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The nutrient-sensing TOR (target of rapamycin) pathway is involved in cellular and organismal aging. Rapamycin, an inhibitor of TOR, extends lifespan in yeast, fruit flies and genetically heterogeneous mice. Here, we demonstrate that lifelong administration of rapamycin extends lifespan in female 129/Sv mice characterized by normal mean lifespan of 2 y. Importantly, rapamycin was administrated intermittently (2 weeks per month) starting from the age of 2 mo. Rapamycin inhibited age-related weight gain, decreased aging rate, increased lifespan (especially in the last survivors) and delayed spontaneous cancer. 22.9% of rapamycin-treated mice survived the age of death of the last mouse in control group. Thus we demonstrated for the first time in normal inbred mice that lifespan can be extended by rapamycin. This opens an avenue to develop optimal doses and schedules of rapamycin as an anti-aging modality.

Introduction

Until very recently, the prospect of pharmacological deceleration of mammalian aging appeared remote.[1] In 2009, it was demonstrated that rapamycin, known as an immunosuppressant, extended lifespan in mice when it was fed to them late in life.[2] Yet this result was not unanticipated.[3] The target of rapamycin (TOR) pathway is involved in senescence of yeast[4-7] and mammalian cells in culture.[8-16] Rapamycin decelerates yeast chronological senescence[5] and suppresses the conversion of cell cycle arrest into senescence in human cells.[8-10,16] TOR accelerates aging in diverse organisms, from worms to mammals.[17-25] In mammals, mTOR (mammalian TOR) controls cellular mass growth, functions and metabolism in response to nutrients, hormones, cytokines and growth factors.[26-28] Rapamycin decelerates senescence in normal[9] and progeric[29] human cells. mTOR is also involved in age-related diseases, such as atherosclerosis, metabolic syndrome, osteoporosis, neurodegeneration and macular degeneration.[30-33] Thus, the mTOR pathway links cellular senescence, organismal aging and diseases of aging.[34]

Remarkably, rapamycin is a clinically approved drug that has been used for a decade in renal transplant patients. Therefore, it was suggested that rapamycin could be used for extension of healthy lifespan and prevention of age-related diseases by slowing down the aging process.[3] This may become one of the major breakthroughs in medicine since the discovery of antibiotics. The main concern, however, is that rapamycin may exert undesirable effects precluding both its lifelong use and life extension. Therefore, it is important to investigate lifelong use of rapamycin in defined strains of mice to develop regiments (doses and schedules) that extend lifespan. Previously, we have demonstrated that lifelong administration of rapamycin in an intermittent fashion (every other 2 weeks) extends lifespan in short-lived cancer-prone mice.[35] However, one may argue that this life extension is due to cancer prevention in short-lived mice. Here, we present a 2-year-long study in inbred 129/Sv mice that received rapamycin intermittently (three times a week for 2 weeks, followed by a 2 week break) starting from the age of 2 mo. 129/Sv mice have a normal lifespan and cancer incidence and are most often used to produce knockout mice. We found that rapamycin significantly increased lifespan and delayed spontaneous cancer. This is the first report of extension of lifespan by rapamycin in normal inbred mice as well as the first report on lifelong administration of rapamycin.

Results

Treatment with rapamycin significantly inhibited age-related weight gain in female mice (**Fig. 1A**). While in control, mice constantly gained weight during their lifespan; mice that received rapamycin demonstrated a significantly smaller weight increase. As a result, from the 5th to 23rd months, body weight was increased by 21.9% and by 12.4% in the control and rapamycin-treated group, respectively. The body weight in the rapamycin-treated

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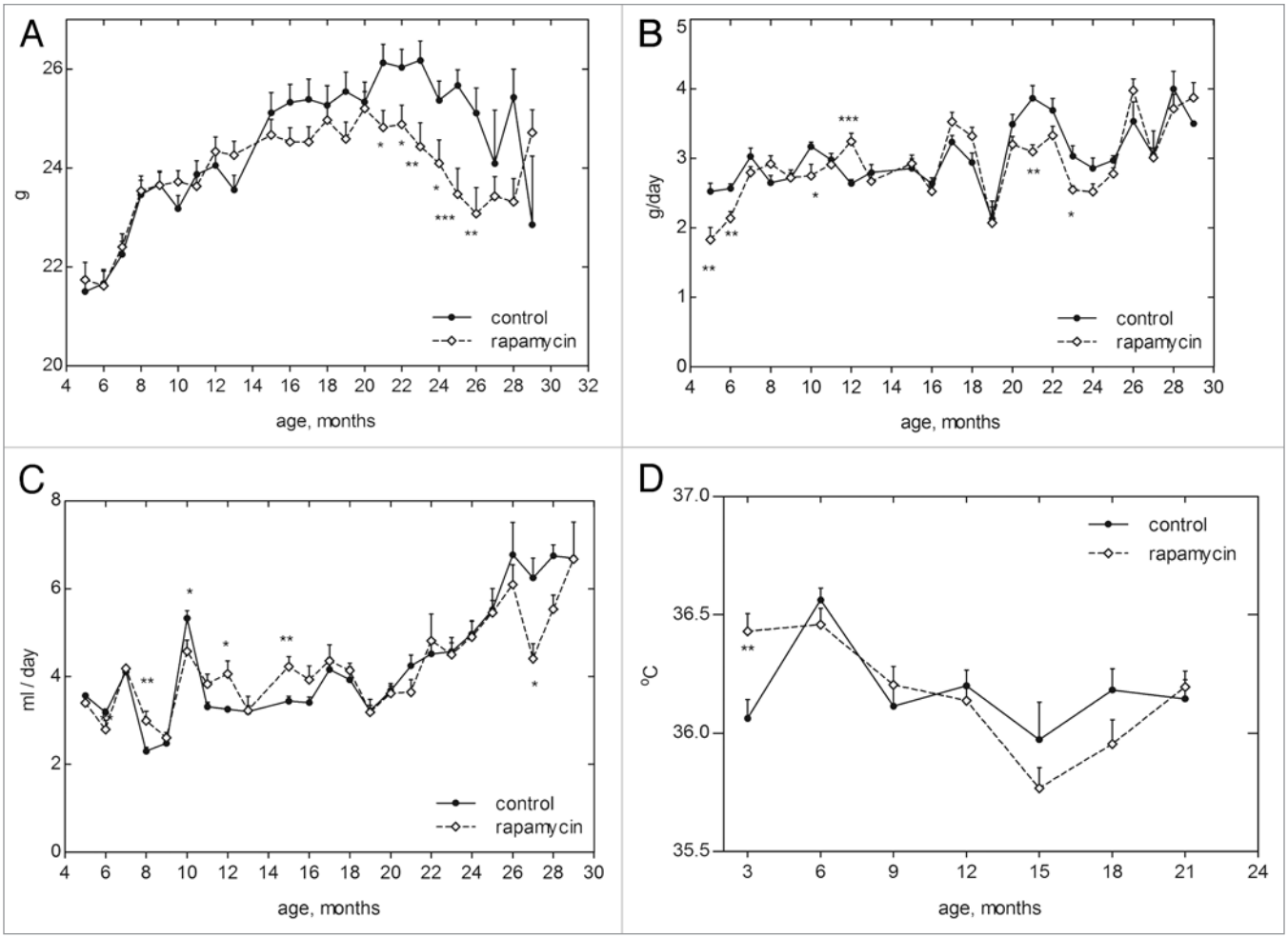

Figure 1. Effects of rapamycin weight gain, food consumption and body temperature in female 129/Sv mice. (A) Body weight. (B) Food consumption. (C) Water consumption. (D) Body temperature.

animals was significantly less as compared with the control between the age of 20 and 27 mo (**Fig. 1A**). Rapamycin slightly affected food consumption in young mice and decreased food consumption by 23% in old mice (**Fig. 1B**). Rapamycin did not affect water consumption (**Fig. 1C**) and insignificantly decreased body temperature (**Fig. 1D**). There was no significant difference in age-related dynamics of the length of estrous cycle and in the ratio between the estrous cycle phases in the control and rapamycin-treated groups. However, at the age of 18 mo, 46% and 65% mice had regular estrous cycle in control and rapamycin-treated groups, respectively (**Table 1**).

The total incidence of chromosome aberrations (ChA) in bone marrow cells and fragments was increased in 26-mo-old treated and non-treated mice compared with 4-mo-old controls. Levels of total ChA were higher in old rapamycin-treated mice than in 26-mo-old controls (**Table 2**).

Most importantly, in control, 11 mice survived until the age of 800 d (35.5%) compared with 19 (54.3%) in the rapamycin-treated group ($p > 0.05$; **Table 3**), while survival until the age of 900 d was 9.7% in control and 31.4% in the rapamycin-treated group ($p < 0.01$, $t = 3.44$). Eight female mice exposed to rapamycin survived the age of the death of the last mouse in the control group (22.9%).

Treatment with rapamycin shifted the survival curve to the right (**Fig. 2A**) and increased the mean lifespan by 7.8% of the last 10% survivors. Rapamycin also increased the median lifespan by 10.1% as well as the maximum lifespan by 9.3% by increasing the average lifespan of the long-lived mice, decreasing initial mortality (β) when treatment began in midlife and decreasing the Gompertz parameter α (increases MRDT) (**Table 4**). According to the Fischer exact test for count data, rapamycin significantly decreased the number of tumor-bearing mice (p-value = 0.0004578) (**Table 5**). Rapamycin also shifted the tumor-yield curve to the right (**Fig. 2B**) and prolonged mean lifespan of long-lived tumor-bearing mice.

According to the log-rank test for the conditional lifespan distributions (given all groups survived the age of 130 d), the difference is significant between treated and untreated groups of mice (**Table 6**). According to the estimated parameters of the Cox regression model, rapamycin treatment decreases the relative risk of death compared with the control group (**Table 7**). The log-rank test for the conditional lifespan distributions shows

Table 1. Effect of rapamycin on parameters of estrous function in female 129/Sv mice

Age (months)	Number of mice	Length of estrous cycles (days)	Rate of estrous cycles of various length			Fraction of mice with regular estrous cycles (%)
			< 5 days	5–7 days	> 7 days	
Control						
3	30	5.7 ± 0.19	19	74	7	100 ± 0
6	29	5.3 ± 0.20	24	66	10	79 ± 7.5
9	29	6.1 ± 0.33	22	53	25	96 ± 3.4
12	29	5.6 ± 0.24	12	82	6	45 ± 9.2
15	26	6.0 ± 0.49	17	67	17	39 ± 9.5
18	24	6.2 ± 0.41	8	62	31	46 ± 10.2
21	21	6.4 ± 0.49	14	64	21	48 ± 10.9
Rapamycin						
3	30	5.7 ± 0.24	16	70	14	90 ± 5.5
6	27	5.9 ± 0.32	17	71	13	63 ± 9.3
9	26	5.8 ± 0.29	21	69	10	77 ± 8.1*
12	26	6.8 ± 0.32	8	71	21	42 ± 9.7
15	26	6.6 ± 0.33	0	77	23	39 ± 9.5
18	26	6.5 ± 0.31	14	59	27	65 ± 9.4
21	26	7.3 ± 0.47	0	66,7	33	46 ± 9.8

Significant in comparison with control, *p<0,05.

Table 2. The incidence of chromosome aberrations (ChA) in bone marrow cells in female 129/Sv mice treated or non-treated with rapamycin

Parameter	Intact control (4 mo.)	Control (26 mo.)	Rapamycin (26 mo.)
Total incidence of ChA (%)	14.2 ± 1.06	23.1 ± 1.60[a]	28.1 ±1 .70[ab]
Incidence of single bridges (%)	7.1 ± 0.9	7.7 ± 1.70	15.6 ± 2.80[ab]
Incidence of multiple bridges (%)	7.3 ± 1.2	11.5 ± 2.3	9.4 ± 1.70
Incidence of fragments (%)	0	3.8 ± 0.90[a]	3.1 ± 0.70[a]

[a]The difference with the intact controls (4-month-old) is significant, $p < 0.05$.
[b]The difference with the controls (26-month-old) is significant, $p < 0.05$. There were four mice in each group.

Table 3. Effect of rapamycin on survival distribution in female 129/Sv mice

Group	Number of survivors at the age of: (days)										
	100	200	300	400	500	600	700	800	900	1000	1100
Control	31	30	30	30*	28	26	20	11	3	0	0
Rapamycin	35	32	32	31*	30	28	25	19	11a	2	0

*The first tumor detected in this interval. [a]The difference with the controls of corresponding age is significant, $p < 0.05$; Fischer exact test.

the significant difference in tumor-bearing between treatment and control groups. According to the estimated parameters of the Cox regression model, rapamycin decreased risk of death in the group of tumor-bearing mice. The increase in lifespan was observed only in the tumor-bearing cohort.

Mice survived the age of 435 d (effective mice). Taking into account the effective number of mice, we consider only those who survived the age of 435 d (the age of the first tumor detection). Rapamycin significantly increased the mean lifespan of long-lived mice. As for the Gompertz model, the initial risk of death (evaluated as parameter β) was 2.5 times lower in the rapamycin-treated group as compared with the controls, whereas the rate of aging (α) was 1.4 times lower. According to the log-rank test for the conditional lifespan distributions, the difference is significant between treated and control groups. According to the estimated parameters of the Cox regression model, rapamycin decreased the relative risk of death. According to the Fischer exact test for count data, the number of tumor-bearing mice under the rapamycin treatment drops significantly (p-value = 0.0007602). Mean lifespan of tumor-bearing mice (as well as long-lived mice) increased under rapamycin treatment. Rapamycin treatment significantly decreased the relative risk of death for tumor-bearing mice (**Table 7**).

Discussion

Evidence is emerging that mTOR is involved in cellular senescence, organismal aging and age-related diseases.[3] Rapamycin is

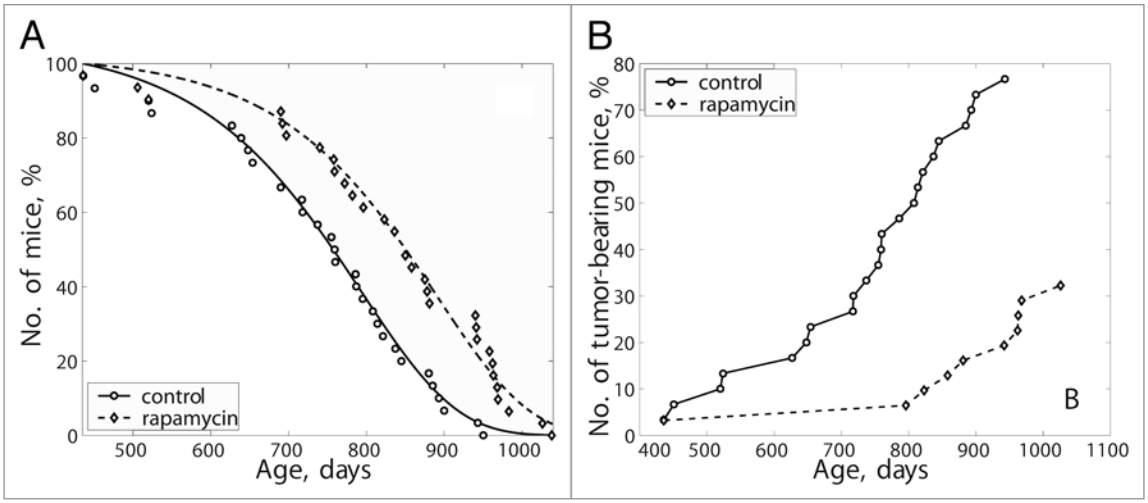

Figure 2. Effects of rapamycin on lifespan and spontaneous carcinogenesis. (A) Effect of rapamycin on mice survival. (B) Effect of rapamycin on tumor incidence.

Table 4. Parameters of the lifespan in female 129/Sv mice treated and non-treated with rapamycin

Parameters	Control	Rapamycin	Ratio
Number of mice	31	35	
Mean lifespan (LS), days	723 ± 30.6	754 ± 43.0	4.3%
Median	759	836	0.1%
Mean LS of last 10% survivors	931 ± 15.6	1004 ± 16.6[a]	7.8%
Maximum lifespan, days	950	1038	9.3%
Initial mortality rate (β), days^{-5}	3.6 (3.0; 3.9)	8.4[a] (7.9; 8.8)	2.3 times
Aging rate (α), days^{-3}	8.2 (7.8; 9.1)	5.9[a] (5.0; 6.1)	-1.4 times
MRDT, days	85.0 (75.9; 88.5)	118.2[a] (113.6; 139.4)	1.4 times

The difference with the control of the same sex: [a]$p < 0.05$; [b]$p < 0.001$.

a clinically approved drug used in high doses in combinations with other immunosuppressants in patients after organ transplantations. Here, we demonstrated that lifelong administration of rapamycin extends lifespan and postpones cancer in an inbred stain of female mice with normal lifespan. Cancer is an age-related disease, and conditions that slow down the aging process are associated with decreased cancer incidence.[36-40] In comparison, calorie restriction (CR), the proven modality that slows down aging, postpones cancer in mice.[38-42] Since nutrients activate the nutrient-sensing mTOR pathway, CR deactivates mTOR.[41] In this sense, rapamycin could be considered as "pharmacological CR." Also, metformin (an anti-diabetic drug that is an indirect inhibitor of the mTOR pathway) moderately increased lifespan in female mice.[43-45] In cancer-prone rodents, metformin delayed cancer, prolonged lifespan or both.[43-48]

In our study, mice received high doses of rapamycin from the age of 2 mo. Therefore, a certain overdose of rapamycin may moderate a lifespan extension because of an increased mortality at a young age. In theory, anti-aging agents should decrease fitness in young animals, especially in young males.[49] Still, even taking this into account, our study demonstrated that lifelong administration of rapamycin significantly prolonged lifespan and dramatically decreased spontaneous carcinogenesis in 129/Sv female mice.

Further investigations of a low-dose or an intermittent administration of rapamycin starting from an older age are warranted. Use of inbred strains of mice will allow one to compare results and to choose the most beneficial schedules of rapamycin administration. Furthermore, combinations of rapamycin with metformin, resveratrol, lipid-lowering agents, diet and physical exercise may afford additional benefits.

Material and Methods

Animals and experimental design. Inbred 129/Sv female mice, originally obtained from Jackson Laboratory by the International Agency for Research on Cancer (IARC), were housed and bred in the Department of Carcinogenesis and Oncogerontology, N.N. Petrov Research Institute of Oncology.[44] The animals were kept 5–7 in polypropylene cages (30 x 21 x 10 cm) under a standard light/dark regimen (12 h light: 12 h darkness) at temperature 22 ± 2°C and received standard laboratory chow[43,50] and tap water ad libitum. Animals were checked daily by animal care personnel and weekly by a veterinarian. All studies were conducted in accordance with ethical standards and according to national and international guidelines and have been approved by the authors' institutional review board.

Table 5. Parameters of the tumorigenesis in female effective 129/Sv mice treated and non-treated with rapamycin

Parameters	Control	Rapamycin	Ratio
The age of the 1st tumor detection, days	436	436	0
Effective number of mice	30	31	
Mean LS of effective mice, days	742 ± 24.8	829 ± 27.0[b] (+10.5%)	10.5%
Initial mortality rate (β), days^{-5}	44.9 (42.8; 46.2)	18.1 (15.3; 19.1)[a]	-2.5 times
Aging rate (α), days^{-3}	8.1 (7.8; 9.1)	5.9[a] (5.0; 6.1)	-1.4 times
MRDT, days	85.0 (75.9; 88.5)	118.3[a] (113.6; 139.4)	1.4 times
Number of tumor-bearing mice	23 (76.7%)	10 (32.3%)[b]	-2.4 times
Number of tumors	27	11	-2.4 times
Mean LS of tumor-bearing mice, days	732 ± 29.8	866 ± 52.9	18.3%
Number of tumor-free mice	8	25	
Mean LS of tumor-free mice, days	698 ± 85.8	710 ± 54.3	1.7%
Localization of tumors			
Uterus	23	9[b]	-2.6 times
Ovary	3	2	
Liver	1	0	

The difference with the control, [a]$p < 0.05$; [b]$p < 0.001$.

Longevity study. Sixty six female 129/Sv mice at the age of 2 mo were randomly divided into two groups. The first group of animals received 1.5 mg/kg rapamycin (LC Laboratories) subcutaneously (s.c.) three times a week for a period of 2 weeks, followed by 2 weeks without rapamycin. Mice in the second group received s.c. 0.1 of solvent without rapamycin and served as a control. Rapamycin was dissolved in 95% ethanol and then diluted with apyrogenic sterile water to a final concentration of 38 μg in 0.1 ml of 2% ethanol.

Once a month, simultaneously with weighing, the amount of drinking water and consumed food was measured and the rate of consumed food (grams) per mouse was calculated. Once every 3 mo, vaginal smears, taken daily for 2 weeks from the animals, were cytologically examined to estimate the phases of their estrous function. In the same period, rectal body temperatures of the mice were measured with an electronic thermometer, TPEM-1 (KMIZ, Russia). The animals were observed until their natural death or were sacrificed when moribund. The date of each death was registered, and the mean lifespan, the age at which 90% of the animals died, and the maximum lifespan were estimated.

Cytogenetic study. Chromosome aberrations in bone marrow cells were studied by the modified Ford method.[51] Mice were sacrificed with ether anesthesia. Both femurs of each mouse were dissected, and bone marrow cells were flushed gently with 0.56% KCl solution into a centrifuge tube. Cells were treated for 20 min with hypotonic solution and fixed with ethanol:acetic acid mixture (3:1). Slides were stained with 4% acetoorseine. 20–30 well-spread anaphases were analyzed for each animal, and cells with chromosome breaks, acentric fragments and other aberrations were evaluated on 1,000x magnification with a light microscope (Leitz).

Pathomorphological examination. All the animals that died or that were sacrificed when moribund were autopsied.

Table 6. Log-rank test estimation of differences in the lifespan distribution between treated and non-treated with rapamycin female 129/Sv mice

Mice survived the age of	Groups of mice	χ^2	p-value
130 days	All mice	25.4	4.55e-07
	Tumor-bearing mice	9.4	0.0022
	Tumor-free mice	0.6	0.458
435 days	All mice	9.5	0.00206
	Tumor-bearing mice	9.4	0.0022
	Tumor-free mice	0.9	0.348

At autopsy, their skin and all internal organs were examined. Revealed tumors were classified according to the recommendation of the IARC as "fatal" (i.e., those, that directly caused the death of animal) or as "incidental" (for the cases in which animal died of a different cause). All tumors as well as the tissues and organs with suspected tumor development were excised and fixed in 10% neutral formalin. After the routine histological processing, the tissues were embedded into paraffin. Thin (5–7 μm) histological sections were stained with hematoxylin and eosine and were microscopically examined by a blind process. Tumors were classified according to the IARC recommendations.

Statistics. Experimental results were statistically processed by the methods of variation statistics with the use of STATGRAPH statistic program kit. The significance of the discrepancies was defined according to the Student t-criterion, Fischer exact method, χ^2, non-parametric Wilcoxon-Mann-Whitney and Friedman RM ANOVA on Ranks. Student-Newman-Keuls method was used for all pair-wise multiple comparisons. Coefficient of correlation was estimated by the Spearman method, as previously described. Differences in tumor incidence were evaluated by the Mantel-Hansel log-rank test. Parameters of the Gompertz model

Table 7. Parameters of the Cox regression model for the relative risk of death compared to the control group in female 129/Sv mice

Mice survived the age:	Groups of mice	Females			
		β	exp (β)	se (β)	P
130 days	All	-0.674	0.51	0.27	0.013
	Tumor-bearing	-1.38	0.252	0.478	0.004
	Tumor-free	-0.314	0.73	0.417	0.45
435 days	All	-0.851	0.427	0.285	0.0028
	Tumor-bearing	-1.38	0.252	0.478	0.004
	Tumor-free	-0.426	0.653	0.451	0.34

were estimated using the maximum likelihood method, a nonlinear optimization procedure and self-written code in "Matlab." Confidence intervals for the parameters were obtained using the bootstrap method.[35]

For the experimental group, the Cox regression model was used to estimate relative risk of death and tumor development under the treatment compared with the control group: $h(t, z) = h_0(t) \exp(z\beta)$, where $h(t, z)$ and $h_0(t)$ denote the conditional hazard and baseline hazard rates, respectively; β is the unknown parameter for treatment group, and z takes values 0 and 1, being an indicator variable for two samples, the control and treatment group.

A semiparametric model of heterogeneous mortality was used to estimate the influence of the treatment on frailty distribution and baseline hazard.[35]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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