro Isidoro, Luigi Murri, Stefano Ruggieri, and Antonio Paparelli, which appeared in issue 6, February 12, 2008, of Proc Natl Acad Sci USA (105:2052–2057; first published February 4, 2008; 10.1073/pnas.0708022105), the authors note that portions of Fig. 2e and of supporting information (SI) Figs. 11b, 12c, 13c, 17c, 19a and b, 20c, and 23b were assembled from multiple sources or include images moved from their original orientation. The rearrangements were done to provide clarity and consistency within the figures. We regret not explicitly noting this rearrangement in the text or figure legends. We are providing corrected figures, with the rearrangements indicated by thin vertical lines, in accordance with PNAS policies. In addition, SI on the web site has been updated to include the revised figures. The corrected figures and their legends appear below.

Fig. 2. Neuroprotective effects of lithium on medium-size lamina VII neurons. (a) Representative micrographs of those H&E-stained lamina VII neurons that were selected for the count based on size specificity (diameter from 10 to 20 μm). (b) Graph indicates the severe loss of these neurons in G93A mice, which exceeded the loss of MN. Remarkably, the G93A mice treated with lithium showed a much higher number of lamina VII medium-size neurons, even compared with saline-treated WT mice. (c–e) These results were confirmed by gephyrin immunostaining, as shown here, and by all of the staining procedures summarized in SI Fig. 16. Counts represent the mean ± SEM of 62,000 cells per group (3,100 per mouse in groups of 20 mice). Comparison among groups was made by using one-way ANOVA. *, P ≤ 0.05 compared with WT saline-treated group. #, P ≤ 0.001 compared with G93A saline-treated groups. (Scale bars: 17 μm.)
SI Fig. 11. Lithium treatment decreases GFAP immunostaining. (a) Immunofluorescence of cervical and lumbar spinal cords of WT and G93A lithium-treated mice compared with the saline-treated mice showing GFAP staining. At the end of the disease, GFAP immunofluorescence appears highly intense in G93A saline-treated mice, whereas lithium treatment decreases GFAP-positive cells in both tracts of the spinal cord compared with saline-treated G93A mice. No GFAP staining was observed in age-matched, saline- or lithium-treated WT mice. (b) Representative immunoblots and relative densitometric analysis of GFAP expression in the spinal cord of saline- and lithium-treated WT and G93A mice. The significance of these findings is discussed in SI Discussion. Values represent the mean ± SEM of five repeated analyses compared by one-way ANOVA. *, P < 0.05 compared with WT saline-treated group; **, P < 0.01 compared with WT saline-treated group; #, P < 0.05 compared with WT and G93A saline-treated groups. (Scale bars: 28 μm.)

SI Fig. 12. Lithium treatment increases the number of NeuN-positive neurons within Lamina VII. (a) Representative images showing NeuN-positive neurons in the Lamina VII of WT and G93A saline- and lithium-treated mice. (b) Histogram shows the number of NeuN-positive neurons within the Lamina VII of the lumbar spinal cord increased in the G93A mice after lithium treatment. Remarkably, this effect not only consisted of protection from the loss that occurred in the G93A saline-treated mice, but extended to a robust increase in neuron number compared with saline-treated controls. The number of NeuN-positive neurons overlaps that reported within Lamina VII after H&E staining, calbindin 28K, and gephyrin immunostaining. The counting criteria were the same under comparable stereological conditions. (c) Representative Western blot and densitometric analyses of NeuN expression in the lumbar spinal cord of WT and G93A mice, which confirms the increase in NeuN staining after lithium treatment. Values represent means ± SEM, analyzed by one-way ANOVA. *, P < 0.05 compared with WT saline-treated group; #, P < 0.05 compared with WT and G93A saline-treated groups. (Scale bars: 13 μm.)
Lithium treatment increases the number of calbindin 28K-positive neurons in Lamina VII. (a) Lumbar spinal cord sections from saline- and lithium-treated WT and G93A mice immunostained for calbindin 28K. (b) Histogram shows the significant loss of Lamina VII calbindin 28K-positive neurons in the G93A mutant mice compared with WT. Even in this case, the increase in the number of neurons in G93A mice after lithium treatment significantly surpasses that counted in controls. As observed after the other staining procedures, lithium induced no effect on neuron number in WT mice. (c) Representative Western blot and densitometric analyses of calbindin 28K expression in lumbar spinal cord in WT and G93A mice confirm the loss of the calbindin 28K-positivity in G93A saline-treated mice and the increase in the calbindin 28K-positivity after lithium treatment. Values represent means ± SEM, analyzed by one-way ANOVA.

Effects of lithium on α-synuclein immunostaining. (a and b) Representative images of Lamina IX (a) and Lamina VII (b) of α-synuclein immunostaining of WT and G93A mice after saline and lithium treatment. Images suggest an increase in α-synuclein staining in G93A mice, which is reverted by lithium administration. (c) Representative Western blot and densitometric analyses of α-synuclein expression confirming the lithium-induced occlusion in α-synuclein increased positivity of G93A mice. Values represent means ± SEM, analyzed by one-way ANOVA. *, P < 0.05 compared with WT saline-treated group; #, P < 0.05 compared with WT and G93A saline-treated groups.

Lithium reduces both α-synuclein and ubiquitin in the spinal cord of G93A mice. Representative Western blots and densitometric analysis of α-synuclein (a) and ubiquitin (b) expression in the cervical and lumbar tracts of WT and G93A saline- and lithium-treated mice. Lithium significantly decreased the expression of α-synuclein in the lumbar tract, whereas the expression of ubiquitin decreased in both the cervical and lumbar tracts. Values represent means ± SEM, analyzed by one-way ANOVA. *, P < 0.05 compared with WT saline-treated group; #, P < 0.05 compared with WT saline-treated mice and G93A lithium-treated mice; ##, P < 0.01 compared with WT saline-treated mice and G93A lithium-treated mice.
Si Fig. 20. Effects of lithium on SOD1 immunostaining. (a–d) Immunostaining for SOD1 is lighter in WT (a and b) compared with G93A (c and d) mice. In particular, G93A mice treated with saline show intense and aggregated SOD1 immunostaining (c), which, similarly to ubiquitin, was also found in the axon of motor neurons (arrow). This SOD1 accumulation was markedly cleared after lithium administration (d). (Scale bars: 33 μm.) (e) Representative Western blot and densitometric analyses of a triplicate of SOD1 expression shows an accumulation of the protein in G93A saline-treated mice, whereas this is suppressed after lithium administration. Values represent means ± SEM, analyzed by one-way ANOVA. * P < 0.05 compared with WT saline-treated group; #, P < 0.05 compared with WT and G93A saline-treated groups.

Si Fig. 23. Effects of lithium administration on mitochondria in motor neuron cell cultures and cell lines. (a) SH-SY5Y cells plated on sterile coverslips were either not treated (saline) or were treated for 72 h with 1 mM lithium (changing the medium and readding the substance every 24 h), stained for 60 min with 300 nM MitoTracker Red or MitoTracker Green (Invitrogen), and observed under the confocal fluorescence microscope. Arrows point to cytoplasmic regions of high fluorescence intensity that indicate the accumulation of mitochondria. (Scale bars: 17 μm.) (b) Immunoblotting of cytochrome C (monoclonal antibody; Alexis) in homogenates of SH-SY5Y cells treated for 72 h with 1 mM lithium or not treated (saline). Using densitometry, the amount of cytochrome C increased in treated vs. nontreated cells by a factor of 2.5. (c) Flow cytometry analysis of control and lithium-treated (1 mM for 72 h) SH-SY5Y cells labeled with rhodamine-123 (100 nM for 20 min), a fluorescent dye that accumulates specifically in healthy mitochondria. Cytofluorimetry profiles show consistent differences between the two populations. In particular, the proportion of cells showing a higher rhodamine labeling (fluorescence > 102 arbitrary units; M2) is increased by 3-fold upon treatment with lithium. Data shown are representative of five independent determinations. (d) Neuronal cultures were treated with 0.5 and 1 mM lithium for 18 h (the time interval used in all of the cell culture experiments), then MitoTracker Red was added to the culture for 1 h (lithium and nontreated (NT)). Labeled mitochondria were quantified on the basis of MitoTracker Red fluorescence in treated and NT cultures. (Scale bars: 40 μm.) (e) Data are expressed in arbitrary units as background-subtracted fluorescence intensity, assessed by selecting three small regions without cells on the coverslip. Images were collected by using a confocal laser microscope (CLSM510; Zeiss) and were analyzed by using the ImageJ analysis program. The values represent means ± SEM, analyzed by one-way ANOVA after Newman–Keuls post hoc test. * P < 0.05 compared with WT.
Lithium delays progression of amyotrophic lateral sclerosis

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ALS is a devastating neurodegenerative disorder with no effective treatment. In the present study, we found that daily doses of lithium, leading to plasma levels ranging from 0.4 to 0.8 mEq/liter, delay disease progression in human patients affected by ALS. None of the patients treated with lithium died during the 15 months of the follow-up, and disease progression was markedly attenuated when compared with age-, disease duration-, and sex-matched control patients treated with rivuleo for the same amount of time. In a parallel study on a genetic ALS animal model, the G93A mouse, we found a marked neuroprotection by lithium, which delayed disease onset and duration and augmented the life span. These effects were concomitant with activation of autophagy and an increase in the number of the mitochondria in motor neurons and suppressed reactive astrogliosis. Again, lithium reduced the slow necrosis characterized by mitochondrial vacuolization and increased the number of neurons counted in lamina VII that were severely affected in saline-treated G93A mice. After lithium administration in G93A mice, the number of these neurons was higher even when compared with saline-treated WT. All these mechanisms may contribute to the effects of lithium, and these results offer a promising perspective for the treatment of human patients affected by ALS.

autophagy | clinical study | G93A mice | morphological analysis

Effects of Lithium on Disease Duration and Survival in G93A Mice. G93A male mice were treated daily with lithium carbonate (1 mEq/kg, i.p.), starting at 75 days of age. Lithium treatment prolonged the mean survival time from 110.8 ± 5.0 days (n = 20) to 148 ± 4.3 (n = 20, ~36% of the life span of these mice; Fig. 1a; P < 0.001) and, most importantly, increased disease duration (from a mean of 9 days to >38 days, >300%; Fig. 1b; P < 0.05) compared with the G93A mice treated with saline. Even when lithium treatment was started at the onset of motor symptoms, the increase in disease duration was still comparable (data not shown). More specifically, lithium delayed the onset of paralysis and limb adduction (Fig. 1c) and significantly improved additional tests we report in SI Fig. 6, such as rotarod, grip strength, and stride length.

Effects of Lithium Treatment on Motor Neuron Survival (Lamina IX of Lumbar and Cervical Spinal Cord and Brainstem Motor Nuclei). These effects were accompanied by a reduced loss of lumbar MN at 90 days of age (SI Fig. 7). However, at the end of disease (which occurred later following lithium), the number of alpha-MN within lumbar lamina IX of the G93A mice treated with lithium was comparable to that found in the saline-treated mice that had died previously (SI Fig. 8). However, even at this stage, we detected a disease modifying effect of lithium. This consisted of (i) preservation of the size of MN (SI Fig. 8d and e); (ii) preservation of MN number and size in those areas [i.e., cervical spinal cord (SI Fig. 9) or the nucleus ambiguous (SI Fig. 10)], which degenerate later compared with lumbar lamina IX (23, 24); (iii) decreased astrocytosis (SI Fig. 11); and (iv) decreased alpha-synuclein, ubiquitin, and SOD1 aggregation (see SI Fig. 6 and Discussion in SI Text).

Effects of Lithium Treatment on the Renshaw-Like Cell Area (Lamina VII). Lamina VII contains a larger number of interneurons, defined as Renshaw cells, which form a collateral circuit that
ImmunoGold TEM (SI Fig. 15) confirmed the increase in and SI Figs. 7a and c in SI Fig. 16. The increase in neuron number produced by lithium calbindin 28k positive neurons in lamina VII. This is summarized Fig. 12. This combination may be necessary to enhance the neurogenetic number compared with controls. In the present study, we logical conditions in the hippocampus (26) but not in the spinal inhibits MN (25). Renshaw neurons are defined by electrophysiological properties but typically stain for gephyrin and calbindin 28k. Therefore, we used the term Renshaw-like neurons to refer to these cells. We focused on these neurons based on findings by Martin et al. (9), who showed that, in G93A mice, interneurons begin to die before MN; further, pioneer electrophysiological studies suggested an early impairment of Renshaw cells in ALS patients (11). We found that neurons within lamina VII of G93A mice were severely decreased (more than MN, 50% loss, from 36 ± 2.97 to 18.53 ± 1.84; Fig. 2a and b). Remarkably, lithium administration led to an increase in the number of these neurons, which became more abundant even when compared with control mice (131% and 100%, respectively). This net increase in neuron number occurred only when lithium was administered to G93A mice; no such effect occurs in WT (Fig. 2b). All of the staining procedures performed to visualize these neurons within lamina VII confirmed these results: H&E (131% of controls), gephyrin (137% of controls; Fig. 2c and d), NeuN (132% of controls; SI Fig. 12a and b) and calbindin 28k (126% of controls; SI Fig. 13a and b). Immunoblotting confirmed all these staining (Fig. 2e and SI Figs. 7c and 13c). Moreover, the procedure of BrdU incorporation at immunofluorescence (SI Fig. 14) and by using ImmunoGold TEM (SI Fig. 15) confirmed the increase in calbindin 28k positive neurons in lamina VII. This is summarized in SI Fig. 16. The increase in neuron number produced by lithium in the spinal cord is disease dependent, because it did not occur in WT. Lithium is known to produce neurogenesis in physiological conditions in the hippocampus (26) but not in the spinal cord. However, in the absence of lithium, occurrence of neurogenesis in the spinal cord of G93A mice is uncertain, and, when occurring (27), it does not produce a net increase in neuron number compared with controls. In the present study, we combined lithium administration with the ongoing disease state. This combination may be necessary to enhance the neurogenetic effects, leading to a robust increase in the number of neurons (see Discussion in SI Text).

Lithium Treatment Rescues Spinal Cord Mitochondria and Facilitates the Clearance of Alpha-Synuclein, Ubiquitin, and SOD1. In ALS, alpha-synuclein and ubiquitin accumulate in affected neurons (9, 28–30). Lithium treatment reduces the accumulation of alpha-synuclein in both MN of lamina IX (SI Fig. 17a) and in neurons of lamina VII (SI Fig. 17b and c). Similarly, lithium reduces ubiquitin (SI Figs. 18 and 19), and SOD1 aggregates in MN (SI Fig. 20).

In G93A mice, MN undergo vacuolation, a process defined as “slow necrosis” (9), in which they appear to be filled with vacuoles (4, 9) due to mitochondrial swelling (Fig. 3 and SI Figs. 8 and 21). Lithium decreases vacuolation (Fig. 3f and h and SI Fig. 21) and

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**Fig. 1.** Effects of lithium treatment on the lifespan and neurological symptoms of G93A mice. (a) Survival curve for saline- and lithium-treated G93A mice. Lithium carbonate (1 mEq/kg, daily, i.p.) treatment significantly increased the survival time of G93A mice compared with saline-treated mice. (b) Effects of lithium on specific symptoms, such as hind limb adduction, gait impairment, and the onset of severe paralysis. (c) Symptomatic effects and prolongation of the life span induced by lithium. Values represent the mean ± SEM of 10 mice per group in two different experiments (total N per group = 20). Comparison was made by using ANOVA with Sheffe’s post hoc analysis. *, P = 0.05 compared with G93A mice administered saline. **, P = 0.001 compared with G93A mice administered saline.

**Fig. 2.** Neuroprotective effects of lithium on medium-size lamina VII neurons. (a) Shows representative micrographs of those H&E-stained lamina VII neurons that were selected for the count based on size specificity (diameter ranging from 10 to 20 μm). (b) Graph indicates the severe loss of these neurons in G93A mice, which exceeded the loss of MN. Remarkably, the G93A mice treated with lithium showed a much higher number of lamina VII medium-size neurons even compared with saline-treated WT mice. (c–e) These results were confirmed by gephyrin immunostaining, as shown here and by all of the staining procedures summarized in SI Fig. 16. Counts represent the mean ± SEM of 62,000 cells per group (3,100 per mouse in groups of 20 mice). Comparison among groups was made by using one-way ANOVA. *, P = 0.05 compared with WT saline-treated group; #, P = 0.001 compared with G93A saline-treated groups. (Scale bars, 17 μm.)
normalizes mitochondrial size (Fig. 3 i and j and SI Fig. 21). Moreover, lithium increases the number of normal mitochondria in both WT and G93A mice. This was counted at ultrastructural level (Fig. 3 k and l) and confirmed by semiquantitative RT-PCR (SI Fig. 22) in vivo. We replicated this effect by cytofluorimetric counts of the mitochondria labeled with MitoTracker Red and MitoTracker Green and by Western blot analysis of cytochrome C (SI Fig. 23 a–c) in SH-SY5Y cells and primary spinal cord cultures (SI Fig. 23 d and e). The increase in mitochondria we found in the spinal cord confirms what recently found in endothelial cells (31). This is very encouraging, considering that the loss of mitochondria may be a risk for drugs acting as autophagy enhancers.

**Lithium Increases the Number of Autophagic Vacuoles.** At the ultrastructural level, we found degenerating mitochondria within autophagic vacuoles and whorl-like autophagosomes within G93A MN (Fig. 4). This led us to explore the hypothesis that lithium may improve MN survival by activating autophagy (18). We stained spinal cord samples with monodansylcadaverine (MDC), a fluorescent dye that incorporates selectively into autophagolysosomes (32). When lithium was administered, there was a marked increase in autophagic vacuoles as confirmed by ImmunoGold-conjugated autophagy markers, such as beclin or the microtubule-associated protein 1 light chain 3 (LC3) (Fig. 4 e and f). In fact, the number of beclin and LC3 positive vacuoles was increased by lithium (Fig. 4 g and h). This effect was confirmed by counting autophagosomes in SH-SY5Y cells stably transfected with LC3 (33). By counting these markers and the vacuoles detected at phase contrast, we found that lithium and rapamycin (used as a positive control for autophagy) increased vacuoles formation (Fig. 4 i–l, arrows) and GFP-LC3 fluorescence (Fig. 4 m–p), whereas asparagine (an amino acid known to down regulate autophagy; see ref. 34) antagonized the effects of lithium. Induction of autophagy by lithium was matched by increased expression of PTEN (the phosphatase acting on PIP3) and a marked reduction in the SER473 phosphorylation of Akt (data not shown). These data confirm the role of autophagy in neurodegeneration (35) and indicate a powerful effect of chronic lithium administration.

**Effects of Lithium on Spinal Cord Cultures from G93A Transgenic Mice.** If autophagy deficiency causes neurodegeneration in ALS, then its blockade is expected to worsen MN death. To test this hypotheses, we treated spinal cord cultures with the autophagy inhibitor 3-MA (36). MN survival was evaluated by counting...
neurodegeneration of ALS (1). Lithium (1 mM) given 20 min to excitotoxicity (37), which is also a mechanism involved in the lithium against kainate, because G93A MN are highly vulnerable to 136% (SI Fig. 24 increased gephyrin-positive G93A neurons dose-dependently up

Norris scale (raw data)

Survival Curve (%)

MRC (raw data)

FVC (%)

FVC (% of basal value)

a Norris scale (raw data)

b ALSFRS-R (raw data)

c Survival Curve (%)

d FVC (%)

e MRC (raw data)

Fig. 5. Effects of lithium treatment on disease symptom progression and survival in patients with ALS. (a, b, d, and e) Symptoms progression (evaluated every 3 months) in controls (riluzole-treated) and treated patients (lithium plus riluzole-treated patients) expressed as raw data, using the Norris (a) and ALSFRS-R (b) rating scales and FVC (d) and MRC (e). There was no significant progression in the lithium-treated patients at any time interval apart from the last two evaluations using the MRC scale. In the riluzole-treated patients, symptoms progressed significantly starting at 3 or 6 months (depending on the scale). (c) Survival curve as normalized data shows the percentage of patients surviving over the 15 months of treatment in the riluzole and lithium groups. None of the patients died in the lithium-treated group; however, in the control group, although the patients had comparable disease severity at enrollment, <30% died. (g) Shows the breakdown between groups for FVC (calculated at 1 month before death) in those patients affected by the bulbar form, *P < 0.05, **P < 0.01 compared with baseline value. #P < 0.05; ##P < 0.01 compared with control patients.

SMI32-positive neurons after drug treatment compared with NT cultures (SI Fig. 24 a-f). 3-MA alone (5 mM for 2 h) increased G93A MN death by 58% compared with NT G93A MN and by 51% compared with lithium-treated G93A MN (SI Fig. 24g). Moreover, 3-MA treatment significantly increased SMI32-positive MN death in cultures from G93A compared with WT, thus supporting a vulnerability of the autophagy pathway in ALS degeneration (SI Fig. 24g). When 3-MA was given 2 h before lithium treatment (18 h, 1 mM), the latter could no longer counteract the effects of 3-MA, confirming that lithium neuroprotection occurs, at least partially, through an autophagic route (SI Fig. 24g). As found in vivo, lithium administration (18 h) increased gephyrin-positive G93A neurons dose-dependently up to 136% (SI Fig. 24). We further tested the protective effects of lithium against kainate, because G93A MN are highly vulnerable to excitotoxicity (37), which is also a mechanism involved in the neurodegeneration of ALS (1). Lithium (1 mM) given 20 min before kainate (100 μM 15 min) fully protected G93A SMI32-positive neurons (SI Fig. 25 a and b).

Clinical Trial. The previous results led us to translate the use of lithium carbonate to human patients affected by ALS. Recruitment began in October 2005. Typically, compliance was high or moderate for most individuals. There were no significant differences in demographic variables between groups (mean age and standard error in the treated group, 66.9 ± 1.9 vs. 70.3 ± 1.6 years; mean age at the ALS onset, 63.8 ± 1.7 vs. 67.1 ± 1.7). Similar baseline values between groups for ALSFRS-R scale, Norris scale, forced vital capacity (FVC), and Medical Research Council were selected.

When we measured the progression of the disability score in the group treated with lithium, ANOVA test for repeated measures did not show any statistical difference according to the Norris scale (basal value, 79.4 ± 2.8; final value, 71.0 ± 3.9; Fig. 5a) and ALSFRS-R scale (basal value, 39.9 ± 1.2; final value, 34.3 ± 2.3; Fig. 5b). Remarkably, all patients treated with lithium were alive at the end of the study. By contrast, in the group receiving riluzole only, 29% of subjects did not survive (Fig. 5c). This difference was significant at 12 and 15 months.

Assessment of disease progression with objective measurements such as the pulmonary function (FVC) provided similar data. In fact, FVC did not progress significantly (basal value, 89.0 ± 2.6; final value, 74.9 ± 3.6; Fig. 5d). The only significant progression compared with baseline values was observed for MRC but only at the end of the follow up (138.5 ± 1.6; final value, 113.4 ± 4.9; P < 0.05; Fig. 5e). No significant decrement in the Short Form Health Survey-36 (SF-36) scale for quality of life was detected during the 15 months of lithium administration (96.2 ± 2.0 vs. 93.4 ± 2.1).

These data indicate that subjects receiving lithium progressed very slowly in the disease during the 15 months of the follow-up. By contrast, in patients receiving only riluzole, the Norris scale significantly decreased (86.6 ± 2.1 vs. 55.3 ± 3.2, P < 0.01), starting from the 3rd month (P < 0.05) (Fig. 5a). Similar results were found for ALSFRS-R score, which significantly decreased (P < 0.01) (basal value, 40.2 ± 0.8; final value, 24.2 ± 1.8; Fig. 5b); again, this decrease was already significant at the first 3 months scoring interval (P < 0.05). Also FVC significantly decreased from 91.0 ± 1.9 to 58.0 ± 3.0, P < 0.01 (Fig. 5d).

Similar results were obtained for the MRC scale (140.7 ± 1.2 vs. 92.0 ± 5.3, P < 0.01), starting from the 6th month (P < 0.05) (Fig. 5e).

When we compared the groups at the end of the follow up, the mean decrement in the normalized Norris score was 46.1% in patients receiving riluzole only and 10.6% in those receiving lithium. Likewise, the decrease was more marked with respect to the baseline in the normalized ALSFRS-R in patients receiving riluzole only compared with lithium (39.8% vs. 14.3%). This was comparable for MRC, which decreased by 34.6% and 18.0%, in riluzole only- and lithium-treated patients, respectively.

In the Fig. 5f the FVC breakdown between lithium- and riluzole-treated patients is shown, and in Fig. 5g this is further detailed for the bulbar form of the disease.
Discussion
Our study indicates that lithium delays ALS progression in human patients. In fact, all subjects treated with lithium were alive at the end of the follow up (15 months), and their quality of life, as measured by SF-36, was not modified. By contrast, 30% of the patients receiving riluzole died during the study. The decreases we observed in the ALS-FRS-R and Norris scales were not statistically significant in the group treated with lithium. The delay in disease progression was also assessed more objectively by quantitative measurement of the muscle strength (by the MRC scale) and the preservation of the pulmonary function (by FVC). By contrast, the disease progressed markedly in the control group from the 3rd month of evaluation.

The analysis in the G93A mice showed that lithium delayed cell death within lamina IX and cranial MN while it increased the number of lamina VII Renshaw-like neurons above control values. In addition, lithium decreased reactive gliosis, rescued spinal cord mitochondria, and produced a marked regression of alpha-synuclein, ubiquitin, and SOD1 aggregates. This latter finding suggests that an increased removal of the mutated SOD1 may contribute to the improvement we observed in G93A mice. Thus, lithium affects multiple targets, all of which are likely to contribute to the improvement of ALS. Although not yet explored, numerous data associate autophagy with ALS. Apart from the autophagy impairment we found in the SOD1 model, another form of ALS, which is characterized by a mutation of dynein (38), likely depends on autophagy failure. In fact, now we know that dynein is critical for delivering the autophagosomes to lysosome to remove protein aggregates (22). In keeping with this observation, mutations in the dynein gene cause an autophagy impairment and reduce the clearance of aggregates (39). Another point mutation in the gene coding for dynactin, which participates in the dynamic of phagosomes, is responsible of fALS (40). Another form of ALS is caused by a mutation in the ALS 2 gene, i.e., the protein alsin (41), which is implicated in endosome trafficking. Interestingly, SOD1 is degraded by the lysosome to remove protein aggregates (22). In keeping with this, the first physician was able to monitor lithium concentration and to adjust the daily dose according to the plasma range of 0.4–0.8 mEq/liter.

At the same time, focusing only on autophagy may be misleading given the multiple targets we found in the present work. For instance, the suppression of glial cells activation we demonstrated in the spinal cord of lithium-treated G93A mice may be critical in view of the recent reports of the detrimental role of glial on MN survival (6–8). This point is addressed further in Discussion in SI Text, which also provides a multifaceted perspective on the potential mechanisms underlying the effects produced by lithium in ALS.

Materials and Methods
Genetic Background and Breeding Protocol of G93A Mice. All of the experiments were carried out in compliance with European Council Directive 86/609/EEC for the use and care of laboratory animals. B6SJL-TgN(SOD1-G93A)1Gur mice expressing the human G93A Cu/Zn superoxide dismutase (SOD1) mutation were obtained from The Jackson Laboratory. For details, see Methods in SI Text.

Methods. Behavior. Observational behaviour at a daily dose of 150 mg for 1 to 2 years. For details, see Methods in SI Text.

Clinical Trial. Study design and patients. We conducted a 15-month parallel-group randomized study of adults with ALS, diagnosed according to the El Escorial criteria, treated with riluzole and lithium for 5 years.

The study protocol was approved by the Neuromed IRCCS Ethical Committee, and all subjects provided written informed consent. Initial statistical analysis determined that at least 40 subjects were needed to determine, with a 95% confidence interval, a survival increase of 10 months.

The present study was performed on 44 patients (20 male and 24 female). No familial case was present. Eleven patients presented the bulbar form of the disease, whereas the remaining ones had the classic onset. Sixteen patients (eight male and eight female, four of whom had the bulbar form) were randomly selected to receive riluzole (Rilutek 50 mg, 1 tablet x 2/day) plus lithium (Carbolithium: two daily 150-mg doses of lithium carbonate), and the remaining (12 male and 16 female, 7 of whom had the bulbar form) received riluzole only (48). In this way, we carefully matched lithium-treated and control patients for bulbar forms and FVC at the time of their inclusion in the study. In particular, the FVC values were 97 ± 10 and 97 ± 10 for lithium-treated and control patients, respectively. Again, the bulbar forms were distributed similarly between the groups (t(16) = 25%) in the treated group and (7/22 – 32%) for controls. One physician was not blind to group assignment; however, clinical evaluation, measurement of FVC, and data analysis were conducted by other physicians who were blind to group identities (single-blind study). In this way, the first physician was able to monitor lithium concentration and to adjust the daily dose from 400 mg up to 450 mg daily when lithium plasma levels were <0.4 mEq/liter. In the daily dose was selected to reach a plasma range of 0.4–0.8 mEq/liter. Compliance and adverse effects were monitored throughout the study period. Subjects were assessed six times (at baseline and every 3 months for 15 months). The primary endpoint of the present study was the survival rate. The secondary outcomes measured changes in global function, as scored by the ALSFRS-R (49), a widely used and extensively validated functional scale for ALS (normal score, 48); and by the Norris ALS scale. This disability score includes evaluation of the functioning of upper and lower limbs, also taking into

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Data analysis. The case analysis used all subjects who entered in the protocol study. Statistical analyses included a descriptive analysis of each group's data.

Kolmogorov–Smirnov test of normality was nonsignificant for all variables. Measures were compared every 3 months with respect to the baseline condition, using an ANOVA for repeated measures. Comparisons between the two groups' mean scores were made by using the unpaired t test and, if necessary, ANOVA, with Bonferroni as post hoc test. Survival analysis was performed by the Kaplan–Meier curve. The null hypothesis was rejected when \( P \leq 0.05 \) for all tests. Statistical analyses were performed by using MedCalc software (version 9.3.6.0). See Methods in SI Text for the detailed methods of the basic study.