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A multi-center screening trial of rasagiline in patients with amyotrophic lateral sclerosis: Possible mitochondrial biomarker target engagement

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Abstract

OBJECTIVE—Rasagiline, a monoamine oxidase B inhibitor, slowed disease progression in the SOD1 mouse, and in a case series of patients with amyotrophic lateral sclerosis (ALS). Here we determine whether rasagiline is safe and effective in ALS compared to historical placebo controls, and whether it alters mitochondrial biomarkers.

METHODS—We performed a prospective open-label, multicenter screening trial of 36 ALS patients treated with 2mg oral rasagiline daily for 12 months. Outcomes included the slope of deterioration of the revised ALS Functional Rating Scale (ALSFRS-R), adverse event monitoring, time to treatment failure, and exploratory biomarkers.

RESULTS—Participants experienced no serious drug-related adverse events, and the most common adverse event was nausea (11.1%). Rasagiline did not improve the rate of decline in the ALSFRS-R; however, differences in symptom duration compared to historical placebo controls differentially affected ALSFRS-R slope estimates. Rasagiline changed biomarkers over 12 months, such that the mitochondrial membrane potential increased (JC-1 red/green fluorescent ratio 1.92, P=0.0001) and apoptosis markers decreased (Bcl-2/Bax ratio 0.24, P<0.0001).

CONCLUSION—Engagement of exploratory biomarkers and questions about comparability of baseline characteristics lead us to recommend a further placebo-controlled trial.

Keywords

Biomarker; rasagiline; apoptosis; clinical trials; mitochondria

INTRODUCTION

Despite more than 30 clinical trials in amyotrophic lateral sclerosis (ALS) over the last 20 years, riluzole, the only FDA approved therapy, prolongs life by only a few months.(1) There is a clear need for new ALS therapies. Rasagiline, a monoamine oxidase B inhibitor approved for the treatment of Parkinson's disease (PD), has demonstrated broad neuroprotective activities in neuronal cell cultures.(2, 3) *In vitro* studies suggest rasagiline may stabilize stressed mitochondria, reduce oxidative damage, and inhibit apoptosis.(4, 5) Rasagiline was also shown to prolong survival by 20% in the ALS-SOD1 mouse model in a dose-dependent fashion.(6) Additionally, a retrospective case series of ALS patients treated with rasagiline showed a slower rate of decline in the revised amyotrophic lateral sclerosis functional rating scale (ALSFRS-R) than patients taking riluzole alone.(7)

Mitochondrial dysfunction in ALS has been shown to feature increased mitochondrial membrane permeability, free radical oxidative stress, and apoptosis signaling.(8) Biomarkers measuring mitochondria function or downstream effects of mitochondrial dysfunction could prove useful in providing an early signal of rasagiline drug-target engagement in clinical trials.

Here we conducted a 12-month, open-label study of rasagiline in ALS patients. Our primary objective was to evaluate rasagiline's safety in this patient population and compare rates of decline in the ALSFRS-R to those seen in the placebo arms of historical randomized, controlled trials.(9-14) This approach of using historical placebo controls has been applied as an early screening technique for candidate ALS drugs.(15, 16) This trial design also allows us to reduce the sample size and eliminate the need for a contemporary placebo group, which is seen as a barrier to recruitment in early studies.(15, 16) Our secondary objective was to determine if rasagiline improves exploratory biomarkers of mitochondrial function and oxidative stress.

METHODS

We performed a 12-month, open-label study at 10 sites in the United States and Canada from February 2009 to October 2011. Rates of decline in the ALSFRS-R were compared to historical placebo controls. Institutional Review Boards at each site approved the trial and all participants provided written informed consent. A safety monitoring committee was formed to review safety data collected monthly by an external safety monitor. The study was registered with ClinicalTrials.gov (NCT01232738).

Participants were treated for 12 months with rasagiline 2mg orally, once daily, with all medication supplied by TEVA Pharmaceuticals. Participants were eligible if they had a clinical diagnosis of laboratory-supported probable, probable, or definite ALS according to modified El Escorial criteria,(17) had onset of weakness within 3 years, were 21–80 years of age, and had a forced vital capacity ≥75% of predicted at enrollment.

Participants were ineligible if they required tracheotomy-assisted ventilation or non-invasive ventilation for > 23 hours per day; were taking concomitant therapy with sympathomimetics or analgesics with serotoninergic properties contraindicated for use with rasagiline; were taking greater than standard doses of serotonergic anti-depressant medications (amitriptyline > 50 mg/day, sertraline > 100 mg/day, citalopram > 20 mg/day, or paroxetine > 30 mg/day); had a history of recent alcohol or drug abuse or noncompliance with treatment; or had received an investigational drug within the past 30 days prior to screening. Pregnant women were not included.

The placebo arms from the following randomized, controlled studies in ALS, performed during 2004-2010, were used for comparison: minocycline, TCH346, celecoxib, lithium, CoQ10, and creatine.(9-14) We selected placebo patients from these trials that matched inclusion criteria of our current study. Exclusion criteria were dictated by the medications being studied, but generally excluded a history of other neurological disease, hepatic or renal disease, or use of medications contraindicated for use with the study medications.

Participants were seen for study visits at baseline and then every 3 months. Monthly telephone calls were used to monitor adverse events. If a subject was unable to appear for the in-person assessments, the ALSFRS-R was performed by phone. The same evaluators performed in-person and phone assessments using a scripted protocol and were blinded to prior results.

The study was conducted in outpatient clinics at private and public academic institutions associated with the Western ALS Study Group (WALS). These 10 institutions included: the University of Kansas Medical Center, Kansas City; California Pacific Medical Center, San Francisco; Phoenix Neurological Associates, Phoenix, Arizona; University of Minnesota, Minneapolis; University of Iowa Health Care, Iowa City; University of Nebraska, Omaha; The Methodist Hospital System, Houston, Texas; University of Pennsylvania, Philadelphia; University of Tennessee, Memphis; and McGill University, Montreal, Quebec, Canada.

Outcomes and Measures

The primary outcome was the slope of deterioration in the ALSFRS-R, a 12-item functional inventory with demonstrated validity in ALS.(18) Telephone administration of the ALSFRS-R has been shown to be reliable and similar to in person administration.(19) The slope of decline of the ALSFRS-R is well characterized in ALS and has been stable over the last decade, with rates of decline averaging 1/month.(20)

Secondary endpoints included reporting adverse events and time to failure (defined as death, endotracheal intubation, tracheostomy-assisted ventilation, or noninvasive ventilation ≥23 hours/day for 14 days).

Exploratory Biomarkers

Blood was collected at baseline, month 6, and month 12 and processed in a central laboratory at the University of Kansas Medical Center. Measures of mitochondrial membrane potential, oxidative stress, and apoptosis or pro-viability were included. Lymphocyte mitochondrial membrane potentials were measured using the dye JC-1 (MitoProbe JC-1 Assay Kit for Flow Cytometry; Invitrogen) with flow cytometry to evaluate the red-green fluorescence ratio.(21) We independently evaluated lymphocyte mitochondrial membrane potential using Mitotracker Red CMXRos (Vybrant Apoptotic Assay Kit #11; Molecular Probes) with flow cytometry to measure fluorescence intensity in order to strengthen our analysis.(22) Lymphocyte apoptosis activity was assessed using a dye-conjugated Annexin V stain quantified using flow cytometry, in which calculating the percentage of Annexin-positive cells approximated the amount of exposed phosphatidylserine.(23) Bcl-2 and Bax proteins were immunochemically detected by Western Blot, quantified through densitometry, and the resultant values were used to calculate Bcl-2/Bax ratios. The ratio of Bcl-2 to Bax provides further information about a cell or tissue's relative state of apoptosis activity.(24) The Oxygen Radical Antioxidant Capacity assay (ORAC; OxiSelect ORAC Activity Assay, Cell Biolabs) has previously demonstrated high specificity for changes in free radical stress in human serum(25) and here was used to measure the antioxidant capacity of blood plasma.

Power and Sample Size

The study sample size was determined by the number needed to observe a significant change in percentage of ALSFRS-R slope of deterioration. A linear mixed effects model was used to estimate ALSFRS-R slopes for each patient. The model included fixed effects for intercept, slope and change in slope for those taking rasagiline. If the "true" effect of rasagiline was a 30% reduction in ALSFRS-R slope, 35 participants would provide 50% power to detect this. If the true effect was a 47% reduction, power was 80% to detect this. A 30% change in slope was expected to correspond to a 3-month increase in median survival.

Statistical Analysis

Baseline characteristics including age, gender, FVC%, ALSFRS-R, symptom duration, and riluzole use were compared using paired student's t-test or Fisher's exact test. Efficacy analyses were based on a linear mixed effects model with a parameter estimated for the rasagiline effect. This model was described by the equation: $Yij = (B0 + b_{0i}) + (B10 + B11*IR + b_{1i})\ tij + eij$; where Yij is a measurement (ALSFRS-R) on the i^{th} patient at time j, B0 is the intercept, B10 is the linear effect for all patients and B11 is an additional linear effect for patients on rasagiline, indicated by IR = 1 (0 otherwise), and lower case terms *boi*, *bli* and *eij* are random effects; tij denotes the time of measurement. The effect of rasagiline was determined by testing B11 = 0 in this model. This model uses only observed data so that subjects with fewer measurements have less weight than subjects with more measurements in estimating the best-fit rate of decline for all subjects. The historical placebo control design is particularly susceptible to differences in population characteristics at baseline.(16) Due to significant differences in baseline symptom duration, age, and riluzole use a post-hoc analysis was performed. For each of these differences a term was added to the linear mixed effects model and the terms were tested to determine whether they had a significant effect on ALSFRS-R slope.

For our secondary endpoints, Kaplan-Meier survival curves were estimated for the rasagiline and the minocycline historical control groups.(9) Difference in the time to failure due to treatment was tested with a two-sided log-rank analysis. A Cox model was used to adjust for baseline age, gender, and forced vital capacity percentage. The 12-month change in mitochondrial biomarkers was evaluated using a paired student's t-test, with a Bonferroni correction for multiple testing and adjusted P-value ≤ 0.01 used for significance. Participants with 12-month biomarker data were included in the analysis with missing data assumed to be missing at random. All statistical tests were two-sided.

RESULTS

Forty-one participants were screened, and 37 meeting eligibility criteria were enrolled (Figure 1). One participant withdrew prior to starting treatment in order to start an excluded medication. Thirty-six participants received treatment with rasagiline. Thirteen participants did not complete the study for the following reasons: 3 participants died due to progression of the disease, 2 were non-compliant with study protocols, 1 was lost to follow up, 2 dropped out due to disease progression and could not return to clinic for an evaluation, and 5 withdrew due to adverse events believed to be related to rasagiline (2 for dizziness, 2 for

headache, 1 for alopecia). 23 participants completed 12 months of follow up. All participants with ≥ 2 monthly ALSFRS-R scores were included in the analysis (n=30 participants).

Our historical controls were selected to meet the same baseline criteria as those enrolled (symptom duration < 3 years and initial FVC 75%); however, we found that the rasagiline group was older (61.1 years vs. 57.0 years, P=0.055), had a lower frequency of riluzole use (46% vs. 68%, P=0.02), and shorter symptom duration (0.65 years vs. 1.47 years, P<0.001, Table 1). Older age and shorter symptom duration have been associated in ALS with faster disease progression.(26, 27) Thus, we included adjustments for these variables in a post-hoc analysis.

Outcomes

In an unadjusted analysis, it appeared that rasagiline slightly increased the average monthly slope of deterioration in the ALSFRS-R compared to historical placebo controls at 12 months (−1.20 per month for treated vs. −0.94 per month for controls, difference −0.25, 95% confidence interval [CI] −0.53, 0.02, P=0.07, Table 2). In a post-hoc analysis, after adjusting for the large differences in symptom durations and its significant effect on ALSFRS-R slope (neither age nor riluzole use had a significant effect on slope) we found that patients taking rasagiline had reduced slopes compared to controls. This effect appeared to be greater at 6 months than at 12 months (the time of follow-up pre-specified in our original protocol). See results detailed in Table 2.

We saw no difference in the time to treatment failure between rasagiline and patients treated with minocycline in our historical control pool (P = 0.58 by log rank test, Figure 2).(9)

Exploratory Biomarkers

Rasagiline showed 12-month changes in most biomarkers with synergistic trends consistent with increased mitochondrial membrane potential, decreased oxidative stress, and an increased ratio of anti-apoptotic to pro-apoptotic proteins (Table 3). We saw consistent changes in our biomarkers from month 6 to month 12. There was a statistically significant increase from baseline in the Mitotracker relative fluorescent intensity [54.14, (95% CI 31.47, 76.81), P<0.001] and the JC-1 red/green fluorescence ratio [1.92, (95% CI 1.14, 2.70), P<0.001], indicating lymphocyte mitochondrial membrane potentials increased over the course of treatment. The Bcl-2/Bax protein ratio was increased, demonstrating a shift in the lymphocyte mitochondria towards a more anti-apoptotic, pro-viability state. Mean ORAC values increased over time and were indicative of reduced free radical production or increased free radical scavenging. There was no statistically significant change in the Annexin V-binding percentage.

Harms

Participants treated with rasagiline reported 13 adverse events (AEs) that were probably or definitely attributed to treatment (Table 4). There were no serious drug-related adverse events and mortality was not attributed to treatment. The frequencies of most AEs were

<6%. The most common adverse events included nausea (11.1%), dizziness (8.3%), headache (5.6%), constipation (5.6%) and anxiety (2.8%).

DISCUSSION

Although rasagiline did not slow the rate of deterioration in the ALSFRS-R slope when compared to historical controls, we did see preliminary evidence for drug-target engagement in biomarkers related to mitochondrial function, oxidative stress, and apoptosis.

A number of factors may be related to the failure of rasagiline to beat historical estimates in rates of decline of the ALSFRS-R: 1) rasagiline may not be effective in ALS; 2) the disease course may have been different in our rasagiline cohort than in prior clinical trial cohorts; and 3) participants were not matched to historical placebo controls in baseline characteristics despite similar inclusion criteria (rasagiline participants were older, less likely to use riluzole, and had shorter symptom duration). One important consideration when using historical controls is how well they match baseline characteristics, particularly those that affect ALSFRS-R progression. The baseline differences seen here would all suggest faster progression in the rasagiline group, and symptom duration in particular was associated with a differential effect on ALSFRS-R slopes of deterioration.

The lack of a clear functional benefit in the ALSFRS-R needs to be balanced against the broad changes in exploratory biomarkers. Although it is impossible to know in this context the functional correlates for the changes in biomarkers seen here, the direction of change could reflect stabilization of the mitochondrial membrane, decreased oxidative stress, and a shift towards pro-viability. Although blood biomarkers may not reflect activity in the central nervous system (CNS), rasagiline showed good CNS penetration in animal models. Subsequent clinical trials showing efficacy for patients with PD suggest rasagiline has good CNS penetration in humans.(28) Because rasagiline has both peripheral and central distribution, it seems likely that a peripheral mitochondrial biomarker would be a good surrogate for CNS activity. In addition, neuroimaging may be a good addition to peripheral biomarkers in future studies as one study showed changes in MRS glutathione measurements in ALS patients possibly reflecting CNS oxidative stress.(29)

A number of neuronal cell culture and animal model studies have reported rasagiline functions as a neuroprotectant.(4, 5) Studies have shown the neuroprotective activity of rasagiline is not dependent on MAO inhibitory activity.(30) Rather, rasagiline suppressed the apoptotic cell death cascade initiated by mitochondria in response to neurotoxins (SIN-1 or N-methyl-R-salsolinol), and prevented preapoptotic swelling of mitochondria and the decline in mitochondrial membrane potential that typically results from increased permeability. *In vitro* studies showed rasagiline increased expression of the anti-apoptotic Bcl-2 and Bcl-xL genes and down-regulates proapoptotic Bad and Bax expression.(31)

Mitochondrial dysfunction may play a critical role in ALS neurodegeneration; this hypothesis is supported by both human and animal model studies.(32) Prior studies showed calcium levels in motor neuron synaptic terminals of ALS participants were elevated despite increased numbers of local mitochondria, suggesting a defect of mitochondrial calcium

sequestration.(33) Increased complex I activity was seen in an individual with familial ALS, (34) and reduced cytochrome oxidase activity was shown in sporadic ALS patients.(35) Abnormal mitochondrial function in ALS may be a component leading to oxidative cytotoxicity. Genetic studies in ALS support this hypothesis. Mutations in CHCHD10, a mitochondrial protein, have been found in a large family with ALS and FTD. Fibroblasts from these patients showed mitochondrial ultrastructural alterations and fragmentation of the mitochondrial network.(36) Depletion of optineurin, due to mutations seen in familial ALS, may inhibit degradation of damaged mitochondria.(37)

The assays selected were meant to detect changes in mitochondrial-related parameters reported from rasagiline-treated cell cultures.(38) Clinical relevance for these observations derives from a prior study of ALS subject platelets, in which JC-1, Mitotracker, and Annexin V assays demonstrated reduced mitochondrial membrane potential and increased apoptosis compared to healthy controls.(39) Our mitochondrial biomarkers showed an effect that could be interpreted as protective. Increases in red/green fluorescence ratios for JC-1 and Mitotracker relative fluorescence could indicate decreased mitochondrial membrane permeability. The increases in the ORAC assay suggest decreased reactive oxygen species production or else increased removal with a reduction in oxidative stress. Finally, an increased Bcl-2/Bax ratio implies apoptosis in peripheral blood cells may be mitigated by rasagiline.

Limitations to the biomarker studies include: 1) although the reproducibility of the biomarker assays is reported by manufacturers to be high, the technical reproducibility of the assays across different subject visits is unknown; and 2) changes in biomarkers seen here may reflect ALS disease progression, or other unknown factors. The changes seen here could be consistent with a genuine rasagiline effect despite these caveats. Use of these biomarkers in a randomized, placebo-controlled trial will more definitively address the question of the relationship between drug-target engagement and ALS disease progression.

In conclusion, we feel a number of key observations support the further investigation of rasagiline in ALS: 1) mitochondrial dysfunction occurs in ALS patients; 2) rasagiline has broad neuroprotective activity in cell culture systems and this activity may arise at a mitochondrial level; 3) rasagiline increased survival in the G93A SOD1 mouse model of ALS (6); 4) rasagiline improved function in a small retrospective case series of ALS patients (7); 5) rasagiline was safe and relatively well-tolerated in ALS participants; 6) rasagiline showed changes in exploratory biomarkers collected here, potentially consistent with mitochondrial stabilization, decreased oxidative stress, and a shift towards increased cell viability; and finally, 7) lack of efficacy in a trial of this size and power should be considered preliminary, and does not exclude a functional effect. Our results also showed the importance of symptom duration on rates of disease progression as measured by the ALSFRS-R. Future studies should take this important variable into account and designs should take advantage of economies offered by restricting enrollees to those with short disease duration. Should rasagiline or mitochondrial manipulation ultimately prove effective in slowing motor function decline and prolong ALS patient survival, it would have a significant impact on the disease.

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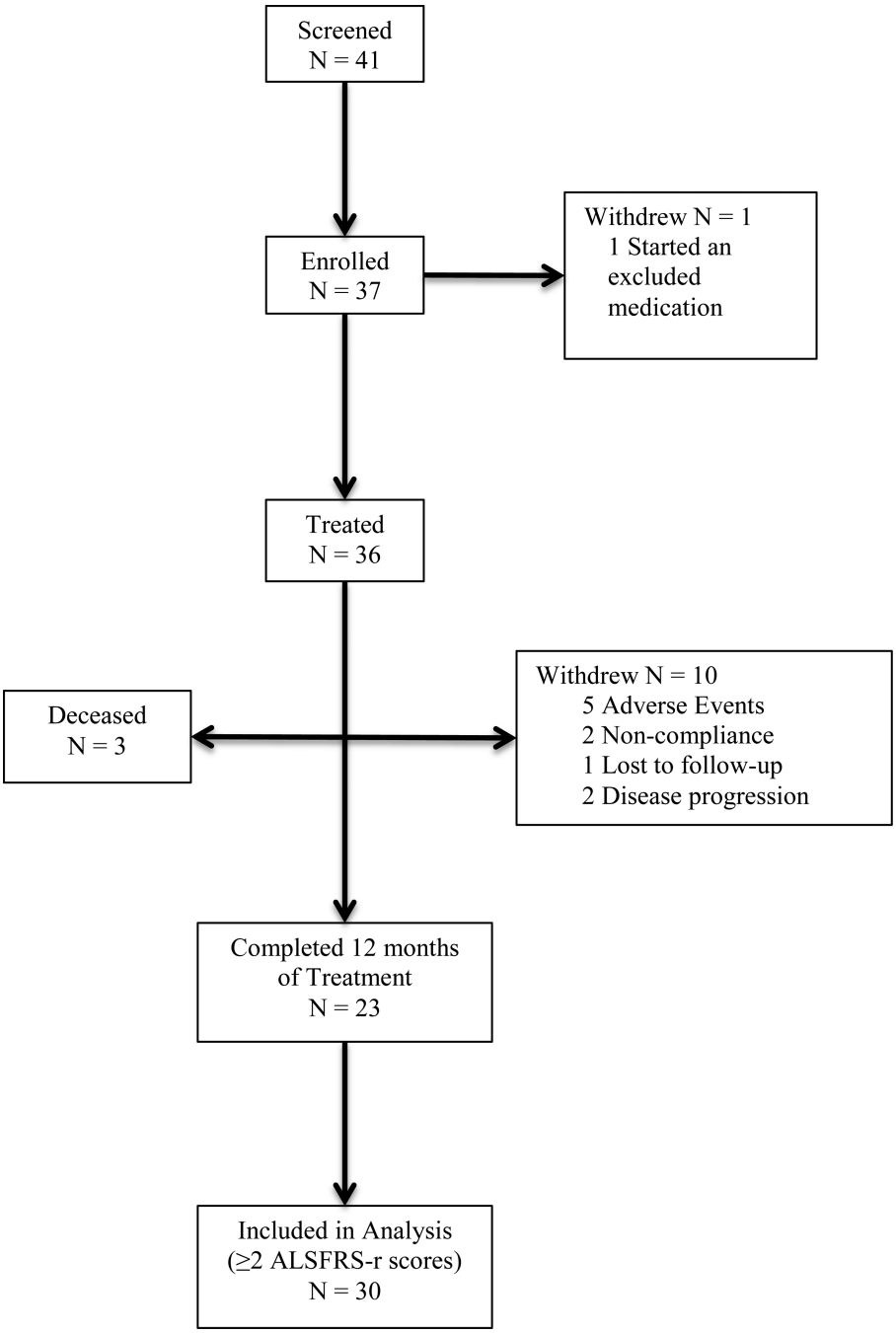

Figure 1.
Study flow diagram.

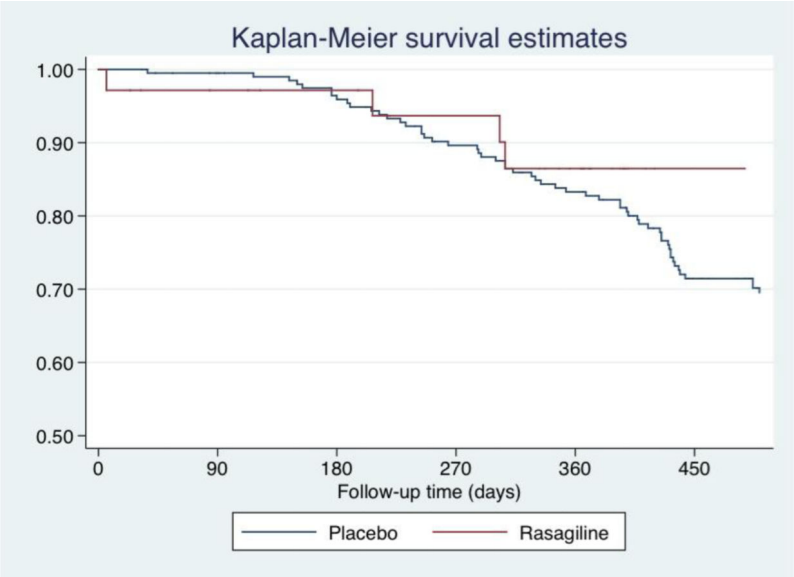

Figure 2. Proportion of Patients with Failure Free Survival at Follow-up

P = 0.58 by log rank test. Failure was defined as death, endotracheal intubation, tracheostomy-assisted ventilation, or use of noninvasive ventilation 23 hours/day for 14 days or more.

Table 1

Patient Baseline Demographics Compared to Historical Controls.(9-14)

	Rasagiline (SD or 95% CI)	Historical Placebo Controls (SD or 95% CI)	P-value
Sample Size	30	478	
Gender (% Female)	53%	36%	P=0.056§
Age, yrs (SD)	61.1 (12.0)	57.0 (11.4)	P=0.055¶
ALSFRS-R (SD)	38.3 (4.3)	38.7 (5.0)	P=0.65¶
FVC (%) (SD)	95.1% (15.7)	94.7% (16.4)	P=0.89¶
Bulbar onset	16.7%	19.5%	P=0.70§
Symptom duration, years (95% CI)	0.65 (0.51, 0.80)	1.47 (1.40, 1.53)	P<0.001*
Riluzole use (%)	46%	68%	P=0.02§

Rasagiline participants with ≥ 2 monthly ALSFRS-R scores were included in the analysis. Abbreviations: Standard Deviation = SD; Confidence Interval = CI; ALS Functional Rating Scale - revised = ALSFRS-R; Forced Vital Capacity (FVC). Historical controls matched for age <80, FVC >75%, and symptom duration <37 months.

§P-value based on Pearson X^2-test with P<0.05 considered significant.

¶P-value based on ANOVA t-test with P<0.05 considered significant.

*P-value based on two-sample t-test with equal variances with P<0.05 considered significant.

Table 2

ALSFRS-R Slope of Deterioration Over 12 Months with Correction for Baseline Symptom Duration.

	ALSFRS-R Slope, Placebo Controls	ALSFRS-R Slope, Rasagiline	Treatment Effect (95% CI)	P-value
6 months				
Uncorrected	−0.90	−0.93	−0.03 (−0.35,0.29)	0.85
With correction for symptom duration[¶]	−1.06	−0.81	+0.26 (−0.06,0.58)	0.11
12 months				
Uncorrected[*]	−0.94	−1.20	−0.25 (−0.53,0.02)	0.07
With correction for symptom duration[¶]	−1.11	−1.09	+0.02 (−0.25,0.30)	0.86

Abbreviations: ALS Functional Rating Scale-revised = ALSFRS-R; Confidence Interval = CI.

[*] Primary endpoint was the difference in 12 month uncorrected average ALSFRS-R slope of deterioration

[¶] Post-hoc analysis adjusted for baseline differences in symptom duration.

Table 3

Biomarker Results Comparing 12 month to Baseline.

	Sample size	Baseline mean (SEM)	6 month mean (SEM)	12 month mean (SEM)	12 month MD (95% CI)	P-value*
JC-1 (Red/Green FR)	14	0.51 (0.04)	0.60 (0.03)	2.43 (0.37)	1.92 (1.14, 2.70)	<0.001
Mitotracker (RFI)	17	18.06 (5.48)	25.66 (3.73)	67.67 (9.62)	54.14 (31.47, 76.81)	<0.001
Annexin V (%)	17	29.39 (5.15)	25.15 (2.58)	22.72 (4.06)	−6.67 (−20.3, 6.7)	0.32
Bcl-2/Bax Ratio	10	0.03 (0.01)	0.05 (0.01)	0.28 (0.02)	0.24 (0.21, 0.27)	<0.001
ORAC (pmol TrolEq)	17	4671.59 (92.99)	6376.59 (180.20)	5700.29 (159.62)	1028.70 (681.09, 1376.31)	<0.001

Abbreviations: Standard Error Measurement = SEM; Mean Difference = MD; Confidence Interval = CI; Fluorescence Ratio = FR; Relative Fluorescent Intensity = RFI; Oxygen Radical Antioxidant Capacity = ORAC; Trolox™ Equivalents = TrolEq.

*P-value taken from paired students t-test. To adjust for multiple comparisons P<0.01 considered significant.

Table 4

Adverse Events Probably Related to Rasagiline Treatment

Adverse Events (AEs)	Incidence
Alopecia	1/36 (2.8%)
Anxiety	2/36 (2.8%)
Constipation	2/36 (5.6%)
Dizziness	3/36 (8.3%)
Headache	2/36 (5.6%)
Hypertension	1/36 (2.8%)
Nausea	4/36 (11.1%)
Xerostomia	1/36 (2.8%)
Lab Abnormality (elevated ALT)	1/36 (2.8%)

Note: All AEs were reported in patients who had received treatment. AEs probably or definitely due to disease progression were not included. Lab abnormality reported was an episode of elevated Alanine Aminotransferase (ALT).