



## Caloric Restriction Delays Disease Onset and Mortality in Rhesus Monkeys

Ricki J. Colman *et al.*

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recommendations. Ongoing full genome sequencing will monitor for the possibility of future reassortment events (39).

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- Materials and methods are available as supporting material on Science Online.
- Single-letter abbreviations for the amino acid residues are as follows: D, Asp; I, Ile; L, Leu; M, Met; N, Asn; P, Pro; S, Ser; T, Thr; and V, Val.
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- Sequences will continue to be uploaded to the sequence databases [NCBI (National Center for Biotechnology Information) ([www.ncbi.nlm.nih.gov/genomes/FLU/](http://www.ncbi.nlm.nih.gov/genomes/FLU/)) and GISAID (Global Initiative on Sharing Avian Influenza Data) (<http://gisaid.org/>)] as they are generated. See table S1 for a list of GenBank accession numbers. Antigenic data will be available at <http://antigenic-cartography.org/>.
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#### Supporting Online Material

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## Caloric Restriction Delays Disease Onset and Mortality in Rhesus Monkeys

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Caloric restriction (CR), without malnutrition, delays aging and extends life span in diverse species; however, its effect on resistance to illness and mortality in primates has not been clearly established. We report findings of a 20-year longitudinal adult-onset CR study in rhesus monkeys aimed at filling this critical gap in aging research. In a population of rhesus macaques maintained at the Wisconsin National Primate Research Center, moderate CR lowered the incidence of aging-related deaths. At the time point reported, 50% of control fed animals survived as compared with 80% of the CR animals. Furthermore, CR delayed the onset of age-associated pathologies. Specifically, CR reduced the incidence of diabetes, cancer, cardiovascular disease, and brain atrophy. These data demonstrate that CR slows aging in a primate species.

Evidence that mammalian longevity could be increased emerged in 1935 in a rodent study showing that caloric restriction (CR), without malnutrition, extended average and maximum life span and delayed the onset of

age-associated pathologies (1). It was not until the 1990s that CR became widely viewed as a scientific model that could provide insights into the retardation of the aging process (2) and thereby identify underlying mechanisms of aging (3). The inverse relationship between caloric intake and increase in life span in mice suggests a role for regulators of energy metabolism in the mechanism of CR. Accordingly, CR-induced metabolic reprogramming may be a key event in the mechanism of life span extension (4). Studies in yeast, worms, flies, and mice point to a role for nutrient-responsive signaling molecules, including SIRT1, mTOR, and PGC-1 $\alpha$ , in aging and CR (5). The relevance of these find-

ings for human aging depends on the conservation of the effects of CR on aging in primates.

The marked anatomical, physiological, and behavioral similarities between human and non-human primates make the latter particularly suited for providing insights into the biology of human aging. Although animals on CR appeared subjectively younger than controls (Fig. 1, A to D), we sought to determine whether they were biologically younger than controls. Two critical indicators of aging retardation are delays in mortality and in the onset of age-associated disease. The incidence of disease increases with age and is a fundamental contributor to mortality (6). Thus, we examined age-associated conditions most prevalent in humans, including diabetes, cancer, cardiovascular disease, and brain atrophy (7).

Our study was begun in 1989 at the Wisconsin National Primate Research Center (WNPRC) (8) (Fig. 2A). Rhesus macaques (*Macaca mulatta*) have an average life span of ~27 years in captivity and a maximal life span of ~40 years. All animals were adults (7 to 14 years old) when introduced into the study. Initially the study included 30 males, and the cohort was expanded in 1994 to include an additional 30 females and 16 males (9). These increased numbers improved statistical power, and the inclusion of females allowed us to monitor gender differences in the effects of CR. The animals were evenly matched and randomized to control or CR diets, taking into consideration baseline food intake, body weight, and age. Individualized food allotments were calculated based on daily food intake data that were collected for each animal over a

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3- to 6-month period. Once animals were assigned to either control or CR groups, each CR animal's individually determined baseline intake was reduced by 10% per month over a 3-month period to reach the desired 30% restriction.

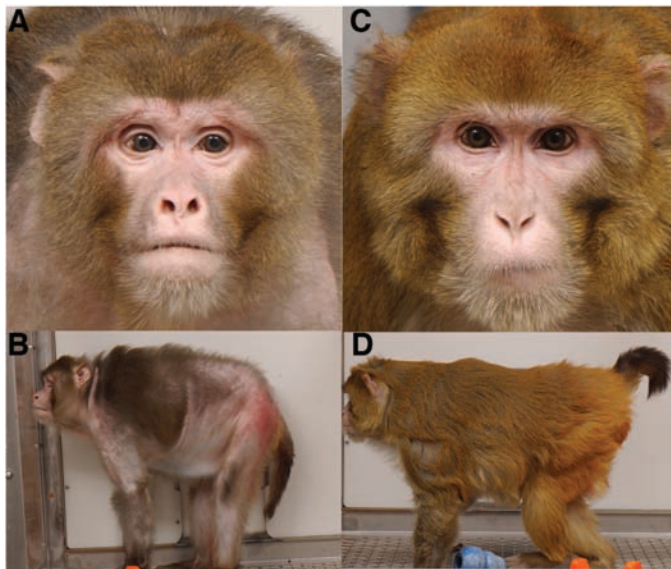
Any animal that died underwent a complete necropsy by a board-certified pathologist (who was not told the animal's diet group), and the

cause of death was determined. On this basis, we distinguished deaths due to age-related causes from those due to acute conditions probably unrelated to aging. Of the original 76 animals, 37% [14 out of 38 (14/38)] of the control animals died of age-related causes as compared to only 13% (5/38) of the CR group. Survival analysis (with a Cox regression) considering only age-

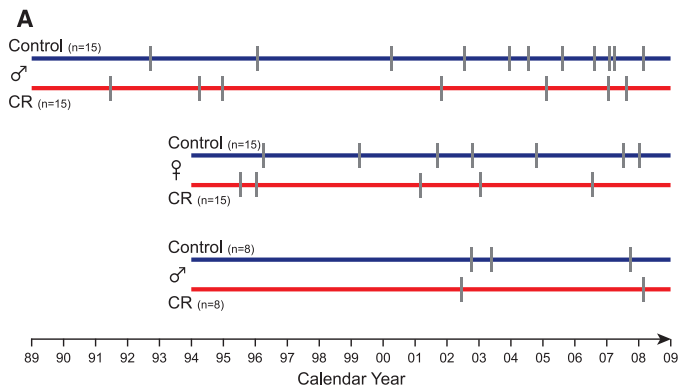
related deaths revealed a statistically significant effect of CR in increasing survival ( $P = 0.03$ ; Fig. 2B) with a hazard ratio (HR) of 3.0, indicating that at any point in time, the control animals had three times the rate of death from an age-related cause when compared to animals under CR. Seven control and 9 CR animals died of non-age-related causes, which included complications of anesthesia, gastric bloat, endometriosis, and injury. The effect of CR on overall mortality is in the predicted direction but is currently not statistically significant ( $P = 0.16$ ; Fig. 2C).

Age-associated diseases in rhesus monkeys have been well documented for animals at the WNPRC and are similar to those observed in humans (10). The most prevalent of such diseases are diabetes, cancer, and cardiovascular disease. To determine the health and aging phenotype of each individual animal, we assessed food intake, body weight, body composition, serum chemistry, glucose regulation, energy expenditure, activity measurement, endocrine profiles, electrocardiogram, blood pressure, brain magnetic resonance imaging, and radiography. These measurements, and a minimum of twice-daily observation of the animals, allowed disease conditions to be identified early and treated appropriately.

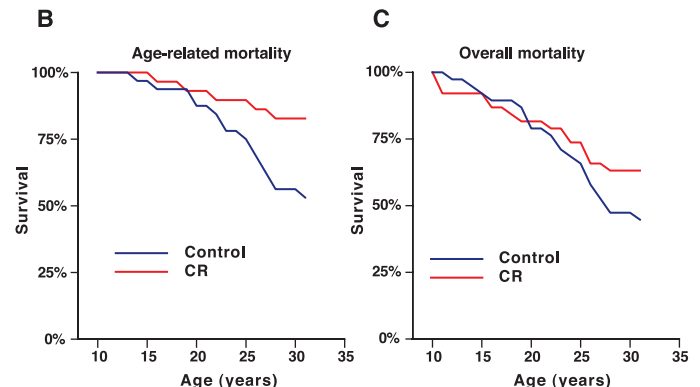
The effects of CR on body composition and metabolic function were robust. Body weight



**Fig. 1.** Animal appearance in old age. (A and B) Photographs of a typical control animal at 27.6 years of age (about the average life span). (C and D) Photographs of an age-matched animal on CR.

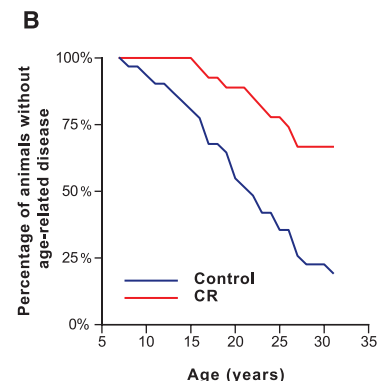
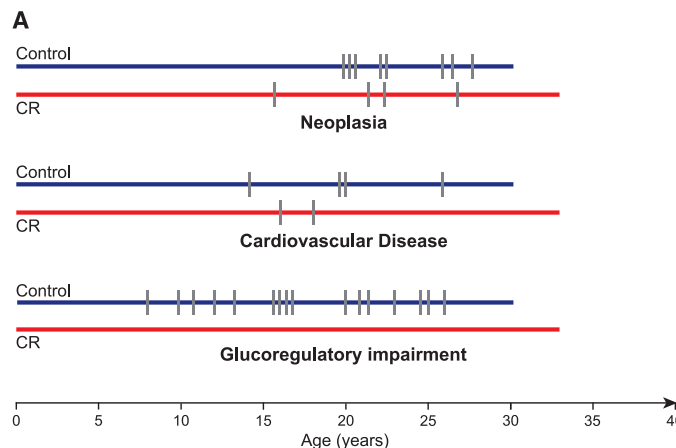


**Fig. 2.** Longitudinal study design and mortality curves. (A) Study design (initial group of 30 males and groups of 30 females and 16 males added in 1994). Hash marks represent deaths. (B) Age-related mor-



tality. Animals that died from non-age-related causes are excluded. (C) Overall mortality. These curves depict data for animals that died from any cause.

**Fig. 3.** Effect of CR on age-associated disease. (A) Incidence of three major age-related conditions. Hash marks represent the age of diagnosis. Individual animals with multiple discrete diagnoses are represented multiple times. (B) Data represent the first occurrence of any age-related disease in each individual animal.



was reduced in animals on CR as compared to that of control animals, primarily due to a decrease in total body fat mass (11). The age-associated decline in muscle mass (sarcopenia) was also attenuated in animals exposed to CR (12). Dual-energy x-ray absorptiometry analysis of lean muscle mass throughout the study revealed the onset of sarcopenia at 15.5 years, with statistically significant maintenance of lean muscle mass in the animals on CR as compared to that of controls, which has been sustained in animals at old age.

Improvements in metabolic function conferred by CR, specifically insulin sensitivity, have been consistent and striking (9, 13). We found that improved glucose homeostasis was maintained and that diabetes was prevented by CR. Of the initial

38 control animals, 5 progressed to diabetes and an additional 11 were classed as pre-diabetic. In contrast, all animals on CR (even those with compromised metabolic function at baseline) showed no impairment of glucose homeostasis (Fig. 3A). These data are consistent with CR providing long-term health benefits in protection against diabetes.

The incidence of cancer increases with age in rhesus monkeys, and intestinal adenocarcinoma is the most commonly diagnosed cancer in these animals (14). The methods used to detect and determine the type of cancer are described (7). The incidence of neoplasia was reduced by 50% in the animals undergoing CR as compared to that in controls (Fig. 3A). The most common form of neoplasia was gastrointestinal adenocar-

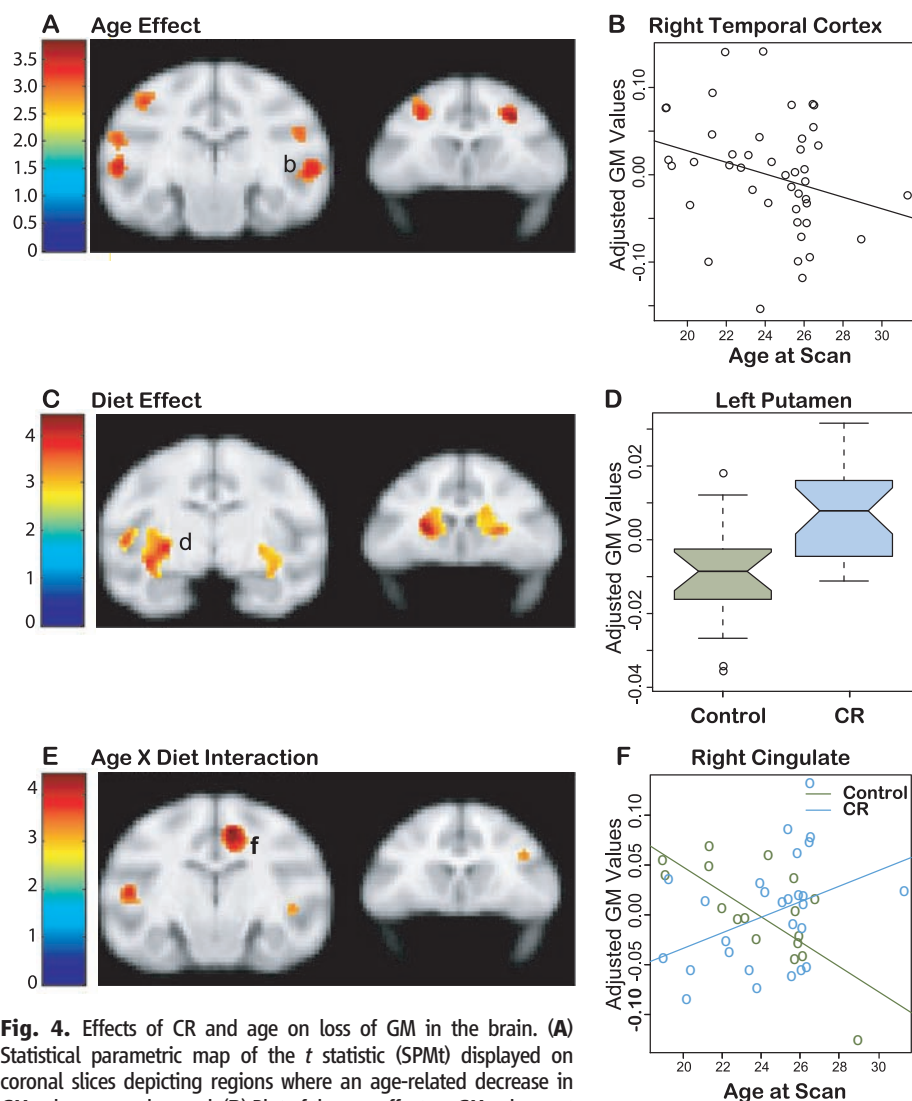
cinoma, which was identified in seven of the eight cases in the control animals and in two of the four cases in the animals on CR.

As it is in humans, cardiovascular disease is a prevalent age-associated disorder in rhesus monkeys. The methods used to diagnose cardiovascular disease are described (7). The most common diagnosis in living monkeys was leak of the mitral valve. The most frequently observed lesions at necropsy were valvular endocardiosis, cardiomyopathy, and myocardial fibrosis. The incidence of cardiovascular disease was reduced by 50% in the animals subjected to CR as compared to that in controls (Fig. 3A).

To assess the overall incidence of age-associated disease, we recorded the age at which animals experienced their first age-associated diagnosis. The diseases mentioned above and other clinical conditions, including diverticulosis and clinically relevant arthritis, were monitored. The effect of CR in reducing disease onset was statistically significant ( $P = 0.008$ , HR of 2.9). Age-related diseases were detected in control animals at about three times the rate they were detected in animals on CR (Fig. 3B). Animals on CR thus appear to be biologically younger than the normally fed animals.

Brain atrophy is a characteristic of human aging that is not accurately reproduced in smaller mammals (15). We therefore determined the regional effects of age, diet, and age by diet interactions on gray matter (GM) volume (16). There were several cortical regions (the bilateral frontal and temporal cortex) where decreases in volume with age were observed independent of diet (Fig. 4, A to C) (17). However, animals subjected to CR had statistically significant preservation of GM volume in subcortical regions (Fig. 4, D to F), including the caudate and putamen and the left insula. The examination of group differences in the slope of age-related GM atrophy (age by diet group interaction) reveals regions where CR significantly modified the aging effect (Fig. 4, G to I) in the midcingulate cortex, lateral temporal cortex bilaterally, and right dorsolateral frontal lobe, indicating relative preservation of volume with age in the CR group. Thus, CR reduced age-associated brain atrophy in key regions that subserve motor function and aspects of executive function.

Our data indicate that adult-onset moderate CR delays the onset of age-associated pathologies and promotes survival in a primate species. In two related nonhuman primate studies, the benefits of CR for health and longevity were less overt, possibly due to differences in study design (18, 19). Given the obvious parallels between rhesus monkeys and humans, the beneficial effects of CR may also occur in humans. This prediction is supported by studies of people on long-term CR, who show fewer signs of cardiovascular aging (20). The effect of controlled long-term CR on maximal life span in humans may never be known, but our extended study will eventually provide such data on rhesus monkeys.



**Fig. 4.** Effects of CR and age on loss of GM in the brain. (A) Statistical parametric map of the  $t$  statistic (SPMt) displayed on coronal slices depicting regions where an age-related decrease in GM volume was observed. (B) Plot of the age effect on GM volume at the labeled location in (A). (C) SPMt indicating regions where CR monkeys exhibited preserved volume relative to controls. (D) Notched box plots for each group at the location labeled in (C) indicating the mean (center line); the 95% confidence interval (notches); and the 5th, 25th, 75th, and 95th percentiles (horizontal lines), representing the range of variability in the data. (E) SPMt depicting regions where the slope between GM volume and age differs as a function of group. (F) Scatter plot of the location labeled in (E). All comparisons included sex and total brain volume as covariates. The probability threshold for each  $t$  contrast was  $P < 0.005$  (uncorrected). The color bars in (A), (C), and (E) represent the value of the  $t$  statistic. The left side of the brain is on the left in the images.

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Supporting Online Material

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References

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# Discovery of Swine as a Host for the *Reston ebolavirus*

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Since the discovery of the Marburg and Ebola species of filovirus, seemingly random, sporadic fatal outbreaks of disease in humans and nonhuman primates have given impetus to identification of host tropisms and potential reservoirs. Domestic swine in the Philippines, experiencing unusually severe outbreaks of porcine reproductive and respiratory disease syndrome, have now been discovered to host *Reston ebolavirus* (REBOV). Although REBOV is the only member of *Filoviridae* that has not been associated with disease in humans, its emergence in the human food chain is of concern. REBOV isolates were found to be more divergent from each other than from the original virus isolated in 1989, indicating polyphyletic origins and that REBOV has been circulating since, and possibly before, the initial discovery of REBOV in monkeys.

Filoviruses are associated with acute fatal hemorrhagic diseases of humans and/or nonhuman primates. The family consists of two genera: *Marburgvirus*, which comprises various strains of the *Lake Victoria marburgvirus* (MARV) discovered in 1967; and the antigenically distinct genus *Ebolavirus* discovered in 1976, which comprises five species including *Sudan ebolavirus* (SEBOV), *Zaire ebolavirus* (ZEBOV), *Ivory Coast ebolavirus* [also known as Cote d'Ivoire Ebola virus (CIEBOV)], *Bundibugyo ebolavirus* (BEBOV), and *Reston ebolavirus* (REBOV) (1). REBOV is the only member of the family thus far not associated with disease in humans (2).

Since the discovery of filoviruses more than 40 years ago, ostensibly random, sporadic, and fatal outbreaks of disease in primates have evoked

interest in delineation of host tropisms, potential reservoirs for disease transmission, and persistence in nature (3). These lines of investigation have recently identified African fruit bats as potential reservoirs for ZEBOV (4, 5) and MARV (6, 7). Similar links to bats have been found for emerging infections in swine and humans involving paramyxoviruses and the severe acute respiratory syndrome (SARS) coronavirus (8, 9).

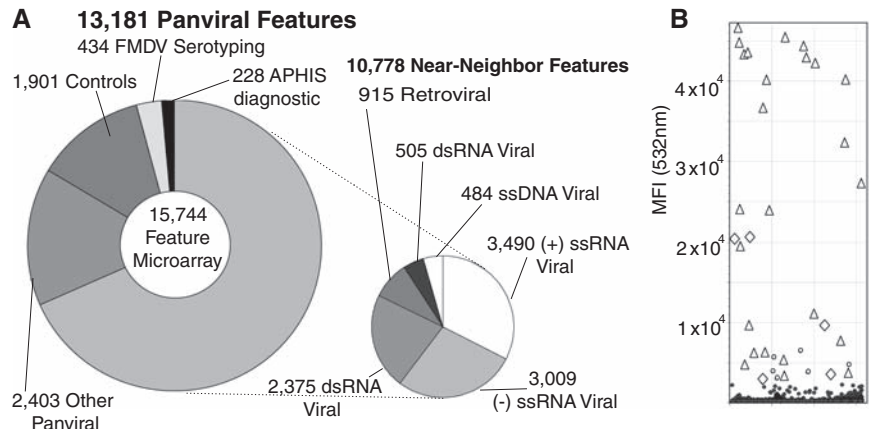
Until now, REBOV has only been associated with disease in nonhuman primates (2, 10). The virus was originally identified in 1989 in the United States from a shipment of cynomolgus monkeys (*Macaca fascicularis*) from the Philippines. Outbreaks of disease occurred in the United States in 1990 and 1996 and in Italy in 1992, which were traced back to a single facility in the Philippines (fig. S1) (11, 12). Here, we report the identification of REBOV infection in domestic swine co-infected with porcine reproductive and respiratory syndrome virus (PRRSV) that were experiencing a severe respiratory disease syndrome.

In July 2008, the Philippine Department of Agriculture requested the assistance of the U.S.

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**Fig. 1.** Detection of REBOV in swine samples from the Philippines. **(A)** Composition of the panviral microarray used to detect REBOV. The microarray feature composition is summarized with reference to the number of unique features for identification of viral pathogens. FMDV, foot-and-mouth disease virus. **(B)** Microarray analysis of Vero cell culture of a swine lymph node from sample group A identified multiple positive features within the genus of Ebola viruses. These features corresponded primarily to sequences from REBOV with minimal reactivity toward SEBOV and ZEBOV. MFI, mean fluorescence intensity (Δ) Positive *Reston ebolavirus* spp. features; (◇) positive *Ebolavirus* genus features; (○) non-*Ebolavirus* features; and (●) negative features.