LETTER

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Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study

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Calorie restriction (CR), a reduction of 10–40% in intake of a nutritious diet, is often reported as the most robust non-genetic mechanism to extend lifespan and healthspan. CR is frequently used as a tool to understand mechanisms behind ageing and age-associated diseases. In addition to and independently of increasing lifespan, CR has been reported to delay or prevent the occurrence of many chronic diseases in a variety of animals. Beneficial effects of CR on outcomes such as immune function[1,2], motor coordination[3] and resistance to sarcopenia[4] in rhesus monkeys have recently been reported. We report here that a CR regimen implemented in young and older age rhesus monkeys at the National Institute on Aging (NIA) has not improved survival outcomes. Our findings contrast with an ongoing study at the Wisconsin National Primate Research Center (WNPRC), which reported improved survival associated with 30% CR initiated in adult rhesus monkeys (7–14 years)[5] and a preliminary report with a small number of CR monkeys[6]. Over the years, both NIA and WNPRC have extensively documented beneficial health effects of CR in these two apparently parallel studies. The implications of the WNPRC findings were important as they extended CR findings beyond the laboratory rodent and to a long-lived primate. Our study suggests a separation between health effects, morbidity and mortality, and similar to what has been shown in rodents[7–9], study design, husbandry and diet composition may strongly affect the life-prolonging effect of CR in a long-lived nonhuman primate.

For over 20 years, the NIA has studied the effects of CR in long-lived nonhuman primates (NHPs) (*Macaca mulatta*, average lifespan in captivity is ~27 years and maximum reported lifespan is ~40 years), to verify whether the life-prolonging effects observed in lower organisms also occur in monkeys and thus, might plausibly translate to human ageing[10,11]. The NIA CR study began in 1987 at the NIH Animal Center[12]. CR was initiated in monkeys of varying ages to evaluate the impact of age of onset of CR on its biological effects. Study design has been reported elsewhere[12,13]. Male and female monkeys were enrolled into the study at young, middle and older ages[12]. Data reported here are grouped as either young-onset (includes juvenile, adolescent and adult) or old-onset monkeys. Supplementary Table 1 reports the current census.

Any animal that died underwent a complete necropsy by a board-certified pathologist. A gross description of the pathology related to each organ was provided along with the probable cause of death and any contributing factors. Survival data were analysed in two ways: all-cause mortality and age-related deaths; a distinction also reported previously[5]. In both studies (NIA and WNPRC), age-related survival excluded deaths due to acute conditions that do not have an age-related increase in risk such as gastrointestinal bloat, anaesthesia, injury or endometriosis. Pathology details are in the Supplementary Information.

Old-onset CR monkeys (16–23 years) did not live longer than controls in either the all-cause (Fig. 1a) or age-related survival analysis (there were three cases of non-age related deaths in the CR group and 2 in the control group, graph not shown). In this group, males had significantly longer survival compared to females ($P = 0.0003$) and neither sex benefitted from CR. To date, four CR monkeys and one control from the old-onset group have lived beyond 40 years. Although CR has not increased mean or maximum lifespan relative to control, 50% survival for the females is 27.8 years and 35.4 years for the males, exceeding the ~27 year median lifespan previously reported for monkeys in captivity[14]. These monkeys may have benefitted from excellent husbandry conditions and thus CR started at older ages provided no additional increase in survival. Furthermore, there were no apparent differences in causes of death between the two diet groups. Neoplasia, cardiovascular disease, amyloidosis and general organism deterioration in the oldest animals were equally represented in both diet groups.

Old-onset CR was beneficial on several measures of metabolic health and overall function. Both male and female CR monkeys weighed less than the control counterparts, although the diet effect was greater in the males. In longitudinal measures from serum of fasted monkeys, triglycerides, cholesterol and glucose levels increased with age for both male and female controls. However, triglycerides were significantly lower in the CR monkeys ($F_{(1,21)} = 5.76$, $P = 0.026$) (Fig. 1b), and cholesterol remained significantly lower in the CR males (Fig. 1c) ($F_{(40,774)} = 1.53$, $P = 0.02$). At the oldest ages, fasting glucose was numerically lower in the CR monkeys (Fig. 1d) and significantly lower in CR males compared to controls ($P = 0.04$). On a single measure of plasma-free isoprostane, an indicator of oxidative stress, control males had significantly higher levels than the CR monkeys (23.24 ± 1.25 versus 15.93 ± 1.97 pg ml^{-1}; $P = 0.009$). In contrast, we previously reported that old-onset CR may negatively affect immune function[15].

Current survival curves for the young-onset male and females are shown in Fig. 2a (all-cause mortality) and Fig. 2b (age-related mortality). No significant diet effects are noted in survival between control and CR

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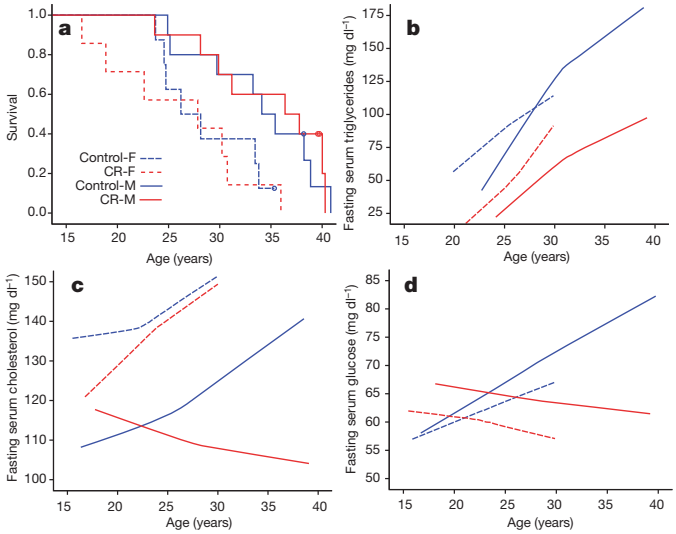

Figure 1 | Survival curve and triglycerides, cholesterol and glucose levels for old-onset monkeys. **a**, Kaplan–Meier survival curve for all-cause mortality for old-onset monkeys. All-cause mortality was analysed using Cox regression with age of onset, sex and diet as predictors. The effect of diet was not significant ($P = 0.934$) and sex was the only significant predictor ($P = 0.003$). Open circles represent alive monkeys. **b**, Fasting serum triglycerides (mg dl^{-1}) predicted from age-dependent individual-specific trajectories for old-onset monkeys. Triglyceride levels increased with age ($F_{(16,162)} = 2.12$, $P = 0.0096$) and CR monkeys had significantly lower levels than control ($F_{(1,21)} = 5.76$, $P = 0.026$). F, female; M, male. Overall triglyceride trajectories were based on 243 observations for 34 monkeys (50 observations for 8 control-F; 81 for 10 control-M; 32 for 7 CR-F; 80 for 9 CR-M). Age breakdowns for all figures are in the supplementary material. **c**, Cholesterol predicted from age-dependent individual-specific trajectories for old-onset monkeys. Cholesterol levels increased with age ($F_{(53,774)} = 1.54$, $P = 0.009$), and male monkeys had significantly lower levels than females ($F_{(1,24)} = 23.60$, $P < 0.0001$). A significant three-way diet–sex–age interaction ($F_{(40,774)} = 1.53$, $P = 0.02$) indicated that cholesterol levels increased with age for control males whereas CR males tended to have a slight reduction in cholesterol. Thus, at older ages (>30 years), CR male monkeys have significantly lower cholesterol levels compared to controls. Overall cholesterol trajectories were based on 994 observations for 28 animals (204 for 7 control-F; 301 for 7 control-M; 134 for 5 CR-F; and 355 for 9 CR-Male). **d**, Fasting serum glucose (mg dl^{-1}) levels predicted from age-dependent individual-specific trajectories for old-onset monkeys. Five glucose measurements above 100 mg dl^{-1} for one diabetic control-M were omitted to remove the influence of these outliers on the analyses and graphs. There were significant changes in glucose over time ($F_{(20,285)} = 10.48$, $P < 0.0001$) and males and females were significantly different in the trends over time ($F_{(18,285)} = 3.58$, $P < 0.0001$) with males having increases in glucose levels over time, whereas the glucose levels of the females slightly decreased. The overall CR difference was not significant, $F_{(1,22)} = 1.18$, $P = 0.288$, and the CR differences in trend over time were not significant, $F_{(20,285)} = 1.23$, $= 0.2259$. Additional analyses stratified by sex conditions showed that control males had significantly higher glucose levels compared to CR males, $F_{(1,\ 14)} = 5.27$, $P = 0.04$. Overall glucose trajectories were based on 387 observations for 34 monkeys (79 observations for 8 control-F; 131 for 10 control-M; 48 for 7 CR-F; 129 for 9 CR-M).

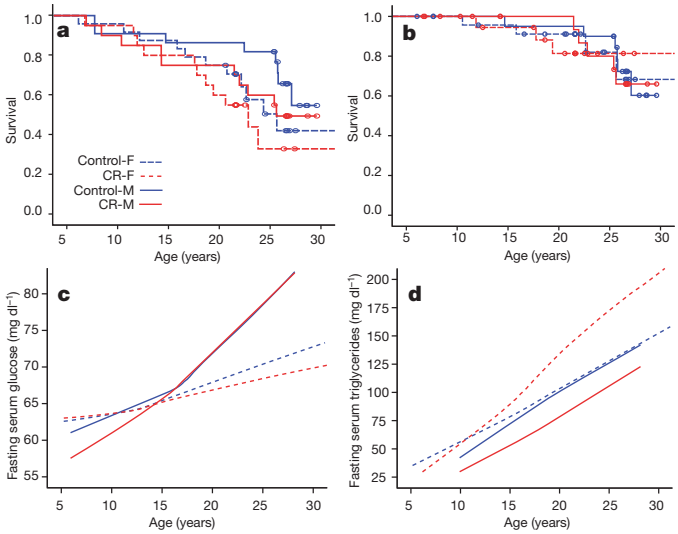

Figure 2 | Survival curves and glucose and triglycerides levels for young-onset monkeys. **a**, **b**, Kaplan–Meier survival curves for all-cause (**a**) and age-related mortality (**b**) for young-onset monkeys. Both were analysed using Cox regression with age of onset, origin, sex and diet ($P = 0.255$ and $P = 0.975$, respectively) as predictors with none of these factors being statistically significant. Open circles represent monkeys that are still alive and non-age related deaths in **b**. **c**, Fasting serum glucose (mg dl^{-1}) levels predicted from age-dependent individual-specific trajectories for young-onset monkeys. 14 glucose measurements above 100 mg dl^{-1} in diabetic monkeys (7 observations for 3 control-M; 5 for 2 CR-M; 2 for 1 control-F) were omitted to remove the influence of these outliers on the analyses and graphs. There were significant changes in glucose over time ($F_{(18,1,112)} = 11.24$, $P < 0.0001$), and males and females were significantly different in the trends over time ($F_{(18,1,112)} = 1.98$, $P = 0.0088$) with males having a larger increase in glucose levels over time. There was no significant difference due to diet group. Overall glucose trajectories were based on 1,260 observations for 81 monkeys (346 observations for 23 control-F; 350 for 20 control-M; 281 for 20 CR-F; 283 for 18 CR-M). **d**, Fasting serum triglycerides (mg dl^{-1}) predicted from age-dependent individual-specific trajectories for young-onset monkeys. There were significant changes in triglycerides over time ($F_{(14,843)} = 17.59$, $P < 0.0001$) and males and females were significantly different in the trends over time ($F_{(14,843)} = 5.36$, $P < 0.0001$). Furthermore, there was a diet–sex interaction indicating that the overall effect of CR on triglycerides was significantly different for male and female monkeys ($F_{(1,68)} = 5.07$, $P = 0.0276$). Specifically, CR males had lower triglycerides than control males. By contrast, CR females had higher triglyceride levels than control females. Overall triglyceride trajectories were based on 973 observations for 81 monkeys (266 observations for 23 control-F; 280 for 20 control-M; 213 for 20 CR-F; 214 for 18 CR-M).

monkeys for either analysis. Statistical controls are described in Methods. Of the original 86 monkeys in the young-onset cohorts, 24% (11/46) of the control animals and 20% (8/40) of the CR group died of age-related causes. The NIA findings contrast with the adult-onset study at WNPRC that demonstrated a beneficial CR effect in which 37% of the control monkeys had died from age-related causes compared to only 13% in the CR group. When accounting for all deaths, the trend persisted with 9 control and 13 CR animals dying of non-age related causes. Survival probabilities for all NIA age groups combined are shown in Supplementary Fig. 1a, b.

Considering that just less than 50% of young monkeys are still alive, these data do not represent final lifespan curves in this study. On the basis of lifespan projections using the hazard function[16], most animals are projected to be dead 10 years from now and the estimated probability statistics indicates a likelihood of less than 0.1% chance that the overall survival outcome would favour the CR group. The probability that a significantly different effect on mean survival will emerge in the next 5–10 years of the study is very low; however, a potential effect on maximum lifespan cannot be ruled out.

As there is a clear difference in CR effect on mortality between the colonies at NIA and WNPRC, further comparisons of these two longitudinal studies are warranted and planned. In an estimate of NIA's current data (as of 1 December 2011) to the published WNPRC data summarized as of 22 February 2008 and reported in ref. 5, NIA monkeys, both control and CR, may have a lifespan advantage comparable to the WNPRC CR monkeys.

Although they eat less (Supplementary Table 2) and weigh less[13], young-onset CR monkeys lack many of the expected CR benefits. Fasting serum glucose levels were not significantly lower in the CR monkeys compared to control (Fig. 2c), and only the CR males had somewhat lower triglycerides compared to respective controls ($P = 0.051$) (Fig. 2d). However, in a ligature-induced model of

inflammation in the oral cavity[1], we have shown an improved immune response in young-onset CR monkeys and beneficial effects in T cells isolated from adolescent-onset males[2].

The incidence of cancer was markedly improved in young-onset CR monkeys ($P = 0.028$ compared to controls); in fact, neoplasia has not been identified in any monkey from this group (Fig. 3a). In contrast, five of the six cases in young-onset control monkeys were considered the cause of death with a mean age at diagnosis of 22.8 ± 1.7 years. Glucoregulatory function was also improved in CR monkeys (Fig. 3a). However, two cases of diabetes have been diagnosed in CR monkeys; thus, the prevention of obesity did not prevent the occurrence of insulin-dependent diabetes and further investigation of the aetiology of such cases is of interest. Interestingly, CR did not reduce the incidence of cardiovascular disease as was reported in the WNPRC colony. Our findings are based on tissue pathology because these diagnoses were identified after death.

An analysis of first occurrence of age-related disease was done on the NIA monkeys using the same disease criteria as defined by the WNPRC study. These conditions included: cancer, diabetes, arthritis, diverticulosis and cardiovascular disease. Although age-related diseases were detected in control monkeys at an earlier age than in CR monkeys, the incident curves were not significantly different ($P = 0.06$) (Fig. 3b).

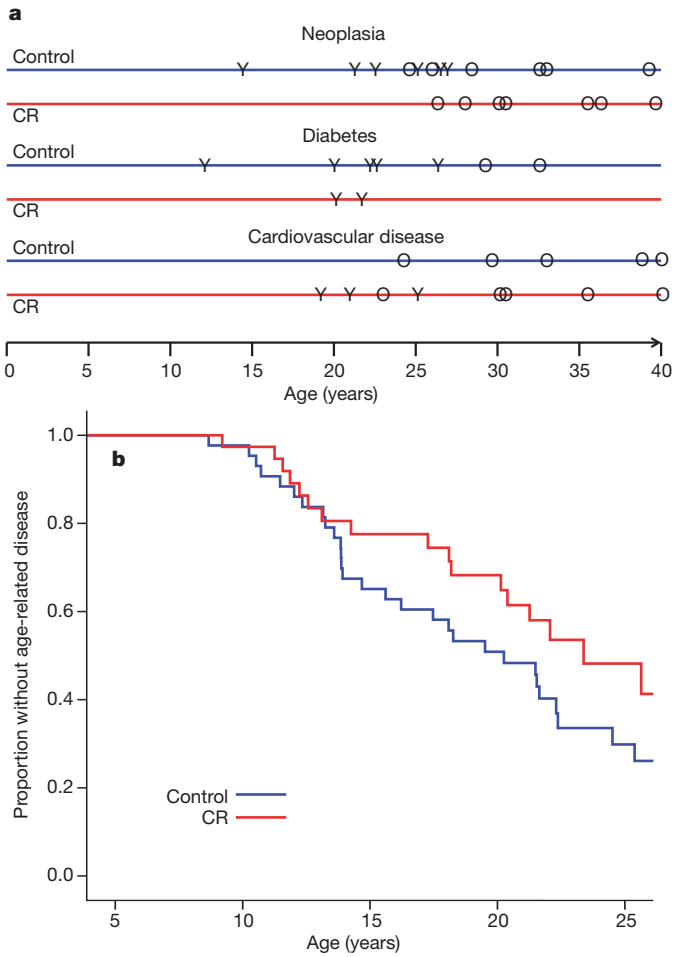

Figure 3 | Incidence and estimated proportions of age-related diseases. **a**, Incidence of three major age-related conditions. Age at diagnosis is represented with a 'Y' for young-onset monkeys and 'O' for old-onset monkeys. Animals may be represented more than once if multiple conditions existed. **b**, Estimated proportions for the first occurrence of any age-related disease in each monkey from the young-onset age group (males and females combined) statistically controlling for sex and sex–CR interaction. These conditions included: cancer, diabetes, arthritis, diverticulosis and cardiovascular disease. The difference between control and CR is not statistically significant, $P = 0.06$. Old-onset monkeys are not represented.

Considering that these two projects maintain high quality veterinary support in comparable experimental settings, study the same species of primates, and test the same intervention, what could account for the differences in survival outcome?

A notable difference between the two studies is diet composition. The NIA-1-87 formulation (Labdiet, PMI Nutrition International) has a natural ingredient base whereas WNPRC diet is purified (Harlan Teklad). Although natural ingredient diets risk having some variation between batches, they contain components that may have an impact on health such as phytochemicals, ultra-trace minerals and other unidentified elements[17]. In purified diets, each ingredient supplies a specific nutrient and each required mineral and vitamin is added as a separate component. Nutrient sources were also different. Protein was derived from wheat, corn, soybean, fish and alfalfa meal for the NIA diet, whereas the WNPRC diet protein source was lactalbumin. The NIA diet also contained flavonoids, known for their antioxidant activity, and fat from soy oil and the oils from the other natural ingredients (that is, corn, wheat and fish). Fish meal contains approximately 8–12% fat and is rich in omega-3 fatty acids. The WNPRC study dietary fat was derived from corn oil. Carbohydrate content was also notably different; although both diets had 57–61% carbohydrate by weight, the NIA study diet was comprised primarily of ground wheat and corn, whereas the WNPRC study diet contained corn starch and sucrose. Indeed, the WNPRC diet was 28.5% sucrose, whereas the NIA study diet was only 3.9% sucrose. This latter point may be particularly important as a diet high in sucrose may contribute to the incidence of type II diabetes[18,19].

The NIA and WNPRC studies also approached vitamin and mineral supplementation differently. The NIA study used one diet for both CR and control monkeys, which was supplemented with an additional 40% of the daily-recommended allowance to insure adequate nutrition for the CR monkeys. Thus, the NIA diet formulation super-supplemented the control monkeys. The WNPRC study fed two different diets and only the CR monkeys were supplemented.

Another important difference in study design was that the NIA study control monkeys were not truly fed *ad libitum*, unlike the WNPRC study. The regulated portioning of food for the NIA control monkeys may be a slight restriction and thus, largely prevented obesity. It has been reported that 10% CR increased lifespan in rats compared to *ad libitum*, even more than 25 and 40% CR[20]. The NIA control monkeys may experience survival benefits from this slight restriction.

Calorie restriction effectively lowered body weight in the NIA and WNPRC monkeys (Supplementary Table 2)[13,21]. However, WNPRC monkeys generally weighed more than corresponding NIA monkeys. For example, at 17 years of age, WNPRC males weighed approximately 12% more than corresponding NIA males and the difference was approximately 18% for the females. Thus, the NIA monkeys may be in an optimal weight range.

NIA monkeys originated from both China and India, and have greater genetic diversity compared to the strictly Indian colony at WNPRC. In rodent studies, genetic differences have affected the survival outcome in CR studies, even shortening it in some recombinant inbred mouse strains[22]. In genetically heterogeneous wild-caught mice, although hormonal and weight loss effects were consistent with CR, there was no overall mean longevity effect[23]. It is apparent that the effect of CR is not straightforward, and genetic differences may have a larger role than has been considered to date. A final analysis which includes all monkeys and controls for genetic origin can address this confounding variable.

Lastly, as in rodent studies[24], the age of onset of the CR regimen for the two studies could certainly impact survival outcome as it has other measures. CR initiated in the youngest male monkeys delayed maturation[25] and slowed skeletal growth[26]. Additionally, only the immune response of the adolescent males was improved by CR[15].

In the first randomized trial in humans, 6 months of CR improved several biomarkers of ageing and improved cardiovascular health, suggesting a reduction in risk of age-related disease. However, a lifespan study in humans is improbable[27]. Even a self-imposed CR regimen in lean individuals improved several metabolic, inflammatory and cardiovascular measures[28]. Current findings show that in nonhuman and human primates, CR evokes very similar metabolic, hormonal and physiological changes that are linked to longevity in CR rodents[28]. It will be valuable to continue to compare findings from ongoing monkey CR studies to dissect the mechanisms behind the improvement in health that occurred with and without significant effects on survival.

METHODS SUMMARY

Approval. This study was approved by the Animal Care and Use Committee of the NIA, NIH and was conducted in an AALAC-accredited facility.

Diagnostics. In live animals, diagnostic evaluations were made on the basis of clinical presentation. Radiographs confirmed conditions of osteoarthritis; endoscopic evaluation of diverticulosis revealed hernia-like outpouching in the mucosa of the descending colon with trapped faecal material in the diverticula; diabetes was confirmed by consistent elevated fasting glucose and glucose response during an intravenous glucose tolerance test; surgical biopsy or removal of tumours confirmed neoplasia. Cardiovascular abnormalities such as myofibre loss and fibrosis were diagnosed at necropsy as well as death due to acute congestive heart failure.

Blood sampling. For longitudinal measures, blood samples were obtained under ketamine (7–10 mg kg^{-1}, intramuscular) or Telazol (3.5 mg kg^{-1}, intramuscular) anaesthesia following an overnight fast. Serum samples were stored at $-80\,^{\circ}$C until analysed. Plasma free isoprostane samples were collected in 2005 and measured according to the description in ref. 29.

Full Methods and any associated references are available in the online version of the paper.

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1. Branch-Mays, G. L. *et al.* The effects of a calorie-reduced diet on periodontal inflammation and disease in a non-human primate model. *J. Periodontol.* **79,** 1184–1191 (2008).
2. Messaoudi, I. *et al.* Delay of T cell senescence by caloric restriction in aged long-lived nonhuman primates. *Proc. Natl Acad. Sci. USA* **103,** 19448–19453 (2006).
3. Kastman, E. K. *et al.* A calorie-restricted diet decreases brain iron accumulation and preserves motor performance in old rhesus monkeys. *J. Neurosci.* **30,** 7940–7947 (2010).
4. Colman, R. J., Beasley, T. M., Allison, D. B. & Weindruch, R. Attenuation of sarcopenia by dietary restriction in rhesus monkeys. *J. Gerontol. A* **63,** 556–559 (2008).
5. Colman, R. J. *et al.* Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science* **325,** 201–204 (2009).
6. Bodkin, N. L., Alexander, T. M., Ortmeyer, H. K., Johnson, E. & Hansen, B. C. Mortality and morbidity in laboratory-maintained Rhesus monkeys and effects of long-term dietary restriction. *J. Gerontol. A* **58,** B212–B219 (2003).
7. Forster, M. J., Morris, P. & Sohal, R. S. Genotype and age influence the effect of caloric intake on mortality in mice. *FASEB J.* **17,** 690–692 (2003).
8. Murtagh-Mark, C. M., Reiser, K. M., Harris, R. Jr & McDonald, R. B. Source of dietary carbohydrate affects life span of Fischer 344 rats independent of caloric restriction. *J. Gerontol. A* **50A,** B148–B154 (1995).
9. Swindell, W. R. Dietary restriction in rats and mice: a meta-analysis and review of the evidence for genotype-dependent effects on lifespan. *Ageing Res. Rev.* **11,** 254–270 (2012).
10. Messaoudi, I. *et al.* in *Calorie Restriction, Aging, and Longevity* (eds Everitt, A. V., Rattan, S., Le Couteur, D. & de Cabo, R.) 55–78 (Springer, 2010).
11. Roth, G. S. *et al.* Aging in rhesus monkeys: relevance to human health interventions. *Science* **305,** 1423–1426 (2004).
12. Ingram, D. K. *et al.* Dietary restriction and aging: the initiation of a primate study. *J. Gerontol.* **45,** B148–B163 (1990).
13. Mattison, J. A. *et al.* Age-related decline in caloric intake and motivation for food in rhesus monkeys. *Neurobiol. Aging* **26,** 1117–1127 (2005).
14. Colman, R. J. & Kemnitz, J. W. in *Methods in Aging Research* (ed. Yu, B. P.) 249–267 (CRC, 1998).
15. Messaoudi, I. *et al.* Optimal window of caloric restriction onset limits its beneficial impact on T-cell senescence in primates. *Aging Cell* **7,** 908–919 (2008).
16. Allison, P. D. *Survival Analysis Using SAS: A Practical Guide* (SAS Institute, 1995).
17. Nadon, N. L. Exploiting the rodent model for studies on the pharmacology of lifespan extension. *Aging Cell* **5,** 9–15 (2006).
18. Lomba, A. *et al.* A high-sucrose isocaloric pair-fed model induces obesity and impairs NDUFB6 gene function in rat adipose tissue. *J. Nutrigenet. Nutrigenomics* **2,** 267–272 (2009).
19. Roncal-Jimenez, C. A. *et al.* Sucrose induces fatty liver and pancreatic inflammation in male breeder rats independent of excess energy intake. *Metabolism* **60,** 1259–1270 (2011).
20. Duffy, P. H. *et al.* The effects of different levels of dietary restriction on aging and survival in the Sprague-Dawley rat: implications for chronic studies. *Aging (Milano)* **13,** 263–272 (2001).
21. Raman, A. *et al.* Influences of calorie restriction and age on energy expenditure in the rhesus monkey. *Am. J. Physiol. Endocrinol. Metab.* **292,** E101–E106 (2007).
22. Liao, C. Y., Rikke, B. A., Johnson, T. E., Diaz, V. & Nelson, J. F. Genetic variation in the murine lifespan response to dietary restriction: from life extension to life shortening. *Aging Cell* **9,** 92–95 (2010).
23. Harper, J. M., Leathers, C. W. & Austad, S. N. Does caloric restriction extend life in wild mice? *Aging Cell* **5,** 441–449 (2006).
24. Speakman, J. R. & Hambly, C. Starving for life: what animal studies can and cannot tell us about the use of caloric restriction to prolong human lifespan. *J. Nutr.* **137,** 1078–1086 (2007).
25. Roth, G. S. *et al.* Age-related changes in androgen levels of rhesus monkeys subjected to diet restriction. *Endocr. J.* **1,** 227–234 (1993).
26. Lane, M. A. *et al.* Aging and food restriction alter some indices of bone metabolism in male rhesus monkeys (*Macaca mulatta*). *J. Nutr.* **125,** 1600–1610 (1995).
27. Redman, L. M. & Ravussin, E. Caloric restriction in humans: impact on physiological, psychological, and behavioral outcomes. *Antioxid. Redox Signal.* **14,** 275–287 (2011).
28. Omodei, D. & Fontana, L. Calorie restriction and prevention of age-associated chronic disease. *FEBS Lett.* **585,** 1537–1542 (2011).
29. Ward, W. F. *et al.* Effects of age and caloric restriction on lipid peroxidation: measurement of oxidative stress by F2-isoprostane levels. *J. Gerontol. A* **60,** 847–851 (2005).

Supplementary Information is available in the online version of the paper.

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Author Contributions G.S.R. and D.K.I. jointly conceived the original study and implemented it. J.A.M., R.d.C., D.K.I. and G.S.R. designed experiments, analysed and discussed data. J.A.M., R.d.C. and D.K.I. wrote the paper. T.M.B. and D.B.A. conducted statistical analyses and consultation. E.M.T., A.M.H. and J.E.Y. provided many years of technical support, data collection and supervision. R.L.H. provided veterinary support. D.L.L. assisted with data interpretation, discussion and paper edits. M.B. performed pathology assessments. D.B. assisted with initial diet formulation and all diet analyses and comparisons. W.F.W. and W.Q. designed and performed the isoprostane assays.

Author Information Reprints and permissions information is available at www.nature.com/reprints. Readers are welcome to comment on the online version of the paper. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com. Correspondence and requests for materials should be addressed to J.A.M. (mattisonj@mail.nih.gov), R.d.C. (deCaboRa@grc.nia.nih.gov) or D.K.I. (Donald.Ingram@pbrc.edu).

METHODS

Animals. With the exception of six old-onset males, all monkeys had known birthdates. Estimated ages were assigned to these six based on dental archives and historical records. No monkey had been used in invasive experiments before procurement. After procurement, monkeys were initiated on the study after required quarantine. Food intake was considered *ad libitum* during this time. Husbandry has been described previously[13]. NIA monkeys were fed a natural ingredient diet containing 56.9% carbohydrate, 17.3% protein and 5% fat.

Statistical methods. A Fisher's exact test was used to compare the incidence of neoplasia in the young-onset cohort. Analyses of age-associated diseases and mortality included all animals with known diagnoses or cause of death before 1 December 2011.

Twenty of the 26 adult-onset females were obtained from a military research facility, and 19 of these monkeys developed severe and rapidly progressing endometriosis. The twentieth monkey of this group died at the age of 12 years from renal necrosis. It seemed apparent that this cohort was differentially affected in terms of long-term health, and thus, an indicator variable that designated the source of this monkey group as 'Aberdeen' was created and was included in most analyses to control statistically for the effects of these animals on the outcomes of interest.

To determine the effect of CR on the onset of age-associated diseases (morbidity) and mortality, a Cox proportional hazard[16] regressions with sex and caloric restriction (CR), a sex–CR interaction term, and a covariate to adjust for whether the animal was obtained from the Aberdeen site as predictors were used to estimate the survival and hazard functions. The proportional hazards (PH) assumption was tested by fitting a non-PH Cox regression with a CR–time interaction, which was not significant for either analysis, and thus, PH models were considered valid. Animals that died of non-age-related causes (for example, death from anaesthesia, gastrointestinal bloat) were censored in both the mortality and morbidity analyses. Their age at death was used as the time variable in the Cox regressions. For the morbidity analysis, the age at which the animal experienced its first age-related diagnosis was used as the time variable in the Cox regression. Animals that received a non-age-related diagnosis were censored and their current age was used as the time variable. Animals that died of an age-related cause without ever receiving an age-related diagnosis were not censored. The designation of 'age-related' was based on the same rationale and list of conditions as reported by WNPRC. Death was considered as their first age-related diagnosis and their age at death was used as the time variable. All analyses were performed in SAS PROC PHREG and likelihood ratio tests were computed to assess statistical significance.

A linear mixed model[30] approach was used to estimate longitudinal trends in the data while accounting for the dependency in the data due to multiple observations per subject. SAS PROC MIXED was used to estimate the trends and group differences among the repeatedly measured outcomes (for example, body weight, glucose, cholesterol and triglycerides across the years of measurement. The effects of CR on overall outcome levels and differences in longitudinal trends were tested by including diet main effect and diet–year interaction terms in the model. Male and female monkeys were analysed together and sex main effects and sex–diet and –year interactions were also included in the models. The young- and old-onset groups were analysed separately. Age at the first measurement (that is, starting age) was used as a covariate to control for differences in age among the animals within a given year of measurement and a lag-1 autoregressive process over time was assumed. For the young-onset group a covariate to adjust for whether they were obtained from the Aberdeen site was added. Outliers were screened and removed. Specifically, a few young animals had glucose levels substantially above 200 mg dl^{-1}. Also one old control male that was eventually diagnosed with diabetes had extremely high triglyceride levels ranging from 342 to 1,314 mg dl^{-1} and these values influenced the significance of some effects. Briefly, the linear mixed model approach estimates a growth trajectory for each individual animal (for example, individual change in weight over time), adjusting for covariates. Then a weighted composite of these individual trajectories is computed to show the average trend over the age of the animals in a particular group (for example, average weight of animals at varying ages for CR-M). The weights for these composites are based on the number of observations each animal contributes to the data. For example, animals that live longer will contribute more data, and therefore will get larger weights. To smooth the trends for plotting graphs, the predicted values from each individual trajectory was averaged, and loess trend lines were constructed.

Competing risk. The analyses in this paper as well as in ref. 5 distinguished between age-related and all-cause mortality. To address the issue that the non-age-related deaths are associated to CR, a competing risks Cox proportional hazard regression models[16] were conducted separately for the young-onset group (9 control and 13 CR non-age-related deaths) and old-onset (2 control and 3 CR non-age-related deaths). Briefly, a competing risks model treats the events as if age-related and non-age-related deaths are mutually exclusive and compared to neither event occurring (that is, animals still alive are censored). These events have competing risks in that if an animal dies from a non-age-related cause they are no longer at-risk for an age-related death (and vice versa). For the old-onset animals, age at start of the experiment was not significantly related to non-age- ($P = 0.188$) or age-related deaths ($P = 0.269$). CR was not significantly related to non-age- ($P = 0.260$) or age-related deaths ($P = 0.490$). Also, sex was not significantly related to non-age- ($P = 0.991$) or age-related deaths ($P = 0.053$); however, this association of sex with age-related mortality is of marginal significance, which is consistent with the trend for males for have higher survival curves (see Figs 1a and 2a, b). For the young-onset animals, age at start of the experiment was not significantly related to non-age- ($P = 0.604$) or age-related deaths ($P = 0.653$). Sex was not significantly related to non-age- ($P = 0.790$) or age-related deaths ($P = 0.480$). CR was not significantly related to non-age- ($P = 0.147$) or age-related deaths ($P = 0.975$). Also, the origin (Aberdeen) of the animal was not significantly related to age-related deaths ($P = 0.513$), and the relationship to non-age-related deaths was not statistically significant. This marginal P value ($P = 0.0889$) could suggest that origin may be a confounding factor.

30. Littell, R. C., Milliken, G. A., Stroup, W. W., Wolfinger, R. D. & Schabenberger, O. SAS for Mixed Models (SAS Institute Inc., 2006).