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ORIGINAL ARTICLE

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Dietary Vitamin D_3 Supplementation at 10× the Adequate Intake Improves Functional Capacity in the G93A Transgenic Mouse Model of ALS, a Pilot Study

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SUMMARY

Background: Vitamin D has antioxidant, anti-inflammatory, and neuroprotective properties, and may mitigate amyotrophic lateral sclerosis (ALS) pathology. **Aims:** To determine the effects of dietary vitamin D_3 (D_3) at 10-fold the adequate intake (AI) on functional and disease outcomes and lifespan in the transgenic G93A mouse model of ALS. **Methods:** Starting at age 40 days, 32 G93A mice (21 M, 11 F) were provided *ad libitum* with either an adequate (AI; 1 IU/g feed) or high (HiD; 10 IU/g feed) D_3 diet. Differences were considered significant at $P \leq 0.10$, as this was a pilot study. **Results:** For paw grip endurance, HiD mice had a 7% greater score between 60–133 d versus AI mice ($P = 0.074$). For motor performance, HiD mice had a 22% greater score between 60–133 days ($P = 0.074$) versus AI mice due to changes observed in male mice, where HiD males had a 33% greater score ($P = 0.064$) versus AI males. There were no significant diet differences in disease onset, disease progression, or lifespan. **Conclusion:** Although disease outcomes were not affected, D_3 supplementation at 10-fold the AI improved paw grip endurance and motor performance in the transgenic G93A mouse model of ALS, specifically in males.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive degeneration of motor neurons in the central nervous system, resulting in muscle weakness, paralysis, and death [1]. Approximately 90% of ALS cases are of unknown etiology (sporadic ALS) whereas the remaining ~10% are known to be due to genetically inherited mutations (familial ALS). Sporadic and familial ALS are clinically and pathologically similar [2]. G93A mice transgenically overexpress the mutant human Cu/Zn-superoxide dismutase (SOD1) gene and follow the same disease pattern as human ALS clinically and neuropathologically [3, 4], and can thus be used as an animal model of ALS. Several different mechanisms are involved in the death of motor neurons in ALS such as oxidative stress, inflammation, and glutamate excitotoxicity [1].

A number of different dietary interventions have shown promise in rodent models of ALS [5]. Vitamin E, folic acid, N-acetyl cysteine, alpha lipoic acid, lyophilized red wine, coenzyme Q10, epigallocatechin gallate, ginkgo biloba, ginseng, melatonin, Cu chelators, catalase, and L-carnitine have shown a delay in disease onset up to 26%, prolongation of lifespan up to 7%, as well as improvement in motor performance up to 2-fold, as compared to controls [5]. Interestingly, both short- and long-term caloric restriction have been demonstrated to hasten disease onset and progression, and shorten lifespan [6–8]. The only treatment approved for use in patients with ALS is the antiglutamate drug Riluzole [1]. Daily 100 mg oral consumption of the drug prolongs the median survival of patients by approximately 2–3 months and increases the likelihood of survival in the first year by 9% [9]. Being modestly effective at best, the drug acts as a voltage-dependent sodium channel blocker while also inhibiting glutamate release from the presynaptic terminal and increasing glutamate re-uptake into the surrounding astrocytes [10]. Vitamin D_3 (D_3) and its metabolites have not been previously investigated in ALS.

In relation to several of the main mechanisms responsible for motor neuron death in ALS, vitamin D_3 and/or its metabolites [$25(OH)D_3$ and $1,25(OH)_2D_3$] have been shown to increase antioxidant enzyme activity [11], decrease inflammation [12], decrease neuronal vulnerability to glutamate excitotoxicity [13],

and prevent neuronal death while increasing expression of neurotrophic factors [14–17].

There currently exists no body of literature on which to base possible clinical trials regarding vitamin D_3 supplementation in ALS. Based on scientific literature, vitamin D_3 supplementation could mitigate the severity of ALS by decreasing oxidative stress, inflammation and glutamate excitotoxicity, and by increasing neuroprotective factors; all mechanisms that directly contribute to ALS disease pathology. Hence, the objective of this study was to examine the effects of high, nontoxic levels of vitamin D_3 (10 IU/g feed) versus the adequate intake (1 IU/g feed) on functional and disease outcomes, as well as lifespan, in the G93A transgenic mouse model of ALS.

Methods

Animals

Male B6SJL-TgN(SOD1-G93A)1Gur hemizygous mice (No. 002726) were harem-bred with female SJL/J nonaffected control mice (No. 000686; Jackson Laboratory, Bar Harbor, ME, USA). Breeding mice consumed LabDiet 5058 (3.3 IU D_3/g feed; LabDiet, Richmond, IN, USA). The expression of the human-derived G93A transgene in the offspring was confirmed using polymerase chain reaction analysis of DNA extracted and amplified from ear tissue as outlined by Sigma–Aldrich (XNAT REDExtract-N-Amp Tissue PCR Kit; XNAT-1KT). All breeding mice were housed three females per one male in a 12-h light/dark cycle. The experimental protocol followed the guidelines of the Canadian Council of Animal Care and was approved by York University Animal Research Ethics Board (protocol # 2007–9). All necessary steps were taken to minimize suffering and distress to the mice in the study.

Study Design

Thirty-two G93A mice (21 males, 11 females) were fed a diet containing adequate amounts of vitamin D_3 (1 IU/g feed; Research Diet AIN-93G; product # D10012G; Research Diets Inc., New Brunswick, NJ, USA [18]) *ad libitum* (AL) after weaning (21 days) until the dietary intervention commenced at age 40 days. At age 35 days, mice were individually caged. At age 40 days, mice were divided into adequate intake (AI, 1 IU D_3/g feed; 9 males, 5 females) and high (HiD, 10 IU D_3/g feed; 12 males, 6 females; product # D08080101; Research Diets Inc.) vitamin D_3 groups (Table 1) and followed to endpoint. The intra-researcher coefficients of variation were 3.2% for body condition, 1.7% for clinical score, and 2.2% for ability to move.

Food Intake and Body Weight

Food intake and body weight measurements began at age 40 days and were recorded twice per week until endpoint.

Table 1 Nutrient composition of the AI and HiD diets

	Diet	
Nutrient	AI	HiD
Energy (kcal/g)	4	4
Carbohydrate (%)	64	64
Protein (%)	20	20
Fat (%)	7	7
Vitamin D_3 (IU/g)	1[a]	10[a,b]
Calcium (%)	0.5[c]	0.5[c]
Vitamin mix V10037 (mg/g)	10	10
Mineral mix S100022G (mg/g)	35	35

Diets provided by Research Diets (based on AIN-93G; New Brunswick, NJ; AI product # D10012G; HiD product # D08080101).
[a]included in vitamin mix V10037.
[b]additional vitamin D_3 was added to reach 10 IU/g feed.
[c]included in mineral mix S100022G.
AI, adequate intake; HiD, high vitamin D_3.

Body Condition (BC)

Beginning at age 60 days, BC was recorded twice per week until mice reached a clinical score of 3.0, thereafter measurements were recorded daily, following a 5-point scale as described elsewhere [6–8].

Clinical Score (CS)

Beginning at age 60 days, CS was assessed daily until endpoint and followed an 8-point scale as described elsewhere [6–8, 19, 20] based on signs exhibited by the mice to establish disease severity. Disease onset was established at CS2, and functional hindlimb paralysis at CS4. At CS5 [considered endpoint; Ref. 21], mice were euthanized using CO_2. For all mice, CS was assessed before all other functional measurements.

Ability to Move (ATM)

Beginning at age 60 days, ATM was recorded following a 5-point scale twice per week as described elsewhere [6–8, 19, 20] until mice reached CS3, thereafter measurements were recorded daily.

Paw Grip Endurance (PaGE)

Beginning at age 60 days, PaGE was measured twice per week using the modified hanging wire test [7, 8, 19, 20, 22, 23].

Motor Performance (MP)

Beginning at age 95 days, motor performance was measured three times per week using the Rotarod test [AccuScan Instruments, Inc., Columbus, OH, USA; Refs. 6, 23, 24], as described elsewhere [19, 20].

Calculations

Human equivalent dosage (HED) was calculated according to the US FDA [25]:

$$\text{HED} = \text{Animal dose (mg/kg)} \times [\text{animal weight (kg)} \div \text{human weight (kg)}]^{0.33}.$$

Statistical analyses

A three-way repeated measures ANOVA (diet, sex, time) was used to determine significant differences over time in food intake, body weight, BC, CS, ATM, PaGE, and MP. Statistical analyses over time for BC, CS, ATM, PaGE, and MP were conducted in two ways: (1) using data from the first day of testing (age 60 days) until the age at which the first group achieved a mean CS5 (endpoint; 133 days) and (2) using data from the age at which the first group achieved a mean CS2 (disease onset; 106 days) until the age at which the first group achieved a mean CS5, that is, during disease progression. A two-way ANOVA (sex and diet) was used to determine significant differences in CS2, CS5, disease progression (between CS2 and CS5), and AUC scores. A Newman–Keuls *post hoc* test was used to determine the source of significant differences. A *t*-test was used to determine differences in AUC within each sex for BC, CS, ATM, PaGE, and MP, and for the correlations, to describe the interrelationship between functional outcomes and disease severity, as well as within the different functional outcomes, and to delineate the improvement in functional outcomes (ATM, PaGE, MP) when corrected for disease severity or other functional outcomes. Statistical analyses for AUC scores for BC, CS, ATM, PaGE, and MP were conducted in two ways: (1) using data from the first day of testing (age 60 days) until individual CS5 specific for each mouse and (2) using data from individual CS2 until individual CS5 specific for each mouse. Scores for each mouse were corrected for the number of days between age 60 days (first day of testing) and CS5 (first approach), or between CS2 and CS5 (second approach). A two-tailed test was used for food intake and body weight. A one-tailed test was used for all other outcome measures, because based on the literature we *a priori* hypothesized that vitamin D_3 supplementation at 10-fold the AI would improve functional outcomes, decrease disease severity, delay onset of disease, and prolong lifespan [26–29]. Statistical analyses were performed using Statistica 6.0 Windows (version 6.0, StatSoft, Tulsa, OK, USA). Data are presented as means ± standard error of the mean (SEM). Significance was considered at $P \leq 0.10$ and trends were at $0.10 > P \leq 0.15$, as this was a pilot study.

Results

Food and Vitamin D_3 Intake

Mean food intake was 3.69 ± 0.07 g/d for the AI mice and 3.51 ± 0.07 g/d for the HiD mice (Figure 1A). HiD mice consumed 5% less absolute feed versus AI mice ($P = 0.093$; Table 2). However, food intake corrected for body weight was not significantly different between the diets (Figure 1B). Males consumed 19% less feed/g body weight versus females ($P < 0.001$).

Body Weight

Mean body weight was 21.5 ± 0.4 g for the AI mice and 20.8 ± 0.4 g for the HiD mice (Figure 1C). Mean body weight was not significantly different between the diets within the sexes or when sexes were combined, indicating that the dose of vitamin D_3 in the HiD mice was not toxic (Table 2).

Body Condition

BC was not significantly different between the diets (Figure 2A, Table 3). However, during disease progression, HiD females showed a trend towards a 31% higher BC over time versus AI females ($P = 0.132$; Fig. 2A). Between 60–133 days, males had a 10% lower BC versus females (Table 4). During disease progression, males had a 37% lower BC over time versus females (Table 4). Between 60–133 days, BC was significantly lower than baseline starting at age 111 days for AI ($P \leq 0.034$) and 111 days for HiD ($P \leq 0.007$). During disease progression, BC AUC positively correlated with ATM ($r = 0.635$; $P < 0.001$) and PaGE ($r = 0.256$; $P = 0.157$) AUC.

Clinical Score

CS between 60–133 days, during disease progression, as well as AUC were not significantly different between the diets within the sexes (Figure 2B) or when the sexes were combined (Table 3). Between 60–133 days, CS was significantly higher than baseline starting at age 97 days for AI ($P \leq 0.017$) and 99 days for HiD ($P \leq 0.013$).

Ability to Move

Between 60–133 days, males had a 10% lower ATM versus females (Figure 2C, Table 4). During disease progression, males had a 34% lower ATM versus females ($P = 0.008$). Between 60–133 days, ATM was significantly lower than baseline starting at age 115 days for AI ($P \leq 0.014$) and 114 days for HiD ($P \leq 0.007$). When corrected for CS (disease severity), HiD mice had a 5% greater ATM versus AI mice ($P = 0.018$). ATM AUC positively correlated with age at CS2 ($r = 0.265$; $P = 0.143$) and CS4 ($r = 0.430$; $P = 0.014$).

Paw Grip Endurance

Between 60–133 days, HiD mice had a 7% greater PaGE score versus AI mice (Figure 3A, Table 3), but not during disease progression or for AUC. Between 60–133 days, PaGE score was significantly lower than baseline starting at age 94 days for the AI mice ($P \leq 0.043$) and 98 days for the HiD mice ($P \leq 0.031$; $P < 0.001$, time × diet interaction). During disease progression, males had a 104% greater PaGE AUC versus females ($P = 0.026$). When corrected for CS, males had a 33% greater PaGE versus females ($P = 0.053$; Figure 4A).

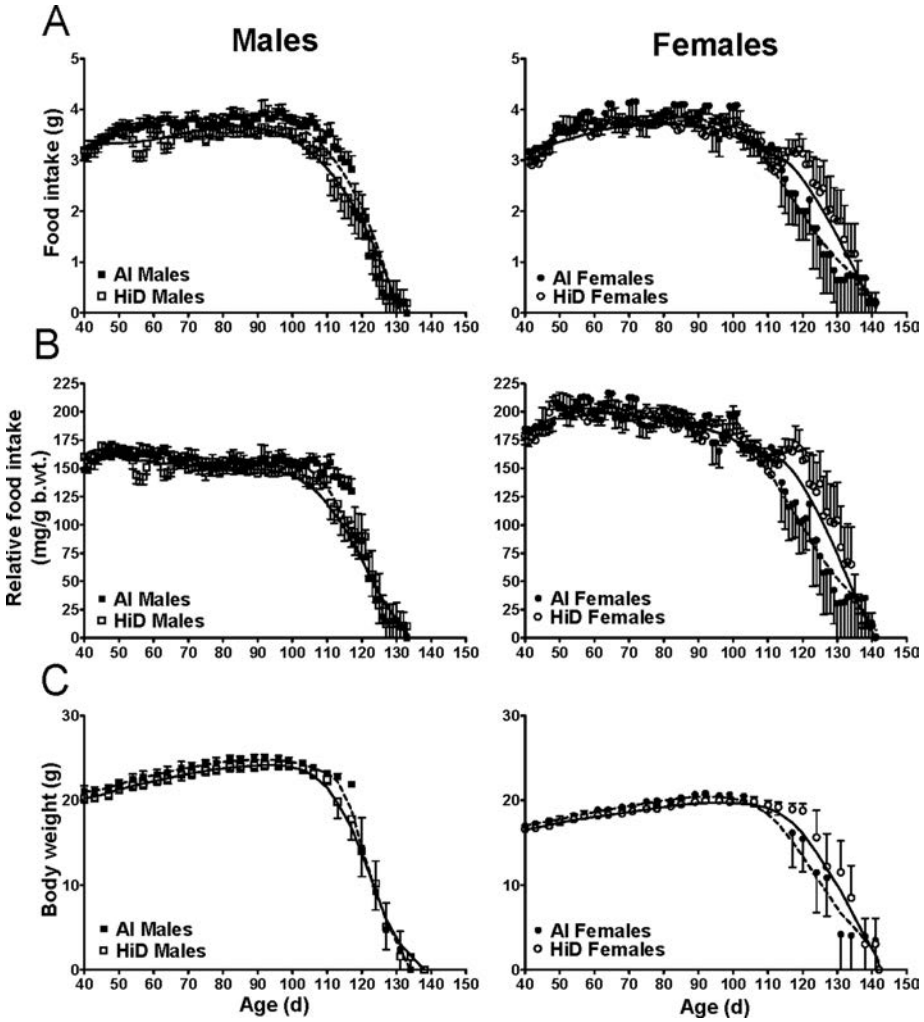

Figure 1 Food intake and body weight for G93A mice on either AI or HiD diets. (A) Food intake (g), (B) relative food intake (food intake corrected for body weight; mg/g body weight/d), and (C) body weight (g) of 14 adequate intake (AI; 1 IU D_3/g feed; ■, 9 males; ●, 5 females) and 18 high (HiD; 10 IU D_3/g feed; □, 12 males; ○, 6 females) vitamin D_3 G93A mice. Relative food intake (food intake adjusted for body weight) and body weight were not significantly different between the diets. Data are means ± SEM. b.wt., body weight.

Motor Performance

Between 95–133 days, HiD mice had a 22% higher MP score versus AI mice (Figure 3B, Table 3), but not during disease progression. This was due to changes in males only, because within males HiD mice had 33% greater MP over time versus AI mice ($P = 0.064$). This was also maintained during disease progression, where HiD males had a 56% greater MP versus AI males ($P = 0.091$). In addition, HiD mice had a 13% greater MP AUC over time versus AI mice ($P = 0.073$); mainly attributed to changes in males only, because within males HiD mice had a 30% greater MP AUC versus AI mice ($P = 0.016$). Between the sexes, males had a 23% lower MP score between 95–133 days versus females (Table 4). During disease progression, males had a 39% lower MP score versus females ($P = 0.010$). Between 95–133 days, MP was significantly lower than baseline starting at age 111 days for AI ($P \leq 0.044$) and 113 days for HiD ($P \leq 0.012$). When corrected for CS, HiD males had an 81% greater MP AUC versus AI males ($P = 0.002$; Figure 4B). CS AUC negatively correlated with MP AUC, with HiD males having 27% greater MP elevation versus AI males ($P = 0.050$). When corrected for PaGE, HiD mice had a 24% greater MP AUC versus AI mice ($P = 0.039$), mainly due to HiD males having a 40% greater MP AUC versus AI males ($P = 0.028$). MP scores did not begin to decline until the average PaGE score declined to ~40 seconds for AI mice ($P = 0.038$) and ~30 seconds for HiD mice ($P = 0.006$; Figure 4C).

[Correction added on 31 May 2012, after first online publication: Three mentions of 60–133 days on this page have been changed to 95–133 days.]

Table 2 Food intake, vitamin D_3 intake, and body weight of 32 G93A mice on either adequate intake or high vitamin D_3 supplementation

	Males		Females	
Measurement	AI	HiD	AI	HiD
Food intake (g/d)	3.68 ± 0.09	3.46 ± 0.08	3.71 ± 0.14	3.55 ± 0.13
Food intake (mg/g b.wt./d)[a]	157 ± 5	152 ± 5	192 ± 4	190 ± 3
Vitamin D_3 intake (IU D_3/d)	3.68 ± 0.09	34.6 ± 0.8	3.71 ± 0.14	35.5 ± 1.3
Vitamin D_3 intake (IU D_3/g b. wt./d)	0.16 ± 0.01	1.52 ± 0.05	0.19 ± 0.00	1.90 ± 0.03
Body weight (g)[a]	23.6 ± 0.6	22.9 ± 0.5	19.4 ± 0.5	18.7 ± 0.5

AI, adequate intake, n = 14; HiD, high vitamin D_3, n = 18; b.wt: body weight.
[a]Males consumed 19% less feed/g body weight and weighed 22% more versus females ($P < 0.001$).
Data are means ± SEM.

Relationship between Functional and Disease Outcomes

For PaGE and CS (Figure 4A), data followed a sigmoidal relationship for males (AI males, $PaGE = (0.31 \pm 0.86) + [(201 \pm 3) - (0.31 \pm 0.86)]/(1 + 10^{[((1.34\pm0.02) - CS)*(-0.78\pm0.03)]}$, $r^2 = 0.997$; HiD males, $PaGE = (-8.97 \pm 2.79) + [(302 \pm 31) - (-8.97 \pm 2.79)]/(1 + 10^{[((0.66\pm0.24) - CS)*(-0.36\pm0.03)]}$, $r^2 = 0.996$; curves were significantly different, $P < 0.0001$) and females (AI females, $PaGE = (2.26 \pm 2.32) + [(199 \pm 9) - (2.26 \pm 2.32)]/(1 + 10^{[((1.14\pm0.06) - CS)*(-0.80\pm0.08)]}$, $r^2 = 0.982$; HiD females, $PaGE = (2.05 \pm 1.25) + [(195 \pm 4) - (2.05 \pm 1.25)]/(1 + 10^{[((1.08\pm0.02) - CS)*(-1.02\pm0.05)]}$, $r^2 = 0.994$; curves were significantly different, $P = 0.0002$). For MP and CS (Figure 4B), data followed a sigmoidal relationship for males (AI males, $MP = (-0.56 \pm 0.45) + [(31.6 \pm 0.9) - (-0.56 \pm 0.45)]/(1 + 10^{[((2.71\pm0.05 - CS)*(-0.79\pm0.07)]}$, $r^2 = 0.988$; HiD males, $MP = (-5.68 \pm 1.61) + [(35.1 \pm 0.59) - (-5.68 \pm 1.61)]/(1 + 10^{[((3.70\pm0.07) - CS)*(-0.62\pm0.06)]}$ $r^2 = 0.992$; curves were significantly different, $P < 0.0001$) and females (AI females, $MP = (1.1 \pm 0.59) + [(31.3 \pm 0.63) - (1.1 \pm 0.59)]/(1 + 10^{[((2.67\pm0.03 - CS)*-1.51\pm0.23)]}$,

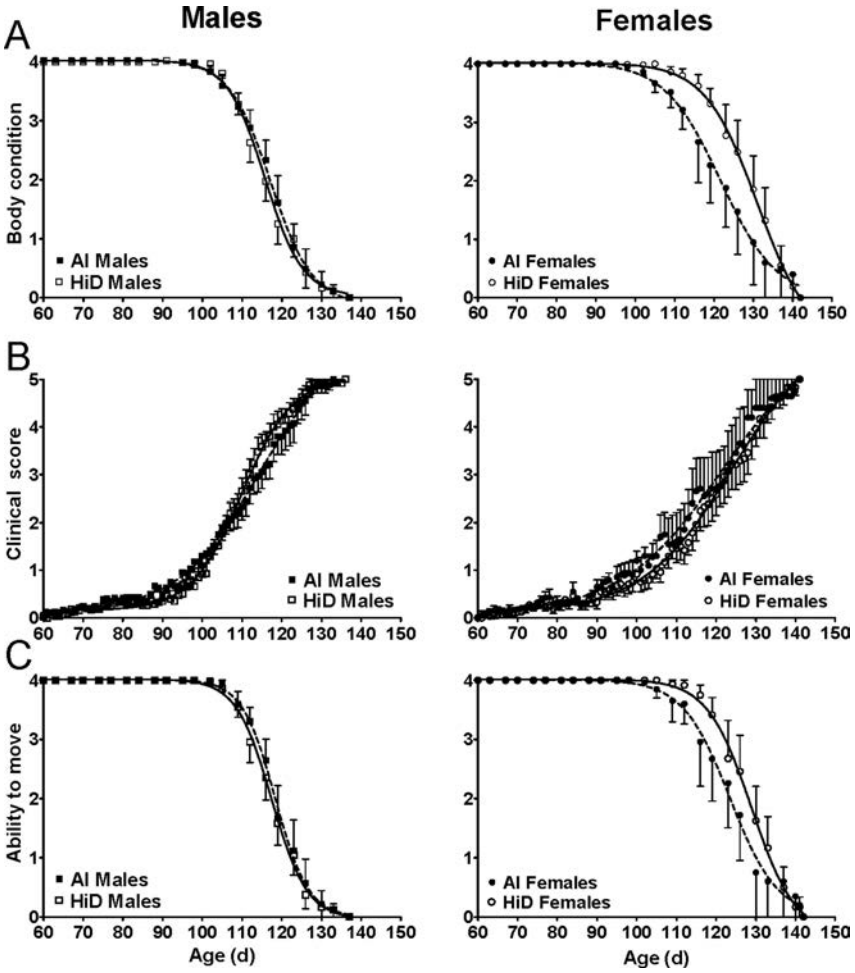

Figure 2 Body condition, clinical score, and ability to move for G93A mice on either AI or HiD diets. (A) Body condition, (B) clinical score, and (C) ability to move of 14 adequate intake (AI; 1 IU D_3/g feed; ■, 9 males; ●, 5 females) and 18 high (HiD; 10 IU D_3/g feed; □, 12 males; ○, 6 females) vitamin D_3 G93A mice. Body condition, clinical score, and ability to move were not significantly different between the diets. Data are means ± SEM.

Table 3 Body condition, functional outcomes, and clinical score between the diet groups in 32 G93A mice

	Age 60 days–133 days			Age 106 days–133 days		
Measurement	AI	HiD	*P* value	AI	HiD	*P* value
Body condition	3.2 ± 0.1	3.3 ± 0.1	NS	1.9 ± 0.2	2.2 ± 0.2	NS
Ability to move	3.3 ± 0.1	3.4 ± 0.1	NS	2.2 ± 0.2	2.4 ± 0.2	NS
Paw grip endurance (s)[a]	100 ± 4	107 ± 3	0.074	17 ± 3	21 ± 3	NS
Motor performance (s)[a,b]	17 ± 2	20 ± 2	0.074	12 ± 2	15 ± 2	NS
Clinical score	1.5 ± 0.1	1.6 ± 0.1	NS	3.2 ± 0.2	3.3 ± 0.2	NS

AI, adequate intake, n = 14; HiD, high vitamin D_3, n = 18.

[a]HiD mice had a 7% greater paw grip endurance between age 60 days–133 days and a 22% greater motor performance between age 95 days–133 days versus AI.

[b]Motor performance measurement began at age 95 days, not 60 days.

Data are means ± SEM.

Table 4 Body condition, functional outcomes, and clinical score between the sexes in 32 G93A mice

	Age 60 days–133 days			Age 106 days–133 days		
Measurement	Males	Females	*P* value	Males	Females	*P* value
Body condition	3.1 ± 0.1	3.4 ± 0.1	0.007	1.6 ± 0.2	2.6 ± 0.3	0.005
Ability to move	3.2 ± 0.1	3.5 ± 0.1	0.004	1.8 ± 0.2	2.7 ± 0.3	0.008
Paw grip endurance (s)	103 ± 3	104 ± 4	NS	14 ± 3	23 ± 4	0.024
Motor performance (s)[a]	16 ± 1	20 ± 2	0.031	10 ± 2	17 ± 2	0.010
Clinical score	1.7 ± 0.1	1.3 ± 0.1	0.004	3.8 ± 0.19	2.7 ± 0.3	0.002

Males, n = 21; females, n = 11.

[a]Motor performance measurement began at age 95 days, not 60 days.

Data are means ± SEM.

$r^2 = 0.974$; HiD females, $MP = (-0.92 \pm 0.72) + [(36.3 \pm 0.9) - (-0.92 \pm 0.72)]/(1 + 10^{[((2.59\pm0.05) - CS)*(-0.60\pm0.05)]}$, $r^2 = 0.993$; curves were significantly different, $P < 0.0001$). For MP and PaGE (Figure 4C), data followed a sigmoidal relationship for males (AI males, $MP = (-986 \pm 11870) + [(30.0 \pm 0.7) - (-986 \pm 11870)]/(1 + 10^{[((-92.7\pm343.1) - PaGE)*(0.02\pm0.004)]}$, $r^2 = 0.988$; HiD males, $MP = (-2089 \pm 40753) + [(33.8 \pm 0.5) - (-2089 \pm 40753)]/(1 + 10^{[((-101\pm502) - PaGE)*(-0.02\pm0.004)]}$, $r^2 = 0.991$; curves were significantly different, $P < 0.0001$) and females (AI females, $MP = (-31.8 \pm 23.06) + [(31.2 \pm 0.5) - (-31.8 \pm 23.06)]/(1 + 10^{[((0.43\pm9.16 - PaGE)*(0.03\pm0.007)]}$, $r^2 = 0.980$; HiD females, $MP = (-1502 \pm 29071) + [(32.0 \pm 0.5) - (-1502 \pm 29071)]/(1 + 10^{[((-49.1\pm263.1 - PaGE)*(0.03\pm0.01)]}$, $r^2 = 0.982$; curves were significantly different, $P < 0.0001$).

Disease Onset (CS2)

CS2 was not significantly different between the HiD and AI mice (Figure 5A, Table 5). Between the sexes, the age of mice at CS2 was 8% sooner for males versus females (Table 5). The Log-rank test revealed that males reached CS2 at a 2.7-fold faster rate (i.e., the hazard ratio) versus females (HR = 2.7, 95% CI: 1.6, 8.1; $P = 0.002$).

Disease Progression

Disease progression was not significantly different between the diets, or between males and females (Table 5).

Functional Hindlimb Paralysis (CS4) and Lifespan

Neither CS4 (data not shown) nor lifespan was significantly different between the diets (Figure 5B). Between the sexes, the age of mice at CS4 was 7% sooner for males versus females (Table 5). The Log-rank test revealed that males reached CS4 at a 2.7-fold faster rate versus females (HR = 2.7, 95% CI: 1.6, 8.0; $P = 0.002$). The age of mice at CS5 was 6% sooner for males versus females (Table 5). The Log-rank test revealed that males reached CS5 at a 2.5-fold faster rate versus females (HR = 2.5, 95% CI: 1.4, 6.8; $P = 0.005$).

Discussion

The improvement in PaGE and MP in the HiD group is corroborated by findings from other studies in both humans and animals. The nuclear vitamin D receptor is involved in regulating a large number of genes (up to 5% of the total human genome [30])

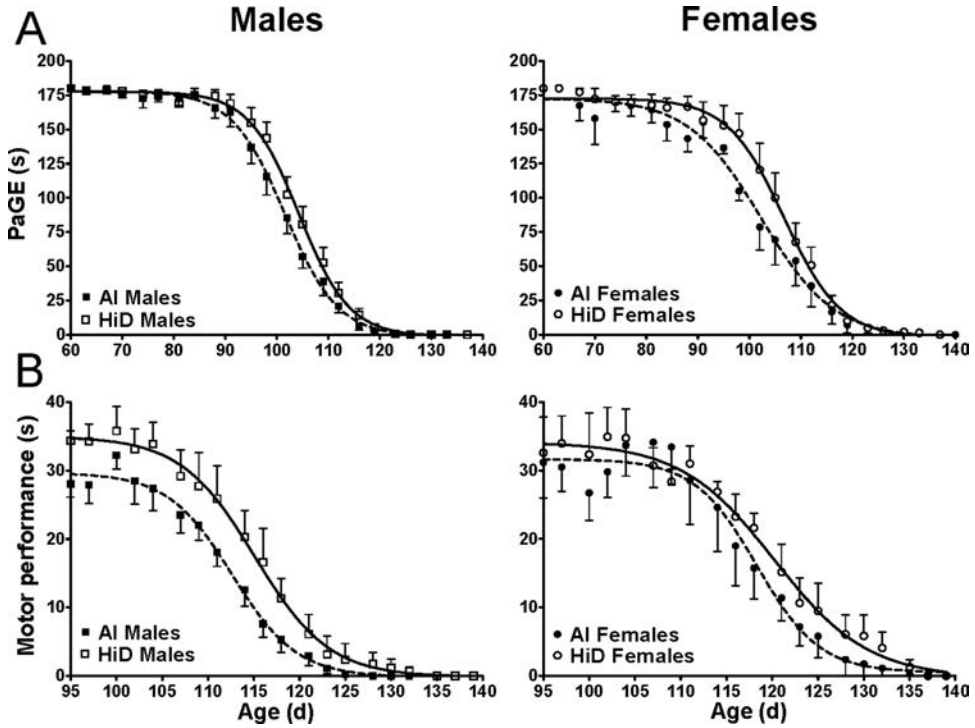

Figure 3 PaGE and motor performance for G93A mice on either AI or HiD diets. (A) PaGE and (B) motor performance in seconds (s) of 14 adequate intake (AI; 1 IU D_3/g feed; ■, 9 males; ●, 5 females) and 18 high (HiD; 10 IU D_3/g feed; □, 12 males; ○, 6 females) vitamin D_3 G93A mice. (A) For PaGE, HiD mice had a 7% greater score over time versus AI mice ($P = 0.074$). (B) For motor performance, HiD mice had a 22% higher score over time versus AI mice ($P = 0.074$), and HiD males had a 33% higher score versus AI males ($P = 0.064$). Data are means ± SEM.

and there is evidence to suggest that it is expressed by human muscle tissue [31]. However, Wang and Deluca recently refuted the presence of VDR in muscle [32], an issue which hence remains equivocal. Notwithstanding, a dose–response relationship was demonstrated between serum 25(OH)D_3 and the ability to walk 8 ft. and the sit-to-stand test in 4100 NHANES III ambulatory adults ≥60 years; those in the highest 25(OH)D_3 quintile scored 6% and 4% higher versus the lowest quintile, respectively [33]. Supplementing 800 IU D_3/d in double-blind, randomized, placebo-controlled trials (RCTs) reduces the risk of falls in the elderly by 27–72% [34–36]. In hemodialysis patients, 1,25$(OH)_2D_3$ supplementation increases 3 RM knee extension, knee extension peak torque and ankle dorsiflexion by 21–38% versus placebo [37], concurrent with an 11% and 15% increase in tibialis anterior and thigh cross-sectional areas, respectively. In a separate study, treating vitamin D deficient elderly women with alphacalcidiol for 6 months increased isometric knee extensor strength by 13% and total walking distance traveled over 2 min by 10% versus baseline [38]. An RCT lasting 3 months comparing 800 IU D_3/d + 1200 mg/d calcium (Ca+D) versus calcium alone (Ca) in elderly women improved musculoskeletal function (knee flexor and extensor strength, grip strength, and the timed up-and-go test) by 4% to 11% in the Ca+D group versus baseline in contrast to the –4% to 1% change in strength demonstrated by the Ca group [34], likely contributing to the 49% lower rate of falls experienced by the Ca+D group versus the Ca group. It is possible that the HiD diet in our study attenuated the disease-induced rapid decline in muscle function and strength by modulating the mechanisms involved in contractile protein synthesis and energy homeostasis. Indeed, vitamin D_3 and/or its metabolites increase troponin C [39], mediate protein synthesis and cellular ATP stores [40], and increase actin and sarcoplasmic protein expression [41] in striated muscle. Vitamin D_3 supplementation above the adequate intake may be a potential therapeutic in disease states involving loss of motor neuron stimuli to voluntary muscles or loss of muscle mass and function due to immobilization, disuse, cachexia, sarcopenia, or neuromuscular disease.

In the current study, only males exhibited an improved MP in response to vitamin D_3 supplementation. It may be that the 10 IU/g feed consumed by the HiD mice is insufficient to induce observable changes in females versus the already adequate 1 IU/g feed consumed by the AI mice using the outcome measures of the current study, despite the female sex having shown synergism with vitamin D_3 [42, 43]. In the B10.PL mouse model of experimental allergic encephalomyelitis (EAE, an animal model of multiple sclerosis), females had similar serum 25(OH)D_3 at baseline but ~40% and 37% higher values at 42 days and 87 days, respectively, following vitamin D_3 supplementation versus males. Interestingly, females had ~67% greater spinal cord 1,25$(OH)_2D_3$ despite similar serum values versus males, indicating local production of 1,25$(OH)_2D_3$ in the CNS. This was also likely due to the slower rate of hormone degradation in females, indicated by the

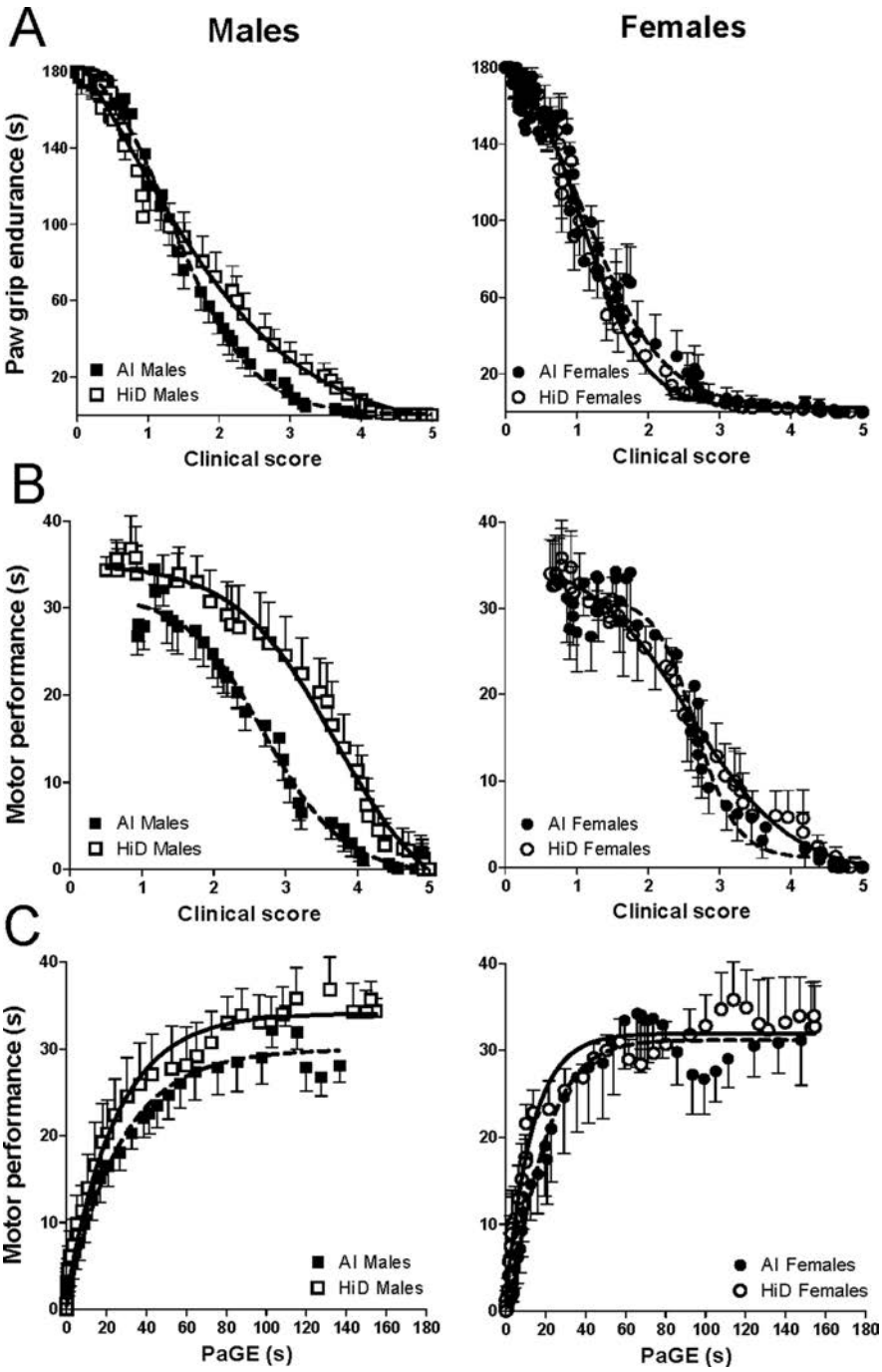

Figure 4 Relationship between functional and disease outcomes for G93A mice on either AI or HiD diets. (A) PaGE and clinical score, (B) motor performance and clinical score, and (C) motor performance and paw grip endurance (PaGE) of 14 adequate intake (AI; 1 IU D_3/g feed; ■, 9 males; ●, 5 females) and 18 high (HiD; 10 IU D_3/g feed; □, 12 males; ○, 6 females) vitamin D_3 G93A mice. Data are means ± SEM.

4-fold lower spinal cord CYP24A1 [the $1,25(OH)_2D_3$ inactivating enzyme] transcripts. There is a calcium- and sex-dependent relationship with vitamin D, with B10.PL males requiring 17- and 2-fold greater dietary $1,25(OH)_2D_3$ to suppress disease at 0.47% and 1.0% dietary calcium, respectively, versus females, indicating that females require less $1,25(OH)_2D_3$ to inhibit the disease [26]. Based on this, it is possible that vitamin D_3 intake in our AI females is near or at optimal regarding its effects on functional and disease outcomes in the presence of female sex hormones. Indeed, HiD females were unable to significantly increase MP above that

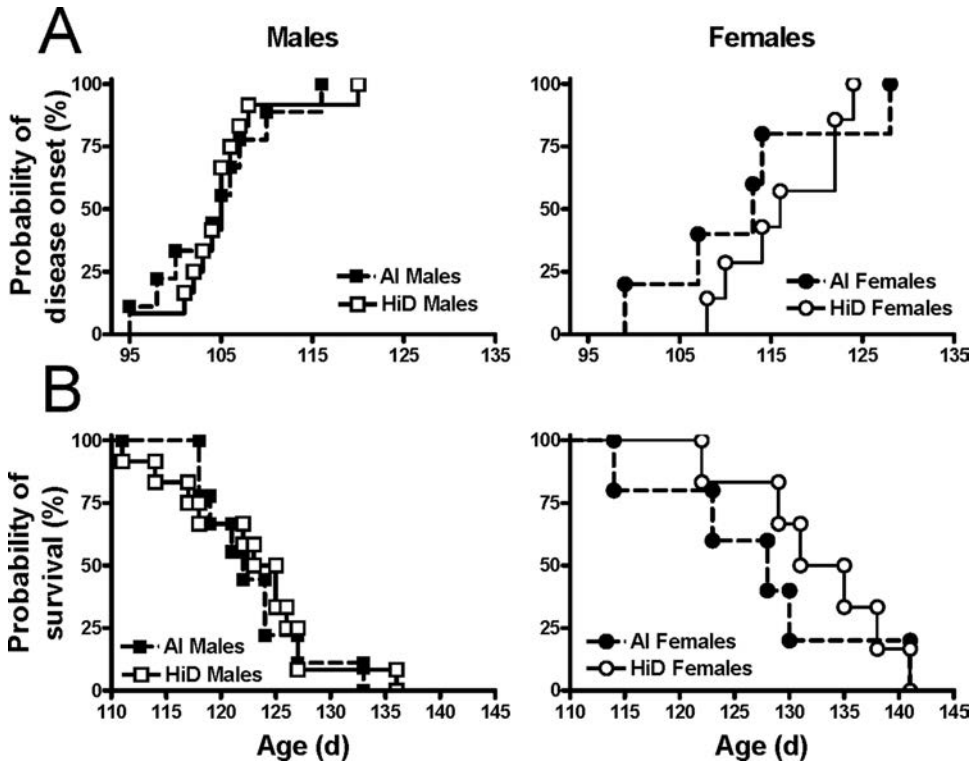

Figure 5 Probability of disease onset and survival for G93A mice on either AI or HiD diets. (A) Probability of disease onset and (B) probability of survival for 14 adequate intake (AI; 1 IU D_3/g feed; ■, 9 males; ●, 5 females) and 18 high (HiD; 10 IU D_3/g feed; □, 12 males; ○, 6 females) vitamin D_3 G93A mice. (A) For disease onset, there were no significant differences between the diets. Males had a 2.7-fold faster rate of reaching disease onset versus females (HR = 2.7, 95% CI: 1.6, 8.1; $P = 0.002$). (B) For survival, there were no significant differences between the diets. Males had a 2.5-fold faster rate of reaching endpoint versus females (HR = 2.5, 95% CI: 1.4, 6.8; $P = 0.005$).

Table 5 Disease outcomes between the diet groups and the sexes in 32 G93A mice

	Diet			Sex		
Measurement	AI	HiD	*P* value	Males	Females	*P* value
Age at disease onset (days)	110 ± 2	108 ± 2	NS	105 ± 2	114 ± 2	0.002
Age at functional hindlimb paralysis (days)	123 ± 2	123 ± 2	NS	119 ± 2	128 ± 2	0.001
Age at endpoint (days)	128 ± 2	125 ± 2	NS	123 ± 2	130 ± 2	0.009
Disease progression (days)	17 ± 1	17 ± 1	NS	18 ± 1	16 ± 1	NS

AI, adequate intake, n = 14; HiD, high vitamin D_3, n = 18.
Males, n = 21; females, n = 11.
Data are means ± SEM.

observed in AI females. In contrast, HiD males had greater MP versus AI males. Given that AI males had a 29% lower MP score versus AI females, whereas HiD males had only 17% lower scores versus HiD females, we conclude that high D_3 supplementation improves functional outcomes when basal performance is suboptimal. HiD males benefited from 10×AI, which may suggest that 10 IU of D_3/g feed may still be suboptimal in males and, therefore, males could further exhibit improvements from even higher supplementation. Additional research is needed to clarify the sexual dichotomy as it pertains to vitamin D_3 supplementation at deficiency and above sufficiency.

PaGE began to decline earlier and at a lower CS versus MP. Specifically, PaGE began to decline at approximately CS 0.5 for males and CS 1.25 for females (94 and 97 days, respectively), whereas MP began to decline at approximately CS 2.25 for both males and females (110 and 116 days, respectively). This suggests that the PaGE test is a more sensitive indicator of disease severity versus MP, and could be used as a predictor or indicator of disease onset and/or severity. This is in accordance with Weydt et al. who concluded that the PaGE test is more diagnostically accurate at detecting early signs of ALS versus MP in the G93A mouse model [21].

The changes detected in the current study are especially important due to the rapid onset and progression of disease and death of this high-copy mutant mouse model, which is most relevant to the rapid progression of ALS in humans. To directly extrapolate to humans, an 80-kg man would need to consume ~8257 IU D_3/d to meet the HED of the 1.52 IU D_3/g body weight/d for HiD male mice, whereas a 70-kg woman would need to consume ~8785 IU D_3/d to meet the HED of the 1.90 IU D_3/g body weight/d consumed by the HiD female mice in the current study. In either case, this amount of vitamin D_3 is far less than amounts reported to be toxic in humans [~30,000–600,000 IU/d; Ref. 44], and is less than the amount of vitamin D_3 produced naturally by the skin (~10,000 IU) after 1 minimal erythemal dose of sunlight (enough to slightly redden the skin while wearing a bathing suit; Ref. 45]. Moreover, the mouse AI HED for men (859 IU/d) and women (901 IU/d) is much higher than the former Institute of Medicine (IOM) AI for young and adult men and women up to 50 years of age (200 IU/d; Ref. 46] and higher than the most recent November 2010 IOM RDA (600 IU/d; Ref. 47]. Had the control AI diet for the G93A mice in the current study truly reflected the former or current IOM stance (former adequate intake: 0.036 IU D_3/g body weight/d for male mice and 0.043 IU D_3/g body weight/d for female mice; current RDA: 0.108 IU D_3/g body weight/d for male mice and 0.129 IU D_3/g body weight/d for female mice), HiD mice could have exhibited more robust differences versus the AI mice.

Despite being inconclusive, the current study demonstrates that 10 IU D_3/g feed (1.52–1.90 IU D_3/g body weight/d) improves PaGE in both sexes and MP specifically in males versus a diet containing the AI of vitamin D_3 (1 IU D_3/g feed; 0.16–0.19 IU D_3/g body weight/d) in the G93A mouse model of ALS. Furthermore, we found that when corrected for disease severity HiD mice had 5% greater ATM versus AI mice, and HiD males had 81% greater MP versus AI males. Indeed, we observed similar functional and disease outcomes in a much larger, higher dose follow-up study using the same mouse model [20]. For humans, this would indicate that vitamin D_3 supplementation far above the adequate intake may improve ambulation at the same stage of disease versus no supplementation. Future research should aim to determine the role of vitamin D_3 supplementation in mitigating the underlying molecular mechanisms contributing to ALS, namely oxidative stress management (oxidative damage and adaptive antioxidant defense), inflammation (pro- and anti-inflammatory makers) and apoptosis in both skeletal muscle and the CNS, as well as skeletal muscle contractile protein and neuronal survival in this animal model. Moreover, we need to delineate the amount of dietary vitamin D_3 required to elicit optimal improvements in functional, disease and molecular outcomes, as well as establish the role of vitamin D insufficiency in ALS.

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Conflict of Interest

The authors declare no conflict of interest.

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