COVER

THE MECHANISMS OF AGING

Studies of living cells and organisms identify genetic and molecular phenomena associated with physical and mental decline

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Never before have so many people lived so long. Of the babies born today, more than half will reach the age of 65 years, living an average of nearly two decades longer than those born in the middle of the last century. Increased longevity and reduced fertility are rapidly increasing the age of the global population. In *Developing in an Ageing World*, a United Nations (UN) report published in 2007, projections indicate that by 2050, there will be approximately 2 billion people aged 60 years and older on the planet (22 percent of the total population). In 2005, 670 million people were in that age category, representing 10 percent of the global population.

The global increase in life expectancy can cause problems, including a rapid increase in the size of the elderly population in many countries—including Brazil. In France, it took almost 150 years for the proportion of elderly individuals to increase from 10% to 20% of the overall population. During that time, the country grew richer, and living conditions improved. China, Brazil, and India will show a similar trend within 25 years (*see the chart on page 7*).

Today, there are 26 million elderly people in Brazil (12.5% of the population). According to projections by the Brazilian Institute of Geography and Statistics (IBGE), the elderly will represent 29% of the Brazilian population by 2050 (66 million people). "Brazil is aging and will face difficulties as a result," says Alexandre Kalache, a doctor and epidemiologist from Rio de Janeiro who headed the World Health Organization (WHO) Global Ageing and Health Program for 13 years and now presides over the Brazilian section of the International Longevity Center (ILC), a non-profit organization that investigates population aging and the strategies countries use to adapt to the so-called old-age revolution. "We already have problems with health, employment, education, and sanitation, and very soon, we will also have to deal with a population composed of many elderly people."

Illnesses associated with aging may become more common. At the same time, more people will remain healthy longer, subsequently changing the work and employment landscape, which will require more flexibility and adaptability from the public, businesses, and the state. "Cities will have to prepare for these new circumstances, creating policies for housing, transportation, social participation, work, and education that consider the elderly," warns the epidemiologist.

Concurrent with these changes, advances have been made in the field of aging over the last century at an unprecedented rate. A simple keyword search for *ageing* or *aging* on PubMed—one of the largest and most important databases of scientific articles related to health—results in approximately 384,000 papers on the subject from 1925 to 2016 (*see the graph on page 9*).

In an article titled *The Hallmarks of Aging*, published in the journal Cell in 2013, researchers in Spain and France presented a summary of the current knowledge regarding the cellular and molecular mechanisms—the deeper causes—of aging. This report revisits the main points of this topic and presents recent advances, including the contributions of Brazilian researchers.

GENES AND TIME

"Good genetics" is perhaps the biological factor most associated with long life. Experiments involving the manipulation of genes have significantly extended the life span of model organisms, including yeasts, flies, worms, and even mammals. Molecular intervention was successful in *Caenorhabditis elegans*, a 1-millimeter-long roundworm whose genome was sequenced in 1998. The worm, whose normal life span is two to three weeks, was able to live for 145-190 days after its genes were altered. The results of experiments on mice (*Mus musculus*) are more modest but equally positive. Genome alterations have been shown to extend rodents' life span by a year, from approximately two years to three years.

These results have led some molecular biologists and geneticists to argue that biological aging is a malleable process that can be controlled to some extent. "We can accelerate or slow down aging in animals," says Portuguese biologist João Pedro Magalhães, head of the Integrative Genomics of Ageing Group at the University of Liverpool in England. "The next step is to repeat this in human beings." According to Magalhães, studies of model organisms have already identified some 2,000 genes that are capable of regulating aging.

One of the strategies used in the pursuit of a longer and healthier life is to search for cellular and molecular mechanisms associated with good health in those who are extremely old. In 2015, Magalhães coordinated the genome sequencing of the bowhead whale (*Balaena mysticetus*), one of the longest-living mammals in the world. The DNA of this 18-meter-long and 100-ton cetacean from the Arctic could provide clues about how to prevent cancer and survive for as long as two centuries. The paper, published in *Cell Reports*, identifies alterations in a gene linked to thermoregulation that may be important to understanding the animal's low metabolism. A slower metabolism could explain how such a large mammal can live for up to three times as long as humans.

DNA from the longest-living individuals of our own species could also be a source of useful information in the fight against diseases associated with old age and the figurative ticking of our biological clocks. This is the goal of ambitious projects such as the Wellderly study, which was initiated in 2007 by the Scripps Research Institute in California. The project sequenced the complete genomes of 600 healthy elderly people (with no chronic diseases) aged between 80 and 105 years and compared them to those of 1,500 younger adults, with the first significant results being published in 2016.

The most significant difference between the healthy elderly group and the young adult group was that Wellderly participants presented a lower genetic risk of developing cognitive problems. In some healthy elderly people, variants of the *COL25A1* gene were identified that may protect against Alzheimer's disease. These individuals also had a low propensity for heart problems, although their genetic risk of tumors, type 2 diabetes, and stroke was equal to that of the control group.

DNA repair failures occur in diseases associated with aging, such as Alzheimer's disease

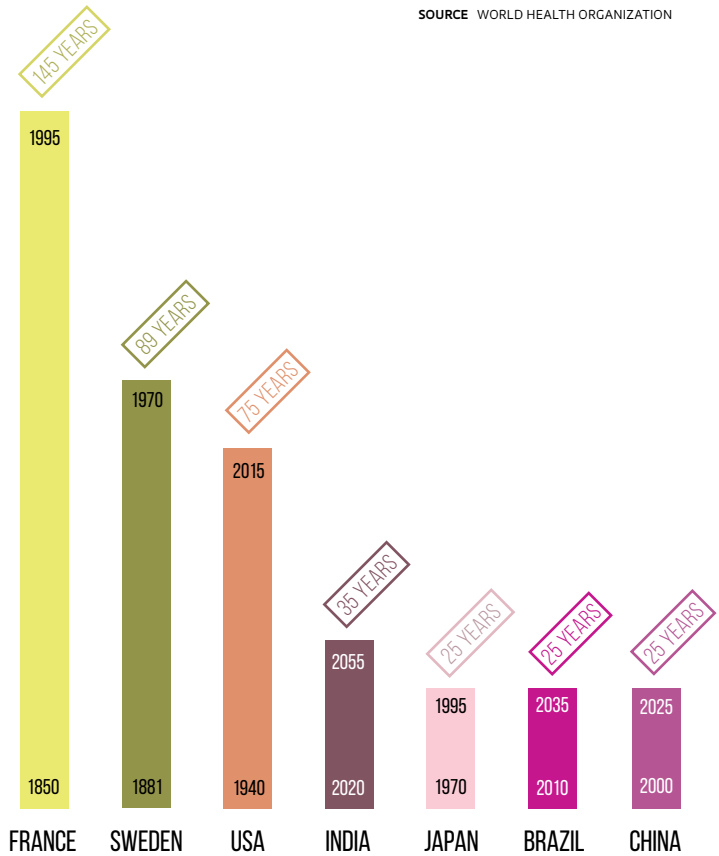
"It was surprising to see no difference in genetic risk for the development of cancers," says Ali Torkamani, director of genome informatics and drug discovery at Scripps. "We also know that there are genetic diseases that influence the speed of aging, usually accelerating it. But overall, aging is a complex process."

The Human Genome and Stem Cell Research Center at the University of São Paulo (CEGH-CEL-USP) is currently coordinating a Brazilian Wellderly project involving two elderly populations. The first of them includes more than 1,300 São Paulo residents who were over 60 years old when they participated in the epidemiological survey Health, Well-Being, and Aging (SABE), which has been conducted by the USP School of Public Health since 1999. The second population, the 80+ group, includes approximately 130 people in their eighties, all in good health, for DNA analysis.

The USP researchers sequenced the exome (the part of the genome that encodes proteins) of the elderly SABE respondents. The first re-

RAPIDLY AGING POPULATION

The chart below indicates the time taken for the proportion of elderly individuals to increase from 10% to 20% of the total population



sults from 609 participants were published in the journal *Human Mutation* in March 2017, demonstrating the unique mixture of populations that characterizes Brazil (African, indigenous, and European). The study identified 207,000 genetic variants that had never been described in international biological databases. "This shows the importance of studying our population," says geneticist Mayana Zatz, co-author of the study and coordinator at CEGH-CEL-USP, one of the Research, Innovation, and Dissemination Centers (CEPID) funded by FAPESP. Each elderly person had an average of 300 genetic alterations, most of which were harmless. Only seven individuals had mutations associated with disease, mostly some form of cancer.

In the coming weeks, geneticist Michel Naslavsky from the USP research center will travel to the United States to begin sequencing the genomes of the 1,300 SABE respondents and the 80+ group subjects. "It will be a long project," says Naslavsky, the first author of the article published in Human Mutation. The data produced by CEGH-CEL-USP are available on the Online Archive of Brazilian Mutations (ABRAOM).

WAYS TO PROTECT DNA

Most biologists and biochemists today accept the idea that organisms age and die because, over time, their cells lose the ability to perform their functions and die faster than they can be replenished.

Chemical reactions in an organism and environmental phenomena can cause lesions in DNA molecules at any time. Experiments conducted by Swedish biochemist Tomas Lindahl in the 1970s showed that the DNA of a human cell undergoes 10,000 small spontaneous alterations per day, almost once every 10 seconds. In the 3.6 billion years of life on earth, proteins have evolved to help genetic material remain intact, allowing cells to produce perfect copies of themselves and continue to exist.

However, nothing is perfect, and these repair mechanisms can fail. In a study on mice published in the journal *Nature* in 2007, researchers from the United States and the Netherlands proved that stem cells accumulate genetic defects over time and lose their ability to reproduce and maintain tissues unaltered and functioning. Further studies have shown that the same process occurs in human cells, including in syndromes associated with accelerated aging, such as progeria.

At the USP Institute of Biomedical Sciences (ICB-USP), molecular biologist Carlos Menck and his team investigate the causes of genetic alterations that prevent the proper repair of genetic material. For several years now, they have monitored people with the hereditary disease *xeroderma pigmentosum* (*see* Pesquisa FAPESP, *issue No. 199*). Sufferers develop skin cancer very easily when exposed to the sun because their cells do not repair the damage caused by ultraviolet radiation. Some may also experience neurological problems and other symptoms similar to those seen in accelerated aging syndromes, which in some cases can lead to death in the first year of life. Faults in these same genes lead to the delayed physical and mental development observed in Cockayne syndrome.

Years ago, Menck began collaborating with a former student, Brazilian biologist Alysson Muotri of the University of California San Diego (UC San Diego), to study phenomena that may affect the neurons of people with Cockayne syndrome. They used chemical compounds to regress skin cells collected from these people to the stem cell stage, from which they can create

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other tissues. They then stimulated the cells to transform into neurons and observed that they formed irregular connections with other cells. "The defects observed in the lab-created neurons could at least partially explain the origin of the neurological problems suffered by these individuals," says Muotri, one of the authors of the article published in the journal *Human Molecular Genetics* in 2016.

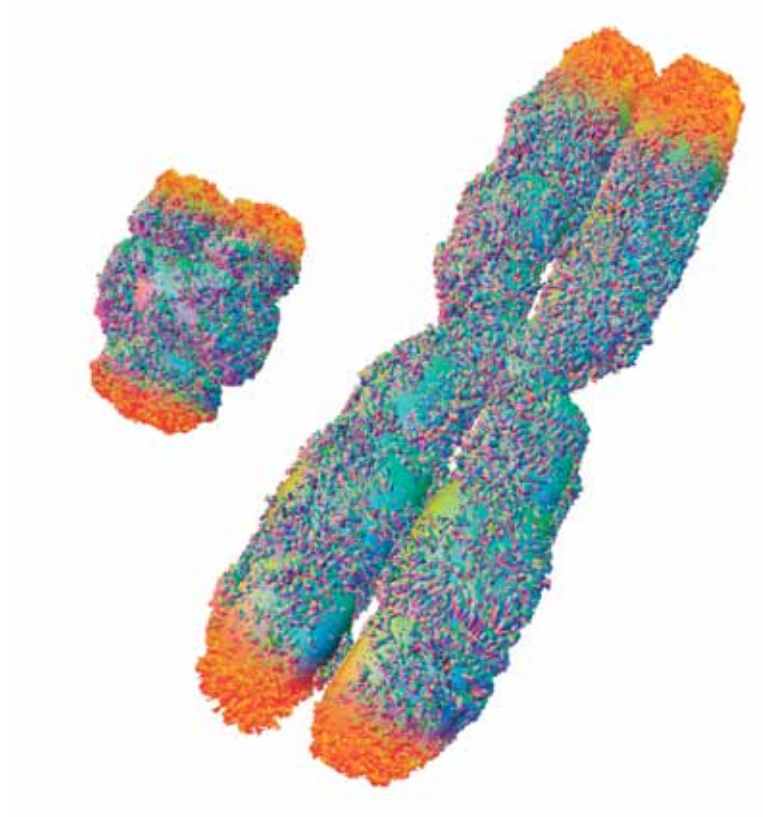
Menck and Muotri also found that these neurons accumulated reactive oxygen species, or free radicals, compounds containing a form of oxygen that easily interacts with and damages DNA and proteins. They suspect that these molecules are produced in defective versions of the mitochondria that are responsible for the production of energy in cells. "We believe that this is the connection to progeria, since high levels of reactive oxygen species have already been linked to aging," says Muotri. "We are now trying to reverse that process using antioxidant compounds."

At USP, Menck is working to amplify the damage that reactive oxygen species inflict on the genetic material of people with Cockayne syndrome while trying to determine which parts of the cellular machinery these damages affect. If this strategy is able to replicate the effects of Cockayne syndrome, Menck and Muotri will have a model of accelerated aging that can be used to understand the process of aging in healthy people.

Problems in DNA repair also occur in other diseases associated with aging, such as Alzheimer's disease, which is more common in people over 80 years old. At the USP campus in Ribeirão Preto, geneticist Elza Sakamoto Hojo and her team have been analyzing the efficiency of DNA repair in people with and without Alzheimer's disease. They collected blood samples from 13 people aged 65 to 90 years who suffer from the disease (and from 14 people who do not) and subjected cells to high concentrations of reactive oxygen species using hydrogen peroxide, simulating conditions of stress. In an article published in the *International Journal of Molecular Sciences* in 2013, the authors show that the Alzheimer's cells took three times as long to recover from the free-radical bath as those from the healthy participants.

SHORTENED TELOMERES

During the life of a cell, genetic damage does not occur uniformly throughout the whole DNA molecule; it more commonly affects the two ends, in regions known as telomeres. These segments of genetic material serve to protect the rest of the DNA strand (their role has been compared to the plastic tip on the end of a shoelace). Each time the genetic material doubles and the cell divides, the telomeres become shorter by approximately 2%. Only one enzyme, telomerase, is capable of restoring telomere length. In mammals, however, most adult cells do not produce telomerase, which is usually synthesized by stem cells. With a restricted capacity for recovery, telomeres shorten with age. Researchers at Harvard University have shown that telomeres can be artificially lengthened by introducing extra copies of the telomerase gene into cells. But this strategy can be risky, with tumors sometimes becoming malignant after the production of the telomerase enzyme has been reactivated.



Artistic representation of two chromosomes, the structures that package DNA into cells. The telomeres (*in orange*) protect the ends of the chromosomes

In some diseases, telomeres become shortened faster than normal. One example is dyskeratosis congenita, a rare disease characterized by difficulties in producing blood, skin, and lung tissue cells, which can lead to accelerated aging, as seen in progeria. It has been known for some time that the telomeres of dyskeratosis sufferers are shorter than normal. Brazilian biologist Luis Francisco Batista, a former student of Menck and a professor at Washington University in Saint Louis, confirmed the cause: failure in the proper functioning of telomerase.

Using skin cells from people with dyskeratosis, he generated stem cells and found more severe forms of the disease associated to a greater inability to produce active telomerase. Since publishing his results in *Nature* in 2011, Batista has dedicated himself to studying how a lack of telomerase and the shortening of telomeres af-

ALFRED PASIEKA / SCIENCE PHOTO LIBRARY

fect the number of stem cells stored in tissues. "We are trying to uncover the chain of events that follows," says Batista.

EXHAUSTION WITHOUT REPLACEMENT

In the elderly, there is an accumulation of cells that have reached the end of their life cycle and have lost the ability to copy their own DNA and generate clones of themselves. This symptom of aging has a positive impact: cells that do not multiply can be eliminated by the immune system, preventing the development of tumors.

The problem is that the body's ability to defend itself against external threats, such as viruses and bacteria, is also diminished, affecting the efficacy of vaccines. "In Japan, which has a large elderly population, they have tested the effects of administering three smaller doses of the influenza vaccine instead of just one," says biologist Valquiria Bueno, a professor at the School of Medicine of the Federal University of São Paulo (UNIFESP).

Bueno, an expert in immunosenescence, compared the production of defense cells in six men and six women aged 88 to 101 years (participants of the SABE survey) with that of university students under the age of 30 years. The generation of leukocytes (a type of defense cell) in bone marrow was 40% lower in the elderly on average, which is similar to the findings of international studies. In addition to these findings, which were presented in 2016 in a book titled *The Ageing Immune System and Health*, the blood of these elderly participants was also found to exhibit increased production of another type of cell that can reduce resistance to infections and promote the development of cancer.

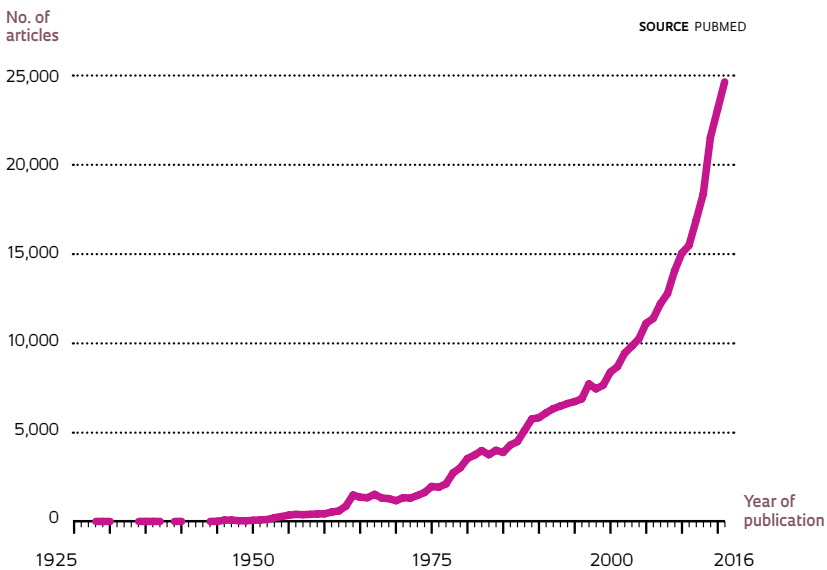
There are other more controversial approaches. Recent experiments on animals suggest that replacing old cells with new ones could delay aging or partially reverse deterioration in certain organs. Some of these studies use a controversial technique developed in the mid-nineteenth century called parabiosis, where a young rodent is surgically attached to an old one in order for the latter to receive a transfusion of new blood.

In 2013, a team led by Amy Wagers, a specialist in regenerative medicine at Harvard University, published an article in *Cell* describing how they used parabiosis to identify an increased level of a protein that can combat cardiac dysfunctions linked to old age in old mice that received blood from young mice. Further studies have reported the benefits of this method on brain and muscle tissues.

Last November, however, an article published in *Nature Communications* reported that the administration of new blood did not significantly improve biological parameters in old mice and that the health of the young rodents deteriorated as a result of receiving transfusions of old blood. "Our study suggests that new blood alone does not work as a treatment," said Irina Conboy, a professor at the University of California Berkeley (UC Berkeley) and the lead author of the paper. "It would be more accurate to say that there are inhibitors in the old blood that we need to mitigate in order to reverse aging."

GROWING SCIENTIFIC OUTPUT

The PubMed research database hosts approximately 384,000 studies on aging published from 1925 to 2016



DAMAGE TO THE ENERGY CENTERS

For many years, mitochondria were regarded as the villains of aging. In 1956, American chemist and physician Denham Harman, at the time a researcher at UC Berkeley, proposed that one cause of the deterioration and death of cells could be the production of free radicals. He suspected that these molecules were able to interact with and damage DNA, proteins, and other cell components. Later experiments supported Harman's arguments and even led some researchers to recommend avoiding physical exercise, which increases energy consumption and cellular respiration. The opinion today is quite different.

Over the last two decades, experiments have indicated that free radicals play a dual role in cells. At low concentrations, they induce the production of antioxidant compounds, protect cells from aging, and even stimulate cell proliferation. At high levels, however, they cause cell death.

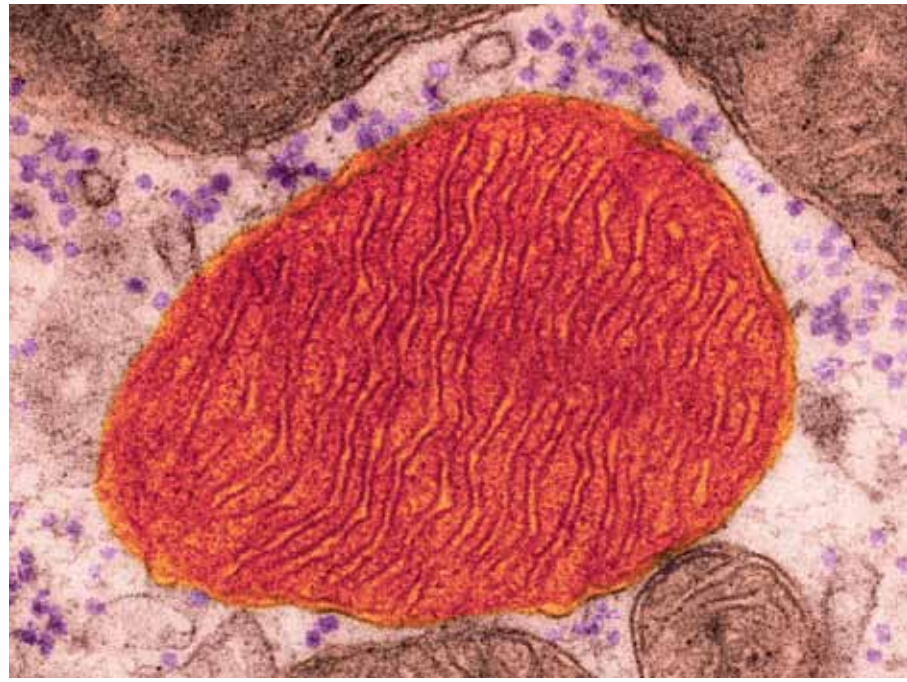
In the late 1970s, during a postdoctoral fellowship at Johns Hopkins University, biochemist and physician Aníbal Vercesi noticed that certain conditions created pores on mitochondria membranes, killing them. Later, after returning to the University of Campinas (UNICAMP) where he is a professor, he found that this effect is due to an increased concentration of free radicals.

In experiments conducted with his team—including biochemists and physicians Roger Castilho and Alicia Kowaltowski, then doctoral students and now professors at UNICAMP and USP, respectively—Vercesi found that inside the mitochondria, the accumulation of calcium stimulates the over-production of free radicals, leading to cellular damage. Proteins, genetic material, and free radicals escape through the pores that open in the membrane of the mitochondria. "We proposed this hypothesis in 2001," says the biochemist. "Today, it is widely accepted and even used to explain the damage that occurs during heart attacks and strokes, as well as the development of age-related illnesses such as diabetes and Alzheimer's."

Biochemist Nadja de Souza Pinto, a former doctoral student of Vercesi's and now a professor at USP, studies the consequences of excessive free radical production in mitochondrial DNA. While working at the National Institute on Aging in the United States, she studied the brains of people with Alzheimer's and observed that repair of the DNA lesions caused by free radicals is less efficient in people with more severe symptoms. Back in Brazil, she is working with geriatrician Wilson Jacob Filho and gerontologist José Marcelo Farfel, both from USP, to evaluate DNA repair in the mitochondria of two groups: people with typical Alzheimer's and asymptomatic carriers who do not develop cognitive problems. In studies on rats, Pinto found that mitochondrial DNA repair increased until midlife and then deteriorated. "We are proposing that the low activity of these repair mechanisms may be a risk factor for Alzheimer's," she says.

EAT LESS AND LIVE LONGER

The effect of diet on the life spans of different organisms may be the longest-studied topic related to aging. For nearly a century, we have known that reducing the amount of energy consumed by an animal prolongs its life span. In 1933, American biochemist and gerontologist Clive McCay, a researcher at Cornell University, published a short article in *Science* comparing the longevity of the rats studied by his team in New York with the rats kept in the laboratory of physiologist James Slonaker at Stanford University in California.



Mitochondrion, a cellular organelle that converts nutrients into energy, as observed under a microscope

McCay's rodents, fed a more nutritious diet, grew and reached sexual maturity faster, but they only lived half as long as the rats from Slonaker's laboratory, which gained weight and matured more slowly and lived for an average of 1,200 days. "It is possible that longevity and rapid growth are incompatible and that the best chance for an abnormally long life span belongs to the animal that has grown slowly and attained a late maturity," argued McCay, hypothesizing that reduced calorie intake would favor longevity to the detriment of reproductive capacity.

For the next 50 years, aging was understood as inevitable and inherent to life. This view began to change in the 1990s with the findings of molecular biologist Cynthia Kenyon. A professor at the University of California San Francisco (UC San Francisco) and vice president of aging research at Calico, a company founded by Google, Kenyon found that alterations in a certain gene doubled the life span of the *C. elegans* roundworm without affecting its fertility.

It was later discovered that this gene encoded a cell-surface protein—a receptor—to which insulin-like peptides are connected. This receptor functions as a nutrient sensor in the extracellular environment. "These advances have sparked a race to study caloric restriction from a molecular perspective," says biomedical scientist Marcelo Mori from UNICAMP, who investigates life

span-increasing mechanisms that are activated by caloric restriction and physical exercise.

One such mechanism is the production of microRNAs, molecules that modulate the functioning of genes and the production of proteins. In studies on mice during his postdoctoral fellowship at Harvard that continued at UNIFESP and UNICAMP, Mori found that the main source of circulating microRNAs in mammals is adipose tissue (where fat is stored) and that microRNA production decreases with age. In experiments with mice and *C. elegans*, he also found that caloric restriction increases life span by boosting Dicer enzyme activity, which transforms long RNA molecules into microRNAs (*see* Pesquisa FAPESP, *issue No. 212*). "Aging decreases the production of the Dicer enzyme and microRNAs and reduces life span, but caloric restriction does the opposite," says Mori.

At USP, Alicia Kowaltowski and her team are interested in how reduced calorie intake affects the functioning of mitochondria. In an article published this year in the journal *Mechanisms of Ageing and Development*, the researchers noted that in animals fed a restrictive diet, mitochondria are more elongated, less damaged, and replaced more quickly than those in animals fed a normal diet. Previous experiments presented in *Aging Cell* in 2016 indicated that caloric restriction improves the functioning of mitochondria in neurons and increases their resistance to cellular stress, such as increased levels of calcium and free radicals. According to another study by the group, eating less also improves the functioning of insulin-producing cells in the pancreas, protecting the body against diabetes, a disease associated with aging.

IMAGE DENNIS KUNKEL MICROSCOPY / SCIENCE PHOTO LIBRARY **PHOTO** LÉO RAMOS CHAVES

Although these findings are encouraging, whether they can be applied to human health remains unknown. "It is difficult to transfer the results of animal models to humans," says Kowaltowski. In the laboratory, animals are protected, sedentary, and eat at will, which makes them obese compared to those living in the wild. "Even people who live sedentary lives perform physical activity and they do not feed continuously," says the researcher. "In humans, it is possible that simply maintaining a healthy weight is equivalent to caloric restriction in laboratory animals," she says.

Marcelo Mori from UNICAMP believes that it is not feasible for most humans to maintain radical caloric restriction throughout their entire lives without damaging their health. He proposes searching for pharmacological or dietary interventions that mimic the effects of caloric restriction in a safe and less demanding way together with regular physical activity, which also seems to increase average life span and is more easily adopted. "Despite recent advances," Mori says, "the fact remains that we are still a long way from proposing viable strategies for increasing the longevity of human life." ■

Projects

1. CEGH-CEL – Human Genome and Stem Cell Research Center (No. 13/08028-1); Grant Mechanism Research, Innovation, and Dissemination Centers (CEPID); **Principal Investigator** Mayana Zatz (USP); **Investment** R$26,897,714.59.

2. Consequences of lesion repair deficiencies on the genome (No. 14/15982-6); **Grant Mechanism** Thematic Project; **Principal Investigator** Carlos Frederico Martins Menck (USP); **Investment** R$2,451,302.99.

3. Genomic instability and molecular signaling involving responses to DNA damage and repair (No. 13/09352-7); **Grant Mechanism** Regular Research Grant; **Principal Investigator** Elza Tiemi Sakamoto Hojo (USP-RP); **Investment** R$624,252.12.

4. Energy metabolism, redox state, and mitochondrial functionality in cell death and in cardiometabolic and neurodegenerative disorders (No. 11/50400-0); **Grant Mechanism** Thematic Project; **Principal Investigator** Aníbal Eugênio Vercesi (UNICAMP); **Investment** R$3,019,922.94.

5. Dicer, miRNAs and the control of mitochondrial function in the context of aging and caloric restriction (No. 15/01316-7); **Grant Mechanism** Regular Research Grant; **Principal Investigator** Marcelo Alves da Silva Mori (UNICAMP); **Investment** R$292,429.97.

6. Bioenergetics, ion transport, redox balance, and DNA metabolism in mitochondria (No. 10/51906-1); **Grant Mechanism** Thematic Project; **Principal Investigator** Alicia Juliana Kowaltowski (USP); **Investment** R$2,210,658.64.

7. Study of cellular responses to mitochondrial DNA damage in the cells of mammals (No. 08/51417-0); **Grant Mechanism** Regular Research Grant; **Principal Investigator** Nadja Cristhina de Souza Pinto (USP); **Investment** R$292,654.45.

8. Evaluation of myeloid-derived suppressor cells in the elderly: Brazilian and British population (No. 14/50261-8); **Grant Mechanism** Regular Research Grant; University of Birmingham Agreement; **Principal Investigator** Valquiria Bueno (UNIFESP); **Investment** R$64,197.47.

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