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# Resveratrol does not mimic the positive effects of calorie restriction on lifespan in *Microcebus murinus*



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Resveratrol (RSV), a polyphenol with anti-aging properties affecting metabolism and energy balance, is considered as a mimetic candidate to calorie restriction (CR). However its potential effects on delaying the onset of age-related diseases and increasing longevity have not yet been demonstrated in non-obese models close to humans such as non-human primates. The longitudinal monitoring of cognitive and motor performances, occurrence of age-related pathologies, age-related brain atrophy and mortality was performed from adulthood to death in a cohort of male grey mouse lemurs (*Microcebus murinus*,  $N = 33$ ), all receiving 105 kJ/day of food but with a subset of 18 animals receiving RSV (200 mg.kg<sup>-1</sup> body weight.day<sup>-1</sup>). RSV supplementation improved cognitive and motor performance at middle age as compared to control (CTL) animals. Median-lifespan was greater in RSV-fed animals (7.9 years compared to 6.4 years for CTL) but long-term RSV supplementation did not significantly affect all-cause nor age-related mortality. Also, brain grey matter atrophy accelerated in the RSV group at old age as compared to the CTL group. Altogether, these results suggest that long-term RSV supplementation procures health benefits at middle-age in male mouse lemurs but has limited long-term effects on health and longevity and might even impair brain integrity at older ages.

Calorie restriction (CR) is the most effective non-genetic intervention known to reduce the emergence of age-related diseases and to increase life span in several animal species<sup>1</sup>. Although considered an “anti-aging” paradigm for decades<sup>2</sup>, it is only recently that CR has proven its beneficial impact in long-lived species such as non-human primates<sup>3,4</sup> and humans<sup>5</sup>. In the latter, CR reduced the loss of autonomy caused by either disabilities or incapacity and delayed the onset of chronic age-associated diseases such as type 2 diabetes and heart diseases<sup>6,7</sup>. However, chronic CR might be difficult to establish in humans on a daily basis, mainly for cultural reasons<sup>1</sup>. During the last two decades, the development of CR mimetics has increased significantly<sup>1,7</sup>, among which resveratrol (RSV; 3,5,40-trihydroxy-trans-stilbene), a natural polyphenolic compound abundant in peanuts, berries, and grapes, which reproduces some of the beneficial outcomes of CR<sup>8</sup>. But whether the adaptive metabolism responses generated by CR are similar under RSV remains controversial<sup>9</sup>. The CR mimetic action of RSV may

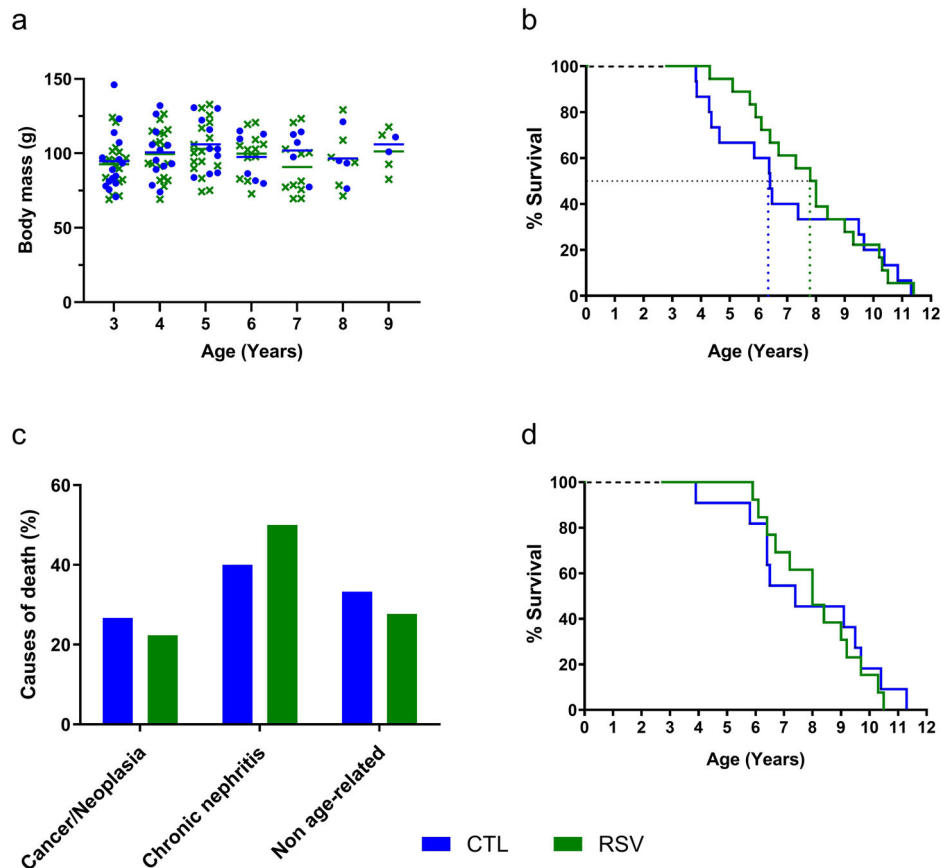
depend on its cardioprotective<sup>10</sup> and neuroprotective effects<sup>11</sup>. Despite such benefits, the capacity of RSV to mimic the long-term effects of CR on longevity remains somewhat unclear, particularly in long-lived species, and even more in aging primates<sup>12</sup>. Some studies even suggest potential adverse effects of long-term RSV supplementation<sup>13</sup>. Such controversies might be due to the high range of supplementation levels, the lowest dose (5.2 mg/k.day<sup>-1</sup>) having limited effects on health and longevity<sup>14</sup> while the highest (up to 5000 mg/k.day<sup>-1</sup>) induced adverse effects<sup>13,15,16</sup>. Thus, the effects of long-term supplementation with intermediate dose of RSV (i.e. 200 mg/kg.day<sup>-1</sup>), were investigated on the health and lifespan of the grey mouse lemur (*Microcebus murinus*), a small lemuriform primate from Madagascar with a median lifespan of 5.7 years in captivity and a maximum lifespan of 12 years in laboratory-controlled conditions<sup>17</sup>. Mouse lemurs are a very suitable species to study ageing. They display age-related alterations of their sensory system, motor functions, biological rhythms, immune and endocrine

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**Fig. 1 | Effects of resveratrol on body mass, pathologies and survival.** Effects of resveratrol (RSV) supplementation (a) on body weight, b and d lifespan and c age-associated pathologies in mouse lemurs. a Mean body mass was averaged during the 6-month summer period of each year of mouse lemurs on a chronic RSV supplemented diet ( $n = 18$ , green) or a control (CTL) diet ( $n = 15$ , blue). Horizontal bars represent means of each group. b Kaplan–Meier survival curves for overall mortality ( $p$ -logrank = 0.76) and d age-related disease mortality ( $p$ -logrank < 0.64). In b Median survival in CTL (6.4 years) and RSV-supplemented animals (7.9 years) is shown with dotted lines for overall mortality. c Incidence of the indicated age-related diseases (including cancer and nephritis) and other non-age-related causes of death (from accidents, infections or undetermined causes) in the RSV and CTL cohorts. The data were obtained after pathological analysis of post-mortem tissues.



systems, in a similar manner to humans<sup>17</sup>. In the mouse lemur, aging also leads to an increased prevalence of diseases such as neoplasia or sarcopenia<sup>18</sup> and alterations of glucoregulatory function<sup>19</sup>, such as observed in aged human subjects. Finally, mouse lemurs display age-related cognitive alterations associated with cerebral atrophy<sup>20</sup> as well as Alzheimer's disease-like amyloid lesions<sup>21</sup>. Like humans and other non-human primates, they are genetically heterogeneous, providing a natural diversity of aging profiles. Because of their relatively lower life span (as compared to macaques), cohorts of lemurs can be easily followed on a long-term basis to evaluate anti-aging strategies. In a previous study, we demonstrated that a chronic moderate (30%) CR significantly extended lifespan (by up to 13.8 years old) and healthspan of mouse lemurs, and impaired brain grey matter integrity without affecting cognitive performance<sup>3,7</sup>.

The aim of the present study is to provide the first complete set of survival data in a non-human primate chronically supplemented with RSV, in association with a longitudinal follow-up of age-associated alterations in motricity, cognition, and brain volumes. Body weight, lifespan, age-related pathologies, cognitive abilities, motor performances, and cerebral atrophy of all animals were then followed until their natural death.

## Results

Resveratrol supplemented animals did not exhibit differences in body mass as compared to CTL animals ( $p = 0.39$ ; Fig. 1a). Median-lifespan in RSV-supplemented animals increased by 1.5 year compared to CTL (7.9 years and 6.4 years for RSV and CTL groups, respectively). However, the maximal life span of the animals remained unchanged between the two groups ( $p = 0.64$ ; Fig. 1b). Incidence of age-related pathologies such as cancer and chronic nephritis was not significantly changed by RSV supplementation (Fig. 1c). Indeed, 72% of deaths (13 of 18) were attributable to these diseases in the RSV group compared to 67% of deaths (10 of 15 deaths) in CTL animals ( $p = 0.96$ ). Also, no significant effect of long-term RSV

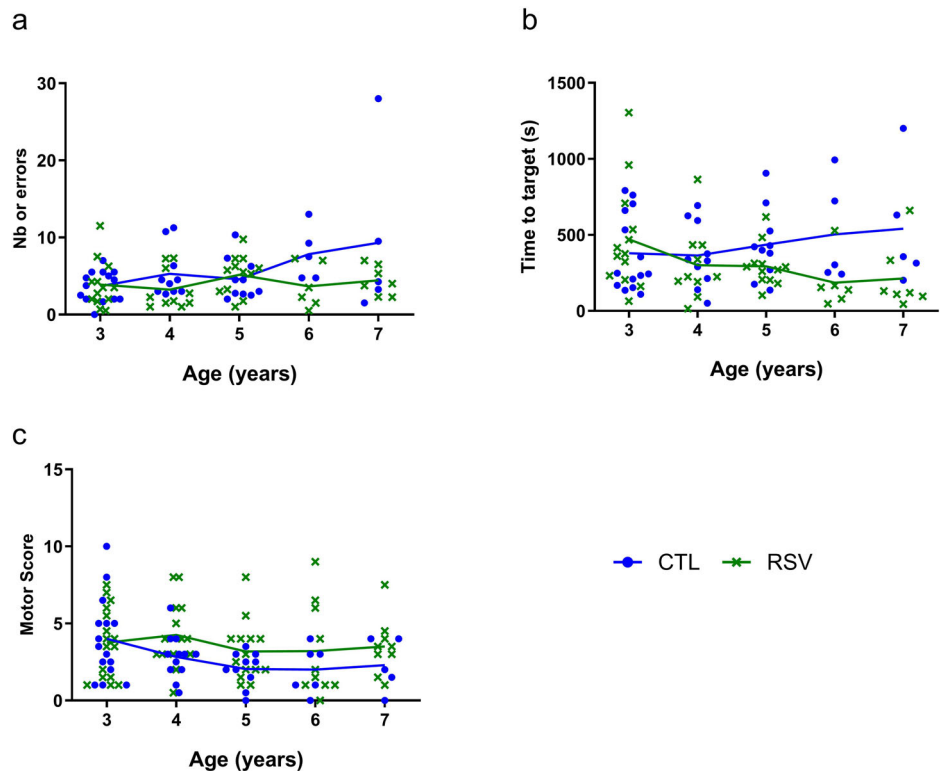
supplementation could be observed on mortality curves when animals dying from age-related pathologies only were included in the analysis ( $p = 0.76$ ; Fig. 1d).

Cognitive performance was assessed by testing working memory using the Barnes-maze task<sup>17</sup>. The number of errors before retrieving the previously learnt target was significantly decreased in RSV-supplemented animals compared to CTL (Fig. 2a,  $p = 0.03$ ), as was the time before reaching the target (Fig. 2b,  $p = 0.01$ ). The difference between the two groups was further emphasized later in life (after 6 years of age), when cognitive decline is more likely to be observed in CTL animals. Indeed, RSV-fed animals had about half the number of errors and time to reach the target as compared to CTL animals after the age of 6 years. However, there was no observable effect of age on these tasks, which is in contrast to results observed in several previous transversal studies, where age-related cognitive changes were detected using the same Barnes-maze task<sup>20</sup>. One possible explanation for the lack of aging effects in the present study might be the occurrence of practice effect that could prevent the detection of age-related decline<sup>22</sup>, as well as the high interindividual variability observed in the CTL animals aged 6 years and more (Fig. 2a and b).

Motor performance, important for physical independence and healthy aging in humans<sup>23</sup>, was assessed using the accelerating rotarod task and a high jump task<sup>17</sup>. Results from these two tasks were computed to calculate a motor score, representative of motor coordination, endurance and muscular strength (see supplementary methods). RSV animals exhibited better motor abilities than the CTL group ( $p = 0.03$ , Fig. 2c), an effect that started to be visible early in life (at 4 years of age).

In humans and non-human primates, including mouse lemurs, aging induces a cerebral atrophy that is associated with cognitive impairments<sup>20,24,25</sup>. Serial magnetic resonance imaging (MRI) of mouse lemur brains was performed once a year for 4 years, beginning at age  $6.8 \pm 0.2$  years (mean  $\pm$  SEM), i.e. approximately 3.5 years after the

**Fig. 2 | Effects of resveratrol on cognitive and motor functions.** Effects of resveratrol (RSV) supplementation on **a**, **b** cognitive and **c** motor coordination performances in mouse lemurs. Cognitive and motor coordination performances were tested either on a chronic RSV supplemented diet ( $n = 18$ , green) or a control (CTL) diet ( $n = 16$ , blue). **a**. Number of errors in the Barnes maze (spatial memory task). A significant effect of treatment was observed between CTL and RSV groups ( $p = 0.03$ ). **b** Time (in seconds) to reach the correct target in the Barnes maze. A significant effect of treatment was observed between CTL and RSV groups ( $p = 0.01$ ). **c** Motor score was calculated by the combination of motor coordination and endurance, measured during a rotarod task, and performances during a high jump task. A significant effect of treatment was observed between CTL and RSV groups ( $p = 0.03$ ).



beginning of the treatment to investigate grey matter and white matter atrophy. Voxel-based morphometric (VBM) analysis of the initial MR images revealed similar grey matter volumes in most brain regions of CTL and RSV animals except a very focal lower grey matter volume in the primary motor cortex (Brodmann area 4) in CTL animals (Fig. 3a, b, Supplementary Table 1). We then focused on the longitudinal change in atrophy from 6.8 to 10 years of age, i.e., between approximately 3.5 and 7 years after the initiation of the treatment (Fig. 3c–h, Supplementary Table 2). This analysis revealed that in RSV animals, an age-related grey matter atrophy occurred in the amygdala as well as in Brodmann areas 13–16, 1–3 (primary somato-sensory area), and to a lesser extent in area 24 (anterior cingulate cortex) (Fig. 3f–h). In CTL animals, the septum, and to a lesser extent the amygdala, were the only regions presenting significant age-related atrophy (Fig. 3c–e). Direct comparison of age-related grey matter loss in RSV compared to CTL highlighted a stronger focal cortical loss in the retrosplenial area only of RSV animals (voxel-based morphometric analysis of time of treatment  $\times$  diet group interaction,  $p < 0.005$ ,  $n = 11$  for RSV and  $n = 7$  for CTL animals) (Supplementary Fig. 1, Supplementary Table 3).

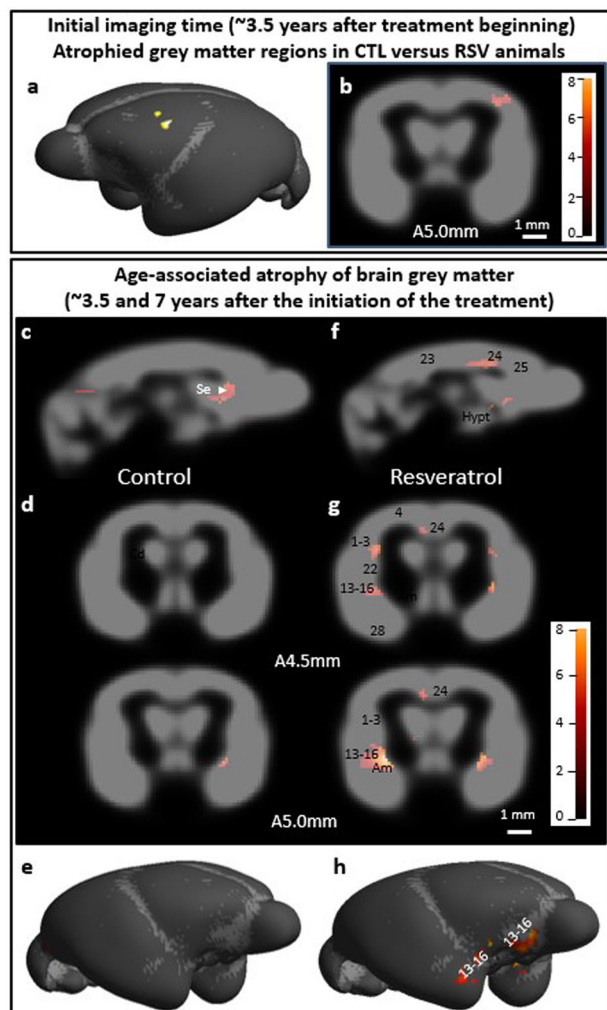
VBM analysis of white matter revealed similar volumes in CTL and RSV animals in initial MR images, except a slightly lower white matter volume in the occipital part of the corpus callosum in CTL animals (Fig. 4a, b, Supplementary Table 4). The longitudinal evaluation of aging effects revealed widespread loss of white matter in most parts of the corpus callosum and the internal capsule of both CTL and RSV animals (Fig. 4c–j, Supplementary Table 5). White matter areas from posterior brain regions presented a marked atrophy in RSV (Fig. 4i, j) but not in CTL animals (Fig. 4e, f). Direct comparison of age-related white matter atrophy (time of treatment by diet group interaction) over 6.8- to 10-year-old follow-up period was similar in RSV compared to CTL animals (not shown).

## Discussion

The present results provide evidence that chronic RSV supplementation, when started early in adult life, sustains better motor and cognitive status compared to CTL. Three and a half years after the initiation of the treatment, CTL and RSV animals had similar grey and white matter volumes in most

brain regions except for focal higher volume of the primary motor cortex as well as of the posterior white matter area in RSV animals. Unexpectedly, RSV animals displayed an age-related atrophy in several grey matter regions, not yet affected in CTL. Such an age-related acceleration of grey matter atrophy provides a cautionary note for using RSV on a long-term basis in humans, as also suggested by studies in AD patients exhibiting accelerated brain atrophy under RSV supplementation<sup>26</sup>.

Both CTL and RSV animals displayed an age-related atrophy in several white matter regions. Such an age-related white matter atrophy has already been reported under normal condition in mouse lemurs<sup>27</sup>. The location of the atrophied region differed in both groups, with the RSV-treated group displaying more severe white matter losses in posterior areas. Differences observed on brain atrophy did not seem to be associated either with measurable cognitive impairments, or with motor deficits. However, a caution note should be made for the interpretation of such lack of association, as behavioural and MRI measurements were not performed in the same time window. Indeed, cognitive and motor measurements were performed until 7 years old while MRI study started at the age of 7. At that age, there was no significant difference in grey and white matter volumes between the two groups, except very focal lower grey matter volume in the primary motor cortex in CTL animals (very small spot, see Fig. 3a, b), which cannot explain by itself the differences observed in motor and cognitive performances between CTL and RSV animals. It is highly probable that better motor performances rely more on better preservation of muscle strength than on brain modifications. Decline in muscle strength, as well as in motor coordination (assessed in the rotarod task) starts around 4 years old in mouse lemurs<sup>18</sup>, an age at which animals are still considered young<sup>17</sup>. Interestingly, RSV was described as being able to reduce age-associated muscle loss in rodents<sup>28</sup>, suggesting that the beneficial effects of RSV on motor performances during aging may rely more on reduced muscle loss than on brain atrophy. As a consequence, we cannot completely exclude that part of the better performance in the Barnes maze task in the RSV group relies on better motor condition. In addition, the observation of better cognitive performances under RSV supplementation is based on a single cognitive task (Barnes maze task, assessing mostly spatial reference memory and working

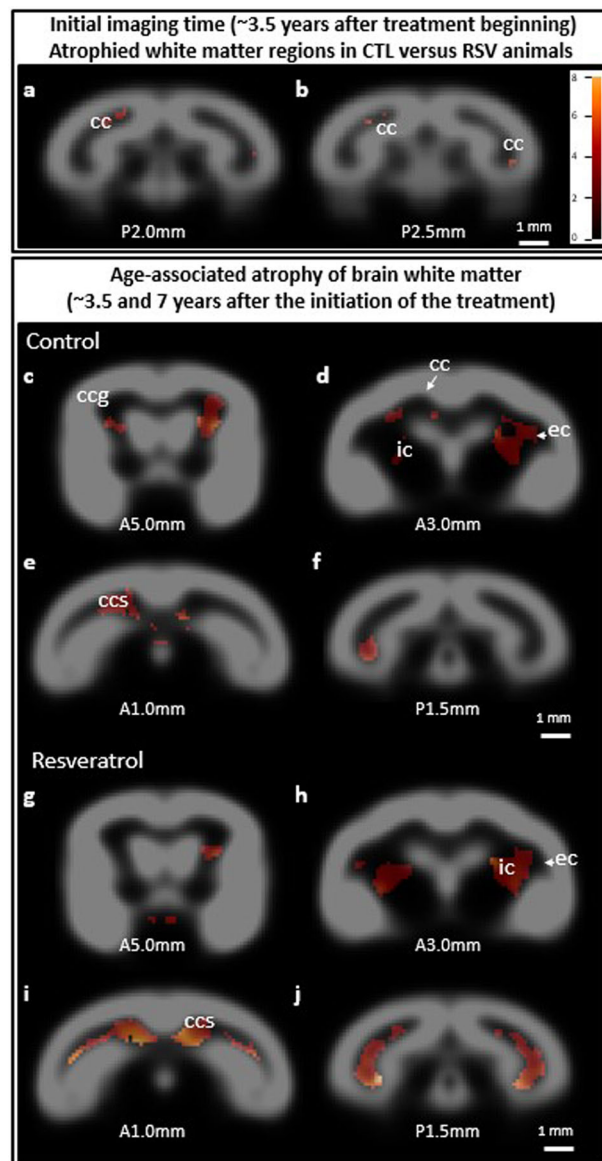


**Fig. 3 | Grey matter atrophy in resveratrol (RSV) mouse lemurs evaluated from voxel-based morphometric analysis of serial magnetic resonance (MR) images.** **a, b** At initial imaging time, i.e., after approximately 3.5 years of treatment, grey matter volumes were almost similar in most brain regions of CTL and RSV animals except a very focal lower grey matter volume in the primary motor cortex in CTL animals as seen on **a** surface rendering and **(b)** coronal section (voxel-based morphometric analysis,  $p < 0.005$ ,  $n = 5$  for CTL and  $n = 11$  for RSV animals). **c–h** Longitudinal follow-up of animals thereafter revealed a widely distributed age-associated grey matter atrophy in **(f–h)** RSV mouse lemurs and a more focal atrophy in **c–e** CTL animals. Coloured spots display regions with statistically significant age-related atrophy obtained ( $p < 0.005$ ,  $n = 7$  for CTL and  $n = 11$  for RSV animals). Unlike CTL animals, RSV individuals displayed atrophy in the amygdala as well as in Brodmann areas 13–16, 1–3 (primary somato-sensory area), and to a lower extent in area 24 (anterior cingulate cortex). Colour bars represent the value of the  $t$ -statistic (no unit). Numbers represent the Brodmann area of mouse lemur brain according to Brodmann and Le Gros Clark classification<sup>37,38</sup>. Am amygdala, Hypt hypothalamus, Se septum. Sections refer to antero-posterior coordinates.

memory). Additional tests to assess other cognitive domains may have led to other conclusions.

Finally, the present study also demonstrated, for the first time in a primate, that long-term RSV supplementation does not mimic the lifespan extension provided by chronic CR, such as recently reported in mouse lemurs<sup>3,7</sup>, and does not reduce either the risk of age-associated diseases or age-associated mortality.

This conclusion must be taken with caution since the dose of RSV we tested herein may not be optimal from a longevity perspective. At the start of the study (in 2006), a dose of 200 mg/kg.day<sup>-1</sup> was considered to be



**Fig. 4 | White matter atrophy in resveratrol (RSV) mouse lemurs evaluated from voxel-based morphometric analysis of serial magnetic resonance (MR) images.** **(a, b)** At initial imaging time, i.e., after approximately 3.5 years of treatment, white matter volumes were almost similar in most brain regions of control (CTL) and resveratrol (RSV) animals except a slightly lower white matter volume in the occipital part of the corpus callosum in CTL as compared to RSV animals (voxel-based morphometric analysis,  $p < 0.005$ ,  $n = 5$  for CTL and  $n = 8$  for RSV animals). **c–j** Longitudinal follow-up of animals thereafter revealed age-associated atrophy of brain white matter in both CTL and RSV mouse lemurs ( $p < 0.005$ ,  $n = 7$  for CTL and  $n = 11$  for RSV animals). Brain levels refer to antero-posterior coordinates. The colour bar represents the value of the  $t$ -statistic (no unit). ccg genu of the corpus callosum, cc body of the corpus callosum, ec external capsule, ccs splenium of the corpus callosum.

intermediate, based on studies in rodents available in the literature. Recent observations in humans suggest that RSV might be efficient at much lower doses (between 500 mg and 1 g/day for an adult, what would mean a dose between 7.15 and 14.3 mg/kg.day<sup>-1</sup> if we consider a 70 kg human adult<sup>29</sup>). Such a low dose, compared to high doses commonly applied in rodents<sup>13</sup> and the current study, could limit the potential adverse effects of RSV<sup>15,30</sup>. RSV bioavailability was also verified<sup>31</sup> and free RSV and its two main metabolites (trans-resveratrol-3-O-glucuronide and trans-resveratrol-3-sulfate) were present several hours after ingestion in lemurs' plasma. Free



dihydro-RSV was not detectable whatever the time of plasma collection, while hydrophilic metabolites were present at 24 h after intake. Ideally, further studies focusing on the optimal dose would be needed to limit the risk of cerebral atrophy and to optimize RSV biological effects. Finally, it must be noted that the present study focused on males, and we cannot rule out the possibility of a differential effect on health or longevity outcomes between males and females as observed with RSV<sup>32</sup> or CR<sup>33</sup> in humans.

Taken together, these results suggest that very long-term RSV supplementation results in health benefits at middle-age in male mouse lemurs, as demonstrated here for motor and cognitive capacities, but has limited long-term effects on health and longevity and might even impair brain integrity at older ages.

## Material and methods

### Animals and breeding conditions

Male grey mouse lemurs were born in the laboratory breeding population of Brunoy (UMR 7179 CNRS/MNHN, France; European Institutions Agreement # 962773). They were integrated in the RESTRIKAL study<sup>34</sup> at  $40 \pm 6$  months of age which is considered an adult age in this species and they weighed  $90 \pm 5$  g at the beginning of the study. Animals were housed individually in cages ( $50 \times 60 \times 70$  cm) given with wooden branches and wooden nests, in standard and constant conditions of temperature ( $24\text{--}26^\circ\text{C}$ ) and of relative humidity (55%). In captivity, seasonal variations of physiological and behavioural functions were entrained by alternating between 6 months of a long-day photoperiod (LD; 14:10 light:dark) and 6 months of a short-day photoperiod (SD; 10:14 light:dark), under artificial light (white light, 250 lux, wavelength peak at 488 nm). Animals were fed fresh fruit and a daily mixture freshly made up of ginger bread, cereals, milk and eggs; water was given *ad libitum*. All of the data presented in this manuscript were collected during the summer season, i.e. when animals were behaviourally and reproductively active. All described procedures were approved by the Animal Welfare board of the UMR 7179 and complied with the European ethic regulation for the use of animals in biomedical research and all relevant ethical regulations for animal use.

### Dietary interventions

Animals were randomly distributed into two different dietary groups: a control group (CTL) of 15 animals and a group of 18 animals supplemented with 200 mg of resveratrol (RSV) per kilogram of body weight per day (Sequoia Research Products, United Kingdom). This dose was selected from the literature based on studies in rodents, and was intermediate between the doses of  $5.2 \text{ mg/kg.day}^{-1}$ <sup>114</sup> and the  $400 \text{ mg/kg.day}^{-1}$ <sup>135</sup> reported in the literature at the start of the project (2006). All animals from both groups received the same number of calories per day (15 g of freshly made mixture and 6 g of fresh fruit per day, equivalent to 105 kJ/day on average), which was estimated from previous internal studies of the Brunoy laboratory (yearly spontaneous food intake in isolated control adult animals; unpublished data).

### Circular platform task (Barnes-like maze) for cognitive skill evaluation

The apparatus consisted of a white circular rotating platform (diameter, 100 cm) placed at 60 cm above the floor. The platform contained 12 equally spaced circular holes (each 5 cm in diameter) at 3 cm from the perimeter. A cardboard nest-box ( $10 \times 10 \times 20 \text{ cm}^3$ ) was inserted beneath one hole and served as a refuge (target box). A small black plywood box was placed beneath the other (non-target) holes to prevent lemurs exiting the apparatus through a non-target exit. The platform was surrounded by a 25 cm high white wall and covered with a transparent Plexiglas cap, allowing the mouse lemurs to discriminate visual cues outside the maze. The apparatus was surrounded by a black curtain hung from a square metallic frame, in the centre of which there was a one-way mirror that allowed observation. Twenty-four evenly spaced 2-Watts lights were affixed around the perimeter of the maze 50 cm above the platform to illuminate the maze. The centre of the maze was also illuminated by a 60-watt light. Between the one-

way mirror and the upper edge of the wall, various objects were attached along the inner surface of the curtain to serve as visual cues. The starting box was an open-ended dark cylinder positioned in the centre of the platform. Transparent radial Plexiglas partitions were placed between the holes to prevent the strategy used by some mouse lemurs to go directly to the periphery of the platform, then walk along the barrier wall and inspect each hole one by one. Consequently, animals had to return to the centre of the platform after each hole inspection.

Animals were given 1 day of training (day 1) and 1 day of testing (day 2). Each day was comprised of four trials, each of which began with the placement of the animal inside the starting box. After 30 s, the box was lifted to release the animal. For the animals, the objective was to reach the target box positioned beneath one of the 12 holes. When it entered the target box, the trial was stopped, and it was allowed to remain in its own nest for 1 min. After each trial, the platform was randomly rotated on its central axis to avoid the use of intra-maze cues, although the position of the target box in the room was kept constant.

On day 1, trials 1 and 2 consisted of placing the animal in the maze centre while only one corridor, containing only the opened target hole, was accessible (one-choice test). For trials 3 and 4, the platform comprised six reachable corridors among which only one hole was opened (six-choice test). These two trials permitted the animal to explore the maze, observe the visual cues and further learn the position of the target box.

On day 2, all 12 corridors were accessible, with only one hole open during the four trials. Performance was assessed by the time required for the animal to reach the correct exit and by the number of errors prior to reaching the target box. An error was defined as an inspection of an incorrect hole. Only data from animals that reached the target box before 20 min of testing were included in the behavioural analyses. This inclusion criterion and the increasing prevalence of ocular pathologies with age accounted for the difference between the total number of animals in the study and the number of animals from which data were analyzed.

The parameters measured to evaluate spatial memory were the number of errors before finding the correct exit on day 2 and the time (in seconds) to reach the correct exit (target). Spatial memory was assessed from year 1 of treatment until natural death.

### Accelerating rotarod task for motor performance evaluation

For each trial, an animal was placed on a rotarod (model 7750, Ugo Basile, Italy), which was a motor-driven treadmill with a 5-cm-diameter cylinder. The speed of rotation was increased from 17 to 40 rpm until the animal could no longer perform the running response without falling or gripping the rod on at least three consecutive turns, and the time spent on the cylinder was used as a measure of motor performance. Animals underwent five consecutive trials, and the best result was retained. Only data from animals that stayed on the rotarod for more than 1 s during at least 1 trial were included in the analyses. The time was converted into a score, based on the following scale:  $\text{time} < 1 \text{ s} = \text{excluded}$ ;  $1 \text{ s} < \text{time} < 10 \text{ s} = 1$ ;  $10 \text{ s} < \text{time} < 20 \text{ s} = 2$ ; ...;  $80 \text{ s} < \text{time} < 90 \text{ s} = 9$ ;  $\text{time} > 90 \text{ s} = 10$ . The score was then used was then used in the calculation of high jump score. The system was cleaned between each trial. Motor performances were assessed from year 1 of treatment until natural death.

### High jump task

The test was performed in a closed wooden box (200 cm high  $\times$  50 cm long  $\times$  50 cm wide). The apparatus was affixed 30 cm above the floor. A one-way mirror covered one side of the closed box, which allowed observation by the experimenter without disturbing the mouse lemurs. In the closed box, an adjustable metal rod was installed that allowed to progressively increase the height of this rod of 10 cm between each effective test. A hatch located at the base allowed to introduce the animal in the closed chamber. To motivate the animal to jump, a 20 W bulb was placed below the system and its nestbox was installed 10 cm above the metal rod.

The animal was introduced in the closed box for a test lasting maximum 5 minutes. For the first trial, the metal rod was placed at a height of

20 cm. When the animal succeeded to reach its nestbox, the trial was stopped, and the animal was allowed to remain in its nestbox for 1 min. The height of the metal rod was then raised by 10 cm between each successful trial. If the animal did not reach its nestbox after 5 minutes, the test was stopped and the animal was manually removed from the apparatus. The maximum height (cm) reached by the mouse lemur was collected. The system was cleaned between each trial. Data from animals that did not jump after 5 min were excluded of the behavioural analyses. The height of jump was converted into a score, based on the following scale: 10 cm = 1; 20 cm = 2; ...; 100 cm = 10. The score was then used to be computed with rotarod score (motor score = high jump score + rotarod score).

### MRI acquisition and analysis

All the animals involved in the current study were studied by MRI from the age of  $6.8 \pm 0.2$  years ( $n = 18$  animals, 7 CTL, 11 RSV ( $7.2 \pm 0.3$  versus  $6.5 \pm 0.2$  years at inclusion, respectively) and once a year for 4 years unless they died before. The average age of the animals at the different imaging time points was similar in the two groups ( $7.7 \pm 0.3$  versus  $7.4 \pm 0.2$  years). Brain images were recorded on a 7.0 Tesla spectrometer (Varian) using a four-channel phase surface coil (RapidBiomedical, Rimpf, Germany) actively decoupled from the transmitting birdcage probe (RapidBiomedical, Rimpf, Germany). Briefly, animals were anaesthetised by isoflurane (4% for induction and 1–1.5% for maintenance). Respiratory rate was monitored to insure animal stability until the end of the experiment. Body temperature was maintained by an air-heating system. Two-dimensional fast spin-echo images were recorded with an isotropic nominal resolution of  $230 \mu\text{m}$  (128 slices, TR/TE = 10000/17.4 ms; rare factor = 4; acquisition time = 32 min). MRIs were zero-filled to reach an apparent isotropic resolution of  $115 \mu\text{m}$ .

Thirty-eight images were analysed using voxel-based morphometry by applying SPM8 (Wellcome Trust Institute of Neurology, University College London, UK, <http://www.fil.ion.ucl.ac.uk/spm/>) with the SPMouse toolbox (<http://spmhouse.org>) for animal brain morphometry<sup>25</sup>. The brain images were segmented into grey and white matter tissue probability maps using locally developed priors<sup>25</sup>, then spatially transformed to the standard space defined by Sawiak et al. using a grey matter mouse-lemur template<sup>25</sup>. Affine regularisation was set for an average-sized template, with a bias non-uniformity cut-off full-width half-maximum of 10 mm, a 5 mm basis-function cut-off, and a sampling distance of 0.3 mm. The resulting grey matter and white matter portions were output in rigid template space, and DARTEL<sup>26</sup> was used to create non-linearly registered maps for each subject and common templates for the cohort of animals. The warped grey matter portions for each subject were modulated with the Jacobian determinant from the DARTEL registration fields to preserve tissue amounts ('optimised voxel-based morphometric analysis'<sup>27</sup>) and smoothed with a Gaussian kernel of  $600 \mu\text{m}$  to produce maps for analysis.

A general linear model was evaluated with a design based on multiple regressions with the diet group effect and time of treatment of the animals of each group (control, caloric restriction) as variables of interest. This type of regression technique produces t-statistic and colour-coded maps that are the product of a regression model performed at every voxel in the brain. Contiguous groups of voxels that attain statistical significance, called clusters, are displayed on brain images.

With the general linear model, the brain of one animal is defined by the number "j", and the location of a pixel is defined as "k". The signal (i.e., the probability for the signal to be grey matter or white matter) within a pixel ( $Y_{jk}$ ) can be explained by the following equation (equation 1)

$$Y_{jk}^k = \beta_1^k + x_{j,1}\beta_2^k + x_{j,2}\beta_3^k + T_j^1\beta_4^k + \dots + T_j^{18}\beta_{21}^k + TIV_j\beta_{22}^k + \epsilon_j^k$$

With  $\beta_1^k$  = Mean image;  $\beta_2^k$  = Evolution of the signal according to time of treatment for CTL animals (i.e. slope of signal evolution for  $n = 13$  images);  $\beta_3^k$  = Evolution of signal according to time of treatment for RES animals (i.e. slope of signal evolution for  $n = 25$  images);  $\beta_4^k$  = Longitudinal follow-up for CTL animal #1; ...;  $\beta_{10}^k$  = Longitudinal follow-up for CTL animal #7;  $\beta_{11}^k$  =

Longitudinal follow-up for RES animal #1; ...;  $\beta_{21}^k$  = Longitudinal follow-up for RES animal #11;  $\beta_{22}^k$  = effect of total intracranial volume (TIV) on the signal for each animal. In the matrix for analysis, the  $T_j^x$  is 1 or 0 if the animal #x is analysed or not. TIV corresponds to the TIV value for each animal. It was similar for the different images from the same animal followed-up longitudinally.

The time of treatment effect within each group is defined by  $x_{j,1}\beta_2^k$  and by  $x_{j,2}\beta_3^k$  for the CTL and RES animals, respectively.  $x_{j,1}$  and  $x_{j,2}$  represent the age of the animals in the CTL and RES groups, respectively. In other words,  $x_{j,1}$  = age of the animal if the  $j^{\text{th}}$  animal is a CTL animal, 0 otherwise and  $x_{j,2}$  = age of the animal if the  $j^{\text{th}}$  animal is a CR animal, 0 otherwise. The term  $\epsilon_j^k$  corresponds to the "error" of the measure for each animal.

A contrast defines a linear combination of the  $\beta$  as  $c^T\beta$ . For example, the time of treatment-related reduction of GM in the CTL animals would be defined using a contrast  $c^T\beta = [0 \ -1 \ 0 \dots]^T$ . The Null hypothesis is  $H_0 : c^T\beta = 0$ , while the alternative hypotheses is  $H_1 : c^T\beta > 0$ . This hypothesis is tested with (equation 2):

$$T = \frac{c^T\beta}{\sqrt{\sigma^2 c^T (X^T X)^{-1} c}} = \frac{\text{contrast}}{\sqrt{\text{estimated variance}}}$$

This analyze allows to remove confounding effects such as repetition of the measures during longitudinal evaluation of the same animal or TIV from the raw data  $Y_j^k$ . Voxels with a modulated GM value below 0.2 were not considered for analysis. In other words, volumetric scans were entered as the dependent variable. Time of treatment of the animals and groups (CTL or RSV) were the independent variables. Longitudinal follow-up effect and TIV were covariates.

One-tailed t-tests contrasts were set up to find areas where GM and WM values were different in CTL and CR animals at the beginning of the MRI study. Then other one-tailed t-tests were used to compare the slopes (i.e.  $\beta_2^k$  for CTL and  $\beta_3^k$  for CR animals) of the evolution of GM and WM values with aging in CTL and CR animals during the 4 years of the MRI study (time of treatment x diet group interaction effect). Time of treatment effects were also evaluated in animals from the two groups. In this case, the model estimates whether the slope of the GM or WM evolution within the two group (i.e.  $\beta_2^k$  for CTL or  $\beta_3^k$  = CR animals) were different from zero. It is the term  $\epsilon_j^k$  corresponding to the error of the measure for each animal that is adjusted to fit the model.

The threshold to consider a voxel as different between two groups was set at  $p < 0.005$  (uncorrected for multiple comparisons) as in Colman et al.,<sup>5</sup> and Pifferi et al.<sup>3</sup> Clusters required 75 contiguous voxels to be selected as relevant. Clusters fulfilling these conditions were displayed on brain sections or 3D views of the brain. Adjusted GM or WM values were also presented to display time of treatment effect in CTL or RES animals on which statistical analysis were performed. For each animal, they correspond to equation 3:

$$Y_j^k - \beta_1^k - \epsilon_j^k = x_{j,1}\beta_2^k + x_{j,2}\beta_3^k + T_j^1\beta_4^k + \dots + T_j^{18}\beta_{21}^k + TIV_j\beta_{22}^k$$

$\beta_{4to22}^k$  and  $TIV_j$  were constant for a given animal studied in a longitudinal way. Also seen in the equation,  $\beta_2^k$  and  $\beta_3^k$  i.e., the slopes of evolution of adjusted GM or WM values with time were similar for the different animals from a single group (CTL or RSV, respectively).

### Mortality data

Animals were followed until their spontaneous death. Based on specific criteria (rapid body mass loss, anaemia, difficulty breathing), euthanasia was also performed when necessary to shorten animal suffering; moribund animals were deeply anaesthetized with 100 mg/kg of pentobarbital, intraperitoneally. All organs were harvested and kept for future analysis.

## Pathophysiological analysis of post-mortem tissues

After the death of an animal, a post-mortem analysis of tissues was performed whenever possible ( $n = 13$  control,  $n = 13$  RSV). Samples from liver, kidney, spleen, small intestine, lungs, heart, stomach and pancreas were collected from each animal. Other organs (bladder, brain or colon) were collected if a macroscopic lesion was observed. All tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4  $\mu\text{m}$  and stained with haematoxylin, eosin and saffron.

## Data analysis and statistics

Data are given as mean  $\pm$  standard error of the mean (SEM). The Shapiro–Wilk goodness-of-fit test was applied to determine whether the sample data were likely to derive from a normally distributed population. Data were analysed with LME models, built with the ‘lmer’ function from package lme4 v 1.1–13 in R 3.0.2 (R Development Core Team, Vienna, Austria). The residuals of each model were checked for normality by plotting normal quantile–quantile and Q–Q line. Explanatory variables were the fixed effects of treatment (CTL versus RSV) and of treatment duration (age effect) and their interaction. Inter-individual variability as well as repetition of measurements over the years of the study were included in the random effect. P-values were calculated by performing an analysis of variance on the model using the package ‘lmerTest’ v 2.0–33.

The effects of treatments (i.e., CTL versus RSV) on both overall and age-related mortalities were investigated using Kaplan–Meier curves and Cox proportional hazard (PH) regressions. Survival time was the time between onset of treatment and any cause of death for overall mortality analyses or age-related death for age-related mortality analyses. The cut-off date was set as December 1, 2016. The PH assumption was tested by fitting a PH Cox regression with linear treatment–time interactions; these interaction terms did not significantly differ from zero for both analyses, and the proportional hazard assumptions were therefore considered as valid. SAS V9.1 (SAS Institute, Cary, NC) was used for survival analyses. Type-1 error was set at 0.05 level. Median lifespan determination was based on a single observation for each group.

## Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

## Data availability

The data sets generated during and/or analysed during the current study are available using the following link: <https://doi.org/10.48579/PRO/GHZWX436>. The file named “Supplementary Data 1” is an excel file containing the numerical source data for Figs. 1 and 2 of the manuscript. It is composed of 7 excel sheets including the data used to create the 7 panels of Figs. 1 and 2. Numerical source for Figs. 2 and 3 in the manuscript (MRI figures) cannot be provided under the form of a supplementary file.

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### Author contributions

F.A., S.B. and J.E. designed all experiments described here. F.D., J.M., A.D.-P., F.P., J.T. executed the experiments. F.D., M.D., J.M., A.D.-P., J.-L.P., F.P. and J.T. conducted data analyses. F.A., S.B., M.D., J.E., F.P., J.-L.P., M.P. and J.T. wrote the manuscript.

### Competing interests

The authors declare no competing interests.

### Additional information

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