

# Pyrimethamine Significantly Lowers Cerebrospinal Fluid Cu/Zn Superoxide Dismutase in Amyotrophic Lateral Sclerosis Patients with *SOD1* Mutations

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**Objective:** Cu/Zn superoxide dismutase (SOD1) reduction prolongs survival in SOD1-transgenic animal models. Pyrimethamine produces dose-dependent SOD1 reduction in cell culture systems. A previous phase 1 trial showed pyrimethamine lowers SOD1 levels in leukocytes in patients with *SOD1* mutations. This study investigated whether pyrimethamine lowered SOD1 levels in the cerebrospinal fluid (CSF) in patients carrying *SOD1* mutations linked to familial amyotrophic lateral sclerosis (fALS/SOD1).

**Methods:** A multicenter (5 sites), open-label, 9-month-duration, dose-ranging study was undertaken to determine the safety and efficacy of pyrimethamine to lower SOD1 levels in the CSF in fALS/SOD1. All participants underwent 3 lumbar punctures, blood draw, clinical assessment of strength, motor function, quality of life, and adverse effect assessments. SOD1 levels were measured in erythrocytes and CSF. Pyrimethamine was measured in plasma and CSF. Appel ALS score, ALS Functional Rating Scale–Revised, and McGill Quality of Life Single-Item Scale were measured at screening, visit 6, and visit 9.

**Results:** We enrolled 32 patients; 24 completed 6 visits (18 weeks), and 21 completed all study visits. A linear mixed effects model showed a significant reduction in CSF SOD1 at visit 6 ( $p < 0.001$ ) with a mean reduction of 13.5% (95% confidence interval [CI] = 8.4–18.5) and at visit 9 ( $p < 0.001$ ) with a mean reduction of 10.5% (95% CI = 5.2–15.8).

**Interpretation:** Pyrimethamine is safe and well tolerated in ALS. Pyrimethamine is capable of producing a significant reduction in total CSF SOD1 protein content in patients with ALS caused by different *SOD1* mutations. Further long-term studies are warranted to assess clinical efficacy.

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Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive neurodegenerative disease of upper and lower motor neurons causing progressive weakness of limbs, swallowing, and breathing, resulting in death

within 3 to 5 years.<sup>1</sup> The cause is uncertain in most patients but in approximately 10% of patients, the disease is familial.<sup>2</sup> Since 1993, mutations in > 36 genes have been associated with causing ALS.<sup>3</sup> Mutations in

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the cytoplasmic free radical scavenging enzyme Cu/Zn superoxide dismutase (SOD1) account for 3 to 23% of familial cases (familial ALS [fALS]) and 1 to 3% of sporadic cases.<sup>2-4</sup> Transgenic mice overexpressing mutant human SOD1 develop a progressive motor neuron degenerative disease mimicking human ALS, whereas knockout of the murine *SOD1* gene does not result in a similar phenotype.<sup>5</sup> These findings combined with the observation that there is no relationship between the level of SOD1 activity and patient prognosis suggest that there is a toxic gain of function for the SOD1 mutant molecule with a predilection for the motor system.<sup>3</sup> Reducing the content of mutant SOD1 attenuates disease progression proportionate to the suppression of mutant protein using interfering RNA.<sup>6</sup> Collective evidence supports the hypothesis that lowering the total SOD1 protein content may be beneficial and influence the disease course in ALS. Attempts to lower SOD1 expression are currently being pursued using antisense oligonucleotides<sup>7</sup> and by increasing consumption of SOD1 by activating heat shock proteins<sup>8</sup> via the drug arimoclomol. Using U.S. Food and Drug Administration (FDA)-approved drugs that also have the ability to lower SOD1 content is another approach.<sup>9</sup> Monitoring the CSF SOD1 protein level has been identified as a reliable biomarker for SOD1 reduction within the anterior horn cell in transgenic rats with *SOD1*-mediated ALS.<sup>7</sup> In humans with ALS and *SOD1* mutations, CSF SOD1 shows minimal variability and is a reliable biomarker for *SOD1*-mediated fALS.<sup>10</sup> We have previously reported that oral treatment with pyrimethamine in ALS patients with a mutation in *SOD1* resulted in a reduction of SOD1 levels in peripheral blood leukocytes and, in both patients studied, a reduction in SOD1 protein content and activity in the cerebrospinal fluid (CSF).<sup>9</sup> We now report a phase 1/2 study whose primary aim was to determine whether pyrimethamine lowers SOD1 in the CSF in ALS patients with a wide variety of *SOD1* mutations and over a longer period of time, with a secondary aim to establish safety and tolerability.

## Patients and Methods

The institutional review board at Weill Cornell Medicine approved this study, followed by approval by the relevant institutional or national ethical review boards at the participating institutions in the USA, Italy, Germany, and Sweden, following FDA and European Medicines Agency regulations and adhering to the Principles of the Declaration of Helsinki (World Medical Association, 1964). The study was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) as NCT01083667. This was a single-arm, open-label study with the primary endpoint of determining whether oral medication with pyrimethamine results in a

reduction of CSF SOD1 levels in ALS patients with different types of *SOD1* mutations. Based on our earlier study,<sup>9</sup> the target dose was set at 75mg per day supplemented with 10mg of leucovorin. However, different dosing was achieved due to reductions required to maintain tolerability. We enrolled 32 patients (Table 1).

Inclusion criteria were: the presence of objective weakness in at least 1 neural segment and a pathogenic mutation in *SOD1*, (El Escorial Definite ALS revised<sup>11</sup>), age of 18 years or older, capable of providing written informed consent and complying with trial procedures, not taking riluzole or on a stable dose for 30 days or more, and not taking coenzyme Q10 or on a stable dose and brand for 30 days or more. Exclusion criteria were: history of malabsorption syndrome, exposure to any other agents considered a therapeutic target for ALS within 30 days of entry into this study, women who were pregnant or planning on becoming pregnant, women who were breastfeeding, alcoholism, taking phenytoin or other medications that may interfere with folate levels, seizures, megaloblastic anemia, folate deficiency, cardiac rhythm disorders, impaired renal or liver function, tracheostomy, mechanical ventilation, and use of any of the following medications: cytosine arabinoside, methotrexate, daunorubicin, sulfonamides, zidovudine, lorazepam, warfarin, sulfamethoxazole, trimethoprim, and lithium.

There were 10 visits: screen/baseline (week 0) and weeks 3, 6, 9, 12, 15, 18, 24, 30, and 36. Visits at weeks 0, 18, and 36 were critical visits for data acquisition. At all visits, weight, vital signs, and concomitant medication screen combined with adverse effect assessment occurred. Blood for SOD1 and pyrimethamine levels was obtained. At weeks 0, 18, and 36, a lumbar puncture (LP) was performed. At weeks 0, 6, 18, and 36, we measured the ALS Functional Rating Scale–Revised (ALSFRS-R), Appel ALS (AALS), and the McGill Quality of Life Single-Item Scale (MQOL-SIS) scores. The ALSFRS-R score is a questionnaire-based assessment of motor function that has been validated in natural history studies of ALS and therapeutic trials.<sup>12</sup> The AALS score is an objective measure of global motor function that has been validated in natural history studies and therapeutic trials.<sup>13</sup> The MQOL-SIS is a single question in which the patient rates their overall quality of life (QOL) on a scale from 1 to 10 (10 being the best possible and 0 being the worst possible) for the past 48 hours. MQOL-SIS has good correlation with ALS QOL.<sup>14</sup>

## Summary of Dose Escalation and Algorithm for Reduction

Pyrimethamine was supplied in 25mg tablets (CorePharma, Middlesex, NJ). The target dose was 75mg, based on our experience in the first study of pyrimethamine,<sup>9</sup> where 100mg was poorly tolerated but 75mg was deemed to be a dose that most patients could tolerate over an extended period of time. The escalation of dose was as follows. At baseline, patients started taking a 25mg tablet daily together with 5mg leucovorin twice daily. The leucovorin remained at the same dose for the duration of the study. Pyrimethamine dose increased to 37.5mg at 3

**TABLE 1. List of Mutations and Clinical Features**

Number	Mutation	Gender	Age at Onset, yr	Site of Onset	AALS Entry
1	A4T	M	50	LE	37
2	A4V	M	51	LE	37
3	A4V	F	36	LE	42
4	A4V	F	49	LE	93
5	A4V	M	40	LE, B	90
6	A4V	F	49	LE, L	111
7	L8Q	F	33	UE	82
8	H46R	F	38	LE	37
9	H46R	M	37	LE	72
10	H80Y <sup>a</sup>	M	51	UE	109
11	L84F	F	36	LE	100
12	L84F	M	32	LE	46
13	N86K	M	53	UE	58
14	D90A (heterozygous)	M	55	LE	53
15	D90A (heterozygous)	M	48	UE	52
16	G93A	F	37	LE	57
17	G93D	F	19	LE, R	115
18	A95T	F	45	LE, L	61
19	E100G	M	57	LE	79
20	D109Y	F	59	LE, L	45
21	I113T	M	67	LE	50
22	I113T	F	61	UE	63
23	L117V (homozygous)	M	44	LE, L	91
24	R115G	M	54	LE	72
25	R115G	F	48	LE	76
26	V119F <sup>a</sup>	M	53	BU	51
27	G127Gfs*7	F	21	LE	36
28	G127Gfs*7	F	48	UE	34
29	E133K <sup>a</sup>	M	62	LE	59
30	G147C	F	61	LE	70
31	L144F	M	58	LE, R	48
32	L144F	F	53	LE, L	84

<sup>a</sup>Novel mutations.

AALS = Appel ALS; B = bilateral; BU = Bulbar; F = female; L = left; LE = lower extremities; M = male; R = right; UE = upper extremities.

weeks, 50mg 6 weeks, 62.5mg at 9 weeks, and 75mg at 12 weeks. Patients remained at 75mg for the duration of the study (36 weeks) if tolerated.

Adverse effects were graded according to the Common Terminology Criteria for Adverse Events version 4.0. A defined algorithm existed to limit dose escalation or dose reduction.

Escalation was stopped if any of the following symptoms achieved a grade 2 or above level: nausea, diarrhea, vomiting, glossitis, heart rate change (>130 beats per minute or >50% over baseline on 2 separate measurements).

Reduction of dose occurred as follows: 75mg to 50mg, 50mg to 37.5mg, 37.5mg to 25mg. Reduction was mandated if either 1 grade 3 or  $\geq 2$  grade 2 of the adverse effects were identified, including: persistent nausea, persistent diarrhea, persistent vomiting, persistent heart rate change, and persistent anemia (>1 study visit). Once dose reduction occurred or escalation was stopped, there were no further attempts to change the dose of medication until the end of the study.

### Collection of Blood

Venous blood samples were collected in vacuum tubes containing ethylenediaminetetraacetic acid as anticoagulant. They were centrifuged at  $1,500 \times g$  for 15 minutes and were separated into plasma, buffy coat, and erythrocytes. The samples were stored at  $-80^{\circ}\text{C}$  until analysis.

### Collection of CSF

LP to obtain CSF was performed in all patients at baseline, visit 6 (18 weeks), and visit 9 (36 weeks). The samples were stored at  $-80^{\circ}\text{C}$  until analysis. Specimens from tube #3 were sent for SOD1 analysis.

### SOD1 Enzymatic Activity in Erythrocytes

The SOD1 enzymatic activity was analyzed in erythrocytes by the direct spectrophotometric method using potassium superoxide as previously described.<sup>15,16</sup> The activity was related to the content of hemoglobin in the erythrocyte lysates. There is a strong correlation between amounts of SOD1 protein measured by enzyme-linked immunosorbent assay (ELISA) and the SOD1 enzymatic activity.<sup>16</sup>

### SOD1 Protein in CSF

For analysis of SOD1 protein, an ELISA based on rabbit and goat anti-native human SOD1 antibodies was used.<sup>16</sup> The rabbit antibody was used as a primary antibody and the goat antibody as the secondary antibody. The ELISA was standardized with a human hemolysate, with the SOD1 content calibrated against pure human SOD1, the concentration of which was determined by quantitative amino acid analysis.<sup>17</sup>

### Measurement of Pyrimethamine in Plasma and CSF

A liquid chromatography/mass spectroscopy method was used for analysis of pyrimethamine in both plasma and CSF. Protein precipitation method was used for sample extraction with a sample size of 50  $\mu\text{l}$  (Primer Analytical Solutions, Princeton, NJ).

### Statistical Methods

Based on previous data, the mean  $\pm$  standard deviation baseline SOD1 content was estimated to be  $112 \pm 30\text{ng}/\text{cm}^3$ .<sup>9</sup> It was determined that enrollment of 34 patients with ALS and mutations in *SOD1* would provide 80% power at a 2-sided alpha level of 0.025 to detect a 15% reduction in SOD1 content

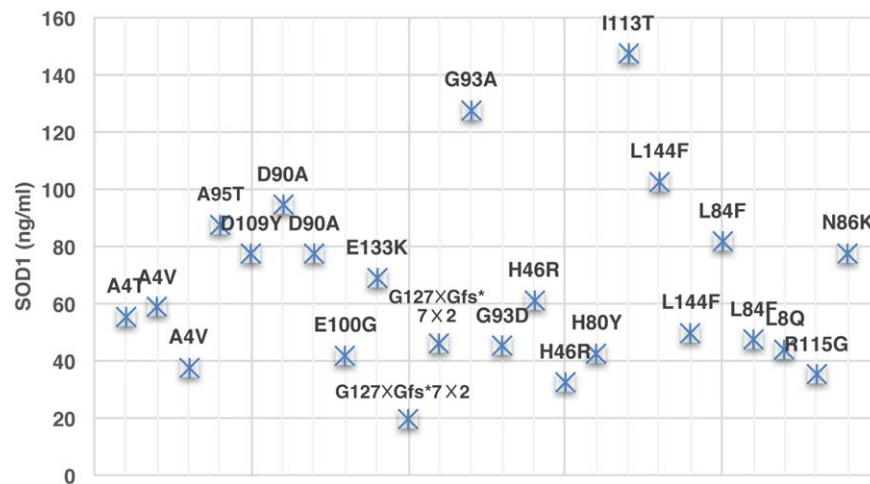
from baseline at 18 weeks and/or 36 weeks after study commencement. A total of 40 patients were to be enrolled to account for potential dropouts. However, the trial was terminated after the enrollment of 32 patients due to a sudden and exorbitant increase in the cost of pyrimethamine.<sup>18</sup>

Continuous variables are presented as means with standard deviations or medians with 1st and 3rd quartiles. Kaplan–Meier analysis was used to estimate the median, 1st quartile, and 3rd quartile duration of disease. Categorical variables are presented as counts and percentages. Continuous baseline variables were compared between patients with complete versus incomplete followup data using 2-sample *t* tests or Wilcoxon rank sum tests, depending upon the distribution of the data. Categorical baseline variables were compared between patients with complete versus incomplete followup data using chi-square or Fisher exact tests, as appropriate. The change in continuous outcomes from baseline across repeated measurements was modeled using linear mixed effects modeling to a Gaussian random intercept, producing equivalent point estimates to a generalized estimating equations approach with a compound symmetric correlation structure. Given the limited sample size, each model contained time point as the sole predictor (treated as a fixed effect). A compound symmetric correlation structure was employed because the models would not converge with more complex correlation structures. As a sensitivity analysis, the mean change in continuous outcomes over time was calculated after multiple imputation of missing values. Specifically, age, sex, and baseline ALSFRS-R, AALS, and MQOL-SIS scores were used to create 10 imputed datasets. Results were combined to obtain pooled mixed effects model estimates of the change in continuous outcomes from baseline to 6, 18, and 36 weeks. All statistical hypothesis tests were 2-sided, with  $p < 0.05$  considered statistically significant. Statistical analyses were performed with SAS version 9.3 (SAS Institute, Cary, NC) and the ICE package implemented in STATA version 14 (Stata Corporation, College Station, TX).

### Results

After signing informed consent, 32 patients were screened, 16 men and 16 women whose symptoms started at a mean age of 47.0 years (range = 19–67). All patients had confirmed mutations in *SOD1*; 21 were unique, and 3 were novel (H80Y, V119F, E133K; see Table 1). At baseline, the mean AALS score was 65.6 (range = 34–115). The mean ALSFRS-R score was 40.1 (range = 27–47). Twenty-two patients were on a stable dose of riluzole at entry and continued throughout the study; 8 were riluzole free.

Of the 32 patients enrolled, 8 dropped out before the scheduled 2nd LP: 6 due to disease progression and 2 due to inability to tolerate medication. Therefore, 24 samples were available to analyze at week 18 (Fig 1). In the final 18 weeks of the study, 2 additional patients dropped out (leaving 22 potential CSF samples for analysis). However, 1 patient declined the last LP and AALS



**FIGURE 1:** Baseline values of cerebrospinal fluid SOD1 protein content according to mutation in each subject with 2 lumbar punctures. [Color figure can be viewed at [www.annalsofneurology.org](http://www.annalsofneurology.org)]

score, leaving 21 patients with 3 longitudinal CSF, blood, and clinical assessments.

The results from all measures obtained during the study, expressed as change from baseline, are presented in Table 2. The primary aim of the study was to determine whether patients with ALS associated with an *SOD1* mutation taking pyrimethamine showed a reduction in the level of CSF SOD1. The mean reduction of CSF SOD1 in all patients at 18 and 36 weeks was 13.5% (raw mean reduction = 8.8ng/ml, 95% confidence interval [CI] = 5.5–12.1,  $p < 0.001$ ) and 10.5% (6.8ng/ml, 95% CI = 3.4–10.3,  $p < 0.001$ ; see Table 2). Achieving a significant reduction in CSF SOD1, an accepted biomarker for SOD1-associated fALS, over the 9-month study period fulfilled the primary aim of this protocol. Peripheral blood SOD1 did not change during the study. Pyrimethamine levels in the CSF approximated 10% of that seen in the plasma. During the final 18 weeks of the study, the mean dose of pyrimethamine declined as did CSF SOD1 levels, suggesting a dose–response effect (Fig 2).

Of the 32 patients enrolled, 24 completed 18 weeks of treatment with 2 LPs. The change in CSF SOD1 from baseline in each patient per mutation, undergoing 2 LPs 4.5 months apart (18 weeks), is shown in Figure 3. There is substantial variation between patients and between patients with the same mutation. Nine patients (39%) showed a reduction of 20% or more from baseline.

Of the 22 patients completing the study (36 weeks of treatment), 13 were able to continue at the protocol-directed 75mg. One patient refused the last LP, leaving 12 patients with CSF to analyze. In 9 (75%), SOD1 reduction exceeded 14% (14.3–24.3%). In one, a woman heterozygous for A4V, the visit 9 (36 week) SOD1 level increased by >20% from baseline and >40% from the SOD1 measurement at week 18. Excluding that patient,

protocol-compliant patients showed a mean decrease of 15.6% at 36 weeks.

CSF SOD1 normal variation is minimal. One study showed that CSF SOD1 varies by  $\pm 7.1\%$  on repeated analysis.<sup>10</sup> Moreover, it appears that CSF SOD1 content does not decrease over time in ALS patients not treated with pyrimethamine. For comparison, we analyzed CSF SOD1 in 12 untreated ALS patients without *SOD1* mutations who had LPs on 2 occasions. The SOD1 content increased by an average of 2.8% over a mean interval of 13.8 months (from  $98.3 \pm 33.5$  to  $102.9 \pm 35.7$ ng/ml).

There were 11 dropouts (34%). We compared baseline characteristics between patients who did and did not complete the study (Table 3) and performed multiple imputation analyses on all measures (Table 4). Point estimates from multiple imputation and available case analyses were fairly similar for most outcomes and time points. However, multiple imputation analysis did show a less pronounced decrease in CSF SOD1 levels between baseline and 36 weeks compared to complete case analysis (9.0% vs 10.5%, respectively) and a greater increase in AALS score over the same time period (29 vs 18, respectively). Comparing baseline features between patients who completed relative to those who dropped out showed that those who did not complete the 36-week protocol were often women with greater burden of disease (admission AALS/ALSFRRS-R in fully protocol compliant = 53/42 vs 76/39 in dropouts). There was no change in self-perceived QOL between baseline and completion.

### **Does Pyrimethamine Slow Progression of fALS?**

Although the primary aim of the study was to determine whether pyrimethamine significantly lowers CSF SOD1

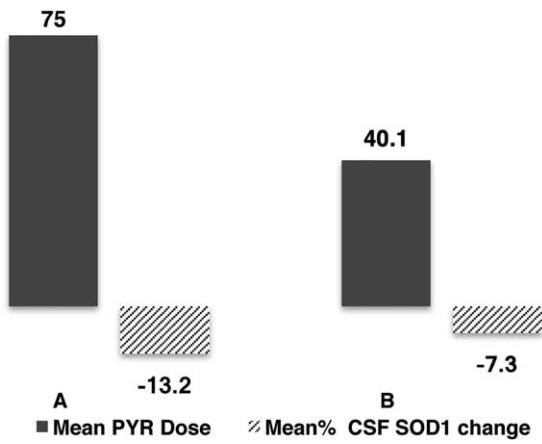
TABLE 2. Change from Baseline for All Measures Studied

Measure	No.	Mean Change from Baseline (95% CI)	<i>p</i>
SOD1 CSF			
18 weeks	24	−8.8 (−12.1 to −5.5); −13.5% (−18.5 to −8.4)	<0.001
36 weeks	21	−6.8 (−10.3 to −3.4); −10.5% (−15.8 to −5.2)	<0.001
Pyrimethamine CSF			
18 weeks	24	273 (242 to 304)	<0.001
36 weeks	22	240 (208 to 272)	<0.001
SOD1 peripheral blood, PRBC			
6 weeks	15	−0.3 (−2 to 1.5)	0.773
18 weeks	14	0.6 (−1.2 to 2.4)	0.501
36 weeks	14	0.3 (−1.4 to 2.1)	0.707
Pyrimethamine blood, plasma			
6 weeks	20	1,935 (1,534 to 2,336)	<0.001
18 weeks	23	2,851 (2,466 to 3,237)	<0.001
36 weeks	22	2,435 (2,045 to 2,825)	<0.001
AALS			
6 weeks	24	2 (−3 to 8)	0.410
18 weeks	23	8 (2 to 14)	0.008
36 weeks	22	18 (12 to 24)	<0.001
ALSFRS-R			
6 weeks	24	−2 (−4 to 1)	0.192
18 weeks	23	−4 (−6 to −1)	0.004
36 weeks	24	−7 (−9 to −4)	<0.001
MQOL-SIS			
6 weeks	23	0 (−1 to 1)	0.782
18 weeks	19	−1 (−2 to 0)	0.157
36 weeks	23	0 (−1 to 0)	0.411

AALS = Appel ALS; ALSFRS-R = ALS Functional Rating Scale–Revised; CI = confidence interval; CSF = cerebrospinal fluid; MQOL-SIS = McGill Quality of Life Single-Item Scale; PRBC = packed red blood cells.

levels, we found patients on pyrimethamine may have progressed slower than expected based on data from historical studies of mutant *SOD1* patients and placebo groups from other therapeutic trials. The mean change

from baseline showed a significant progression using both AALS and ALSFRS-R measures at 18 and 36 weeks (see Table 2), but the rate of change was 2 points per month (PPM) and −0.7 PPM, respectively, for all



**FIGURE 2:** Dose-dependent change in cerebrospinal fluid (CSF) SOD1 content in patients with amyotrophic lateral sclerosis at (A) visit 6 (18 weeks) and (B) visit 9 (36 weeks). PYR = pyrimethamine.

patients. Of note, disease progression in patients with A4V/A4T mutations needs to be considered separately because of marked differences in progression and survival.<sup>19</sup> In one study, survival in patients with non-A4V mutations showed a mean disease duration of  $6.6 \pm 7$  years compared to  $1.4 \pm 0.7$  years in A4V and  $0.8 \pm 0.1$  months in A4T patients.<sup>19</sup>

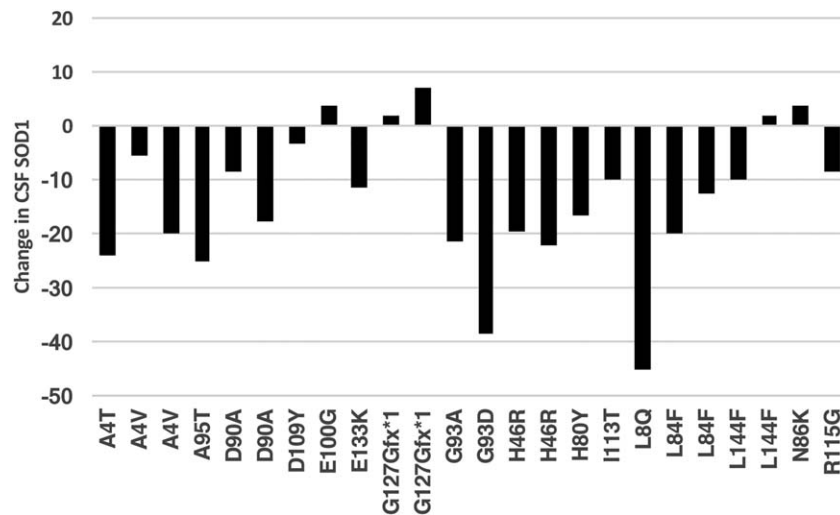
Of our non-A4V patients, mean progression was 0.52 PPM on the ALSFRS-R scale and 0.92 PPM on the AALS score (Fig 4). In comparison, placebo and historical controls from non-fALS patients show average ALSFRS-R/AALS change to be  $1.18 \pm 0.85$  and 4.70 PPM, respectively.<sup>20,21</sup>

We had 4 patients who showed no change in ALSFRS-R or AALS over the 9-month period (ie, non-progressors). The mutations were H46R, I113T, L144F,

and A95T. All of these mutations are known to produce a progressive motor neuron disorder with either a protracted or a variable time course.<sup>22,23</sup>

Progression is more uniform in rapidly progressive mutations, such as A4V and A4T. Our population included 3 patients with A4V (n = 2; Patients 2 and 3; see Table 1) and A4T (n = 1; Patient 1; see Table 1) who were fully compliant with the protocol and completed the entire 36-week treatment period with ALSFRS-R and AALS scores and CSF SOD1 values (3 other A4V patients dropped out because of disease progression). For the first 18 weeks, the rate of change of AALS/ALSFRS-R was 2.5/1.0 PPM for the patients with A4V and 4/2 PPM for the patient with A4T; the last 4 months showed an increase to an average of 12/4.35 PPM for A4V and 6.25/1.5 PPM for A4T (Fig 5). The median duration of disease for the 2 A4V patients was 20 months (interquartile range = 16–22), and the duration of disease for the A4T patient was 24 months.

There was a proportionate reduction in SOD1 levels in all 3 compliant A4V/T subjects during the first 18 weeks of the study (mean = 16.6ng/ml). One A4V patient showed a reduction in SOD1 content of 20% during the first 4.5 months. However, during the last 4.5 months of the study, SOD1 content increased 47%. This subject had the shortest disease duration of 19 months and a change in AALS rate of change from 4.0 PPM during the first half to 6.25 PPM during the last 18 weeks. She expired from disease-related respiratory failure 2 months after completing the study. The 2nd A4V patient showed a slower but progressive decline in SOD1 content, and he lived 22 months, that is, almost double the mean survival time for patients with A4V.<sup>19</sup> The patient



**FIGURE 3:** Percentage change from baseline in cerebrospinal fluid (CSF) SOD1 content at 18 weeks of treatment according to mutation.

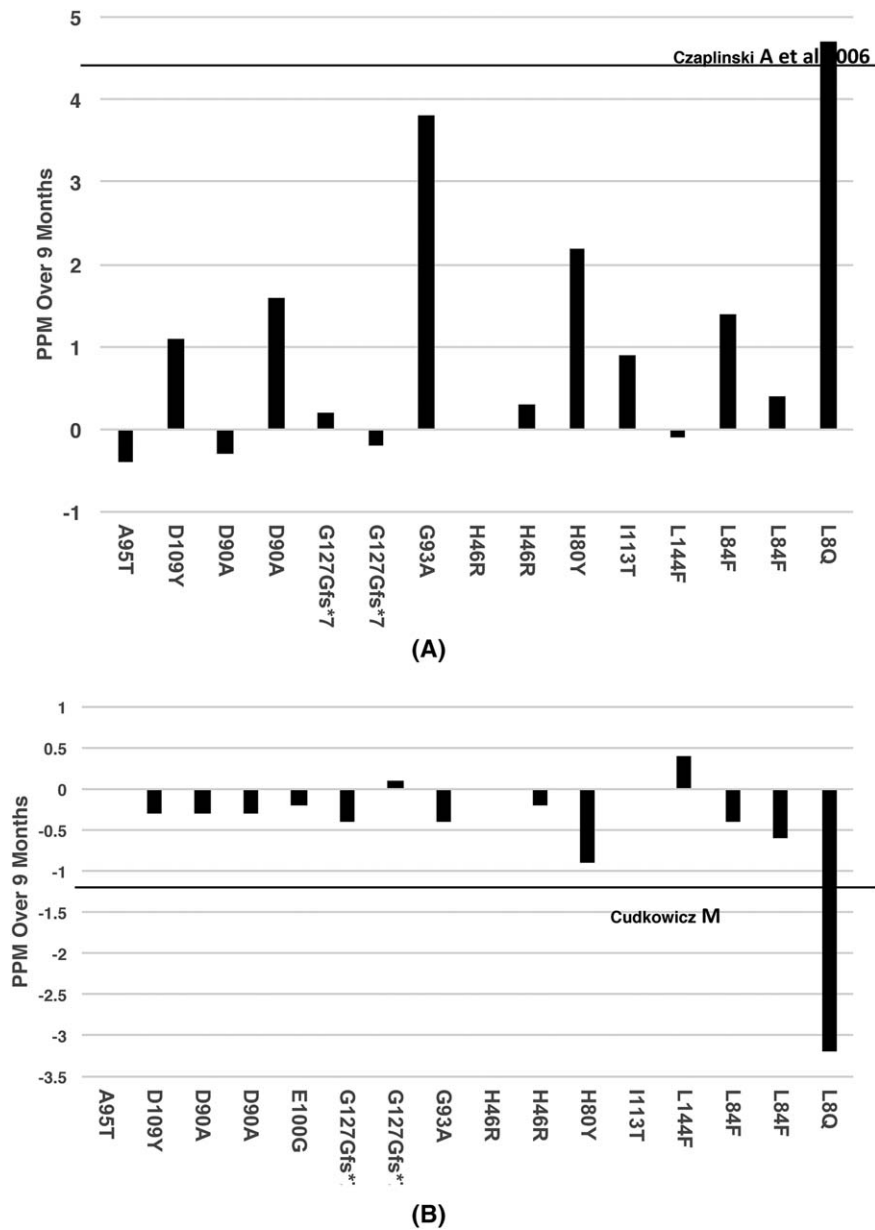


FIGURE 4: Rate of change expressed as points per month (PPM) in Appel ALS score (A) and ALS Functional Rating Scale-Revised (B) over 9 months (36 weeks) in non-A4V patients. Bold horizontal lines represent change of controls in Czaplinski et al<sup>21</sup> (A) and Cudkowicz et al<sup>20</sup> (B).

Measure	CSF SOD1 Level at 36 Weeks, n = 21	No CSF SOD1 Level at 36 Weeks, n = 11	<i>p</i>
Age, yr, mean ± SD	49.7 ± 13.0	52.7 ± 9.7	0.506
Female, No. (%)	8 (38.1)	8 (72.7)	0.063
AALS, median [IQR]	53 [42–72]	76 [61–91]	0.066
ALSFRS-R, median [IQR]	42 [38–45]	39 [37–43]	0.186
MQOL-SIS, median [IQR]	8 [8–8]	7 [6–8]	0.099

AALS = Appel ALS; ALSFRS-R = ALS Functional Rating Scale-Revised; CSF = cerebrospinal fluid; IQR = interquartile range; MQOL-SIS = McGill Quality of Life Single-Item Scale; SD = standard deviation.



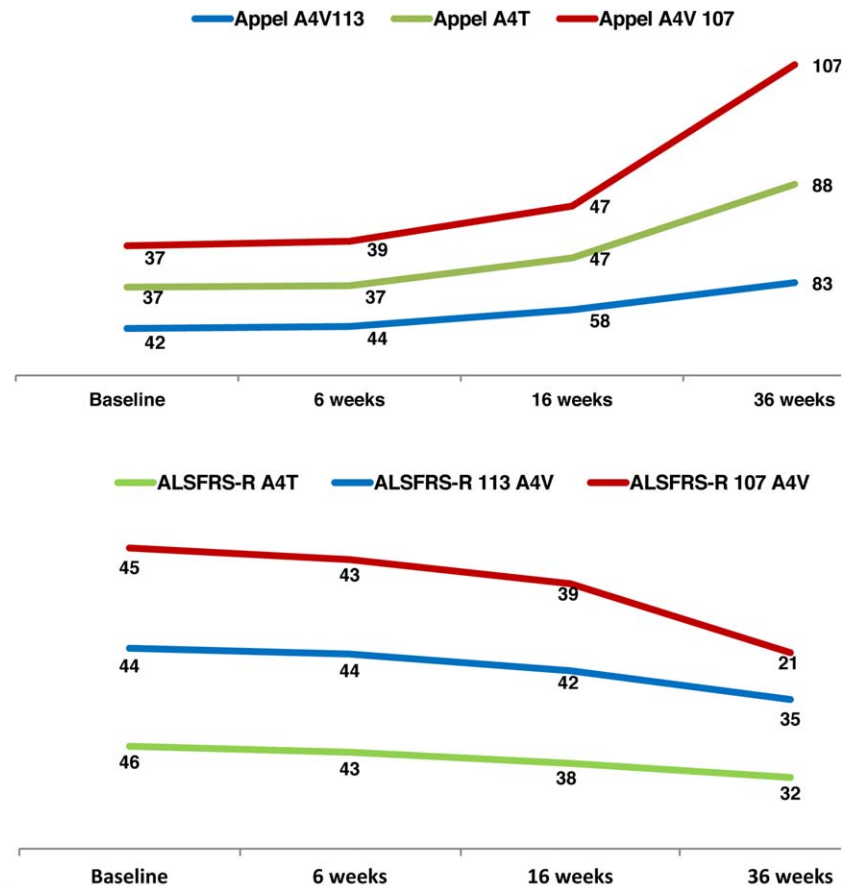


FIGURE 5: Rate of change expressed as points per month (PPM) in Appel ALS score (A) and ALS Functional Rating Scale-Revised (ALSFRS-R; B) over 9 months (36 weeks) in 3 A4V/A4T patients.

with A4T, which is often more aggressive than A4V, lived the longest at 24 months from symptom onset. Clearly, no conclusions can be drawn from these 3 A4V/T patients, but these observations seem to suggest a slowing of progression in this most aggressive of familial diseases.

### Adverse Events

There were 30 unique symptoms classified as adverse effects, 77 in total. Among the most common were headache ( $n = 13$  of 32 patients; most associated with LP); nausea (14 of 32); diarrhea (6 of 32); pain (5 of 32); falls, upper respiratory tract infections, shortness of breath/aspiration pneumonia, and fatigue (4 of 32); weight loss (3 of 32); decreased appetite and rash (2 of 32); and nightmares, lightheadedness, hunger, and dysgeusia (1 of 32). Seven patients had grade 3 adverse effects encompassing headache ( $n = 3$ ), aspiration pneumonia, weight loss, shortness of breath, and urinary tract infection. One patient had shortness of breath deemed to be grade 4. There was no increase in adverse events in the patients taking riluzole relative to those not taking that medication.

### MQOL-SIS

The mean score from the MQOL-SIS showed no significant change during the duration of the study (see Table 4). However, when individual patients with more aggressive mutations such as A4V and A4T were measured, there was a decline in MQOL-SIS.

### Discussion

To our knowledge, this is the first study in humans with ALS that targeted and achieved a significant reduction of an ALS biomarker in the CSF: the content of SOD1.<sup>10</sup> The degree of reduction varied among patients, but on average, the reduction of 13.5% relative to baseline was statistically significant, with the greatest reduction noted being 46%. There was also a dose response observed with respect to mean dose of pyrimethamine relative to mean reduction of SOD1 CSF content.

In humans, it is unknown whether lowering of CSF SOD1 attenuates disease progression and if it does, what the critical threshold is, if any. We originally targeted 15% based on our observations in our first study. However, subsequent studies showed that normal

TABLE 4. Multiple Imputation Analysis

Measure	Mean Change from Baseline (95% CI)	<i>p</i>
SOD1 CSF		
18 weeks	−8.6 (−12.0 to −5.3); −13.6% (−18.8 to −8.3)	<0.001
36 weeks	−5.7 (−9.8 to −1.7); −9.0% (−15.4 to −2.6)	0.007
Pyrimethamine CSF		
18 weeks	289 (244 to 334)	<0.001
36 weeks	258 (212 to 305)	<0.001
SOD1 peripheral blood, PRBC		
6 weeks	−4.0 (−11.3 to 3.2)	0.260
18 weeks	2.1 (−3.5 to 7.7)	0.452
36 weeks	1.3 (−3.6 to 6.3)	0.592
Pyrimethamine blood, plasma		
6 weeks	1,809 (1,137 to 2,481)	<0.001
18 weeks	2,837 (2,152 to 3,523)	<0.001
36 weeks	2,359 (1,783 to 2,935)	<0.001
AALS		
6 weeks	3 (−15 to 21)	0.734
18 weeks	12 (−9 to 33)	0.250
36 weeks	29 (−14 to 72)	0.170
ALSFRS-R		
6 weeks	−2 (−7 to 4)	0.548
18 weeks	−5 (−13 to 3)	0.188
36 weeks	−8 (−18 to 3)	0.142
MQOL-SIS		
6 weeks	0 (−4 to 3)	0.892
18 weeks	1 (−4 to 6)	0.712
36 weeks	1 (−2 to 5)	0.455

AALS = Appel ALS; ALSFRS-R = ALS Functional Rating Scale–Revised; CI = confidence interval; CSF = cerebrospinal fluid; MQOL-SIS = McGill Quality of Life Single-Item Scale; PRBC = packed red blood cells.

variation in CSF does not exceed 7.1%.<sup>10</sup> Total CSF SOD1 content includes both wild-type and mutant proteins. Owing to widely variable stabilities, the mutant SOD1s are degraded in the central nervous system (CNS) at variable rates. Depending on the mutation, the mutant SOD1 in the CNS has, in earlier studies, been found to vary from nearly 100% (for stable mutants) to

around 0.5% (highly unstable truncating mutations) of the amount of wild-type SOD1 in heterozygous mutant *SOD1* patients.<sup>24,25</sup> This is reflected in our highly variable amounts of SOD1 in CSF from our patient population, which overall are lower than those found in the time-course study in ALS patients without *SOD1* mutations. Therefore, a small reduction in total SOD1 may

cause a large or minimal change in mutant SOD1. The technology to reliably measure the relative composition of wild-type and mutant SOD1 in the CSF is not yet available.

Our findings suggest that there may be slowing of progression using historical controls. However, patients with fALS may have a more variable course than sporadic disease, sometimes unusually long. Therefore, drawing any conclusion about pyrimethamine's impact on disease progression from these studies would be incorrect and misleading. Our findings should only serve as a basis for further studies sufficiently powered to determine whether pyrimethamine will change rate of progression.

The mechanism behind the lowering of the SOD1 content by pyrimethamine is not known. If the synthesis of the protein is reduced, it is likely that the wild-type and mutant SOD1s are affected to equal extents. However, our results show excellent CNS penetration. Pyrimethamine is one of the lipophilic antifolates.<sup>26</sup> More than 90% of pyrimethamine<sup>27</sup> is bound to plasma proteins, suggesting there is 10% free pyrimethamine in the plasma. This equals the 10% of plasma levels we found in the CSF. The proportion of pyrimethamine bound to protein in the CSF should be negligible given the very low CSF protein content.

A single study found that pyrimethamine has no impact on SOD1 levels when administered to cell cultures or homogenates of liver, spinal cord, and brain of wild-type mice.<sup>28</sup> Both our previous study and the present study find that there are no significant changes in SOD1 content in erythrocytes. However, our previous work showed a reduction in leukocytes and now in CSF, a very different tissue from the ones tested by Wright et al.<sup>28</sup> Perhaps of importance, different methodologies to assay SOD1 were used by Wright et al and by us.

In our first study, pyrimethamine was poorly tolerated at higher doses, especially at the 100mg dose, causing multiple adjustments in dose.<sup>9</sup> We therefore set 75mg as the maximum dose and established strict criteria for dose reduction after which the tolerated dose would not be altered. This allowed us to discern a possible dose impact on CSF SOD1 levels, which we did observe. Therefore, it would be important to try to achieve the target of 75mg per day. When factors that impacted dose reduction were reviewed, the severity of illness at entry seemed to be the best predictor of successful escalation to and maintenance of the maximum dose. In future studies, a maximum level of disease severity prior to entry should be established.

MQOL-SIS is a validated scale found to be useful in patients with ALS.<sup>14,29</sup> Our results show that over a 9-month period, there was no difference in MQOL-SIS,

and in some patients, there was a self-perceived improvement in QOL. Of import, there was no significant decrease in QOL over the 9-month study. However, in rapidly progressive forms (A4V/A4T), weakness and QOL declined, as observed by others.<sup>30</sup>

This is the largest prospective clinical and biological investigation of patients with fALS in the literature to date. There are, however, limitations to this study. We admitted all patients with varying levels of disease severity, and patients with higher disease burden were more likely to drop out. Our multiple imputation results suggest the possibility that results from the available case analysis were biased toward a greater average decrease in CSF SOD1 or lesser average worsening in disease than would have been observed had these sicker patients remained in the trial. Future power analyses should likely target levels exceeding 7% change in CSF content. We are not certain that patients willing to undergo 3 or 4 LPs might in some way be different from those not willing to have invasive procedures. We also did not study patients with sporadic ALS, in whom misfolded wild-type SOD1 may act like the mutant protein, justifying use of potential SOD1 reducing therapies in this disease.<sup>31-33</sup> Despite these limitations, we showed, for the first time in humans, a pharmacologic agent caused a significant reduction in CSF SOD1 in patients with ALS and *SOD1* mutations. Although no comment can be made with confidence regarding impact on disease progression, slowing relative to historical controls may be present, and further study regarding impact on disease progression and its relation to SOD1 reduction is needed.

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## Author Contributions

Study concept and design: D.J.L., M.S., S.H.A., P.M.A., S.M., V.S., A.C.L., S.A.-D., S.M.L. Data acquisition and analysis: D.J.L., V.S., A.C.L., J.H.W., S.A.-D., C.M., A.D., L.M., S.M., U.W., S.M.L., K.G.F., R.R. Drafting of manuscript and tables: D.J.L., P.M.A., S.M., V.S., A.C.L., S.M.L., K.G.F.

## Potential Conflicts of Interest

Nothing to report.

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