Fig S1A. Kaplan-Meier Survival Curves for All-Cause Mortality for all (young- and old-onset) Monkeys. All-cause mortality was analyzed using Cox regression with age of onset, age group, sex, and diet as predictors. Sex was the only significant predictor ($p = 0.009$), therefore Kaplan-Meier survival curves for the four (diet-by-sex) groups were plotted to display the results. Open circles represent monkeys that are still alive.

Fig S1B. Kaplan-Meier Survival Curves for Age-Related Mortality for all (young- and old-onset) Monkeys. Age-related mortality was analyzed using Cox regression with age of onset, origin, sex, and diet as predictors with none of these factors being statistically significant. Therefore Kaplan-Meier survival curves for the two diet groups were plotted to display the results.

Supporting Online Material

Material and Methods

Animals and diet

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 prior to 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 [9]. NIA monkeys were fed a natural ingredient diet containing 56.9% carbohydrate, 17.3% protein, and 5% fat.

Supplement Table 1. Monkey Census

N (start)	N (as of 12/1/11)	Sex	Diet	Age Group
22	14	M	CON	Young
20	10	M	CR	Young
10	1	M	CON	Old
10	2	M	CR	Old
24	12	F	CON	Young
20	9	F	CR	Young
8	1	F	CON	Old
7	0	F	CR	Old

Diagnostics

In live animals, diagnostic evaluations were made based on clinical presentation. Radiographs confirmed conditions of osteoarthritis; **endoscopic evaluation of diverticulosis revealed hernia-like outpouching in the mucosa of the descending colon with trapped fecal 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 tumors confirmed neoplasia. Cardiovascular abnormalities such as myofiber loss and fibrosis were diagnosed at necropsy as well as death due to acute congestive heart failure.

Pathology Summary

Cancer was the leading cause of death for the old-onset monkey group, listed as the primary cause of death for 5 CON and 4 CR monkeys; however an incidental finding of neoplasia was identified in one CON and 3 CR monkeys at necropsy. For the old-onset group age of diagnosis was 30.6 $\pm$2.3 years and 33.0 $\pm$1.9 years for CON and CR, respectively. For incidental cancers identified on necropsy, age of diagnosis was death age.

In all cases, cardiovascular abnormalities were identified at necropsy and were considered the cause of death for 3 old-onset CON, 2 old-onset CRs, and 2 young-onset CRs. For 2 CON and 4 CR monkeys, myocardial fibrosis and myofiber degeneration were listed as significant pathological changes in the necropsy report although this condition was not the primary cause of death. In a 40-year old CR monkey, heart failure was listed as his cause of death and the primary pathological condition in a 40-year old CON was right ventricular necrosis. Interestingly, two young-onset CR monkeys had pathological changes in the heart tissue that were considered the primary cause of death at a mean age of 20.5 $\pm$ 1.1 years. For the third young-onset CR, changes associated with previous myocardial infarcts were documented.

Diabetes was suspected when fasting serum glucose exceeded 90 mg/dL, and a diagnosis was confirmed by persistent glucose in the urine and lack of an insulin response. There have been 7 cases in the CON monkeys, 2 old-onset and 5 in the young group, 3 young-onset CR monkeys were also diagnosed. Monkeys were euthanized when the disease could not be adequately managed and weight loss persisted.

When considering just the young-onset monkeys, the average age at diagnosis for both CON and CR was 21 years.

Medical management

As a study of lifespan and health span, medical treatment of both acute and chronic medical conditions alters the outcome. At the outset, NIA followed a policy of no treatment in the cases of long-term chronic diseases. Monkeys were maintained on the study and monitored closely until they could not be adequately maintained free of pain. This course of action relieved the investigators from trying to decipher the best course of medical treatment available at any given time, the length of time to administer, and assuming the role of studying a treatment modality rather than absolute lifespan.

This policy contrasted with that followed by the WNPRC CR study, in which drug interventions were pursued and thus, decreased the likelihood of endometriosis-induced mortality. Unfortunately, without some treatment, the NIA study ran the risk of losing a large percentage of females to a single disease not particularly relevant to human mortality and thus compromised the ability to accurately assess the effect of CR on lifespan. Therefore in 2004, NIA instituted a policy change to treat and prevent the development of endometriosis and thus reduce the bias created by this condition which can be treated if diagnosed early.

Acute conditions such as infection, wounds, gastrointestinal distress, and dental problems were always medically treated.

Blood sampling

For longitudinal measures, blood samples were obtained under ketamine (7-10 mg/kg, IM) or Telazol (3.5 mg/kg, IM) anesthesia following an overnight fast. Serum samples were stored frozen at -80° C until analyzed. Plasma free isoprostane samples were collected in 2005 and measured according to the description in Ward, et al.[43]

Statistical Methods

Analyses of age-associated diseases and mortality included all animals with known diagnoses or cause of death prior to 12/1/2011.

Twenty of the 26 adult-onset females were obtained from a military research facility, 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 statistically control 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 [20] regressions with Sex and Caloric Restriction (CR), a Sex-by-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 PH assumption was tested by fitting a non-PH Cox regression with a CR by Time interaction, which was not significant for either analysis, and thus, PH models were considered valid. Animals that died of non-age-related causes (e.g., death from anesthesia; 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

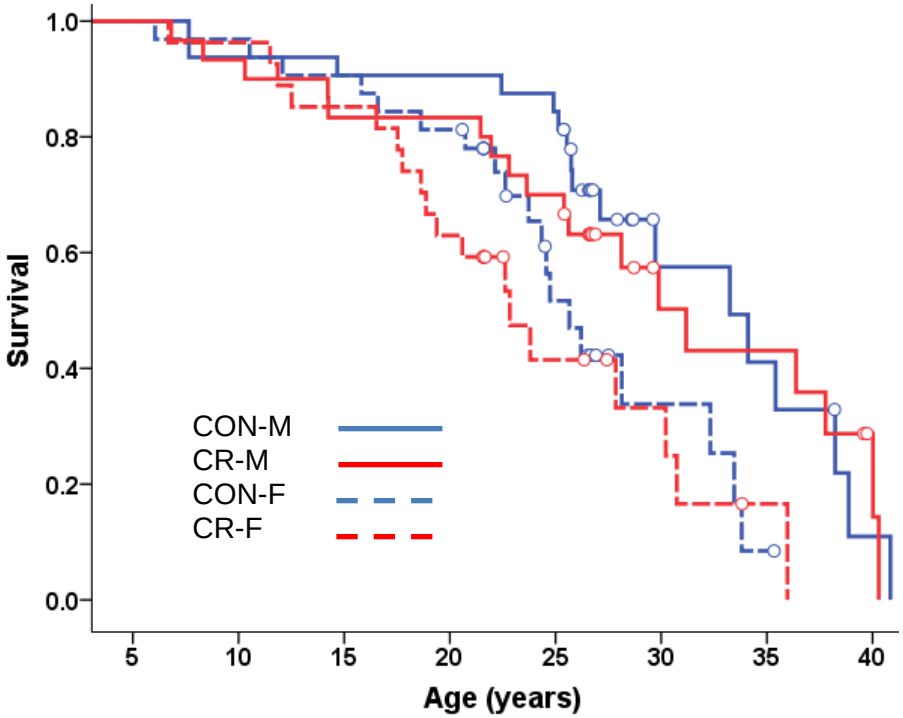
used as the time variable. All analyses were performed in SAS PROC PHREG and Likelihood Ratio Tests were computed to assess statistical significance.

A linear mixed model 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 (e.g., 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-by-Year interaction terms in the model. Male and Female monkeys were analyzed together and Sex main effects and Sex by Diet and Year interactions were also included in the models. The young- and old-onset groups were analyzed separately. Age at the first measurement (i.e., 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. Also one Old Control male that was eventually diagnosed with diabetes had extremely high triglyceride levels ranging from 342 to 1314 mg/dl and these values influenced the significance of some effects. Briefly, the linear mixed model approach estimates a growth trajectory for each individual animal (e.g., 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 (e.g., average weight of animals at varying ages for CR-Males). 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 Colman et al. distinguished between age-related and all-cause mortality. To address the issue that the nonage-related deaths are associated to CR, a competing risks Cox proportional hazard regression models 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 (i.e., 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 Figures 1 and 3). 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.

Supplemental Figure 1A



Supplemental Figure 1B