

ABSTRACT: Long-term caloric restriction (CR) prolongs the lifespan in healthy insects, rodents, and nonhuman primates. We previously reported that long-term CR improves motor performance but hastens clinical onset of disease in an animal model of amyotrophic lateral sclerosis (G93A mice). G93A mice overexpress the mutant human Cu/Zn-SOD gene and show progressive lower motor neuron weakness and increased oxidative stress. To study short-term (15 days) CR in the same animal model, we investigated the effect of transient caloric restriction (TCR) on paw grip endurance, clinical onset, disease progression (time from clinical onset to endpoint), and lifespan. Starting at age 40 days, 32 separately caged G93A mice were randomly divided into two groups: ad libitum (AL, $n = 17$; 10 females, 7 males) and TCR ($n = 15$; 6 females, 9 males) with a diet equal to 60% of AL. When the TCR mice lost 30% of their weight they were offered food AL until endpoint, otherwise all TCR mice were provided food AL from age 55 days until endpoint (i.e., range of TCR = 13–15 days). Paw grip endurance started to decrease significantly at age 96 days compared with baseline values for all the groups. TCR males reached clinical onset 5 days sooner than TCR females. Disease progression was 8 days faster in TCR mice than AL mice and 6 days faster in male mice than female mice. The probability of survival was significantly different between the groups, with the TCR males having a faster rate of reaching endpoint than TCR females, AL males, and AL females. We conclude that TCR hastens clinical onset of disease and shortens the lifespan in male, but not female, G93A mice. Moreover, TCR hastens progress of disease but has no effect on paw grip endurance. The female sex is protective against the detrimental effects of short-term CR in G93A mice. Assuming we can extrapolate these results to humans, short-term CR should be avoided in patients with amyotrophic lateral sclerosis, especially men.

Muscle Nerve 34: 709–719, 2006

TRANSIENT CALORIC RESTRICTION IN EARLY ADULTHOOD HASTENS DISEASE ENDPOINT IN MALE, BUT NOT FEMALE, Cu/Zn-SOD MUTANT G93A MICE

MAZEN J. HAMADEH, PhD, and MARK A. TARNOPOLSKY, MD, PhD

Departments of Pediatrics and Medicine, Rm. 2H26, McMaster University Medical Center, 1200 Main St. West, Hamilton, Ontario L8N 3Z5, Canada

Accepted 13 July 2006

Long-term caloric restriction (CR) attenuates age-related pathologies and increases survival in different phyla.^{3,7,8,38,39,59} In contrast to studies conducted in healthy animals, we previously reported that long-term CR in G93A mice hastens clinical onset of

disease.²⁰ The G93A mouse is a transgenic model that overexpresses a mutant human Cu/Zn-superoxide dismutase (*SOD1*) gene that possesses enhanced free radical-generating function compared with normal human SOD1.^{22,23,25,40,63} These mice serve as a model of amyotrophic lateral sclerosis (ALS) since they follow a similar disease pattern clinically and neuropathologically.^{16,17} Along with defects in mitochondrial respiration, there is increased generation of free radicals and enhanced susceptibility to physiologically produced oxidants, making the G93A mouse a primary model of oxidative stress.^{1,4,19,23,29,33,34,40,42,63}

Our previous findings of a more rapid clinical onset in G93A mice with CR appears to be at odds with the biochemical consequences of CR. CR in-

Abbreviations: AL, ad libitum; ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; CR, caloric restriction; GPx, glutathione peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SOD1, Cu/Zn-superoxide dismutase; SOD2, Mn-superoxide dismutase; TCR, transient caloric restriction

Key words: amyotrophic lateral sclerosis; caloric restriction; G93A mice; lifespan; paw grip endurance

Correspondence to: M. A. Tarnopolsky; e-mail: tarnopol@mcmaster.ca

© 2006 Wiley Periodicals, Inc.

Published online 29 August 2006 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mus.20630

duces a decrease in the production of reactive oxygen species (ROS), lipid peroxidation, protein oxidation, and oxidative damage to DNA.^{9,10,15,28,30,39,49,59} Transcription of genes coding for free radical scavenging enzymes (Mn-SOD, Cu/Zn-SOD and glutathione peroxidase I [GPx-I]) is increased, whereas the transcription of genes involved in stress response (heat shock protein 70, stress-inducible protein GrpE, DnaJ-like protein, and chaperonin 60) is decreased.⁵⁰ CR also abolishes the increase in protein oxidation, lipid peroxidation, and superoxide anion radical generation observed in aging mice.³¹ Furthermore, CR in rats decreases mitochondrial SOD and GPx activities and H₂O₂ production, increases mitochondrial and cytosolic catalase activity, and has no influence on cytosolic SOD and GPx activities in the heart.²⁸ Alternatively, protein carbonyl content is significantly elevated in both the mitochondria and cytosol of the CR rats as compared with rats feeding ad libitum. In G93A mice fed ad libitum, gastrocnemius muscle malondialdehyde (27%–29%) and protein carbonyls (30%) are elevated with upregulation of antioxidant enzyme activities (total SOD; SOD1, 7–10-fold; SOD2, 4–5-fold; and catalase, 2–2.5-fold), compared with wildtype controls.³⁷ We hypothesized that CR may have attenuated the normal adaptive compensatory upregulation in SOD2 and catalase activities.²⁰ Furthermore, the CR-induced reduction in free radicals in the mitochondria may have been of little consequence to the several magnitude increase in free radical generation by the mutant SOD1 located in the cytosol. Therefore, CR may have exacerbated the imbalance between oxidant production and antioxidant enzyme activity, favoring an enhanced oxidative environment.

In our pilot study, we did not find an effect of long-term CR on survival but only on clinical onset, mainly due to the small sample size and lack of statistical power.²⁰ We wondered whether short-term CR, a more clinically relevant scenario, could affect this animal model of oxidative stress. Short-term CR is more frequently observed in humans than chronic CR and can occur for many reasons, including illness, surgery, dieting, and short-term limitation in food availability (famine, war). We therefore investigated the effect of short-term CR on body condition, ability to move, paw grip endurance, clinical onset, disease progression, and lifespan in G93A mice. We also studied whether there were sex differences in the main outcome variables, based on our previous observation of sex-specific differences when the same animal model was subjected to high-intensity exercise.³⁶ CR would address the alternative approach to inducing energy imbalance, and hence metabolic stress, mainly reducing caloric intake as

opposed to increasing energy expenditure. A secondary objective was to validate the use of different measures of endpoint.

MATERIALS AND METHODS

Breeding. G93A mice were bred from a colony maintained at our institution. Male B6SJL-TgN-(SOD1-G93A)1Gur autosomal hemizygous mice (No. 002726) were harem-bred with female B6SJL nonaffected control mice (Jackson Laboratory, Bar Harbor, Maine). The presence of the human G93A transgene was confirmed using polymerase chain reaction amplification of DNA extracted from tail samples as outlined by Jackson Laboratories. All animals were housed 3–5 per cage on a 12-h light/dark cycle. The study was approved by our institutional review board and conducted in accordance with guidelines of the National Institutes of Health.

Study Protocol. Thirty-two G93A mice (16 females, 16 males) were fed ad libitum (AL) after weaning (21 days) until the study commenced at age 40 days. At age 35 days the mice were housed in individual cages. At age 40 days the mice were randomly divided into two groups. The first group (AL, *n* = 17; 10 females, 7 males) was provided AL feeding with the standard rodent diet (Harlan Teklad, Madison, Wisconsin; 22/5 Rodent Diet (W), product 8640). The second group (transient caloric restriction, TCR, *n* = 15; 6 females, 9 males) was provided with food (NIH-31/NIA Fortified Diet) equivalent to 60% of the average intake of the AL group. When the TCR mice lost 30% of their weight, they were provided food AL until they reached endpoint. Otherwise, all TCR mice were provided food AL at age 55 days until they reached endpoint. As a result, the range of the TCR was 13–15 days (Fig. 1).

When mice reached a clinical score of 2.5 (see below), food and gel (Harlan-Gel, Harlan Teklad) were placed on the floor of the cage to fulfill the requirements of the ethics committee. The gel contained corn syrup, TIC 10,002 natural gum, potassium sorbate, and phosphoric acid. All measurements were conducted by two researchers in order to minimize variability. The researchers were not blinded to the treatment protocol. The inter-researcher variability was 3.2% for body condition, 2.2% for ability to move, and 0.2% for clinical score.

Measurements. *Body Weight and Body Condition.* For the AL mice, body weight was recorded daily from age 35–50 days, after which it was recorded twice per week. For the TCR mice, body weight was

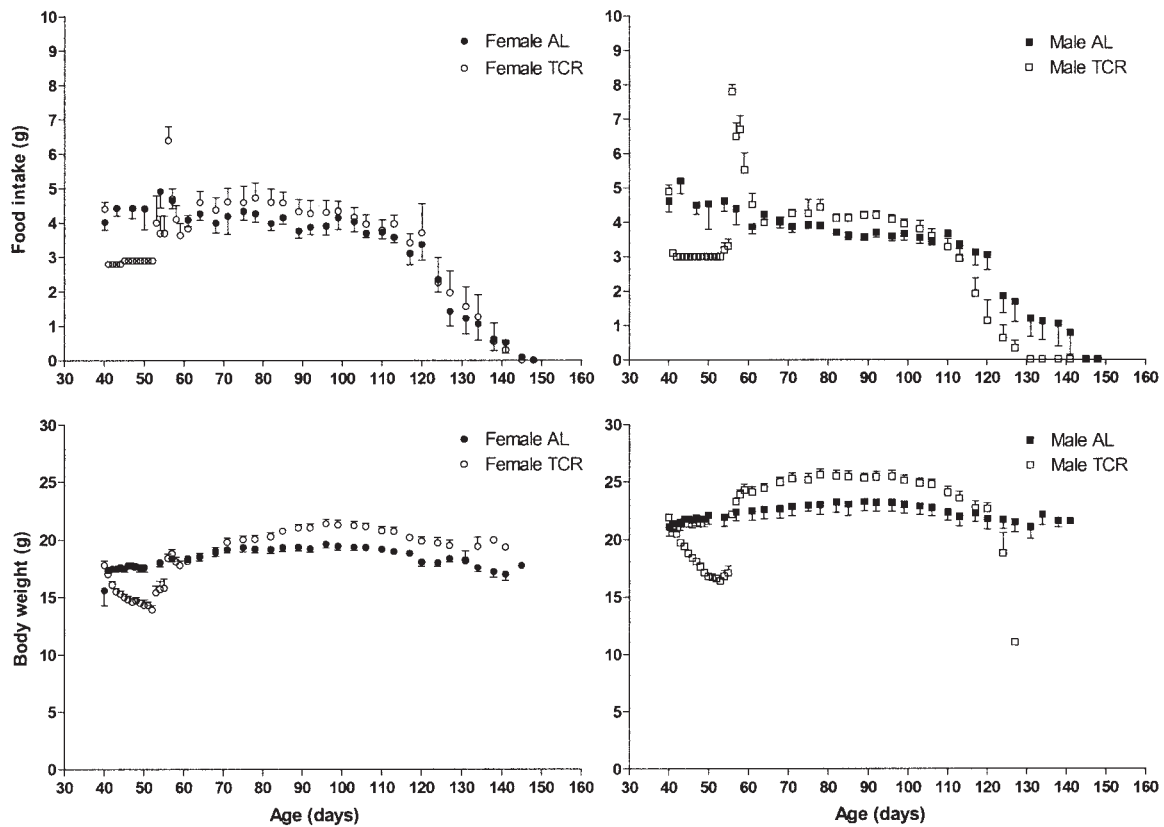


FIGURE 1. Food intake and body weight of 17 ad libitum (AL; filled circle, 10 females; filled square, 7 males) and 15 transient calorie-restricted (TCR; open circle, 6 females; open square, 9 males) G93A mice. Between age 56 days and clinical score of 2, food intake was 4.28 ± 0.05 g/d for AL females, 4.25 ± 0.06 g/d for AL males, 4.49 ± 0.09 g/d for TCR females, and 4.62 ± 0.09 g/d for TCR males. Maximum weight loss prior to refeeding was 4.1 ± 0.6 g for the females and 5.7 ± 0.3 g for the males. Data are means \pm SEM.

recorded daily from age 35–59 days, after which it was recorded twice per week. When the TCR mice were provided with CR food, body weight and body condition were recorded before mice were provided with their daily food portions. Starting at age 50 days, body condition was assessed using a 5-point scale: 5, obese mice; 4, overconditioned mice (spine is a continuous column and the vertebrae are palpable only with firm pressure); 3, well-conditioned mice (vertebrae and dorsal pelvis are not prominent and are palpable with slight pressure); 2, underconditioned mice (segmentation of the vertebral column is evident and the dorsal pelvic bones are readily palpable); and 1, emaciated mice (skeletal structure is extremely prominent and vertebrae are distinctly segmented).

Ability to Move. Starting at age 50 days, the ability to move was assessed using a 5-point scale: 4, normal mobility; 3, move with limited use of the hindlegs; 2, move with the use of the forelegs; 1, move only for a short period with the use of the forelegs; and 0, unable to move. For the TCR group,

ability to move during CR was recorded before mice were provided with their daily food portions.

Paw Grip Endurance. Starting at age 50 days, paw grip endurance was measured twice per week using the modified hanging wire test.^{46,61} For this test, a wire-lid of a housing cage was used at a height of 40 cm. The mice were individually placed on the wire-lid and were allowed to hold tightly in response to a gentle shake of the lid. The lid was inverted and the time until the mouse fell off the lid was recorded, for a maximum of 90 s. The test was performed in triplicate, with the best result recorded. For the TCR group, paw grip endurance during CR was recorded before mice were provided with their daily food portions.

Clinical Score. Starting at age 50 days, clinical score was assessed using an 8-point scale dependent on signs exhibited to identify the severity of the disease: 0, no evidence of disease; 1, shaking of the hindlegs or splaying of the hindlegs when suspended by the tail (an indication of weakness in the hindlegs); 1.5, weakness in one hindlimb (compensation

for footdrop); 2, change in gait (used as clinical onset); 2.5, extreme weakness in one hindlimb (inability to dorsiflex); 3, extreme weakness in both hindlimbs; 3.5, functional paralysis in one hindlimb; 4, functional paralysis in both hindlegs but can right themselves in less than 20 s after being placed on their side; and 5, cannot right themselves within 20 s after being placed on their side (clinical endpoint). When mice reached endpoint they were euthanized using gaseous CO₂. For the TCR group, clinical score during CR was recorded before mice were provided with their daily food portions.

Endpoint. Traditionally, we have used a clinical score of 5 to indicate when mice reached endpoint and were subsequently euthanized. The righting reflex measures proprioceptive (awareness of posture, movement, and changes in equilibrium) and motor (assessment of motion) functions. The G93A mouse expresses progressive phenotypic degeneration of the white matter in the brainstem and spinal cord and, consequently, neurological deficits, such as hindleg weakness that progresses to functional paralysis. These neurological and phenotypic changes coincide with progressive deterioration of proprioception and motor function. We use the righting reflex to assess the level of neurological deterioration that occurs throughout the disease. The more advanced the neuromotor degeneration, the worse is the righting reflex, i.e., the longer it takes the mouse to right itself to sternum from recumbency. However, due to more recent recommendations by our institutional animal research ethics committee, the mice were considered to have reached endpoint when they reached a clinical score of 4 in conjunction with one or more of the following additional clinical signs: (1) functional paralysis in both hindlegs, so mice could not right themselves in less than 20 s after being placed on their side (considered a clinical score of 5); (2) reduced intake of food and water even when food and gel was placed at cage floor level, indicated by weight loss of 20% or more, compared with weight immediately prior to clinical onset; (3) loss of bladder function or urinary bladder infection (the former was indicated by urine wetting of the anterior and lateral sides of the hindlegs, whereas the latter was veterinarian-diagnosed and was indicated by either observing pus or frank blood exuding from the urinary tract or complete blockage of the urinary bladder); (4) complete lethargy and depression so mice no longer attempted to move about the cage and drag themselves to the floor location of the food and gel; and (5) loss of body condition to a level below a score of 2, where the

cervical or thoracic vertebrae were protruding significantly.

Statistical Analyses. A two-way analysis of variance (ANOVA) was used to determine significant differences in disease progression (between clinical onset and endpoint) and age of mice at clinical onset and death, the factors being gender and diet (TCR vs. AL) (Statistica v. 5.0; StatSoft, Tulsa, Oklahoma). A three-way repeated-measures ANOVA was used to determine significant differences in body weight, body condition, ability to move, and paw grip endurance, the factors being gender, diet (TCR vs. AL), and time. When significance occurred, Tukey's honestly significant difference test was used post hoc to determine the source of difference. The Logrank test (GraphPad Prism v. 4.0; GraphPad Software, San Diego, California) was used to determine significant differences in survival curves, whereas the hazard ratio was used to compare the rate of attaining endpoint between the different groups (TCR females, TCR males, AL females, and AL males), i.e., how rapidly the TCR mice were attaining endpoint compared with the AL mice.

In conformity with increasing demands to limit unnecessary suffering in mice, we used a clinical score of 4 in conjunction with additional clinical symptoms as a proxy measure of endpoint. To validate the new measure, we also conducted statistical analyses using the traditional approach, i.e., a clinical score of 5 as a measure of endpoint. Furthermore, we also investigated whether using a clinical score of 4 was a valid measure of endpoint, which could potentially further reduce unnecessary suffering in mice in future studies. Disease progression was considered as the number of days between clinical onset and endpoint. Again, disease progression was analyzed three times, with endpoint being defined as a clinical score of 5 (the traditional approach), clinical score of 4 in conjunction with additional clinical symptoms (current approach), and clinical score of 4. Differences were considered significant at $P \leq 0.05$. Results are presented as means \pm SEM unless otherwise indicated.

RESULTS

Food Intake and Body Weight. Actual food intake of the TCR mice between age 40–55 days was 2.97 ± 0.01 g/d (females, 2.87 ± 0.01 g/d; males, 3.03 ± 0.01 g/d), ~64% that of the AL mice (4.67 ± 0.07 g/d: females, 4.55 ± 0.10 g/d; males, 4.83 ± 0.10 g/d) (Fig. 1). The range of the TCR was between 13–15 days. From age 56 days until mice reached a

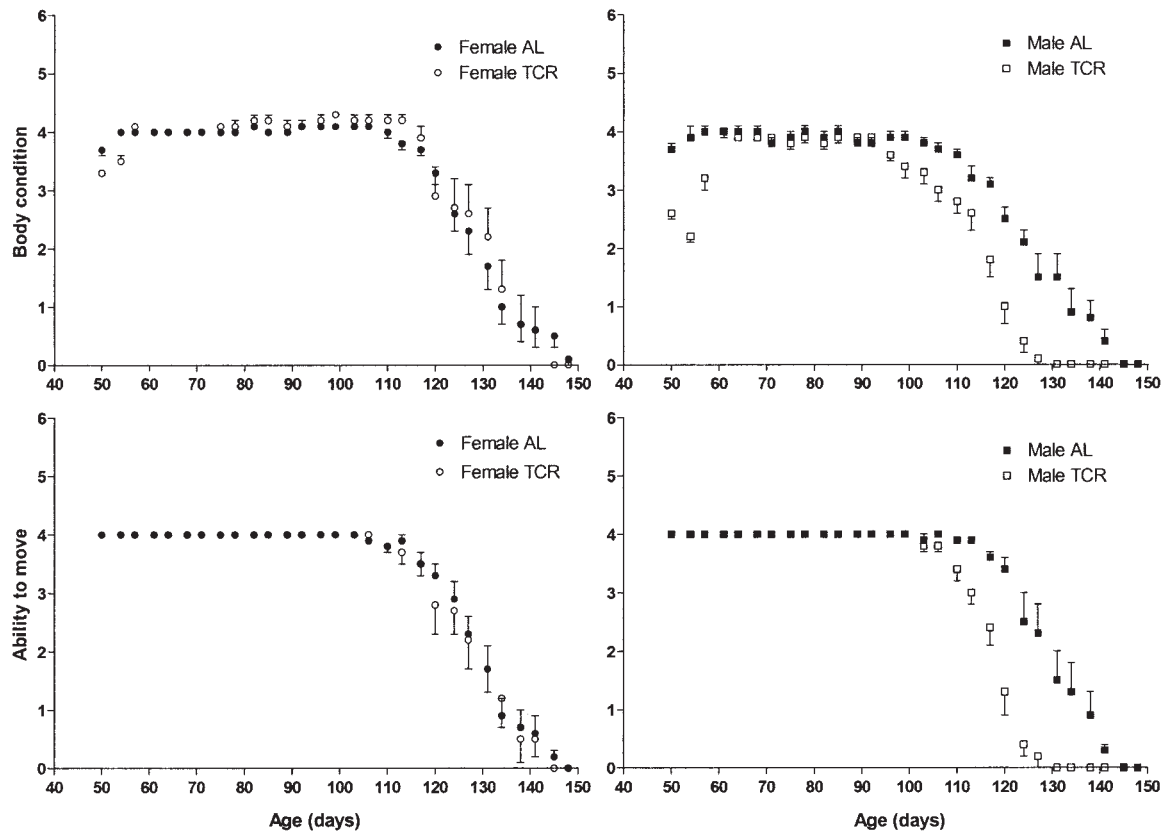


FIGURE 2. Body condition and ability to move in 17 ad libitum (AL; filled circle, 10 females; filled square, 7 males) and 15 transient calorie-restricted (TCR; open circle, 6 females; open square, 9 males) G93A mice. Data are means \pm SEM.

clinical score of 2, food intake of the TCR mice was 4.56 ± 0.07 g/d vs. 4.27 ± 0.04 g/d for the AL mice.

Maximum weight loss in mice during the first 15 days of CR was $25 \pm 1\%$, with females losing a maximum of $23 \pm 3\%$ and males $26 \pm 1\%$ prior to refeeding, similar to values previously reported for CR mice.^{32,47,52,62} The weight of TCR mice at maximum weight loss was 13.6 ± 0.5 g for the females and 16.0 ± 0.3 g for the males, compared with 18.2 ± 0.4 g for AL females and 22.2 ± 1.0 g for AL males at age 55 days (main effects for diet and gender, $P < 0.0001$ for both). Overall, males (21.9 ± 0.1 g) had significantly higher body weights than females (18.1 ± 0.1 g) ($P < 0.0001$), and there was a main effect for time ($P < 0.0001$). TCR females had significantly lower weights at age 44–55 days than AL females ($P \leq 0.002$ for all). TCR males had significantly lower weights at age 44–55 days ($P \leq 0.0002$ for all) and higher weights at age 68–106 days ($P \leq 0.016$ for all) than AL males.

Body Condition and Ability to Move. For body condition, there were main and interaction effects for gender, diet, and time. Body condition was higher in

females (3.2 ± 0.1) than males (2.6 ± 0.1) ($P = 0.0001$), and in the AL (3.1 ± 0.1) than TCR (2.7 ± 0.1) mice ($P = 0.025$) (Fig. 2). TCR males (2.4 ± 0.1) had lower body condition than AL males (2.9 ± 0.1 , $P = 0.003$), AL females (3.2 ± 0.1 , $P = 0.0002$), and TCR females (3.2 ± 0.1 , $P = 0.005$). Within the same gender, TCR males had lower body condition at age 117–131 days than AL males ($P \leq 0.0011$), with no differences between the female groups.

For ability to move, there were main and interaction effects for gender, diet, and time. Ability to move was higher in females (3.1 ± 0.1) than males (2.9 ± 0.1) ($P = 0.04$), and in the AL (3.2 ± 0.1) than TCR (2.9 ± 0.1) mice ($P = 0.018$). TCR males (2.7 ± 0.1) had a lower ability to move than AL males (3.2 ± 0.1 , $P = 0.022$) and AL females (3.2 ± 0.1 , $P = 0.007$), but a trend for lower ability to move compared with TCR females (3.1 ± 0.1 , $P = 0.063$). Within the same gender, TCR males had a lower ability to move at age 120–131 days than AL males ($P \leq 0.0024$), with no differences between the female groups.

There was a strong correlation between body condition and ability to move. The slopes and cor-

relation coefficients were, respectively, 0.988 and 0.998 for the AL females ($P < 0.0001$), 1.000 and 0.994 for the AL males ($P < 0.0001$), 0.998 and 0.993 for the TCR females ($P < 0.0001$), and 1.066 and 0.975 for the TCR males ($P < 0.0001$). The slopes between the four groups were similar, with a common slope of 0.997 ($r = 0.982$), but the elevations were different ($P < 0.0001$).

Paw Grip Endurance. There were no gender and diet differences in paw grip endurance (Fig. 3). Starting at age 96 days, paw grip endurance was significantly lower than at baseline ($P < 0.0001$).

Clinical Onset. The clinical score was higher in TCR than AL mice ($P < 0.047$). There was no effect of diet on age of mice at clinical onset, whereas there was a trend for a gender effect ($P = 0.083$). There was a significant gender \times diet interaction ($P = 0.022$), with TCR males having the earliest age of clinical onset (AL females, 106 ± 1 day; AL males,

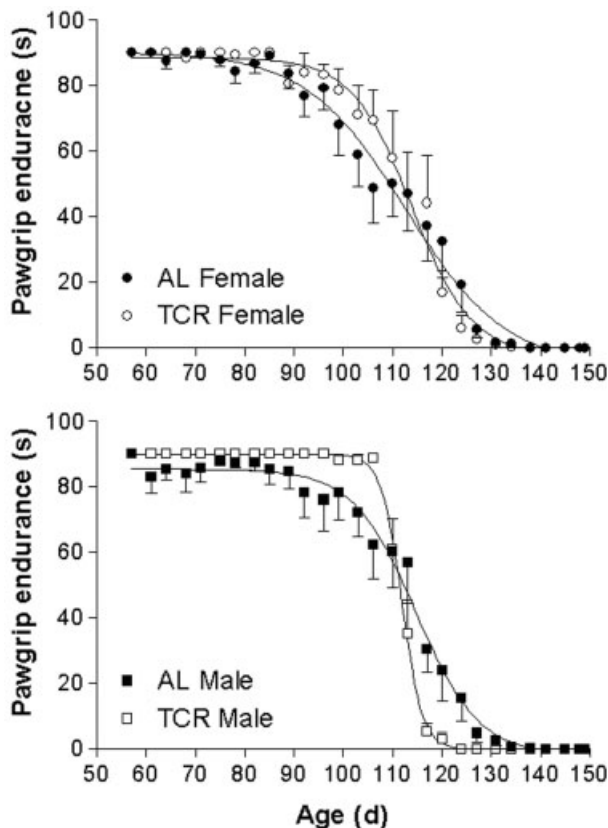


FIGURE 3. Paw grip endurance time (seconds) in 16 female (filled circle, 10 AL; open circle, 6 TCR) and 16 male (filled square, 7 AL; open square, 9 TCR) G93A mice on either ad libitum (AL) or transient caloric restriction (TCR). Data are means \pm SEM.

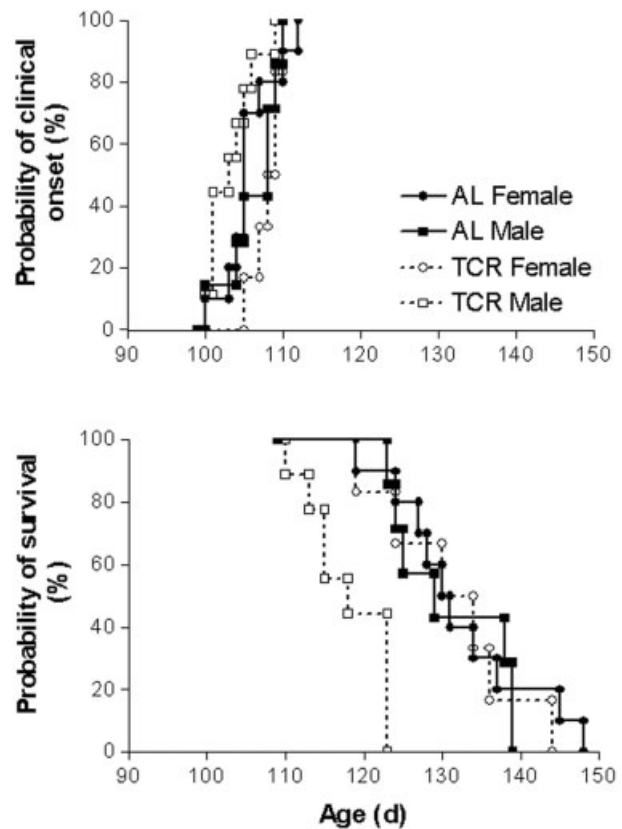


FIGURE 4. Probability of clinical onset and survival in 17 ad libitum (AL; filled circle, 10 females; filled square, 7 males) and 15 transient calorie-restricted (TCR; open circle, 6 females; open square, 9 males) G93A mice.

106 ± 1 day; TCR females, 108 ± 1 day; TCR males, 103 ± 1 day), the TCR males reaching clinical onset 5 days sooner than TCR females ($P = 0.0355$) (Fig. 4).

Disease Progression. There was no gender difference in disease progression. Progression of the disease from clinical onset to endpoint (clinical score of 4+ additional clinical symptoms, see Materials and Methods) was 8 days faster for the TCR (19 ± 2 days) than AL (27 ± 2 days) mice ($P = 0.023$), a reduction of 30%. There was a trend for the males to have a faster progression than the females (females, 26 ± 2 days; males, 20 ± 2 days; $P = 0.076$).

Between clinical onset (clinical score of 2) and clinical score of 5, the ability to move strongly and negatively correlated with clinical score. For the females the relationship followed a second-order polynomial (AL females, $r^2 = 0.992$; CR females, $r^2 = 0.996$; there was a trend for the curves to be different, $P = 0.086$). For the males, the relationship was linear (AL males, $r = -0.986$; CR males, $r = -0.991$; there was no difference between the curves).

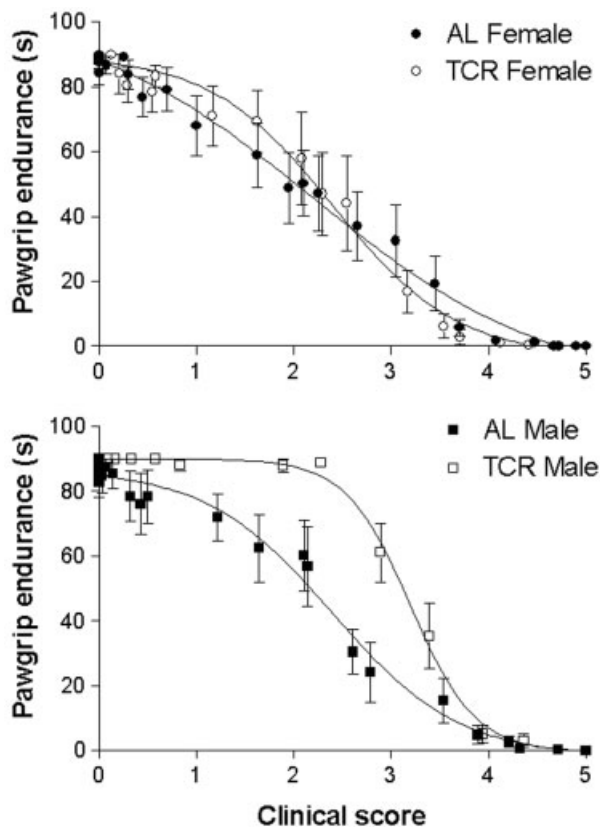


FIGURE 5. Relation between clinical score and paw grip endurance time (seconds) in 16 female (filled circle, 10 AL; open circle, 6 TCR) and 16 male (filled square, 7 AL; open square, 9 TCR) G93A mice on either ad libitum (AL) or transient caloric restriction (TCR). For the AL female mice, paw grip endurance time = $(-13.4) + (118.7)/[1 + \exp((2.2 - \text{clinical score})/-1.2)]$; for the TCR female mice, paw grip endurance time = $(-3.3) + (93.3)/[1 + \exp((2.4 - \text{clinical score})/-0.64)]$; for the AL male mice, paw grip endurance time = $(-2.2) + (89.7)/[1 + \exp((2.4 - \text{clinical score})/-0.66)]$; for the TCR male mice, paw grip endurance time = $(-0.5) + (90.5)/[1 + \exp((3.2 - \text{clinical score})/-0.33)]$, with the data presented as means. Within the same gender, the slopes were significantly different between the AL and TCR groups ($P \leq 0.015$). Data are means of each group on the same day.

There was a strong relation between clinical score and paw grip endurance (Fig. 5). For the females, the relationship was sigmoidal (AL females, $r^2 = 0.993$; CR females, $r^2 = 0.994$; curves were significantly different, $P < 0.007$). For the males, the relationship was sigmoidal (AL males, $r^2 = 0.991$; CR males, $r^2 = 0.999$; slopes were significantly different, $P < 0.001$).

We conducted statistical analyses on disease progression using different definitions of endpoint (see Materials and Methods). All three measures gave similar results (<http://www.fhs.mcmaster.ca/pediatrics/pages/neuromuscularmanuscript.htm>).

Survival. The age of mice at endpoint (clinical score of 4 plus additional clinical signs, see Materials and Methods) was 132 ± 2 days for the AL mice and 123 ± 2 days for the TCR mice ($P = 0.022$), and 132 ± 2 days for the females and 124 ± 2 days for the males ($P = 0.019$). This is equivalent to a 6%–7% decrease in lifespan. A significant gender \times diet interaction ($P = 0.039$) indicates that TCR males (118 ± 2 days) were 14 days younger than AL females (132 ± 3 days, $P = 0.004$) and 13 days younger than AL males (131 ± 3 days, $P = 0.022$) and TCR females (131 ± 4 days, $P = 0.034$) at endpoint, equivalent to a 10%–11% decrease in lifespan. Survival curves were different between the groups ($P < 0.0001$; Fig. 4), with the rate of reaching endpoint (i.e., the hazard ratio) in the TCR males being 3.1-fold higher (95% CI: 1.9, 29.5; $P = 0.004$) than the TCR females, 3.5-fold higher (95% CI: 2.8, 48.9; $P = 0.0007$) than the AL males, and 3.9-fold higher (95% CI: 3.7, 58.2; $P = 0.0001$) than the AL females.

To validate the use of different measures to define endpoint, we conducted statistical analyses using the traditional approach (clinical score of 5), the current definition (see Methods) and a clinical score of 4 (i.e., functional paralysis in both hindlegs) as proxy measures of endpoint. All three measures gave similar results (<http://www.fhs.mcmaster.ca/pediatrics/pages/neuromuscularmanuscript.htm>).

DISCUSSION

We found an earlier clinical onset by 5 days in G93A male mice than females, with no effect on paw grip endurance, in response to TCR. TCR also accelerated disease progression by 30%, with a trend for the disease to progress 23% faster in the males than females. Overall, TCR decreased average lifespan by 10% in the males, with no effect in the females. Furthermore, the rate of dying in TCR males was 3–4-fold faster than TCR females and the AL group. Together, these data suggest that the female sex is protective against transient nutritional stress induced by TCR.

The present study builds on our previous observation in the same animal model that nutritional insufficiency in G93A mice hastens clinical onset of disease.²⁰ In that study, we reported that long-term CR improved motor performance but, paradoxically, hastened clinical onset of disease, but could not speculate on gender differences, disease progression, or lifespan due to lack of power.²⁰ In the current study, we observed that TCR hastened the onset of disease in males only. In contrast, the age at clinical onset of males provided the AL diet was

similar to that of females. The sex difference observed with the TCR is consistent with our group's previous observation of an earlier clinical onset in male G93A mice exposed to high-intensity treadmill exercise training.³⁶ Furthermore, we observed that TCR showed a trend for males to have a faster disease progression, as compared with females, and that TCR males had a 10%–11% decrease in lifespan as compared with the AL group and their female counterparts. This indicates that G93A females are protected against the damaging effects of TCR, probably due to the sex hormone estrogen. In corroboration, supplementing G93A mice with the phytoestrogen genistein delayed disease onset and prolonged lifespan in males only.⁵⁵

In humans, women have longer life expectancies than men, with a difference of 5–10 years, equivalent to 6%–11% of lifespan.^{2,13,51} The percentage of men surviving to age 65 years and 85 years of age is lower than those in women in the general population and vegetarian subpopulation, with the difference becoming greater with older age.¹³ In rodents, female rats have a 21% longer lifespan than male rats, in concordance with females exhibiting 30%–50% lower mitochondrial H₂O₂ production in the liver and brain, 73% lower mitochondrial DNA damage (8-oxo-dG), 53% higher mitochondrial reduced glutathione (GSH), a 2-fold increase in Mn-superoxide dismutase (Mn-SOD) expression and activity, and up to a 2.8-fold increase in glutathione peroxidase (GPx) expression and activity.^{5,56} This gender bias is mainly due to estrogen, since mitochondria from ovariectomized female rats generate similar amounts of peroxides and have similar levels of GSH as males.⁵ In addition, females exhibit 4-fold higher levels of 16S rRNA, a marker that decreases significantly with age, than males of the same age.⁵ Moreover, estrogen supplementation in ovariectomized female rats improves antioxidant capability *in vivo*, reduces muscle damage, and hastens muscle regeneration following muscle strain injury¹¹; 17- β -estradiol results in a cascade of pathways by increasing the activation of MAPK and NF κ B, with the upregulation of Mn-SOD and GPx.⁶ Estrogen also increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels.⁵³

In cultured rat vascular smooth-muscle cells, 17- β -estradiol decreases the angiotensin II-induced increase in reactive oxygen species (ROS), increases ecSOD and MnSOD transcription rate (146% and 172%, respectively), and mRNA expression (219% and 255%, respectively) and activity, increases Mn-SOD protein content by 185%, and stabilizes the mRNA of ecSOD.⁵⁴ Cells from ovariectomized fe-

male rats exhibit a 2-fold increase in ROS and a reduction in ecSOD (63%) and MnSOD (43%) mRNA expression, all of which are corrected following 17- β -estradiol replacement.⁵⁴ These effects may not be limited to estrogen, but also to phytoestrogen supplementation.^{55,57}

In G93A mice, the effects of TCR are in contrast to what has been observed in different phyla and other murine strains.^{3,7,8,38,39,42} CR delays the onset of age-related diseases and extends lifespan in healthy animals, and this is attributed to a decrease in oxidative stress at the level of the mitochondria. In contrast, the G93A mouse model is not healthy since it has an underlying genetic abnormality. The G93A mouse model transgenically overexpresses the mutant human Cu/Zn-superoxide dismutase (*SOD1*) gene that results in a mutant SOD1 enzyme, normally a cytosolic enzyme, which is abnormally colocalized with the mitochondria in the CNS and possesses enhanced free radical-generating function when compared with the normal human SOD1.^{22,23,25,40,63} Defects in mitochondrial respiration and increased generation of free radicals present with an animal model of a heightened level of basal oxidative stress.^{1,4,19,23,29,33,34,40,42,63} In comparison with their wildtype counterparts, 95-day-old AL G93A mice exhibit increased levels of malondialdehyde (lipid peroxidation; 27%–29%) and protein carbonyls (protein oxidation; ~30%) and significant upregulation of antioxidant enzyme activities in the gastrocnemius muscle.³⁷ The increase in SOD2 and catalase activities is a compensatory adaptive response to the enhanced oxidative environment observed in G93A mice. Moreover, in a similar animal model of ALS, 90- and 105-day-old G86R mice exhibit an increase in the mRNA expression of GPx and γ -glutamylcysteine synthetase, a response mainly attributed to denervation.²⁷

It is possible that in the G93A mouse CR, both short- and long-term, exacerbates the transgene-induced increase in oxidative stress. Healthy rats exposed to CR show decreases in mitochondrial SOD (SOD2) and GPx activities and H₂O₂ production, increases in mitochondrial and cytosolic catalase activity, and no change in cytosolic SOD and GPx activities in the heart.²⁸ It is possible that TCR caused similar changes in the skeletal muscle of G93A mice. The fact that the mutant human SOD1 is mainly located in the cytosol, along with evidence that CR does not affect the activities of the cytosolic antioxidant enzymes SOD1 and GPx, suggests that, at least in the cytosol, TCR has no significant effect on the production of free radicals generated by the overexpression of the mutant human SOD1 enzyme

in the G93A mouse. CR is expected to reduce the flux through the electron transport chain and the activities of its free radical-generating enzymes, resulting in a reduction in mitochondrial oxidative stress.²⁸ Furthermore, the energy/protein malnutrition caused by TCR may have attenuated the normal adaptive compensatory upregulation of SOD2 and catalase activities observed in AL G93A mice as compared with their wildtype counterparts.³⁷ Given that the cytosolic mutant SOD1 is the major free radical-generating enzyme in this animal model, in contrast to healthy normal animals, where oxidative stress is primarily due to mitochondria-derived radicals, the CR-induced reduction in free radicals in the mitochondria may be undermined by the proportionately greater ROS generation in the cytosol, or in the mislocalized mitochondrial SOD1, unrelated to that from complex I or III. Future studies should investigate the changes in antioxidant enzyme activity and net free radical production following CR in the G93A mouse model.

Would short-term CR for a mere 15 days result in long-lasting adaptive dysregulation in antioxidant enzyme activities? For serious nutritional perturbations to exert lasting effects on the phenotype, the changes must be occurring at the level of the genotype. Metabolic imprinting, or epigenetic remodeling, due to environmental, including nutritional, stress occurs during embryogenic development, perinatally, and in early childhood.^{12,35,41,43,60} It is possible that, at least in the G93A mouse, the window for epigenetic remodeling is extended until early adulthood, since in the present study the mice were transiently (15 days) exposed to a nutritionally stressful environment at age 40 days, an age equivalent to early puberty. Future studies in this mouse model should attempt to delineate the biochemical and epigenetic modifications following transient periods of CR.

In the present study, TCR mice had a significantly younger age-specific clinical onset (in males), faster disease progression, and a shorter lifespan (mainly due to TCR males) as compared with the AL mice, in line with our previous research²⁰ and the only other research showing that intermittent feeding in G93A mice hastened disease progression by 55% and shortened the estimated lifespan by 14%.⁴⁵ More recent data from our laboratory further strengthen the current results by showing that CR hastened the rate of reaching clinical onset 2-fold, disease progression by 18%, and endpoint by 3.1-fold compared with the AL mice.²¹ Although Pedersen and Mattson⁴⁵ and Hamadeh and Tarnopolsky²¹ provided the groups with CR until endpoint,

short-term CR has been shown to exhibit effects similar to those observed with long-term CR. Short-term CR initiated at any age prolongs survival in *Drosophila*,³⁸ whereas in rats short-term CR decreases liver mitochondrial and nuclear DNA damage¹⁴ and mitochondrial H₂O₂ production in the heart and liver.^{14,28} The latter occurred at complex I, not due to a decrease in oxygen consumption, but rather to less ROS released per unit electron flow.¹⁴ In a mouse model of Alzheimer's disease, short-term CR decreases A β -plaque deposition.^{44,58}

Traditionally, we have used a clinical score of 5 to define endpoint, the point when mice could not right themselves to sternum from recumbency in less than 30 s when placed on their sides.^{18,24,26,48} However, institutional limitations compelled us to shorten the time from recumbency to 20 s.^{20,36} Moreover, we are now ethically obligated to define endpoint as a clinical score of 4 in conjunction with additional clinical symptoms (see Materials and Methods). In this study, we conducted statistical analyses using both the traditional and current definitions of endpoint. We also conducted statistical analysis using a clinical score of 4 as a proxy measure of endpoint. We found similar results for all three approaches. In future studies, it is thus acceptable to use a clinical score of 4 as a measure of endpoint in the G93A mouse to meet the most stringent ethical standards.

Presented at the 48th annual meeting of the Canadian Federation of Biological Societies, Guelph, Canada, June 2005. We thank Bart Hettinga for technical assistance, Alissa Aboud and Julie Hall for assisting with the PCR analysis and animal handling, Dr. Jan Kaczor for supervising the PCR analysis, and Adeel Safdar, Gillian Mazzetti, Jennifer Day, and Ursula Kazmierski for assisting with animal handling and food preparation. Funded by the National Sciences and Engineering Research Council of Canada.

REFERENCES

1. Andrus PK, Fleck TJ, Gurney ME, Hall ED. Protein oxidative damage in a transgenic mouse model of familial amyotrophic lateral sclerosis. *J Neurochem* 1998;71:2041–2048.
2. Ballesteros RF, Nicolas JD, Torres AR. Aging in Europe. In: Schroots JFF, Ballesteros RF, Rudinger G, editors. *Biomedical and health research*, vol. 17. Amsterdam: IOS Press; 1999. p 107–121.
3. Bodkin NL, Alexander TM, Ortmeyer HK, Johnson E, Hansen BC. Mortality and morbidity in laboratory-maintained rhesus monkeys and effects of long-term dietary restriction. *J Gerontol Series A Biol Sci Med Sci* 2003;58:212–219.
4. Bogdanov MB, Ramos LE, Xu Z, Beal MF. Elevated "hydroxyl radical" generation in vivo in an animal model of amyotrophic lateral sclerosis. *J Neurochem* 1998;71:1321–1324.
5. Borrás C, Sastre J, Garcia-Sala D, Lloret A, Pallardo FV, Vina J. Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free Radic Biol Med* 2003;34:546–552.

6. Borrás C, Gambini J, Gomez-Cabrera MC, Sastre J, Pallardo FV, Mann GE, et al. 17beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade. *Aging Cell* 2005;4:113–118.
7. Bruce-Keller AJ, Umberger G, McFall R, Mattson MP. Food restriction reduces brain damage and improves behavioral outcome following excitotoxic and metabolic insults. *Ann Neurol* 1999;45:8–15.
8. Dirx MJ, Zeegers MP, Dagnelie PC, van den Bogaard T, van den Brandt PA. Energy restriction and the risk of spontaneous mammary tumors in mice: a meta-analysis. *Int J Cancer* 2003;106:766–770.
9. Drew B, Phaneuf S, Dirks A, Selman C, Gredilla R, Lezza A, et al. Effects of aging and caloric restriction on mitochondrial energy production in gastrocnemius muscle and heart. *Am J Physiol Regul Integr Comp Physiol* 2003;284:R474–480.
10. Dubey A, Forster MJ, Lal H, Sohal RS. Effect of age and caloric intake on protein oxidation in different brain regions and on behavioral functions of the mouse. *Arch Biochem Biophys* 1996;333:189–197.
11. Feng X, Li GZ, Wang S. Effects of estrogen on gastrocnemius muscle strain injury and regeneration in female rats. *Acta Pharmacol Sin* 2004;25:1489–1494.
12. Frankel S, Gunnell DJ, Peters TJ, Maynard M, Davey Smith G. Childhood energy intake and adult mortality from cancer: the Boyd Orr Cohort Study. *Br Med J* 1998;316:499–504.
13. Fraser GE. The longevity of Adventists as compared with others. In: Diet, life expectancy, and chronic disease: studies of Seventh-Day Adventists and other vegetarians. New York: Oxford University Press; 2003. p 45–58.
14. Gredilla R, Barja G, Lopez-Torres M. Effect of short-term caloric restriction on H₂O₂ production and oxidative DNA damage in rat liver mitochondria and location of the free radical source. *J Bioenerg Biomembr* 2001;33:279–287.
15. Gredilla R, Sanz A, Lopez-Torres M, Barja G. Caloric restriction decreases mitochondrial free radical generation at complex I and lowers oxidative damage to mitochondrial DNA in the rat heart. *FASEB J* 2001;15:1589–1591.
16. Gurney ME. Transgenic-mouse model of amyotrophic lateral sclerosis. *N Engl J Med* 1994;331:1721–1722.
17. Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 1994;264:1772–1775.
18. Gurney ME, Cutting FB, Zhai P, Doble A, Taylor CP, Andrus PK, et al. Benefit of vitamin E, riluzole, and gabapentin in a transgenic model of familial amyotrophic lateral sclerosis. *Ann Neurol* 1996;39:147–157.
19. Hall ED, Andrus PK, Oostveen JA, Fleck TJ, Gurney ME. Relationship of oxygen radical-induced lipid peroxidative damage to disease onset and progression in a transgenic model of familial ALS. *J Neurosci Res* 1998;53:66–77.
20. Hamadeh MJ, Rodriguez MC, Kaczor JJ, Tarnopolsky MA. Caloric restriction transiently improves motor performance but hastens clinical onset of disease in the Cu/Zn-superoxide dismutase mutant G93A mouse. *Muscle Nerve* 2005;31:214–220.
21. Hamadeh MJ, Tarnopolsky MA. Long-term caloric restriction hastens clinical onset, disease progression and endpoint in the Cu/Zn-SOD mutant G93A mouse, an animal model of ALS. *Ann Nutr Metab* 2005;49(Suppl 1):164.
22. Higgins C, Jung C, Xu Z. ALS-associated mutant SOD1G93A causes mitochondrial vacuolation by expansion of the intermembrane space and by involvement of SOD1 aggregation and peroxisomes. *BMC Neurosci* 2003;4:16.
23. Higgins CM, Jung C, Ding H, Xu Z. Mutant Cu, Zn superoxide dismutase that causes motoneuron degeneration is present in mitochondria in the CNS. *J Neurosci* 2002;22:RC215.
24. Hottinger AF, Fine EG, Gurney ME, Zurn AD, Aebischer P. The copper chelator d-penicillamine delays onset of disease and extends survival in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Eur J Neurosci* 1997;9:1548–1551.
25. Jaarsma D, Rognoni F, van Duijn W, Verspaget HW, Haasdijk ED, Holstege JC. CuZn superoxide dismutase (SOD1) accumulates in vacuolated mitochondria in transgenic mice expressing amyotrophic lateral sclerosis-linked SOD1 mutations. *Acta Neuropathol* 2001;102:293–305.
26. Jiang F, DeSilva S, Turnbull J. Beneficial effect of ginseng root in SOD-1 (G93A) transgenic mice. *J Neurol Sci* 2000;180:52–54.
27. Jokic N, Di Scala F, Dupuis LUC, Rene F, Muller A, De Aguilar J-LG, et al. Early activation of antioxidant mechanisms in muscle of mutant Cu/Zn-superoxide dismutase-linked amyotrophic lateral sclerosis mice. *Ann N Y Acad Sci* 2003;1010:552–556.
28. Judge S, Judge A, Grune T, Leeuwenburgh C. Short-term CR decreases cardiac mitochondrial oxidant production but increases carbonyl content. *Am J Physiol Regul Integr Comp Physiol* 2004;286:R254–259.
29. Jung C, Higgins CM, Xu Z. Mitochondrial electron transport chain complex dysfunction in a transgenic mouse model for amyotrophic lateral sclerosis. *J Neurochem* 2002;83:535–545.
30. Lambert AJ, Merry BJ. Effect of caloric restriction on mitochondrial reactive oxygen species production and bioenergetics: reversal by insulin. *Am J Physiol Regul Integr Comp Physiol* 2004;286:R71–79.
31. Lass A, Sohal BH, Weindruch R, Forster MJ, Sohal RS. Caloric restriction prevents age-associated accrual of oxidative damage to mouse skeletal muscle mitochondria. *Free Radic Biol Med* 1998;25:1089–1097.
32. Lee CK, Pugh TD, Klopp RG, Edwards J, Allison DB, Weindruch R, et al. The impact of alpha-lipoic acid, coenzyme Q10 and caloric restriction on life span and gene expression patterns in mice. *Free Radic Biol Med* 2004;36:1043–1057.
33. Liu R, Althaus JS, Ellerbrock BR, Becker DA, Gurney ME. Enhanced oxygen radical production in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Ann Neurol* 1998;44:763–770.
34. Liu R, Li B, Flanagan SW, Oberley LW, Gozal D, Qiu M. Increased mitochondrial antioxidative activity or decreased oxygen free radical propagation prevent mutant SOD1-mediated motor neuron cell death and increase amyotrophic lateral sclerosis-like transgenic mouse survival. *J Neurochem* 2002;80:488–500.
35. Lucas A. Programming by early nutrition: an experimental approach. *J Nutr* 1998;128:401S–406S.
36. Mahoney DJ, Rodriguez C, Devries M, Yasuda N, Tarnopolsky MA. Effects of high-intensity endurance exercise training in the G93A mouse model of amyotrophic lateral sclerosis. *Muscle Nerve* 2004;29:656–662.
37. Mahoney DJ, Kaczor JJ, Bourgeois J, Yasuda N, Tarnopolsky MA. Oxidative stress and antioxidant enzyme upregulation in SOD1-G93A mouse skeletal muscle. *Muscle Nerve* 2006;33:809–816.
38. Mair W, Goymer P, Pletcher SD, Partridge L. Demography of dietary restriction and death in *Drosophila*. *Science* 2003;301:1731–1733.
39. Matsuo M, Gomi F, Kuramoto K, Sagai M. Food restriction suppresses an age-dependent increase in the exhalation rate of pentane from rats: a longitudinal study. *J Gerontol* 1993;48:B133–136.
40. Mattiazzi M, D'Aurelio M, Gajewski CD, Martushova K, Kiaei M, Beal MF, et al. Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. *J Biol Chem* 2002;277:29626–29633.
41. Maynard M, Gunnell D, Emmett P, Frankel S, Davey Smith G. Fruit, vegetables, and antioxidants in childhood and risk of adult cancer: the Boyd Orr cohort. *J Epidemiol Community Health* 2003;57:218–225.
42. Menzies FM, Cookson MR, Taylor RW, Turnbull DM, Chrzanoska-Lightowlers ZM, Dong L, et al. Mitochondrial dys-

- function in a cell culture model of familial amyotrophic lateral sclerosis. *Brain* 2002;125:1522–1533.
43. Ness AR, Maynard M, Frankel S, Smith GD, Frobisher C, Leary SD, et al. Diet in childhood and adult cardiovascular and all cause mortality: the Boyd Orr cohort. *Heart* 2005;91:894–898.
 44. Patel NV, Gordon MN, Connor KE, Good RA, Engelman RW, Mason J, et al. Caloric restriction attenuates A β -deposition in Alzheimer transgenic models. *Neurobiol Aging* 2005;26:995–1000.
 45. Pedersen WA, Mattson MP. No benefit of dietary restriction on disease onset or progression in amyotrophic lateral sclerosis Cu/Zn-superoxide dismutase mutant mice. *Brain Res* 1999;833:117–120.
 46. Sango K, McDonald MP, Crawley JN, Mack ML, Tiff CJ, Skop E, et al. Mice lacking both subunits of lysosomal beta-hexosaminidase display gangliosidosis and mucopolysaccharidosis. *Nat Genet* 1996;14:348–352.
 47. Seng JE, Agrawal N, Horsley ET, Leakey TI, Scherer EM, Xia S, et al. Toxicokinetics of chloral hydrate in ad libitum-fed, dietary-controlled, and calorically restricted male B6C3F1 mice following short-term exposure. *Toxicol Appl Pharmacol* 2003;193:281–292.
 48. Snow RJ, Turnbull J, da Silva S, Jiang F, Tarnopolsky MA. Creatine supplementation and riluzole treatment provide similar beneficial effects in copper, zinc superoxide dismutase (G93A) transgenic mice. *Neuroscience* 2003;119:661–667.
 49. Sohal RS, Agarwal S, Candas M, Forster MJ, Lal H. Effect of age and caloric restriction on DNA oxidative damage in different tissues of C57BL/6 mice. *Mech Ageing Dev* 1994;76:215–224.
 50. Sreekumar R, Unnikrishnan J, Fu A, Nygren J, Short KR, Schimke J, et al. Effects of caloric restriction on mitochondrial function and gene transcripts in rat muscle. *Am J Physiol Endocrinol Metab* 2002;283:E38–43.
 51. StatisticsCanada. Canada at a glance. <http://www.statcan.ca/cgi-bin/downpub/listpub.cgi?catno=12-581-XIE2004001>: Statistics Canada; 2005.
 52. Stein O, Dabach Y, Halperin G, Ben-Naim M, Stein Y. Calorie restriction in mice does not affect LDL reverse cholesterol transport in vivo. *Biochem Biophys Res Commun* 2003;308:29–34.
 53. Stirone C, Duckles SP, Krause DN, Procaccio V. Estrogen increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels. *Mol Pharmacol* 2005;68:959–965.
 54. Strehlow K, Werner N, Berweiler J, Link A, Dirnagl U, Priller J, et al. Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation. *Circulation* 2003;107:3059–3065.
 55. Trieu VN, Uckun FM. Genistein is neuroprotective in murine models of familial amyotrophic lateral sclerosis and stroke. *Biochem Biophys Res Commun* 1999;258:685–688.
 56. Vina J, Borras C, Gambini J, Sastre J, Pallardo FV. Why do females live longer than males? Importance of the upregulation of longevity-associated genes by oestrogenic compounds. *FEBS Lett* 2005;579:2541–2545.
 57. Vina J, Borras C, Gambini J, Sastre J, Pallardo FV. Why females live longer than males? control of longevity by sex hormones. *Sci Aging Knowl Environ* 2005;2005:pe17.
 58. Wang J, Ho L, Qin W, Rocher AB, Seror I, Humala N, et al. Caloric restriction attenuates beta-amyloid neuropathology in a mouse model of Alzheimer's disease. *FASEB J* 2005;19:659–661.
 59. Wang K, Li D, Sun F. Dietary caloric restriction may delay the development of cataract by attenuating the oxidative stress in the lenses of brown Norway rats. *Exp Eye Res* 2004;78:151–158.
 60. Waterland RA, Jirtle RL. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition* 2004;20:63–68.
 61. Weydt P, Hong SY, Kliot M, Moller T. Assessing disease onset and progression in the SOD1 mouse model of ALS. *Neuroreport* 2003;14:1051–1054.
 62. Wu A, Sun X, Liu Y. Effects of caloric restriction on cognition and behavior in developing mice. *Neurosci Lett* 2003;339:166–168.
 63. Yim MB, Yim HS, Chock PB, Stadtman ER. Enhanced free radical generation of FALS-associated Cu,Zn-SOD mutants. *Neurotox Res* 1999;1:91–97.