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A Mouse Model of Familial ALS Has Increased CNS Levels of Endogenous Ubiquinol$_{9/10}$ and Does Not Benefit from Exogenous Administration of Ubiquinol$_{10}$

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Abstract

Oxidative stress and mitochondrial impairment are the main pathogenic mechanisms of Amyotrophic Lateral Sclerosis (ALS), a severe neurodegenerative disease still lacking of effective therapy. Recently, the coenzyme-Q (CoQ) complex, a key component of mitochondrial function and redox-state modulator, has raised interest for ALS treatment. However, while the oxidized form ubiquinone$_{10}$ was ineffective in ALS patients and modestly effective in mouse models of ALS, no evidence was reported on the effect of the reduced form ubiquinol$_{10}$, which has better bioavailability and antioxidant properties. In this study we compared the effects of ubiquinone$_{10}$ and a new stabilized formulation of ubiquinol$_{10}$ on the disease course of SOD1^{G93A} transgenic mice, an experimental model of fALS. Chronic treatments (800 mg/kg/day orally) started from the onset of disease until death, to mimic the clinical trials that only include patients with definite ALS symptoms. Although the plasma levels of CoQ$_{10}$ were significantly increased by both treatments (from <0.20 to 3.0–3.4 µg/mL), no effect was found on the disease progression and survival of SOD1^{G93A} mice. The levels of CoQ$_{10}$ in the brain and spinal cord of ubiquinone$_{10}$- or ubiquinol$_{10}$-treated mice were only slightly higher (≤10%) than the endogenous levels in vehicle-treated mice, indicating poor CNS availability after oral dosing and possibly explaining the lack of pharmacological effects. To further examine this issue, we measured the oxidized and reduced forms of CoQ$_{9/10}$ in the plasma, brain and spinal cord of symptomatic SOD1^{G93A} mice, in comparison with age-matched SOD1^{WT}. Levels of ubiquinol$_{9/10}$, but not ubiquinone$_{9/10}$, were significantly higher in the CNS, but not in plasma, of SOD1^{G93A} mice, suggesting that CoQ redox system might participate in the mechanisms trying to counteract the pathology progression. Therefore, the very low increases of CoQ$_{10}$ induced by oral treatments in CNS might be not sufficient to provide significant neuroprotection in SOD1^{G93A} mice.

Introduction

Coenzyme Q (CoQ) is an amphipathic molecule structurally composed of a quinone ring synthesized from *p*-hydroxybenzoate and of a polyisoprene chain synthesized from acetil-CoA [1]. There are different forms of CoQ, related to the length of polyisoprene chain: the predominant form in humans is CoQ$_{10}$ which contains 10 isoprenoid units, whereas CoQ$_9$ is the predominant form in rodents [2]. CoQ exists in three redox states: a fully oxidized form (ubiquinone), a fully reduced form (ubiquinol) and an intermediate semiquinone radical (CoQ$^{•−}$). The redox state is tissue-specific: for example, in mice ubiquinol is the predominant form in liver and skeletal muscle while ubiquinone is predominant in the brain [3]. Several factors, such as drugs, age, diet and pathologies affect total levels and redox state of CoQ [4,5,6,7] and their alterations may be regarded as markers of oxidative stress and mitochondrial dysfunctions [8].

Amyotrophic Lateral Sclerosis (ALS) is a severe neurodegenerative disorder in which the loss of motor neurons induces a progressive motor paralysis associated with dysphagia, dysarthria, respiratory failure and death within 3–5 years from the diagnosis. Analysis of plasma [9] and cerebrospinal fluid (CSF) [10] of sporadic ALS (sALS) patients have shown an increase of ubiquinone$_{10}$ with a shift in the redox state of the coenzyme, interpreted as an index of oxidative stress.

Because of the substantial involvement of oxidative stress and mitochondrial impairment in this disease [11] different therapeutic approaches have been aimed towards counteracting these mechanisms. Among them, the use of CoQ$_{10}$ has raised some interest for the treatment of ALS. In fact, on the basis of a modest but significant increase of survival observed in a mouse model of familial ALS (fALS) after chronic treatment with ubiquinone$_{10}$ [12], a phase II trial was designed to investigate the effects of a chronic treatment with the same molecule (2700 mg daily) in patients with sALS. Unfortunately, the data collected after 9 months of treatment did not provide sufficient evidence of a benefit to justify a phase III trial [13].

The use of the reduced form ubiquinol, characterized by potent antioxidant properties, was difficult because it is readily oxidized in air. Recently, however, a new stabilized formulation of ubiquinol$_{10}$ has been produced (Kaneka QH^{TM}) which overcomes this problem [14]. Notably, pharmacokinetic studies showed that ubiquinol$_{10}$ has a better oral bioavailability than ubiquinone$_{10}$, in humans [14], dogs and rats [15]. It may be expected that chronic treatment with this formulation results in higher CNS levels of CoQ$_{10}$, with superior neuroprotective properties. Consistently, ubiquinol$_{10}$ was more active than ubiquinone$_{10}$ in protecting mice from striatal neurodegeneration induced by acute treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a model for Parkinson's disease [16].

On this basis, in the present study we evaluated: 1) the effects of a chronic oral administration with ubiquinol$_{10}$ or ubiquinone$_{10}$ on disease progression of transgenic SOD1^{G93A} mice, a model of fALS [11], 2) the endogenous levels of total, reduced and oxidized CoQ$_{10}$ and CoQ$_9$ in plasma, brain and lumbar spinal cord of transgenic SOD1^{G93A} mice at symptomatic stage.

Our data show that exogenous administration of ubiquinol$_{10}$ or ubiquinone$_{10}$ to SOD1^{G93A} mice, starting at the onset of symptoms, had no effect on the disease course. Since the basal levels of reduced CoQ$_{9/10}$ are increased in the CNS of symptomatic SOD1^{G93A} mice, suggesting an attempt of equilibrium toward a protective antioxidant state, we think that the small increase of ubiquinol after the oral treatment is insufficient to exacerbate this effect.

Materials and Methods

Animals and Treatments

Transgenic SOD1^{G93A} and SOD1^{WT} mice on 129S2/SvHsd genetic background, and non-transgenic littermates were used in this study. The SOD1^{G93A} mouse line derives from the line originally obtained from Jackson Laboratories (B6SJL-TgNSOD-1-SOD1G93A-1Gur) expressing approximately 20 copies of mutant human SOD1 with a Gly93Ala substitution (SOD1^{G93A}) or wild-type human SOD1 (SOD1^{WT}) and were maintained on 129S2/SvHsd background for more than 15 generations at Harlan Italy S.R.L., Bresso (MI), Italy [17]. We observed no differences between the males and females as regards the disease progression and survival, as reported in supporting figure 1 (Fig. S1). Therefore, to reduce at minimum the number of transgenic mice used, chronic treatments were carried out in female whereas the biochemical profile was examined in males.

All mice were maintained at a temperature of $21\pm1°C$ with a relative humidity $55\pm10\%$ and 12 h of light. Food (standard pellets) and water were supplied ad libitum.

Ubiquinone$_{10}$ (Ubidecarenone from Kaneka) and ubiquinol$_{10}$ (Kaneka QH^{TM} from Kaneka) were administered by gavage after solubilization in sunflower seed oil (vehicle); preparation procedures were carried out at room temperature protecting from light. All the solutions were administered within four hours from preparation.

Ethics statement. Procedures involving animals and their care were conducted in conformity with the institutional guidelines of "Mario Negri" institute (IRFMN), that are in compliance with national (D.L. no. 116, G.U. suppl. 40, Feb. 18, 1992, Circular No.8, G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 Dec.12, 1987; NIH Guide for the Care and use of Laboratory Animals, U.S. National Research Council, 1996). All the experiments and the protocol proposed in the projects were reviewed and approved by the IRFMN Animal Care and Use Committee (IACUC), that includes

members "ad hoc" for ethical issues. The mice were bred and maintained in a SPF environment. Animals with substantial motor impairment had food on the cage bottom and water bottles with long drinking spouts. The survival time was defined as the time when the animals were unable to right themselves within 10 s after being placed on either side. At this point, the animals were deeply anesthetized with Equithesin and then sacrificed by decapitation before proceeding to the dissection of tissues for biochemical analyses.

Preliminary acute treatment in 129Sv mice. Two preliminary pharmacokinetic studies were carried out in non-transgenic 129Sv female mice aging 9-11 weeks. For the time-course study, mice were treated orally with 200 mg/kg of ubiquinone$_{10}$ or ubiquinol$_{10}$ and killed at various times thereafter (30, 60, 180, 360 min and 24 hours after dosing, four mice/group). In a separate single-dose study, ubiquinone$_{10}$ and ubiquinol$_{10}$ were administered orally at various doses (200, 400, 800 and 1600 mg/kg) and the animals (4 mice/dose) were killed 3 hours after dosing. Four mice were treated with vehicle alone.

Chronic treatment in 129Sv SOD1G93A mice and analysis of their motor dysfunction and survival. Female 129Sv SOD1^{G93A} mice were treated by oral gavage with ubiquinone$_{10}$ or ubiquinol$_{10}$ (800mg/kg), or vehicle (n = 15 each group). The treatment started when the mice were 13 weeks old, which is the mean age that the growth of their body weight starts to differentiate from that of non-transgenic littermates and they show first alterations of hindlimbs abduction when raised by tail. This occurs just before the appearance of motor impairments (onset of motor deficit). Mice were treated daily until the end-stage of the disease, when they were sacrificed (see below).

Disease progression was assessed two times a week from the start of the experiment by an operator blinded to the treatment. The parameters included body weight, latency on rotating bar (rotarod) and paw grip endurance (PaGE) and were evaluated as previously described [17]. The motor symptoms onset was determined by the first impairment in grip strength or on rotarod performance for two consecutive time points. The mice were sacrificed when they were unable to right themselves within 10 seconds after being placed on both sides. This time point was considered as the end-stage of the disease and was used to estimate the survival time.

Statistical analysis of the data of body weight, grip strength and rotarod performance was carried out by Two-way ANOVA for repeated measures (time) and different groups (treatments), followed by *post-hoc* Bonferroni's test to compare the effect of treatments in respect to vehicle at each time point. The analysis was applied at the time points when all animals for each group were still alive in order to maintain the number per group balanced. The age at onset and the survival length were statistically evaluated by the Log-rank test to compare probabilities.

HPLC Analysis of CoQ$_{10}$ and CoQ$_9$ Levels

Animals were sacrificed by decapitation. Blood was collected from the heart in heparinized tubes. After centrifugation at 5000 g for 10 min, plasma was collected and stored at $-80°C$. CNS tissues were removed immediately, blotted with paper to remove surface blood, quickly frozen in dry ice and stored at $-80°C$.

All solvents and reagents used were HPLC grade (Carlo Erba, Milan, Italy). Ubiquinone$_{10}$, ubiquinone$_9$, FeCl$_3$, 1,4-benzoquinone and sodium perchlorate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plasma levels of total CoQ$_{10}$ and CoQ$_9$ in treated SOD1^{G93A} mice. The method described by Mosca *et al.* [18] was used. Fifty µL of plasma were pre-incubated for 10 min at
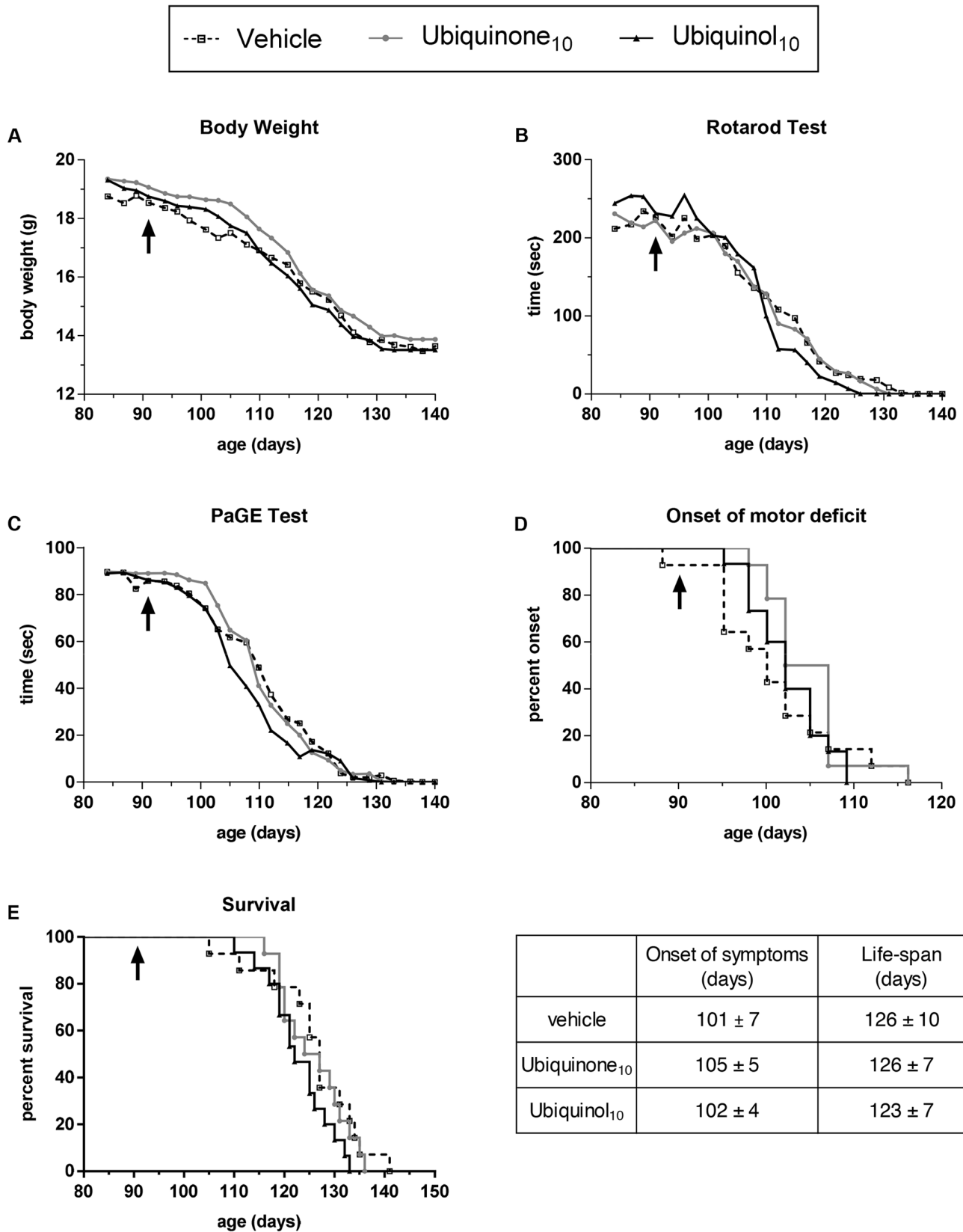
Figure 1. Ubiquinol$_{10}$ chronic treatment has no effect on disease course in SOD1^{G93A} mice. Effect of oral treatment with 800 mg/kg/day ubiquinone$_{10}$, ubiquinol$_{10}$ or vehicle, on motor dysfunction and disease progression of 129Sv SOD1^{G93A} mice (n = 15 mice per group). Treatment started at the age of 91 days (arrows) until the sacrifice (at the end-stage of the disease, when mice were unable to right themselves within 10 seconds after being placed on both sides). Treatment with ubiquinone$_{10}$ or ubiquinol$_{10}$ had no significant effects on body weight (A), on the latency

of rotarod (B) and PaGE test (C) (Two-way ANOVA), or on disease onset (D) and survival length (E). Each point represents the mean; for sake of clarity standard deviations are not indicated but they were always less than 15% of the value. Table reports the mean and standard deviations of symptoms onset and life-span for each group.
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room temperature with 15 µL of 1,4-benzoquinone (2 mg/mL in ethanol), in order to allow a complete oxidation of ubiquinol$_{9/10}$. The samples were then added to 235 µL of 1-propanol, vortexed and then centrifuged at 10000 g for 2 minutes. Two hundred µL of supernatant were then injected into the HPLC system with UV detection at 275 nm (Waters 1525 Binary Pump; Waters 2487 Dual λ Absorbance Detector; Waters mod. 717-plus autosampler). Separation was carried out using a Supelcosil LC 18-DB column (15×0.46 cm, i.d. 5 µm, Supelco Inc., Bellefonte, PA, USA), with a Supelcosil LC 18-DB Supelguard Cartridge (2×0.4 cm, i.d. 5 µm, Supelco Inc., Bellefonte, PA, USA) by isocratic elution with ethanol/methanol (65:35 v/v) at flow rate of 1 mL/min. The total run time was 19 min and the retention times for CoQ$_9$ and CoQ$_{10}$ were 7.1 and 8.9 min, respectively.

Total levels of CoQ$_{10}$ and CoQ$_9$ were quantified by reference to calibration curves with ubiquinone$_{10}$ or ubiquinone$_9$, run in parallel with each analytical session. Calibration curves were linear in the range 0.2–3.2 µg/ml for both coenzymes, with r^2 values >0.99.

CNS levels of total CoQ$_{10}$ and CoQ$_9$ in treated SOD1^{G93A} mice. CoQs were extracted from CNS tissues and measured by HPLC with UV detection at 275 nm, based on the method described by Kitano et al. [14], with slight modifications.

CNS tissues were homogenized in ultrapure water (1 g in 20 mL), aliquots of 0.15 mL were added to 1 mL of acetonitrile and the samples were centrifuged at 2500 rpm for 5 min; the supernatants were collected and 1 ml aliquots were added to 0.1 mL of 2% FeCl$_3$ to allow the oxidation of ubiquinols to ubiquinones. After 20 minutes incubation at room temperature the extraction procedure started by adding 2 ml methanol, 0.5 mL purified water and 5 ml hexane, followed by 15 minutes shaking, centrifugation (1200 g, 5 min) and collection of the organic phase. This procedure was repeated twice and the combined extracts were evaporated to dryness, the residue was dissolved in 150 µL of water/methanol (20:80 v/v), transferred in Eppendorf tubes, centrifuged (2500 rpm, 30 sec) and 100 µl were injected into the chromatographic system (Waters 1525 Binary Pump; Waters 2487 Dual λ absorbance detector; Waters mod. 717-plus autosampler). Separation was carried out on a Kinetex 2.6 µ PFP 100 Å column (100×4.6 mm, Phenomenex Inc., Torrance, CA, USA), with a HPLC KrudKatcher 5 µ ultra column in-line filter (Phenomenex Inc., Torrance, CA, USA), using gradient elution with water/methanol (20:80 v/v; solvent A) and acetonitrile/methanol (4:96 v/v; solvent B). During HPLC analysis the solvent gradient was programmed to operate at a flow rate of 1 mL/min for 25 minutes at the following gradient conditions: step1 - from the initial condition of 100% solvent A to 100% solvent B over 16 min; step 2 - from 100% solvent B to 100% solvent A over 1 min. Retention times of CoQ$_9$ and CoQ$_{10}$ were 17.8 and 18.3 minutes, respectively.

Total levels of CoQ$_{10}$ and CoQ$_9$ were quantified by reference to calibration curves with ubiquinone$_{10}$ or ubiquinone$_9$, run in parallel with each analytical session. Calibration curves were linear in the range 4.0–16.0 µg/ml (CoQ$_{10}$) and 10.0–60.0 µg/ml (CoQ$_9$), with r^2 values >0.99.

CNS levels of ubiquinol$_{9/10}$ and ubiquinone$_{9/10}$. The method described by Takada et al. [19] was used with slight modifications. Tissues were homogenized (1 g in 10 mL of ultrapure water) and divided in two aliquots, one to measure total

CoQ$_{9/10}$ levels (aliquot A) and the other to measure ubiquinone$_{9/10}$ only (aliquot B). 100 µL of aliquot A were added to 10 µL of ultrapure water and 60 µL of 1,4-benzoquinone (2 mg/mL in ethanol) to allow a complete oxidation of ubiquinol. In parallel, 100 µL of aliquot B were added to 10 µL of 10% Na$_2$EDTA (to protect ubiquinol from oxidation), and 60 µL of ethanol. After 10 min, both aliquots were added to 750 µL of ethanol/hexane (2:5, v/v), shaken, and centrifuged at 4000g for 3 min (4°C). The supernatants were dried under nitrogen flow, reconstituted with 100 µL of ethanol and 50 µL were injected into a HPLC system (Waters 1525 Binary pump; Waters mod. 717-plus autosampler) coupled to a coulometric detector (ESA Coulchem 5100A with ESA analytical cell mod. 5011). The electrochemical analytical cell consisted of a series of two coulometric electrodes: the first one (−650mV) was for ubiquinone$_{9/10}$ reduction and the second one (+400mV) was for detection of CoQ$_{9/10}$ in the reduced form. The chromatographic column was a reverse phase Supelcosil LC-18 DB (15×0.46 cm, i.d. 3 µm; Supelco Inc., Bellefonte, PA, USA). The separation was carried out in isocratic operational mode with ethanol/methanol/HClO$_4$ (700:300:1, v/v/v) +7% sodium perchlorate at 0.8 mL/min for 13 minutes. Retention times were 5.3 min (ubiquinol$_9$), 6.3 min (ubiquinone$_9$), 7.1 min (ubiquinol$_{10}$) and 8.8 min (ubiquinone$_{10}$).

Total levels of CoQ$_{9/10}$ (aliquot A), and ubiquinone$_{9/10}$ (aliquot B) were quantified by reference to calibration curves with ubiquinone$_{9/10}$, run in parallel with each analytical session. Calibration curves were linear in the range 5–50 µg/g tissue (ubiquinone$_{10}$) and 20–100 µg/g tissue (ubiquinone$_9$), with r^2 values >0.99. Endogenous levels of ubiquinol$_{9/10}$ were obtained as difference between total CoQ$_{9/10}$ levels (determined in fully oxidized aliquot A) and ubiquinone$_{9/10}$ levels (determined in aliquot B).

All the procedures were carried out rapidly, on ice, with protection from light, and in the presence of Na$_2$EDTA, to limit the possibility that endogenous ubiquinol oxidizes to ubiquinone during the sample preparation. In these conditions, we verified that the storage of CNS homogenate at 4°C, for up to one hour, did not affect ubiquinol/total CoQ ratios (data not shown). We also note that the ubiquinol/total CoQ ratios detected in our study are very similar to published data [3,19,20]. Most importantly, we operated to ensure reliable comparisons between the different groups (SOD1^{G93A}, SOD1^{WT} and non-transgenic), always including matched samples in the same analytical session, with random processing.

Plasma levels of ubiquinol$_9$ and ubiquinone$_9$. The method described by Takada et al. [21] was used with slight modifications. Plasma samples were divided in two aliquots, one to measure total CoQ$_9$ levels (aliquot A) and the other to measure ubiquinone$_9$ only (aliquot B). 50 µL of aliquot A were added to 50 µL of ultrapure water and 25 µL of 1,4-benzoquinone (2 mg/mL ethanol), to allow a complete oxidation of ubiquinol$_9$. In parallel, 50 µL of aliquot B were added to 50 µL of 10% Na$_2$EDTA (to protect ubiquinol from oxidation), and 25 µL of ethanol. After 10 min, both aliquots were added to 750 µL of ethanol/hexane (2:5, v/v), shaken, and centrifuged at 4000 g for 3 min (4°C). The supernatant was dried under nitrogen flow, reconstituted with 200 µL of H$_2$O/2-propanol (1:5, v/v) and 150 µL were injected in a HPLC system as reported for CNS samples. The chromatographic separation was carried out in

isocratic operational mode with ethanol/methanol/H_2O/$HClO_4$ (700:300:10:1, v/v/v/v) +7% sodium perchlorate at 1 mL/min for 15 minutes. Retention times for ubiquinol$_9$ and ubiquinone$_9$ were 6.2 and 8.6 minutes, respectively.

Total levels of CoQ$_9$ (aliquot A), and ubiquinone$_9$ (aliquot B) were quantified by reference to calibration curves with ubiquinone$_9$, run in parallel with each analytical session. Calibration curve was linear in the range 0.05–1.20 μg/mL, with r^2 values >0.99. Endogenous levels of ubiquinol$_9$ were obtained as difference between total CoQ$_9$ levels (determined in fully oxidized aliquot A) and ubiquinone$_9$ levels (determined in aliquot B).

All the procedures were carried out rapidly, on ice, with protection from light, and in the presence of Na$_2$EDTA. The ubiquinol/total CoQ ratios detected in our study are very similar to published data [3,19,20]. Most importantly, we operated to ensure reliable comparisons between the different groups (SOD1^{G93A}, SOD1^{WT} and NTg), always including matched samples in the same analytical session, with random processing.

Results

Mice Treatment with Ubiquinone$_{10}$ and Ubiquinol$_{10}$

Since available data indicated that the stabilized formulation of ubiquinol$_{10}$ has a better oral bioavailability than ubiquinone$_{10}$ in different animal species [14,15], preliminary studies were carried out to verify if this was true also in 129Sv mice. With this aim, plasmatic CoQ$_{10}$ levels were measured in wild-type mice treated acutely with both ubiquinol$_{10}$ and ubiquinone$_{10}$. Fig. S2A shows that, after oral treatment with 200 mg/kg**,** the area under the plasma concentration-time curve over the dosing interval (AUC$_{0–24h}$), was 1.5-fold higher for ubiquinol$_{10}$ (14.2 μg·h/mL) than ubiquinone$_{10}$ (9.8 μg·h/mL). Fig. S2B shows the plasma CoQ$_{10}$ levels in mice treated with different doses of the two compounds, three hours before sacrifice, confirming higher CoQ$_{10}$ levels in the plasma of ubiquinol$_{10}$–treated mice than in the plasma of ubiquinone$_{10}$-treated mice, except at the highest dose tested (1600 mg/kg). In particular, treatment with 800 mg/kg ubiquinol$_{10}$ resulted in CoQ$_{10}$ levels double than those found after treatment with the same dose of ubiquinone$_{10}$ (1.5±0.2 and 0.8±0.4 μg/mL, respectively, p<0.05 Student's t test).

Brain CoQ$_{10}$ levels (13 μg/g tissue on average) were not affected by acute treatment with either ubiquinone$_{10}$ or ubiquinol$_{10}$, at any time-point or any concentration (data not shown).

Chronic Treatment with Ubiquinone$_{10}$ and Ubiquinol$_{10}$ in SOD1^{G93A} Mice

Effect on motor dysfunction, body weight and survival of SOD1^{G93A} mice. Fig. 1 shows that chronic oral treatment of SOD1^{G93A} mice with 800 mg/kg/day ubiquinone$_{10}$ or ubiquinol$_{10}$, started at the onset of disease symptoms, did not modify the motor impairment progression assessed by rotarod performance (fig. 1B) and grip strength (fig. 1C), in respect to the vehicle-treated mice. The progressive loss of body weight (fig. 1A) was also unaffected by both treatments. The lack of effect was clearly evidenced by the overlapping Kaplan-Meyer curves and the similar mean for motor symptom onset (fig. 1D) and survival length (fig. 1E).

Total CoQ$_{10}$ and CoQ$_9$ plasma levels. Table 1 reports the plasma concentrations of total CoQ$_{10}$ and CoQ$_9$ after chronic administration of ubiquinone$_{10}$ and ubiquinol$_{10}$ in the same SOD1^{G93A} mice used for behavioral tests. The last dose of both formulations was administered two hours before sacrifice.

As expected, no detectable levels of CoQ$_{10}$ were found in mice chronically treated with vehicle (<0.20 μg/mL), whereas levels of

Table 1. Levels of CoQ$_{9/10}$ in the plasma of SOD1^{G93A} mice treated chronically with ubiquinone$_{10}$ or ubiquinol$_{10}$.

Chronic treatment	Total CoQ$_{10}$ (μg/mL)	Total CoQ$_9$ (μg/mL)
Vehicle	<0.20 (5)	1.63±0.40 (5)
Ubiquinol$_{10}$	3.40±0.22 (13)	1.25±0.12 (13)
Ubiquinone$_{10}$	3.03±0.26 (9)	1.19±0.20 (9)

Female 129Sv SOD1^{G93A} mice were treated orally with 800 mg/kg of ubiquinol$_{10}$ or ubiquinone$_{10}$, or vehicle (sunflower seed oil), once a day, starting from age of 91 days until the last stage of the disease (age of 125 days, on average) when they were sacrificed, two hours after the last treatment. Values are means±SEM of (n) mice.
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3.0 and 3.4 μg/mL were measured in the mice treated with ubiquinone$_{10}$ and ubiquinol$_{10}$, respectively (difference not statistically significant). Plasma levels of CoQ$_9$ were not significantly affected by treatment with either ubiquinone$_{10}$ or ubiquinol$_{10}$ (table 1).

Total CoQ$_{10}$ and CoQ$_9$ CNS tissues levels. Figure 2 shows the levels of CoQ$_{10}$ and CoQ$_9$ measured in the brain and spinal cord (cervicothoracic, CT-SpC and lumbar, L-SpC) of SOD1^{G93A} mice after chronic treatment with ubiquinone$_{10}$ or ubiquinol$_{10}$, or vehicle.

CoQ$_{10}$ levels in brain, CT-SpC and L-SpC of vehicle-treated mice were 17.6±0.3, 10.8±0.3 and 11.0±0.3 μg/g tissue (mean±SEM, n = 5), respectively; levels were slightly (4–11%) higher in all CNS tissues obtained from both ubiquinone$_{10}$- or ubiquinol$_{10}$-treated mice, with statistically significant differences detected in the brain of mice treated with ubiquinol$_{10}$ only (Fig. 2A). CoQ$_9$ levels were not affected by treatment with ubiquinone$_{10}$ or ubiquinol$_{10}$ (Fig. 2B). This finding prompted us to normalize, for each mouse, the CoQ$_{10}$ level on the corresponding CoQ$_9$ content, in order to minimize variability between mice. After this normalization, the levels of CoQ$_{10}$ (expressed as CoQ$_{10}$/CoQ$_9$ ratio) appeared to be slightly but significantly increased (10%) by treatment with both ubiquinone$_{10}$ or ubiquinol$_{10}$ in all tissues, with the only exception for the levels in CT-SpC after treatment with ubiquinol$_{10}$ (Fig. 2C).

Levels of Reduced and Oxidized Forms of CoQ$_{10}$ and CoQ$_9$ in SOD1^{G93A} Mice

Basal levels of reduced and oxidized forms of both coenzymes were measured in male 129Sv SOD1^{G93A} mice at the symptomatic stage of the disease (16 weeks), and in age-matched 129Sv SOD1^{WT} and non-transgenic 129Sv mice. In figures 3, 4, and 5**,** the levels in transgenic mice are shown as a percentage of levels in non-transgenic mice (absolute values in table S1), in order to highlight the effect of the over-expression of the human SOD1 transgene, and any difference related to the G93A mutation.

Brain. Figure 3 shows that over-expression of human SOD1^{WT} enzyme does not alter the endogenous levels of oxidized and reduced CoQ$_{9/10}$, in comparison with non-transgenic mice. Over-expression of the mutated protein (SOD1^{G93A}) was instead associated with a significant increase (10–25%) of endogenous total levels of both CoQ$_9$ and CoQ$_{10}$, in comparison with age-matched SOD1^{WT} mice (Fig. 3A,D). This effect was associated with a significant increase (30–40%) of the reduced forms only (Fig. 3B,E), whereas no significant changes were observed for the oxidized forms (Fig. 3C,F).

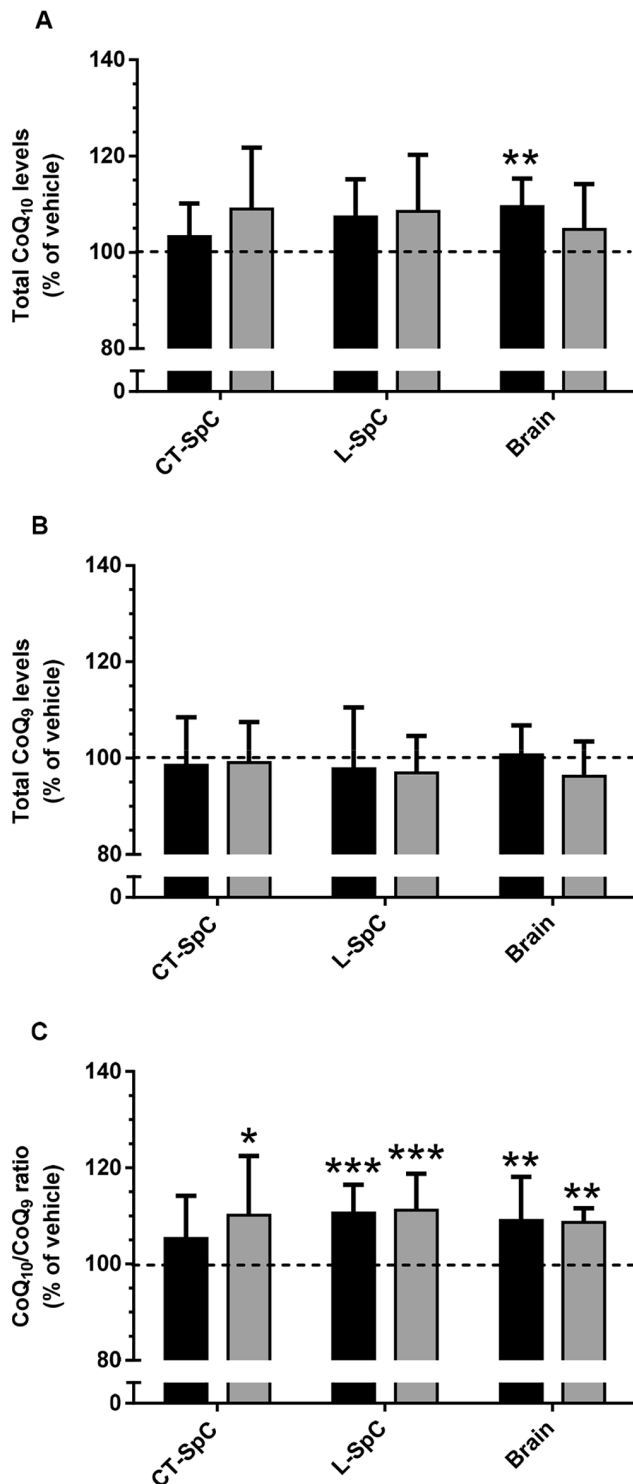**Lumbar spinal cord.** Endogenous levels of CoQ$_9$ and CoQ$_{10}$ in lumbar spinal cord of 16 weeks-old SOD1^{WT} mice

Figure 2. CoQ$_{9/10}$ levels in brain and spinal cord of SOD1^{G93A} chronically treated with ubiquinol$_{10}$ or ubiquinone$_{10}$. Total levels of CoQ$_{10}$ (A) and CoQ$_9$ (B), and CoQ$_{10}$/CoQ$_9$ ratio (C), in brain, and in cervicothoracic (CT-SpC) and lumbar (L-SpC) spinal cord of mice chronically treated with 800 mg/kg ubiquinol$_{10}$ (black) or ubiquinone$_{10}$ (grey). Each value is the mean±SEM of 10–12 mice and is indicated as percentage of levels measured in mice treated with vehicle. * $P<0.05$, ** $P<0.01$ compared to vehicle (Dunnet's multiple comparison tests following One-way ANOVA).
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were significantly lower (−20%), than in age-matched non-transgenic mice, a difference due to a decrease of the oxidized forms. In line with the results found in the brain, basal levels of CoQ$_9$ and CoQ$_{10}$ in SOD1^{G93A} were significantly higher (16–20%) than corresponding levels in age-matched SOD1^{WT} mice (Fig. 4A,D) and this increase was associated with a significant increase (36–60%) of the reduced forms only (Fig. 4B,E), whereas no or much lower changes where observed for the oxidized forms (Fig. 4C,F).

Plasma. Figure 5 shows that over-expression of human SOD1^{WT} enzyme does not alter the endogenous plasma levels of total CoQ$_9$, ubiquinol$_9$ and ubiquinone$_9$, in comparison with non-transgenic mice. Moreover, no significant differences were observed between SOD1^{WT} and SOD1^{G93A} mice. CoQ$_{10}$ levels were under the limit of detection.

Discussion

Oxidative stress and mitochondrial impairment are the most prominent pathogenic mechanisms of ALS. The CoQ complex is a key component of the mitochondrial function, and its reduced form ubiquinol is fundamental for the antioxidant response, by direct interaction with radical species or by regenerating antioxidant agents [22].

It had been previously reported that chronic treatment with ubiquinone$_{10}$ had a significant, although slight, effect on the life-span of a mouse model of familial ALS [12]. Regrettably, this finding was not confirmed in a phase II trial in humans [13]. One of the potential reasons underlying these results regarded the poor pharmacokinetic profile of ubiquinone$_{10}$. In fact, a stabilized formulation of ubiquinol$_{10}$, with a better bioavailability in respect to the oxidized form [14,15] showed a better neuroprotective activity on MPTP-induced degeneration in mice [16].

On these basis we chronically treated a mouse model of familial ALS (SOD1^{G93A}) with ubiquinol$_{10}$, and ubiquinone$_{10}$ in parallel, to increase CNS CoQ$_{10}$ levels with the aim to obtain beneficial effect on the disease course.

Chronic treatment with both formulation resulted in a significant increase of plasmatic CoQ$_{10}$ levels (from <0.2 to ≥3 µg/mL) that was associated to a very mild increase (10% on average) of the CoQ$_{10}$ levels in CNS tissues (brain and spinal cord). Despite the better pharmacokinetic profile of the new formulation of ubiquinol$_{10}$ after acute treatment [14,15 and present data], we found no differences in the CoQ$_{10}$ levels after chronic treatment with both formulations.

No effect of the chronic treatments with either ubiquinone$_{10}$ or ubiquinol$_{10}$ (both at 800 mg/kg/day) was observed on the disease progression or the life-span of SOD1^{G93A} mice.

This finding is at variance with previous data showing a 8% increase of survival after chronic treatment with a lower dose (200 mg/kg) of ubiquinone$_{10}$ [12]. Unfortunately, that study did not report the levels of CoQ$_{10}$ reached in the brain of SOD1^{G93A} mice after the chronic treatment. Two possible reasons may account for the discrepancy: 1) the strain of SOD1^{G93A} mice is different (C57BL6/SJL in [12] and 129Sv in our study), although the disease onset and progression is very similar in the two strains; and 2) in our study, the treatment started at the appearance of the first symptoms of disease (13 weeks of age), while Matthews *et al.* [12] started the treatment when mice were 7-weeks old, well before the onset of symptoms. According to the recent guidelines for preclinical animal research in ALS [23], the poor impact of preclinical studies on clinical practice is in part due to the fact that most of the pharmacological interventions are intended to be disease-preventive rather than being disease-modifying once the
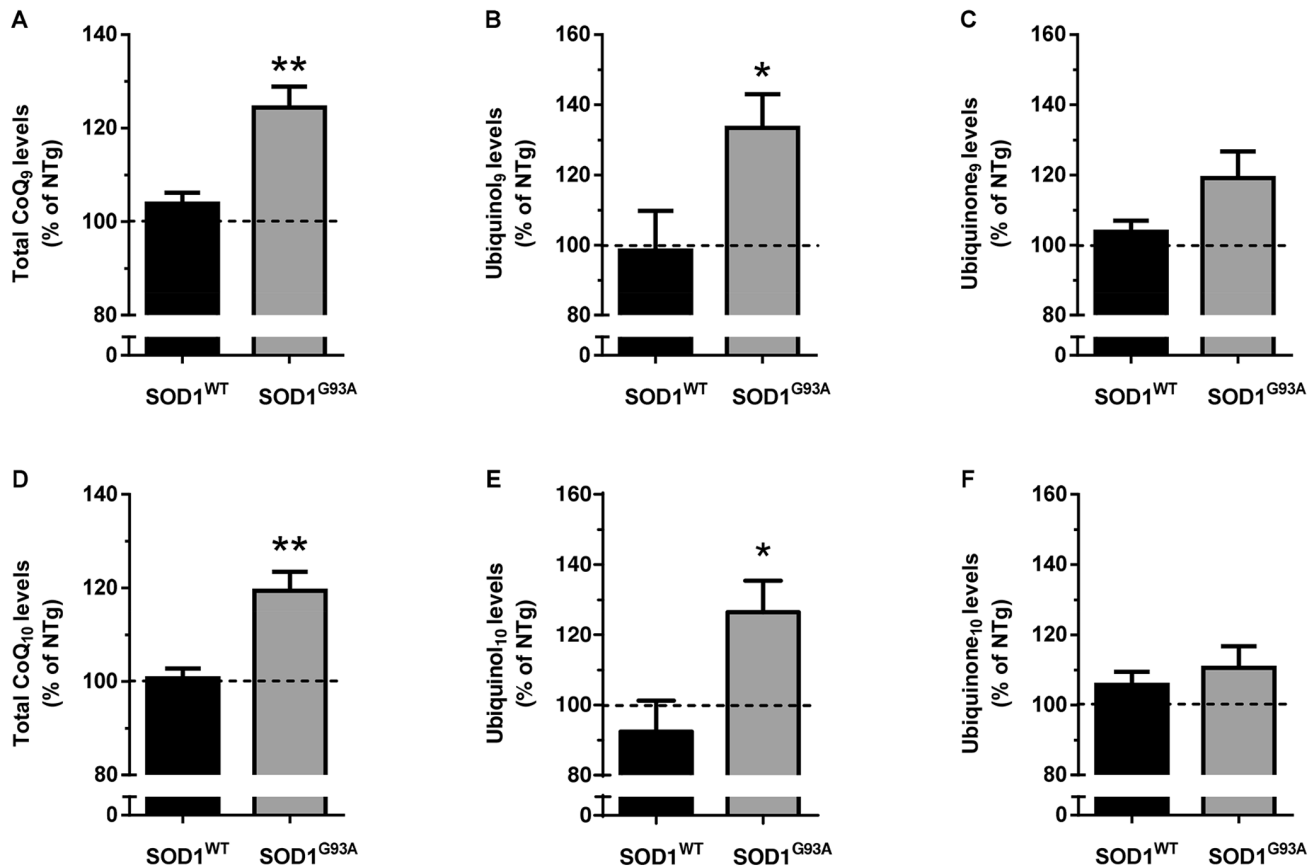
Figure 3. Increased levels of ubiquinol$_{9/10}$ in the brain of symptomatic SOD1^{G93A} mice. Endogenous brain levels of CoQ$_9$ (A–C) and CoQ$_{10}$ (D–F) in 16 week-old male 129Sv SOD1^{WT} and SOD1^{G93A} mice are shown as percentage of the levels measured in age-matched 129Sv non-transgenic mice (absolute values in table S1). Panels A, D show the total CoQs levels, whereas panels B, E and panels C, F show the reduced and oxidized forms, respectively. Each value is the mean±SEM of 4–7 mice. * P<0.05, ** P<0.01 compared to SOD1^{WT} mice (Student's T-test).
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symptoms are evident. In this respect, since we start treatment when the mice exhibited the first disease symptoms, our data are in line with the lack of effect found in phase II clinical trials with ubiquinone$_{10}$ [13].

The observation that the chronic treatments with ubiquinone$_{10}$ and ubiquinol$_{10}$ resulted in a very small increase of CoQ$_{10}$ levels in the CNS of SOD1^{G93A} mice (≤10%) may provide an explanation for the lack of effect on disease progression and life span. In fact, it was previously shown that the neuroprotection observed after 1-week pretreatment of non-transgenic rats with 200 mg/kg ubiquinone$_{10}$, i.e. prevention of the neurotoxicity induced by 3-nitropropionic acid, was associated with a ~30% increase of brain CoQ$_{10}$ [12]. All these data may suggest that an high enough increase of CNS concentrations of CoQ$_{10}$ over basal levels is required to support neuroprotection and that the increase associated with our treatments in 129Sv SOD1^{G93A} mice, started at the time of the onset of symptoms, was too low to provide an efficient neuroprotection.

To better understand this point, in the second part of the study we measured the endogenous levels of CoQ$_{10}$ and CoQ$_9$ in CNS tissues and plasma of symptomatic SOD1^{G93A} mice, in comparison with age- and sex-matched SOD1^{WT} and non-transgenic mice.

Our results show for the first time that the CNS tissues of SOD1^{G93A} mice have CoQ$_{9/10}$ levels 10–25% higher than those of age-matched SOD1^{WT} mice, while no differences were found in

the plasma between the two strains. The increase was due to the reduced form only, ubiquinol, indicating a shift in the redox equilibrium toward a protective antioxidant state, and suggesting an attempt of the CNS to maintain an antioxidant environment to counteract the pathological processes of the disease. According with the antioxidant function of the reduced form of CoQ, it has been shown that ubiquinol$_{10}$ is involved in the control of the mitochondrial levels of nitric oxide [24] and that the increase of ubiquinol pool in mitochondria (by antimycin) results in a decrease of protein nitration induced by ONOO$^-$ [25]. Notably, increased protein tyrosine nitration has been observed in spinal cord of ALS transgenic mice (C57BL/6 SOD1^{G93A}), from the pre-symptomatic till late stages of the disease, suggesting a role of nitrative stress in ALS pathogenesis and progression [26,27]. Therefore, the increased levels of ubiquinol in the spinal cord of SOD1^{G93A} mice are likely not sufficient to counteract the intense nitrative stress occuring in these mice, with consequent lack of effect on their disease progression and life span.

An increase of ubiquinone$_{10}$ with shift in the redox state of the coenzyme has been reported in the plasma [9] and CSF [10] of sALS patients and was suggested as a marker of the underlying oxidative stress. Our data, showing that the increase of ubiquinol$_{9/10}$ in the CNS of SOD1^{G93A} mice was not paralleled by changes in plasma levels, indicate that the peripheral and central compartments react differently to the oxidative stress induced by mutant SOD1, and suggest that plasmatic CoQ levels do not represent
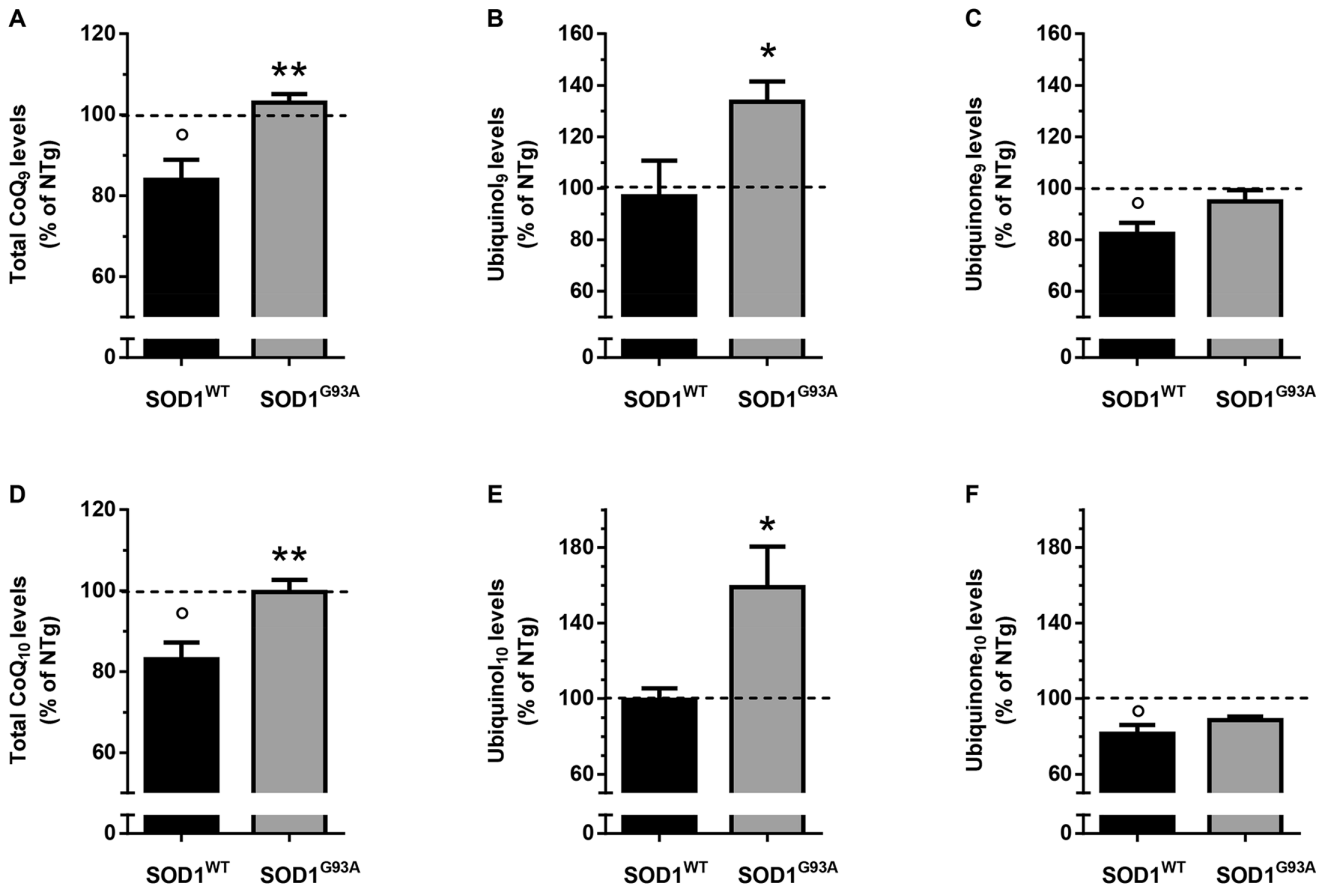
Figure 4. Increased levels of ubiquinol$_{9/10}$ in the lumbar spinal cord of symptomatic SOD1^{G93A} mice. Endogenous levels of CoQ$_9$ (A–C) and CoQ$_{10}$ (D–F) in the lumbar spinal cord of male 16 week-old 129Sv SOD1^{WT} and SOD1^{G93A} mice are shown as percentage of the levels measured in age-matched 129Sv non-transgenic mice (absolute values in table S1). Panels A, D show the total CoQs levels, whereas panels B, E and panels C, F show the reduced and oxidized forms, respectively. Each value is mean±SEM of 4–7 mice. * P<0.05, ** P<0.01 compared to SOD1^{WT} mice; ° P<0.05 compared to 129Sv NTg mice (Student's T-test).
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sensitive markers for the changes associated to oxidative stress in the CNS. This is in line with the observations of Kontush *et al.* [28] who could not detect changes of ubiquinol$_{10}$ in the plasma of

patients with Alzheimer's disease (AD), another neurodegenerative condition associated with increased free radical production in the
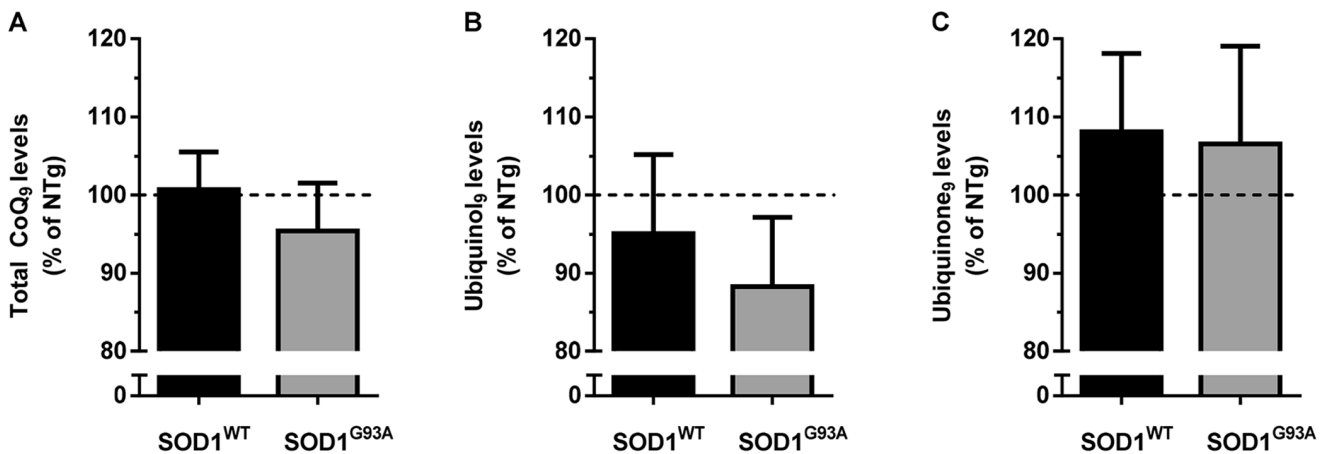

Figure 5. Unchanged plasma levels of ubiquinol$_9$ in symptomatic SOD1^{G93A} mice. Levels of CoQ$_9$ (A), ubiquinol$_9$ (B) and ubiquinone$_9$ (C) in plasma of male 16 week-old 129Sv SOD1^{WT} and SOD1^{G93A} mice are shown as percentage of the levels measured in age-matched 129Sv non-transgenic mice (table S1). Each value is mean±SEM of 4–7 mice.
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CNS, even if higher levels of total CoQ were reported in post mortem brain tissues from AD patients [29].

In summary, our data show that chronic treatment of $SOD1^{G93A}$ mice with ubiquinone$_{10}$ or ubiquinol$_{10}$ had no effect on the disease progression. This lack of efficacy may be due to the negligible changes of CoQ$_{9/10}$ levels in the brain and spinal cord of treated mice, which in turn may be the consequence of a poor CNS availability of the compounds and/or the fact that endogenous CoQ$_{9/10}$ levels are already significantly increased in $SOD1^{G93A}$ mice.

Supporting Information

Figure S1 No gender effect on disease progression in $SOD1^{G93A}$ mice. Disease progression and survival length in males (gray) and females (black) $SOD1^{G93A}$ mice on 129Sv background. Each point represent the mean of n = 10 male and n = 13 female. Table reports the mean and standard deviations of symptoms onset, life-span and disease duration for each group.
(TIF)

Figure S2 Ubiquinol$_{10}$ shows a better oral pharmacokinetic profile than ubiquinone$_{10}$. Plasma CoQ$_{10}$ levels in female 129Sv non-transgenic mice treated orally with ubiquinone$_{10}$ (grey) or ubiquinol$_{10}$ (black). (A) Time-course after acute treatment with 200 mg/kg. (B) Dose-dependency after acute treatment with 200, 400, 800 and 1600 mg/kg and sacrifice 3 hours later. Each value is the mean±SEM of 4 mice. Plasma basal levels of CoQ$_{10}$ were under the limit of quantification (<0.2 μg/mL).
(TIF)

Table S1 Endogenous levels of CoQ$_{9/10}$ in 129Sv NTg mice. Endogenous levels (μg/g tissue or ng/mL) of total CoQ$_{9/10}$, ubiquinol$_{9/10}$ and ubiquinone$_{9/10}$ in brain, lumbar spinal cord (L-SpC) and plasma of 16 week-old male 129Sv non-transgenic mice. Values are expressed as means±SEM of (n) mice.
(DOC)

Author Contributions

Conceived and designed the experiments: JL SC EB CB MG. Performed the experiments: JL MM S. Papa MT GG S. Pozzi. Analyzed the data: JL MM MT VB CB MG. Wrote the paper: JL CB MG.

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