

## Longevity and cause of death in male Wistar rats fed lifelong diets based on virgin olive oil, sunflower oil or fish oil

César L. Ramirez-Tortosa<sup>a#</sup>, Alfonso Varela-López<sup>b#</sup>, Maria D. Navarro-Hortal<sup>c</sup>, FM Ramos-Pleguezuelos<sup>d</sup>, B. Márquez-Lobo<sup>e</sup>, M. Carmen Ramirez-Tortosa<sup>f</sup>, Julio J. Ochoa<sup>c</sup>, Maurizio Battino<sup>b</sup>, José L. Quiles<sup>c</sup>.

<sup>a</sup> UGC de Anatomía Patológica Hospital San Cecilio de Granada, Avda. Conocimiento s/n, 18100 Granada, Spain.

<sup>b</sup> Dipartimento di Scienze Cliniche Specialistiche ed Odontostomatologiche (DISCO)-Sez. Biochimica, Facoltà di Medicina, Università Politecnica delle Marche, 60131 Ancona, Italy

<sup>c</sup> Department of Physiology, Institute of Nutrition and Food Technology “Jose Mataix”, Biomedical Research Center, University of Granada, Avda. Conocimiento s/n, 18100 Armilla, Granada, Spain

<sup>d</sup> Unidad provincial intercentros de Anatomía Patológica de Granada, Granada, Spain.

<sup>e</sup> UGC Anatomía Patológica. Complejo Hospitalario de Jaén. Jaén, Spain.

<sup>f</sup> Department of Biochemistry and Molecular Biology II, Institute of Nutrition and Food Technology “Jose Mataix”, Biomedical Research Center, University of Granada, Avda. Conocimiento s/n, 18100 Armilla, Granada, Spain.

# These authors contributed equally to the work.

\***Corresponding author:** Prof. José L. Quiles. Departamento de Fisiología. Instituto de Nutrición y Tecnología de los Alimentos “José Mataix Verdú”. Laboratorio 250. Parque Tecnológico de

Ciencias de la Salud. Avda del Conocimiento sn. 18100 Armilla (Granada) Spain. Email:

[jlquiles@ugr.es](mailto:jlquiles@ugr.es)

Accepted Manuscript

## Abstract

Extending life by delaying the aging process have proven to be the most effective way to fight multiple chronic diseases in elderly adults. Evidence suggests that longevity is inversely related to unsaturation of membrane phospholipids. The present study investigated how different unsaturated dietary fats affect lifespan and cause death in male Wistar rats fed diets based on virgin olive oil (V), sunflower oil (S) or fish oil (F), which were supplemented or not with Coenzyme Q10 (CoQ<sub>10</sub>). Previous results suggest that individual longevity and survival probability at different ages may be modulated by an appropriate dietary fat treatment. Lifelong feeding with V or F diets would reduce death probability compared to feeding with S diet at certain ages, although the effects of V diet would be maintained for most of life. Furthermore, the addition of lower amounts of CoQ<sub>10</sub> reduced mortality associated with S diet, but CoQ<sub>10</sub> had no effect on survival when combined with virgin olive oil or fish oil. Supplementation with low doses of CoQ<sub>10</sub> failed to increase the maximum lifespan potential of rats fed a V or F diet. No clear evidence showing that MUFA, n-3 PUFA or CoQ<sub>10</sub> exerted the observed effects by modulating the rate of aging has been found.

**Keywords:** longevity, life expectancy, MUFA, PUFA, fat

## 1. Introduction

Longevity is influenced by numerous environmental, sociodemographic, behavioral and dietary factors; therefore, longevity is a very complex phenomenon. It is known that nutrition have an important impact on overall mortality and morbidity, so nutrition may contribute to extend life expectancy. In fact, some components of the diet (i.e. foods or their components), termed “geroprotectors” by the gerontologist Ilya Mechnikov (1), are thought to increase lifespan, at least in experimental models. Currently, some authors have extended this term to any intervention with similar consequences, including modifications on the diet energy and/or macronutrient balance. A good example of this modification is a decrease of 30%–50% (without malnutrition) in food consumption, which consistently increases mean, median and maximum lifespans of both rats and mice (2–5), although this effect was not found for all the strains (6). More recent evidence also attributes this effect to diets low in proteins or even low in methionine (7,8).

Increases in mean and median lifespan are the manifestation of a reduced mortality, particularly at young age and middle age. This increase might be achieved by preventing causes of early mortality or slowing the rate of aging. The latter is expected to extend the maximum lifespan too and to delay the onset of age-related diseases. These effects would occur as a consequence of acting on basic mechanisms of aging such as cellular senescence, mitochondrial dysfunction, impaired proteostasis, inflammation or oxidative stress. Extending life by delaying the aging process *per se*, may prove to be the most effective way to fight multiple chronic and disabling diseases currently present in elderly adults, because this strategy would delay or prevent all age-associated pathologies rather than to overcome them individually, which is the current approach of the disease-based paradigm of drug development. Actually, treatments extending lifespan of animal models often protect against chronic diseases related to aging (9) and there is a reason to believe that a similar approach might work in humans reducing co-morbidities typical of elderly populations (10). Therefore, preventive therapies increasing lifespan by slowing the aging process become a high priority for disease prevention (9). In this context, elucidating the effects of other

dietary macronutrients on aging and health remains a fundamental challenge with profound implications for human health.

Lipids provide energy and play very relevant biological roles as components of the biological membranes. Current guidelines recommend consuming no more than 25–35% of total daily calories from fat and reducing saturated fat intake to 10% of daily calories. Initially, saturated fats were replaced by fats rich in n-6 polyunsaturated fatty acids (PUFA), such as linoleic acid, which consistently decreased plasma cholesterol and slightly reduced triglycerides. Such effects were expected to reduce cardiovascular diseases, the main cause of death globally. More recently, high consumption of monounsaturated fatty acids (MUFA)-rich oils has been shown to be as effective as the consumption of those rich in PUFA in lowering low-density lipoprotein-cholesterol (LDL-C). However, in contrast to the effect of PUFA-rich diets, MUFA-rich diets do not lower high-density lipoprotein-cholesterol (HDL-C), which have a low prothrombotic potential. Furthermore, differences in the chemical properties of different unsaturated fatty acids, as components of membranes phospholipids or lipoproteins, have implications for aging mechanism and longevity determination. The available comparative evidence suggests a higher degree of unsaturation of biological membranes in postmitotic tissues of short-lived homeothermic vertebrates compared to those found in long-lived ones. The latter have membranes highly resistant to damage since highly polyunsaturated acyl chains are very susceptible to peroxidative damage (11–14). Furthermore, this may prevent lipoxidation-derived damage to other macromolecules (15). Consistently, the “membrane theory of aging” proposes that longevity is inversely related to the level of unsaturation of membrane phospholipids. Along with intrinsically high resistance to modification of tissue macromolecules, the low rate of generation of endogenous damage also would be a key feature of animal longevity (11). In fact, long-lived animals also show low rates of reactive oxygen species generation and oxidative damage at their mitochondria. The potential relationship between these rates and the unsaturation degree has been explained by the “membrane pacemaker theory of metabolism”. This theory poses that highly polyunsaturated acyl chains confer

physical properties to cell-membrane bilayers, in particular a high “fluidity” that enhance and “speed up” the molecular activity of membrane proteins and consequently the metabolic activity of cells, tissues and the whole animal (12). Both theories are closely related to the “mitochondrial free radical theory of aging” because both mitochondrial oxygen radical production and susceptibility of the tissue macromolecules to oxidative damage would modulate the rate of aging. Therefore, they would determine the maximum lifespan (11). Membrane lipids are also chemically modified as part of other processes including the use of membrane lipid products for chemical signaling, a phenomenon that is intimately involved in determining the longevity of different animals (16). In this sense, n-3 PUFA may contribute to a lower production of proinflammatory prostanoids compared to n-6 PUFA, which would have beneficial effects on pathologies and conditions associated with inflammation, such as cardiovascular diseases (17).

Many studies have shown that the composition of the acyl chains of phospholipids found in biological membranes is influenced by dietary fatty acids. Therefore, membrane composition could be modified and maintained to increase longevity by long-term consumption of diets rich in a particular unsaturated fat. The main dietary source of MUFA is olive oil. Seed oils such as soybean, corn or sunflower oils are the main dietary sources of n-6 PUFA, whereas linseed, canola and fish oils are the main dietary sources of n-3 PUFA (18). Along with fatty acids, other lipid molecules also present in biological membranes can contribute to modulate both susceptibility to oxidation and functionality of membranes. Coenzyme Q (CoQ) is a key electron carrier in mitochondrial respiratory chain and constitutes an important lipid-soluble antioxidant present in all biological membranes (19,20). Likewise, other interesting physiological roles related to these activities have been suggested for this molecule (19, 20). Levels of this molecule in blood and different tissues (21-25) have been reported to change in response to dietary intake. Therefore, CoQ<sub>10</sub> supplements could prove to be particularly interesting for aging. Several studies conducted in preclinical models have reported positive effects of dietary CoQ<sub>10</sub> regarding aging, particularly under certain conditions associated with oxidative stress (26-34).

Based on the above, the present study aimed to investigate how different unsaturated dietary fats affect lifespan and cause death in male Wistar rats fed from weaning throughout life on isocaloric and normolipid diets according to the AIN-93 rodent diet criteria (35), being virgin olive oil, sunflower oil or fish oil, the only dietary fat used for each experimental group, supplemented or not with CoQ<sub>10</sub>.

## 2. Material and methods

### 2.1 Chemicals

All the chemical products and solvents, of the highest grade available, were acquired from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany).

### 2.2 Animals and diets

One hundred-fifty male Wistar rats (*Rattus norvegicus*) weighing 80–90g were housed 3 to a cage and maintained at 20 °C in a 12-h light/12-h dark cycle, with free access to water. The rats were randomly assigned to six experimental groups. From weaning until death, the animals were fed semi-synthetic and isoenergetic diets formulated according to the AIN-93 rodent diet criteria (35), except for the dietary fat source. From weaning up to 2 months of age, animals received diets formulated according to the AIN-93 diet for growth (AIN-93G), whereas the AIN-93 for maintenance diet (AIN-93M) was followed for the remaining period. Three different experimental diets according to the dietary-fat source were manufactured. The dietary fats used were: extra virgin olive oil (provided by the agricultural research center “Venta del Llano”, Mengibar, Jaen, Spain), sunflower oil (purchased in a local supermarket) and pure fish oil ROPUFA 30 (DSM, Kaiseraugst, Switzerland). Fatty acid profile of experimental oils are shown in Table 1S. At the same time, 2.5

mg/kg per day of CoQ<sub>10</sub> was added to the diet of half of the animals of each group. Therefore, our study was conducted in six different experimental groups: V= virgin olive oil; VQ= virgin olive oil+CoQ<sub>10</sub>; S= sunflower oil; SQ= sunflower oil+CoQ<sub>10</sub>; F= fish oil; FQ= fish oil+CoQ<sub>10</sub>. Diets were provided *ad libitum* for the first two months and then at 25 g/rat/day for the rest of the experiment (in order to avoid overweight). An individual follow-up until death of each animal was performed, body weights were recorded each month and death dates were recorded throughout the study. At the time of death, three experienced pathologists performed a necropsy in parallel following a registered protocol. The animals were treated according to the guidelines of the Spanish Society for Laboratory Animals. The experiment was approved by the Ethics Committee of Laboratory Animals of the University of Granada (permission number 20-CEA-2004). The potential presence of pathogens was evaluated in sentinel rats simultaneously present in the facility where animals were maintained. The analysis of the samples was performed under the supervision of a veterinarian following FELASA guidelines (36).

### **2.3 Pathological evaluation and determination of causes of death**

After death, each animal underwent a complete necropsy conducted by three board-certified pathologists. The main causes of death were classified into five major groups: (i) neoplasm, (ii) infectious/inflammatory, (iii) vascular (hemorrhagic), (iv) other, (v) unknown. When the main cause of death was neoplasm, an additional classification was performed: (a) epithelial, (b) mesenchymal, (c) cerebral, (d) pituitary adenoma, (e) hematolymphoid. These data were used to calculate cumulative incidences of the different causes of death for each dietary group.

### **2.4. Survival study**

The survival time (in days) of each rat is defined as the time elapsed from the date of birth until the date of death. For each dietary group, survival probabilities of the entire lifespan were



nonparametrically estimated with the Kaplan-Meier method using the SPSS statistical software package (SPSS for Windows, 24 SPSS Inc. Chicago, IL, USA). All rats were dead at the time of the analysis, so no censoring was required. Kaplan-Meier survival curves stratified by causes of death were constructed.

## 2.5. Mortality study

Discrete time intervals were set and the number of animals dying at each interval was counted. For each time interval, age-specific mortality ( $q_x$ ) was estimated as the number of animals alive at the end of the interval divided by the number of animals alive at the beginning of the interval. The hazard rate ( $H_z$ ) was estimated by  $H_z = 2q_x/(2 - q_x)$ . The Gompertz equation relating mortality rate ( $R$ ) to age  $t$  is:  $R_t = R_0 e^{\alpha t}$  (a), where the constants  $R_t$  is the chance of dying at age  $t$  (i.e., the  $H_z$  function),  $R_0$  is the non-exponential factor in mortality and  $\alpha$  is the coefficient of age-related rise of mortality rate (20).  $\ln R_t$  was plotted against age and a weighted linear regression curve was calculated from logarithmic formula of Gompertz equation (a):  $\ln(R_t) = \alpha t + \ln(R_0)$  (b) to estimate the constants  $R_0$  and  $\alpha$ . Data clearly located prior to the onset of senescence were excluded. In addition, estimated Gompertz parameters and equation were used to calculate mortality rate doubling time (MRDT) and initial mortality rate (IMR). MRDT was calculated by the formula:  $MRDT = 0.693/\alpha$  (c). The IMR was calculated from the Gompertz equation based on the mortality rate prior to the age-related increase in mortality (37).

## 2.6 Statistical analysis

Data were analyzed using the SPSS statistical software package. Regarding causes of death, the analysis of statistical differences between groups was performed using the  $\chi^2$  test for goodness of fit test. Mean, median and maximum lifespan were estimated from survival time for each dietary group. Normality of the data distribution and homogeneity of variances were checked using the

Shapiro-Wilk and Levene tests, respectively. Differences between survival times were evaluated using a one-way ANOVA followed by a pair-wise *post hoc* Bonferroni's test. Repeated ANOVA was used for time course of body weight data. To estimate potential differences in maximum longevity, the method based on quantile regression described by Wang *et al.* (38) was used. According to that, the proportions of rats alive in each group at the age at which 90% of the pooled population had died must be compared using the Fisher's exact test. Differences among survival distributions of cohorts fed different diets were evaluated using the Log-rank, Breslow and Tarone-Ware tests. The same tests were also performed to construct the Kaplan-Meier survival curves stratified by cause of death. Differences in the regression coefficients  $\ln R_0$  and  $\alpha$  were tested comparing the hazard functions obtained by linear regression from two different experimental groups, as previously described (39). Alternatively, to examine the potential confounding effect of cause of death on lifespan, a second analysis was performed using Cox's regression model as a multivariate approach for analyzing survival time. The cause of death was considered a covariate for each dietary treatment. A *P* value less than 0.05 was considered statistically significant for all the comparisons.

### 3. RESULTS

#### 3.1. Body weight

Body weight curves of the different experimental groups are shown in Figure 1S. Body weight increased in all groups of animals from weaning up to 18 months of age, although it was maintained for two- or three-month intervals several times during this period. There was no difference in body weight between dietary groups at baseline, and from weaning up to 12 months of age; rats from the three cohorts were similar in weight. However, the F group showed higher values of body weight than the V or the S groups between 12 and 25 months of age. Moreover, the F group showed a weight increase that continued until 20 months of age. Differences in weights between the F group and the V or the S groups increased dramatically with age. When animals supplemented

with CoQ<sub>10</sub> were compared with their non-supplemented counterparts, no significant differences were found, except for the F group that presented a lower body weight than FQ at the age of 20-25 months. After those ages, apparently, there is a decline in body weight values, but the standard error of the mean (SEM) was so high, that finding statistically significant differences was difficult. Since the rats are social animals, they should be kept in groups and not in individual cages, which greatly hindered the monitoring of food intake individually. Nevertheless, controlled amounts of diet were daily provided for all cages. No significant amounts of remained food or spillage were found in any cage.

### **3.2. Causes of death**

The apparent causes of death, based on the necropsy findings, are summarized in Table 2S. No major differences in main causes of death were found between groups fed different diets. The most common causes of death in all our experimental groups were neoplasms. The most common neoplasm was the epithelial type. Six individuals were excluded from the statistical analyses and from the survival evaluation because they suffered an accidental death early during the study. No animal showed alterations or signals leading to the application of the end-point protocol established in order to avoid the suffering of the animals.

### **3.3. Survival evaluation**

Mean lifespans estimated for non-supplemented rats were  $894 \pm 44$  days for V group,  $732 \pm 55$  days for S group and  $886 \pm 42$  days for F group. Maximum lifespans registered for the V, S and F groups were 1180, 1138 and 1208 days (39, 38 and 40 months approximately), respectively. The estimated values of median lifespan were  $904 \pm 69$  days (95%CI: 770-1038 days) for the V group,  $766 \pm 28$  days (95%CI: 712-820 days) for the S group and  $931 \pm 64$  days (95%CI: 806-1056 days)

for the F group. Statistical analysis revealed a lower mean lifespan in the S group compared to the V and F groups. No differences in median values were found between these three groups.

Estimated average lifespan of the groups supplemented with CoQ<sub>10</sub> were  $862 \pm 51$  days for the VQ group,  $886 \pm 39$  days for the SQ group and  $873 \pm 37$  days for the FQ group. The median survival of the VQ, SQ and FQ groups were 867 days (95%CI: 799-936 days), 961 days (95%CI: 873-1049 days) and 906 days (95%CI: 825-987 days), respectively. Statistical analyses showed significant differences between the S and the SQ groups. Moreover, a higher maximum lifespan was registered (1185 days) for the SQ group *vs.* the S group; however, no differences were found between the VQ (1143 days) *vs.* the V group or between the FQ (1116 days) *vs.* the F group. No differences were found among the three diets supplemented with CoQ<sub>10</sub>.

The age at which 90% of the rats had died excluding mice removed from the study as defined in the Methods section (90<sup>th</sup> percentile point of the joint survival distribution) was 1114.2 days. A total of 16.2 %, 16.9 % and 16.2 % of animals receiving non-supplemented V, S and F diets, respectively, were alive at that age. In groups fed with supplemented diets, this percentage was 16.9 % for all groups. However, Fisher's exact test showed no statistically differences among these percentages for the considered significance level.

Kaplan-Meier accumulated survival curves are shown in Figures 1, 2, 3 and 4. In non-supplemented groups (Figure 1), data indicate that lifespan would be affected by the dietary fat consumed throughout life. Statistical tests (Figure 2) revealed that survival probability was lower in the S group compared to the V group at any age. However, no differences in survival were found between the S group and the F group according to the results of the Log-rank test. In addition, differences in survival between the S group and the F group were statistically significant for the established significance level according to Wilcoxon–Breslow and Tarone-Ware tests. When animals were fed with CoQ<sub>10</sub>-supplemented diets and they were compared with their respective counterparts (Figure 3), Wilcoxon–Breslow and Tarone-Ware tests also showed statistically

significant differences in survival of those rats fed sunflower oil (S vs. SQ). In contrast, no significant effects were found for virgin olive and fish oil-fed animals whose survival curves were virtually identical between CoQ<sub>10</sub>-supplemented and non-supplemented groups (V vs. VQ, F vs. FQ). Kaplan Meier survival curves stratified by causes of death and subsequent analyses also were carried out; however, no significant differences were found (data not shown).

### 3.4. Mortality study

For the mortality study, time interval length was set at five months to estimate mortality and Hz at different ages. Some intervals were excluded in the regression analysis because no clear increases in Hz were noted and, therefore, data were located prior to the onset of the exponential growth of mortality rate. In turn, no intervals were excluded at old ages when hazard risks did not decelerate or deaths represented more than a 10% of animals per group. Gompertz equation parameters from the weighted linear regression curves obtained for each cohort are included in Figure 5. This figure also shows the linear regression curves of hazard risks as a function of age for the different dietary groups calculated according to Gompertz equation. For animals non-supplemented with CoQ<sub>10</sub>, Ln R<sub>t</sub> was lower in the V group compared to the S and F groups, whereas  $\alpha$  was higher in the V group compared to its value for the S and F groups. Therefore, MRDT was lower in the V group compared to the other two non-supplemented groups. Comparisons between regression curves of CoQ<sub>10</sub>-supplemented and non-supplemented diets revealed that for animal receiving CoQ<sub>10</sub>, Ln R<sub>0</sub> was lower in the SQ group than in the S group, whereas  $\alpha$  was higher in SQ than in the S group. This difference was also found in FQ group vs. F group. A value of 10 months was used to calculate IMR; however, no significant differences in IMR were found among dietary groups.

## 4. Discussion

Because of the rapid aging of the population, there is a strong interest not only in unraveling the causes of aging, but also in discovering how we can manipulate potential causes of aging to decrease, stop, or even revert its rate of progression (40,41). Of note, a number of dietary approaches have shown to be successful in increasing lifespan in some strains of rodents, at least under certain conditions. Effects of this type of interventions are caused by the prevention of the main causes of death at different ages or by slowing the rate of aging by modulating basic mechanisms of aging. The latter would be an efficient strategy to delay or prevent all age-associated pathologies particularly common in the elderly. Elucidating the effects of other dietary macronutrients on aging mechanisms and lifespan is still a critical challenge with profound implications for human health. In particular, the dietary fat consumed has shown to influence and modulate different proposed aging mechanisms, as well as to decrease the incidence of different pathologies and physiological alterations related to aging. The present study aimed to investigate how feeding rats with a virgin olive oil-, sunflower oil- or fish oil-based diet from weaning would have consequences on their lifespan. We also aimed to determine the causes of death.

Dietary interventions extending lifespan in animal models can also be evaluated by their reported positive therapeutic effects on known causes of human mortality. However, an important limitation of different studies on diet and lifespan is the absence of pathological analyses of the animals to elucidate if the treatments affected the causes of mortality. In the present study, despite differences in survival distributions, no effects of dietary treatments on causes of death were found. Furthermore, comparisons of the survival distributions of the three different cohorts using the log-rank test revealed that survival probability was significantly lower in the S group compared to the V group. The null hypothesis for the Log-rank test poses the equality of survival functions by weighting all time points equally. Whereas the null hypothesis for the other two (Wilcoxon–Breslow and Tarone-Ware) tests used poses the equality of survival functions by weighting all time points by the number of cases at risk (Wilcoxon–Breslow) or by the square root of the number of cases at risk (Tarone-Ware), at each point in time. This would imply that survival probability could be

statistically different between the F group and the S group at least during some periods of life. In other words, they do not assume that the difference in risk between the test groups is constant at all ages in contrast to Log-rank test. In addition, the Wilcoxon–Breslow test gives more weight to earlier deaths. The diet consumed would be responsible for the change in lifespan by altering the rate of aging, aging-independent mortality, or both of them. Differences among average lifespans of non-supplemented groups would suggest that lifespan was affected by dietary fat consumed throughout life. In particular, the mean lifespan of the S group was 18% and 17% lower than the mean lifespan of the V group and the F group, respectively. Increasing mean or median lifespan has been largely accomplished by reducing the rate of certain mid-life diseases causing death. Therefore, virgin olive oil- or fish oil-based diets would reduce or delay the onset of certain mid-life diseases causing death compared to the sunflower oil-based diet. However, the absence of differences in causes of death supports the second option, i.e. a delay in the onset of diseases.

Previous studies in rats fed throughout their lives with similar diets have verified a proper adaptation of fatty acid profiles of plasma and mitochondrial membranes to the dietary fat consumed in six- and 24-month-old animals (26,29,46 a 42). In addition, some dietary interventions with positive effects on lifespan has shown to increase MUFA proportion in biological membranes (43,44). In addition, a positive correlation between cardiac MUFA levels and lifespan was found in another study regardless dietary intervention (45). Some authors have suggested that n-6 PUFA used as the main dietary fat were able to reduce mortality, particularly when these interventions involved reducing saturated fat consumption because of its effects on blood lipoproteins and triglycerides, which are known cardiovascular risk factors. The results of the present study suggest that MUFA-rich diets could reduce mortality at most ages compared to diets rich in n-6 PUFA.

As stated above, no differences between our six experimental groups were found in causes of death. Nevertheless, an influence of these diets on the age at onset of the disease causing death or on the progression rate of the disease causing death cannot be discarded. However, evaluating these effects is not possible when pathological analyses are carried out after the animals' death. In this

sense, describing age-related changes found in biochemical and physiological indicators is very relevant. Compared with animals fed on a virgin olive oil-based diet, 24-month-old rats receiving a sunflower oil-based diet showed higher number of  $\beta$ -cells and insulin content at pancreas (46), higher fibrosis levels at liver (42), higher alveolar bone loss (47), and lower femur bone mass density (26). However, regardless the dietary fat, all 24-month-old rats showed steatosis with similar degree of centrilobular inflammation and non-alcoholic steatohepatitis (NASH) grade (42). Oxidative damage markers at hepatic (42), pancreatic (46), gingival (47) and systemic levels (26) in old rats fed diets with sunflower oil were higher than in rats fed diets with virgin olive oil as the unique dietary fat. In another experiment with similar diets but with a high amount of fat (8%), virgin olive oil-rich diets led to lower accumulation of mitochondrial DNA (mtDNA) deletions at Complex I gene in rat heart (27). Overall, all results in conjunction support that lifespan is determined, at least in part, by the extent of fatty acid unsaturation of the mitochondrial membrane. This effect can be explained by the differences between n-6 PUFA and MUFA susceptibility to lipid oxidative damage, and the consequent downstream protein and genome toxicity.

Regarding n-3 PUFA, previous studies have evaluated the effects of supplementation with these FA to modify the n-6 to n-3 PUFA ratio in the diet. Other studies have used combinations of both types of PUFA. In particular, the use of supplements of fish oil have been tested in a large cohort of genetically heterogeneous mice, but the pooled results revealed no significant longevity benefits (48). Some interventions in animal models have even been shown to shorten lifespan (49,50). Likewise, many recent clinical trials have also failed to substantiate the benefits of fish oil. Only 2 studies out of 18 clinical trials and 6 meta-analyses have reported benefits of fish oil (51). Nevertheless, as occur with sunflower oil, a scarce number of studies have compared the effects of diets based on fish oil with those based on virgin olive oil or on a n-6 PUFA-rich source of fat (such as sunflower oil) on lifespan. Regarding PUFA-rich diets, a study compared the effects on lifespan of diets rich in n-3 or n-6 PUFA and a commercial diet, but no significant differences were found. However, those animals began the treatment at 10 months of age (45). In a study conducted in



SAMP8 mice, a model with accelerated senescence, feeding on a mixture of soybean (3%) that is rich in n-6 PUFA and fish oil (2%) from 8 to 12 months of age increased survival probability compared to those mice using lard as the source of fat. However, animals on diets with only soybean oil as the source of fat showed no significant difference in survival compared to animals on diets using lard as the source of fat (52). Memory was also better preserved in animals fed a PUFA-rich diet than in animals fed a lard-based diet (52). This study supports the importance of n-3 PUFA in the diet for longevity compared with n-6 PUFA. According to the results of the present study, the effects of a fish oil-rich diet would reduce mortality compared with a diet rich in sunflower oil, at least during some periods of life. In consistency with this, other studies comparing fish oil-based diets with other diets based on n-6 PUFA have reported that detrimental changes associated with aging are prevented or delayed with fish oil. For instance, age-related alveolar bone loss (42) and liver fibrosis found in old rats were lower (42). In addition, age-associated increase in complex I activity has been shown to be prevented by fish oil-rich diets. This effect could be related with a lower production of reactive oxygen species in animals fed fish oil-rich diets (42).

In spite of the benefits on health of fish oil over sunflower oil, feeding on diets based on virgin olive oil have shown better results than fish oil regarding some alterations associated with aging. In particular, a higher degree of acinar fibrosis and macrophage infiltrates in peri-insular regions were found in the exocrine pancreas of 24-month-old rats fed fish oil compared to rats fed a diet based in virgin olive oil (46). Similarly, a higher age-related alveolar bone loss has been found (47). Moreover, lower levels of oxidative damage at liver (42) and pancreas (46) have been evidenced in rats fed on virgin olive oil compared to those fed on fish oil. It seems that mortality is not influenced by all these differences, although they might affect quality of life. In addition, this could also help to understand why the differences in survival probability between animals fed with virgin olive oil or sunflower oil are maintained throughout life, whereas differences between fish oil and sunflower oil should be referred only to certain periods of life.

Furthermore, changes in maximum lifespan potential, which represents the longest-lived member of the population, have been proposed to be a consequence of acting on the aging process *per se*. Accumulated evidence from comparative studies support that the maximum lifespan found in different vertebrate species has been correlated with the proportion and level of PUFA in membranes and with the degree of unsaturation of PUFA (13,14). However, no differences were found for maximum lifespan in the present study. Similarly to what seems to occur in many human populations during the last decades (53), it is possible that under our experimental conditions rats have reached or are very close to reach their maximum lifespan potential.

A second objective of the present study was to test the effect of dietary CoQ<sub>10</sub> on lifespan and causes of death. Usually CoQ<sub>10</sub> has been provided at very high dosage (100 to 2400 mg/day per person) in humans, but for short periods of time and to old or ill people (19). However, in the present study, a low dosage of CoQ<sub>10</sub> (equivalent to 18.43 mg per day for a 70 kg man) (19) was added to the diets because previous results of our research group suggest that a similar low-dosage supplementation is able to increase CoQ<sub>10</sub> body levels and prevent some deleterious aspects of PUFA-rich diet consumption during aging (26,29). In a previous study, our research group reported that long-term dietary CoQ<sub>10</sub> addition to a n-6 PUFA-rich diet led to higher mean and maximum lifespan (28). Some beneficial effects of CoQ<sub>10</sub> supplementation have been found in those animals fed fish oil-based diets (29), but effects of CoQ<sub>10</sub> supplementation of virgin olive oil-based diets are not so clear. Nevertheless, other studies in rodents have reported that CoQ<sub>10</sub> supplementation with daily doses ranging from 10 to 370 mg/kg in combination with standard diets has no effect on longevity (30,31,54,55), although treatments did not start from weaning in most of these studies (30,31,54). To test if CoQ<sub>10</sub> effects on lifespan are always present regardless the dietary context, CoQ<sub>10</sub> was also added to the other diets in the present study. Again, the addition of CoQ<sub>10</sub> to S diet increased average lifespan (a 17%), but this effect was not found in animals fed VQ or FQ diets. However, differences found between S and SQ groups were only statistically significant with Wilcoxon–Breslow and Tarone-Ware tests, which suggest that the effect of CoQ<sub>10</sub>

supplementation would only be present during some periods of life. The absence of effect on maximum lifespan and the presence of the lack of statistically significant differences among survival curves according to log-rank test in this study and not in the previous one (28) can be explained by the different amount of fat used in each study (4% vs. 8%). Overall, these results suggest that CoQ<sub>10</sub> benefits concerning lifespan are particularly useful under unhealthy dietary conditions. Many age-related alterations present in 24-month-old rats fed a sunflower oil-based diet (femur bone mass density, alveolar bone loss, increases pancreatic  $\beta$ -cell mass) have been reverted with CoQ<sub>10</sub> supplementation resembling findings found in animals fed virgin olive oil-based diets supplemented with or without a similar amount of CoQ<sub>10</sub> (26,32,56). These beneficial effects were related to a decrease in oxidative damage (26,32). In other experiments comparing similar diets but with a high amount of fat (8%), the addition of CoQ<sub>10</sub> also decreased cardiac (27) and brain levels of hydroperoxides (33) and mtDNA deletions at Complex I gene in the heart of old rats (27), but not in the brain (33). Beneficial effects of short-term supplementation with a high amount of CoQ<sub>10</sub> (2.81 g/kg per day) on protein oxidative damage at hepatic and mitochondrial level have also been reported in old mice (34). However, long-term CoQ<sub>10</sub> intake (93 or 371 mg/kg per day) in healthy mice fed a standard diet failed to modulate mitochondrial respiratory capacity in liver or oxidative damage in liver, kidney, skeletal muscle or brain, which correlated with the lack of effect on lifespan (31). Notwithstanding, the treatment began at 3.5 months of age in contrast to the present study that began the intervention at weaning.

Moreover, different authors have argued that changes in average and/or maximum lifespan are not a reliable indicator of alterations in the aging process (37). For this reason, similarly to other studies (39), the simpler Gompertz equation was also used to model the aging process. Comparing the Gompertz parameters between different cohorts has been shown to be a simple but effective method to determine whether the rate of aging was indeed altered. In turn, parameters of the Gompertz curve were used to calculate MRDT and IMR. The relative standard deviation of IMR for each group was very high (55-84%) and no differences were found among groups, but this is

expected to be species-specific and independent of the rate of aging. Furthermore, differences in basal Hz are consistent with virgin olive oil-based diet delay in the onset of diseases causing death at younger and middle ages, but they do not explain the reported differences among animals receiving fish and sunflower oils. Paradoxically, MRDT values suggest that virgin olive oil increases the rate of aging compared to the other dietary fats because an increase in this parameter is expected to reflect a decrease in the rate of aging (37). Estimations of Gompertz parameters point out that CoQ<sub>10</sub> addition caused changes in the mortality patterns of both sunflower and fish oil-fed animals but differences are in a similar way. In addition, previous studies have correlated MRDT with maximum lifespan, but no differences were found when this parameter was evaluated. Importantly, data obtained from this model should be interpreted with caution due to several reasons. On one hand, lower sizes of cohorts may bias early-life and late-life mortality estimates and skew measures of mortality slopes. On the other hand, the choice of onset of senescence and outliers is partly subjective and different plots could be obtained for the same mortality data; so different interpretations could be suggested.

Lastly, given the evidences in favor of caloric restriction role in life-extending experiments and the existence of some mimetic interventions, recording body weight information was very important. Differences in body weight did not correlate with the effect on lifespan when animals were fed with fish oil-based diets reaching higher values at some ages. Interestingly, a dose-dependent increase in body weight has been also reported for male mice receiving fish oil supplementation (48). Consequently, differences in lifespan seem not to be a consequence of body weight in this study, although it is possible that some alterations found in animals maintained on a fish oil-rich diet, as those observed at pancreas (46), involve a higher accumulation of body fat. Moreover, the addition of CoQ<sub>10</sub> to fish oil-based diet prevented an effect on body weight that would be maintained at values similar to those found in the other dietary groups. This might be a consequence of the reported benefits of supplementation with this molecule on pancreas of animals fed on similar diets based on fish oil (56).

In conclusion, the results of the present study suggest that individual longevity and survival probability at different ages may be modulated by an appropriate dietary treatment. Life-long feeding on virgin olive oil or fish oil would reduce death probability compared to sunflower oil, although the effects of virgin olive oil would be maintained during most part of life. The differences between sunflower oil and virgin olive oil could be explained by the susceptibility of membranes to oxidative damage that is reflected by the lower age-related changes and alterations found in different organs and tissues from relatively old animals maintained on virgin olive oil-rich diets compared to animals maintained on a sunflower oil-rich diet. However, diets based on both virgin olive oil and fish oil have failed in increasing maximum lifespan potential in this study, at least when they were administrated in the context of an isocaloric and normolipid diet according to the AIN93 criteria (35). Furthermore, the addition of lower amounts of CoQ<sub>10</sub>, an antioxidant molecule able to transport electrons within biological membranes, would remove part of the detrimental age-associated effects of sunflower oil used as main dietary fat. However, CoQ<sub>10</sub> has no effect on survival when it is combined with virgin olive oil or fish oil. Nevertheless, there is no clear evidence that MUFA, n-3PUFA or CoQ<sub>10</sub> exerted the above-mentioned effects by modulating the rate of aging.

### **Acknowledgements**

This work was supported by R+D grants from the Spanish Ministry of Education and Science [AGL2008-01057] and the Government of Andalusia [AGR832]. Alfonso Varela-López is recipient of a Research scholarship at universities or centers abroad from the foundation “Alfonso Martín Escudero”. María Dolores Navarro-Hortal is a FPU fellow from the Spanish Ministry of Educación y Formación Profesional. The authors thank Nutraceutical Translations for English language editing of the manuscript.

**Conflict of interest**

None

Accepted Manuscript

## References

1. Mechnikov II. Immunity in infective diseases. *Rev Infect Dis*. 1988;10(1):223–227.
2. Swindell WR. Dietary restriction in rats and mice: a meta-analysis and review of the evidence for genotype-dependent effects on lifespan. *Ageing Res Rev*. 2012;11(2):254-270. doi:10.1016/j.arr.2011.12.006.
3. Mercken EM, Carboneau BA, Krzysik-Walker SM, de Cabo R. Of mice and men: the benefits of caloric restriction, exercise, and mimetics. *Ageing Res Rev*. 2012;11(3):390–398. doi:10.1016/j.arr.2011.11.005.
4. Speakman JR, Mitchell SE, Mazidi M. Calories or protein? The effect of dietary restriction on lifespan in rodents is explained by calories alone. *Exp Gerontol*. 2016;86:28-38. doi:10.1016/j.exger.2016.03.011.
5. Anderson RM, Shanmuganayagam D, Weindruch R. Caloric restriction and aging: studies in mice and monkeys. *Toxicol Pathol*. 2009;37(1):47-51. doi:10.1177/0192623308329476.
6. Liao CY, Rikke BA, Johnson TE, Diaz V, Nelson JF. Genetic variation in the murine lifespan response to dietary restriction: from life extension to life shortening. *Aging Cell*. 2010;9(1):92-5. doi: 10.1111/j.1474-9726.2009.00533.x.
7. Zimmerman JA, Malloy V, Krajcik R, Orentreich N. Nutritional control of aging. *Exp Gerontol*. 2003;38(1-2):47–52.
8. Orentreich N, Matias JR, DeFelice A, Zimmerman JA. Low methionine ingestion by rats extends life span. *J Nutr*. 1993;123(2):269–274.
9. Moskalev A, Chernyagina E, Tsvetkov V, et al. Developing criteria for evaluation of geroprotectors as a key stage toward translation to the clinic. *Aging Cell*. 2016;15(3):407–415.

10. Chrysohoou C, Stefanadis C. Longevity and Diet. Myth or pragmatism? *Maturitas*. 2013;76(4):303-307. doi:10.1016/j.maturitas.2013.09.014.
11. Pamplona R, Barja G. Highly resistant macromolecular components and low rate of generation of endogenous damage: two key traits of longevity. *Ageing Res Rev*. 2007;6(3):189–210. doi:10.1016/j.arr.2007.06.002.
12. Hulbert AJ. The links between membrane composition, metabolic rate and lifespan. *Comp Biochem Physiol A Mol Integr Physiol*. 2008;150(2):196-203. doi:10.1016/j.cbpa.2006.05.014.
13. Pamplona R. Membrane phospholipids, lipoxidative damage and molecular integrity: a causal role in aging and longevity. *Biochim Biophys Acta*. 2008;1777(10):1249–1262. doi:10.1016/j.bbabbio.2008.07.003.
14. Hulbert AJ, Kelly MA, Abbott SK. Polyunsaturated fats, membrane lipids and animal longevity. *J Comp Physiol B*. 2014;184(2):149-166. doi:10.1007/s00360-013-0786-8.
15. Pamplona R, Barja G. Mitochondrial oxidative stress, aging and caloric restriction: the protein and methionine connection. *Biochim Biophys Acta*. 2006;1757(5-6):496–508. doi:10.1016/j.bbabbio.2006.01.009.
16. Bustos V, Partridge L. Good Ol' Fat: Links between Lipid Signaling and Longevity. *Trends Biochem Sci*. 2017;42(10):812-823. doi:10.1016/j.tibs.2017.07.001.
17. de Magalhães JP, Müller M, Rainger GE, Steegenga W. Fish oil supplements, longevity and aging. *Ageing*. 2016;8(8):1578-1582. doi:10.18632/aging.101021.
18. Perez-Jimenez F, Alvarez de Cienfuegos G, Badimon L, et al. International conference on the healthy effect of virgin olive oil. *Eur J Clin Invest*. 2005;35(7):421–424. doi:10.1111/j.1365-2362.2005.01516.x.



19. Varela-López A, Giampieri F, Maurizio Battino M., Quiles JL. Coenzyme Q and its role in the dietary therapy against aging. *Molecules*. 2016; 21(3), 373. doi:10.3390/molecules21030373.
20. López-Lluch G, Rodríguez-Aguilera JC, Santos-Ocaña C, Navas P. Is coenzyme Q a key factor in aging? *Mech Ageing Dev*. 2010; 131:225–235.
21. Reahal S, Wrigglesworth J. Tissue concentrations of coenzyme Q10 in the rat following its oral and intraperitoneal administration. *Drug Metab Dispos*. 1992; 20:423–427
22. Kwong LK, Kamzalov S, Rebrin I, Bayne ACV, Jana CK, Morris P, Forster MJ, Sohal RS. Effects of coenzyme Q(10) administration on its tissue concentrations, mitochondrial oxidant generation, and oxidative stress in the rat. *Free Radic Biol Med*. 2002; 33:627–638.
23. Hosoe K, Kitano M, Kishida H, Kubo, H, Fujii K, Kitahara M. Study on safety and bioavailability of ubiquinol (Kaneka QH) after single and 4-week multiple oral administration to healthy volunteers. *Regul Toxicol Pharmacol*. 2007; 47:19–28.
24. Matthews RT, Yang L, Brown S, Baik M, Beal MF. Coenzyme Q10 administration increases brain mitochondrial concentrations and exerts neuroprotective effects. *Proc Natl Acad Sci. USA*. 1998; 95: 8892–8897.
25. Lee BJ, Huang, YC, Chen, SJ, Lin, PT Coenzyme Q10 supplementation reduces oxidative stress and increases antioxidant enzyme activity in patients with coronary artery disease. *Nutrition*. 2012; 28: 250–255.
26. Varela-López A, Ochoa JJ, Llamas-Elvira JM, et al. Loss of Bone Mineral Density Associated with Age in Male Rats Fed on Sunflower Oil Is Avoided by Virgin Olive Oil Intake or Coenzyme Q Supplementation. *Int J Mol Sci*. 2017;18(7):1397.
27. Quiles JL, Pamplona R, Ramirez-Tortosa MC, et al. Coenzyme Q addition to an n-6 PUFA-rich diet resembles benefits on age-related mitochondrial DNA deletion and oxidative stress of a

MUFA-rich diet in rat heart. *Mech Ageing Dev.* 2010;131(1):38-47. doi:10.1016/j.mad.2009.11.004.

28. Quiles JL, Ochoa JJ, Huertas JR, Mataix J. Coenzyme Q supplementation protects from age-related DNA double-strand breaks and increases lifespan in rats fed on a PUFA-rich diet. *Exp Gerontol.* 2004;39(2):189–194. doi:10.1016/j.exger.2003.10.002.
29. Varela-López A, Ochoa JJ, Llamas-Elvira JM, et al. Age-Related Loss in Bone Mineral Density of Rats Fed Lifelong on a Fish Oil-Based Diet Is Avoided by Coenzyme Q10 Addition. *Nutrients.* 2017;9(2). doi:10.3390/nu9020176.
30. Lee C-K, Pugh TD, Klopp RG, et al. The impact of  $\alpha$ -lipoic acid, coenzyme Q10 and caloric restriction on life span and gene expression patterns in mice. *Free Radic Biol Med.* 2004;36(8):1043-1057. doi:10.1016/j.freeradbiomed.2004.01.015.
31. Sohal RS, Kamzalov S, Sumien N, et al. Effect of coenzyme Q10 intake on endogenous coenzyme Q content, mitochondrial electron transport chain, antioxidative defenses, and life span of mice. *Free Radic Biol Med.* 2006;40(3):480-487. doi:10.1016/j.freeradbiomed.2005.08.037.
32. Varela-Lopez A, Bullon P, Battino M, et al. Coenzyme Q Protects Against Age-Related Alveolar Bone Loss Associated to n-6 Polyunsaturated Fatty Acid Rich-Diets by Modulating Mitochondrial Mechanisms. *J Gerontol A Biol Sci Med Sci.* 2016;71(5):593-600. doi:10.1093/gerona/glv063.
33. Ochoa JJ, Pamplona R, Ramirez-Tortosa MC, et al. Age-related changes in brain mitochondrial DNA deletion and oxidative stress are differentially modulated by dietary fat type and coenzyme Q<sub>10</sub>. *Free Radic Biol Med.* 2011;50(9):1053–1064. doi:10.1016/j.freeradbiomed.2011.02.004.

34. Shetty RA, Forster MJ, Sumien N. Coenzyme Q10 supplementation reverses age-related impairments in spatial learning and lowers protein oxidation. *Age Drodr*. 2012;35(5):1821–1834. doi:10.1007/s11357-012-9484-9.
35. Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. *J Nutr*. 1997;127(5):838S-841S. doi: 10.1093/jn/127.5.838S.
36. FELASA working group on revision of guidelines for health monitoring of rodents and rabbits, M Mähler M (Convenor), Berard M, Feinstein R, Gallagher A, Illgen-Wilcke B, Pritchett-Corning K, Raspa M. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. *Lab Anim*.
37. Finch CE, Pike MC, Witten M. Slow mortality rate accelerations during aging in some animals approximate that of humans. *Science*. 1990;249(4971):902–905.
38. Wang C, Li Q, Redden DT, Weindruch R, Allison DB. Statistical methods for testing effects on “maximum lifespan.” *Mech Ageing Dev*. 2004;125(9):629–632.
39. de Magalhães JP, Cabral JA, Magalhães D. The influence of genes on the aging process of mice: a statistical assessment of the genetics of aging. *Genetics*. 2005;169:265-274.
40. Sousa-Victor P, Perdiguero E, Muñoz-Cánoves P. Geroconversion of aged muscle stem cells under regenerative pressure. *Cell Cycle*. 2014;13(20):3183–3190.
41. Li M, Belmonte JCI. Ageing: genetic rejuvenation of old muscle. *Nature*. 2014;506(7488):304.
42. Varela-Lopez A, Pérez-López MP, Ramirez-Tortosa CL, et al. Gene pathways associated with mitochondrial function, oxidative stress and telomere length are differentially expressed in the liver of rats fed lifelong on virgin olive, sunflower or fish oils. *J Nutr Biochem*. 2018;52:36–44.
43. Villalba JM, López-Domínguez JA, Chen Y, et al. The influence of dietary fat source on liver and skeletal muscle mitochondrial modifications and lifespan changes in calorie-restricted mice. *Biogerontology*. 2015;16(5):655-670. doi:10.1007/s10522-015-9572-1.

44. Jové M, Naudí A, Ramírez - Núñez O, et al. Caloric restriction reveals a metabolomic and lipidomic signature in liver of male mice. *Aging Cell*. 2014;13(5):828-837. doi:10.1111/accel.12241.
45. Valencak TG, Ruf T. Feeding into old age: long-term effects of dietary fatty acid supplementation on tissue composition and life span in mice. *J Comp Physiol B*. 2011;181(2):289–298.
46. Roche E, Ramírez-Tortosa CL, Arribas MI, et al. Comparative analysis of pancreatic changes in aged rats fed life long with sunflower, fish, or olive oils. *J Gerontol A Biol Sci Med Sci*. 2014;69(8):934-944. doi:10.1093/gerona/glt157.
47. Bullon P, Battino M, Varela-Lopez A, et al. Diets based on virgin olive oil or fish oil but not on sunflower oil prevent age-related alveolar bone resorption by mitochondrial-related mechanisms. *PLoS ONE*. 2013;8(9):e74234. doi:10.1371/journal.pone.0074234.
48. Strong R, Miller RA, Antebi A, et al. Longer lifespan in male mice treated with a weakly estrogenic agonist, an antioxidant, an  $\alpha$ -glucosidase inhibitor or a Nrf2-inducer. *Aging Cell*. 2016;15(5):872-884. doi:10.1111/accel.12496.
49. Sugawara S, Honma T, Ito J, Kijima R, Tsuduki T. Fish oil changes the lifespan of *Caenorhabditis elegans* via lipid peroxidation. *J Clin Biochem Nutr*. 2013;52(2):139-145. doi:10.3164/jcbtn.12-88.
50. Spindler SR, Mote PL, Flegal JM. Dietary supplementation with Lovaza and krill oil shortens the life span of long-lived F1 mice. *Age*. 2014; 36, 36(3):9659. doi: 10.1007/s11357-014-9659-7.
51. Grey A, Bolland M. Clinical trial evidence and use of fish oil supplements. *JAMA Intern Med*. 2014;174(3):460–462.

52. Ueda Y, Wang M-F, Irei AV, Sarukura N, Sakai T, Hsu T-F. Effect of Dietary Lipids on Longevity and Memory in the SAMP8 Mice. *J Nutr Sci Vitaminol (Tokyo)*. 2011;57(1):36-41. doi:10.3177/jnsv.57.36.
53. Cutler DM, Poterba JM, Sheiner LM, Summers LH, Akerlof GA. An aging society: opportunity or challenge? *Brook Pap Econ Act*. 1990;1990(1):1–73.
54. Sumien N, Heinrich KR, Shetty RA, Sohal RS, Forster MJ. Prolonged intake of coenzyme Q10 impairs cognitive functions in mice. *J Nutr*. 2009;139(10):1926-1932. doi:10.3945/jn.109.110437.
55. Lönnrot K, Holm P, Lagerstedt A, Huhtala H, Alho H. The effects of lifelong ubiquinone Q10 supplementation on the Q9 and Q10 tissue concentrations and life span of male rats and mice. *Biochem Mol Biol Int*. 1998;44(4):727-737.
56. González-Alonso A, Ramírez-Tortosa CL, Varela-López A, et al. Sunflower Oil but Not Fish Oil Resembles Positive Effects of Virgin Olive Oil on Aged Pancreas after Life-Long Coenzyme Q Addition. *Int J Mol Sci*. 2015;16(10):23425-23445. doi:10.3390/ijms161023425.

Accepted Manuscript

## Figure captions

**Figure 1:** Kaplan–Meier survival curves of the indicated cohorts of rats fed diets based on different dietary fats: virgin olive oil (V), sunflower oil (S), and fish oil (F).

**Figure 2:** Kaplan–Meier survival curves of the indicated cohorts of rats fed diet based on virgin olive oil supplemented (VQ) and non-supplemented (V) with CoQ<sub>10</sub>.

**Figure 3:** Kaplan–Meier survival curves of the indicated cohorts of rats fed diet based on sunflower oil supplemented (SQ) and non-supplemented (S) with CoQ<sub>10</sub>.

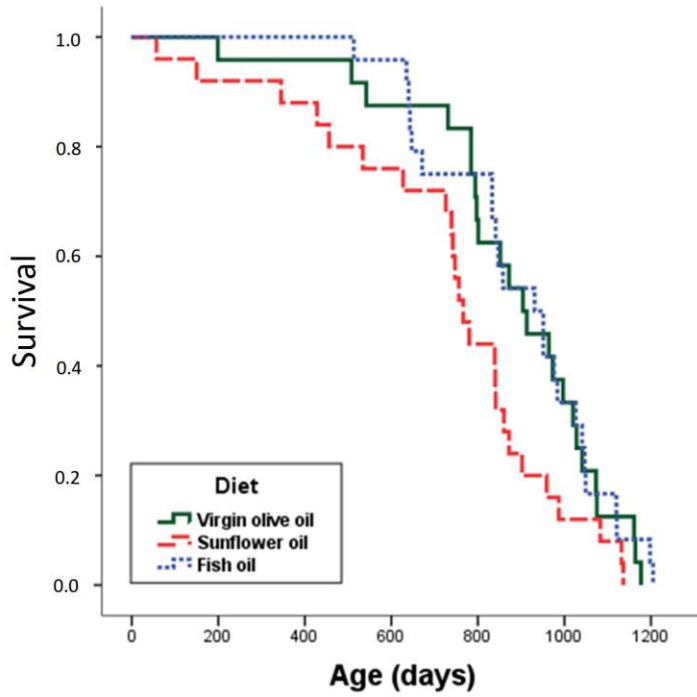
**Figure 4:** Kaplan–Meier survival curves of the indicated cohorts of rats fed diet based on fish oil supplemented (FQ) and non-supplemented (F) with CoQ<sub>10</sub>.

**Figure 5:** a) Natural logarithm of mortality rates (LnHz) at different age intervals for animals fed diets based on virgin olive oil (circles), sunflower oil (triangles) and fish oil (squares) without CoQ<sub>10</sub>. Lines represent estimated adult mortality trajectories based on Gompertz parameters for the different cohorts. b) Natural logarithm of mortality rates (LnHz) at different age intervals for animals fed diets based on virgin olive oil with CoQ<sub>10</sub> (circles). Lines represent estimated adult mortality trajectories based on Gompertz parameters for the two cohorts receiving diets based on virgin olive oil. c) Natural logarithm of mortality rates (LnHz) at different age intervals for animals fed diets based on sunflower oil with CoQ<sub>10</sub> (triangles). Lines represent estimated adult mortality trajectories based on Gompertz parameters for the two cohorts receiving diets based on sunflower

oil. d) Natural logarithm of mortality rates ( $\ln H_z$ ) at different age intervals for animals fed diets based on fish oil with CoQ<sub>10</sub> (squares). Lines represent estimated adult mortality trajectories based on Gompertz parameters for the two cohorts receiving diets based on fish oil. e) Gompertz parameters of the curves obtained from the linear regression analysis for each cohort. Abbreviations:  $\ln R_0$ : Natural logarithm of the non-exponential factor in mortality;  $\alpha$ : coefficient of age-related rise of mortality rate; MRDT: mortality rate doubling time; IMR: initial mortality rate. F: rats fed a fish oil-based diet; FQ: rats fed a fish oil-based diet supplemented with CoQ<sub>10</sub>; S: rats fed a sunflower oil-based diet; SQ: rats fed a sunflower oil-based diet supplemented with CoQ<sub>10</sub>; V: rats fed a virgin olive oil-based diet; VQ: rats fed a virgin olive oil-based diet supplemented with CoQ<sub>10</sub>. Symbols: \* represents statistically significant differences between animals fed with the same dietary fat with and without CoQ<sub>10</sub>; different lowercase letters indicate statistically significant differences between rats fed diets based on dietary fat without CoQ<sub>10</sub>.

Accepted Manuscript

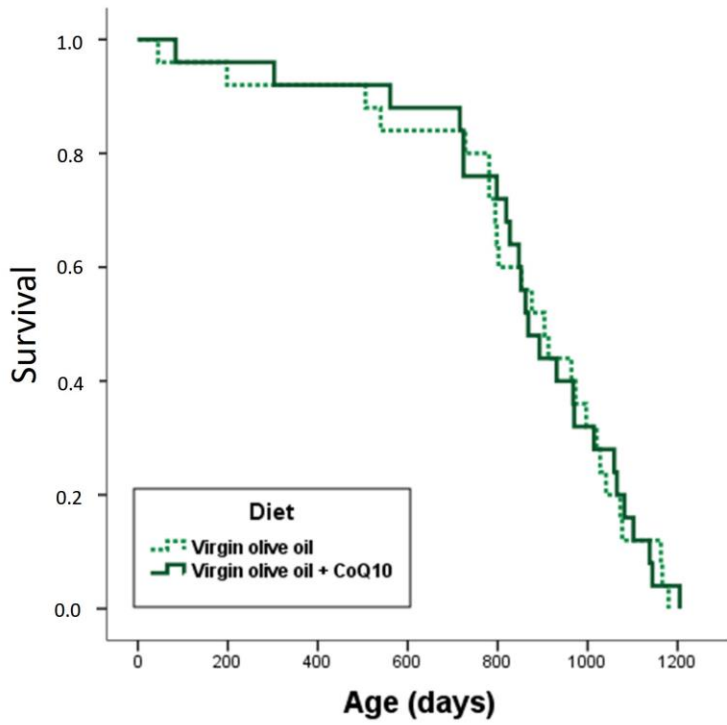
**Figure 1**



	V vs. S		V vs. F		S vs. F	
	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>
Log-Rank	4.738	0.029	0.168	0.682	3.589	0.058
Wilcoxon-Breslow	5.442	0.020	0.024	0.878	4.430	0.035
Tarone-Ware	5.276	0.022	0.049	0.825	4.215	0.040

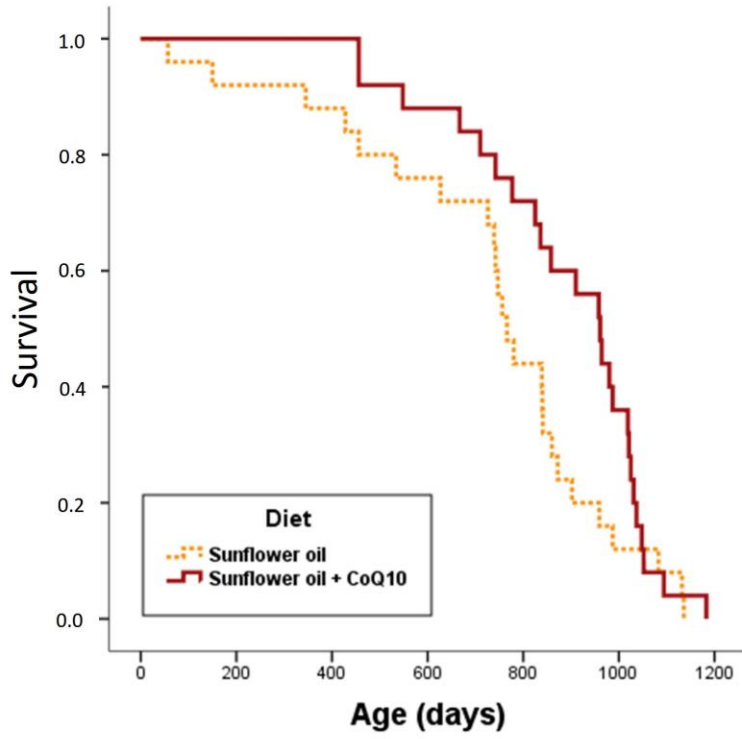


Figure 2



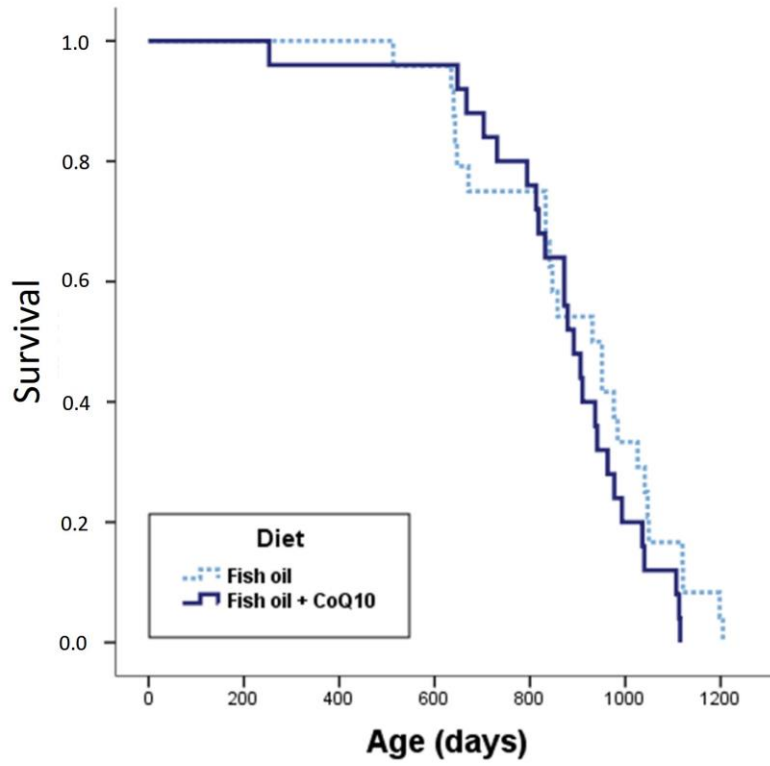
	V vs. VQ	
	$\chi^2$	<i>P</i>
Log-Rank	0.004	0.950
Wilcoxon-Breslow	0.036	0.850
Tarone-Ware	0.026	0.873

**Figure 3**



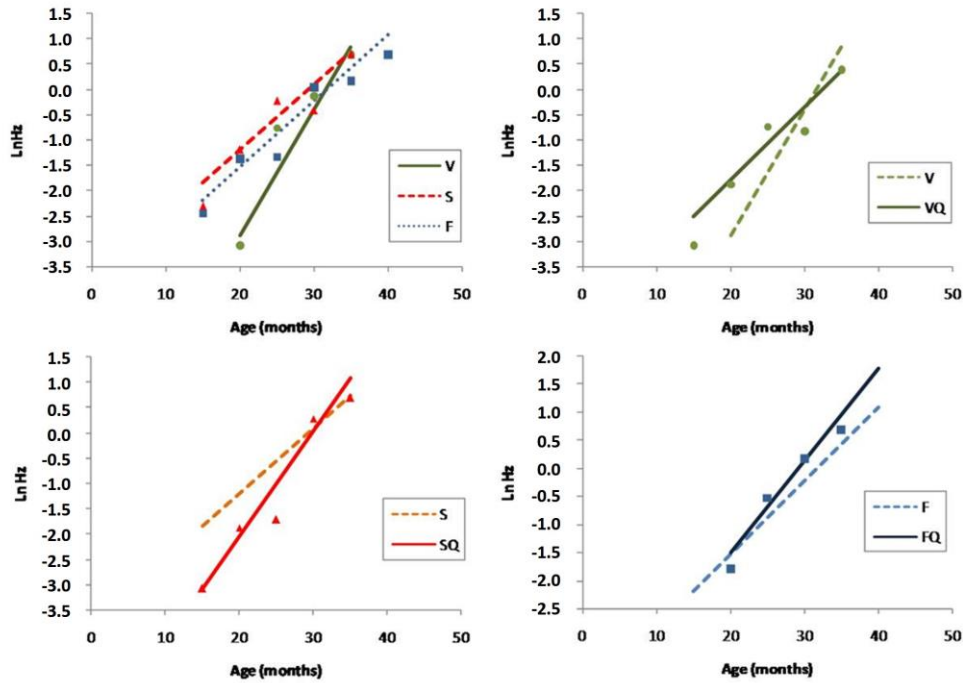
	S vs. SQ	
	$\chi^2$	P
Log-Rank	2.667	0.102
Wilcoxon-Breslow	4.704	0.030
Tarone-Ware	4.027	0.043

**Figure 4**



	F vs. FQ	
	$\chi^2$	<i>P</i>
Log-Rank	1.621	0.203
Wilcoxon-Breslow	0.249	0.617
Tarone-Ware	0.701	0.402

Figure 5



Diet	$\ln R_0$	$\alpha$	MRDT (months)	IMR
V	$-5.44 \pm 0.45^a$	$0.18 \pm 0.02^a$	$3.89 \pm 0.31^a$	$0.026 \pm 0.021$
VQ	$-4.67 \pm 0.34$	$0.14 \pm 0.01$	$4.82 \pm 0.37$	$0.040 \pm 0.023$
S	$-3.78 \pm 0.32^b$	$0.13 \pm 0.01^b$	$5.37 \pm 0.47^b$	$0.083 \pm 0.046$
SQ	$-6.19 \pm 0.21^*$	$0.21 \pm 0.02^*$	$3.33 \pm 0.23^*$	$0.016 \pm 0.013$
F	$-4.16 \pm 0.27^b$	$0.13 \pm 0.01^b$	$5.29 \pm 0.37^b$	$0.058 \pm 0.026$
FQ	$-4.78 \pm 0.22$	$0.16 \pm 0.03^*$	$4.23 \pm 0.69^*$	$0.043 \pm 0.031$