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Mitochondrial ROS and mtDNA fragments inside nuclear DNA as a main effector of ageing: the "cell aging regulation system"

Title in Spanish: *Fragmentos de mitocondria ROS y mtDNA dentro del ADN nuclear como principal efector del envejecimiento: el sistema de regulación celular de envejecimiento*

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ABSTRACT: The updated mitochondrial free radical theory of aging (MFRTA) is reviewed as part of the cell aging regulatory system (CARS). Any valid theory of aging should explain why different animal species age at so different rates. Only two known parameters correlate with species longevity in the right sense: the mitochondrial rate of reactive oxygen species production (*mitROSp*) and the degree of fatty acid unsaturation of tissue membranes calculated as the double bond index (*DBI*). Both are *low* in long-lived animals. *Dietary restriction* (DR), which increases longevity, *also decreases mitROSp* and % free radical leak (FRL) at complex I and *oxidative damage to mtDNA*. This can increase longevity by decreasing mtDNA fragments accumulation inside nuclear DNA which revitalizes MFRTA. Lowered mitROSp and FRL at complex I also occurs during protein or methionine restriction, and rapamycin treatment (which also increases longevity). The decrease in mitROSp during DR (dietary restriction) is due to restriction of a single substance, methionine, and occurs at the matrix domain of complex I. This updated MFRTA focuses on low mitROSp and low sensitivity of membranes to oxidation in long-lived animals. The three best known aging effectors of the genetic *Aging Program* of aerobic tissues are *mitROSp, membrane fatty acid unsaturation*, and *autophagy*. This program reacts to cytoplasmic signaling proteins, influenced by nutrients, drugs and hormones, varying the activity of the mitROSp and macroautophagy aging effectors. An analogous program, although with additional gene clusters of aging involved, and different output activity, can determine longevity in different animal species.

RESUMEN: Se revisa la teoría del envejecimiento por radicales libres de origen mitocondrial (MFRTA) como parte del Sistema de Regulación Celular del Envejecimiento (CARS). Cualquier teoría del envejecimiento debe explicar porqué las especies animales envejecen a velocidades tan diferentes. Solo dos parámetros conocidos correlacionan con la longevidad de las especies en el sentido correcto: la producción mitocondrial de radicales de oxígeno (mitROSp) y el grado de insaturación de los ácidos grasos de las membranas celulares. Ambos están disminuidos en las especies longevas. La restricción calórica, de proteínas, o de metionina, y la rapamicina, que aumentan la longevidad, también disminuyen la mitROSp y la fuga % de radicales libres en el complejo I y el daño oxidativo al ADNmt. Esto puede aumentar la longevidad disminuyendo la acumulación de fragmentos del ADNmt dentro del ADN nuclear con la edad, lo cual revitaliza la MFRTA. El descenso en mitROSp durante la restricción calórica se debe sólo a la restricción de metionina, y ocurre en el dominio de membrana del complejo I. *La mitROSp, el grado de insaturación de los ácidos grasos, y la autofagocitosis* son los tres efectores de envejecimiento conocidos dependientes del programa genético pro-envejecimiento (PAP, que es parte del CARS). El PAP responde a proteínas de señalización celular en función de la disponibilidad de nutrientes y hormonas en los medios ambiente e interno. Este programa, aunque con más clusters génicos del envejecimiento implicados, y con diferente intensidad efectora, podría ser responsable de la regulación de la longevidad de las distintas especies animales.

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1. INTRODUCTION

Aging causes 100.000 people deaths per day and 40 million per year worldwide. It is responsible for 70 % of all human deaths (90 % in developed countries like Spain). The terrible degenerative diseases most cancers and cardiovascular diseases, senile dementias including Alzheimer disease, Parkinson´s disease, osteoporosis, or type-2 diabetes have a common root: aging. Even if some can survive one of these illnesses, another one will come rather soon because the body is old and thus heavily damaged. The large majority of human cancers will never

be eliminated unless aging is defeated. Treating all the degenerative diseases one by one will never eliminate them. However, *defeating aging, all of them will be eliminated with a single manipulation*. This will restart evolution of increasing longevity, which has been among the main causes of the great success of our species in the planet, from our last common primate ancestors, the early small Plesiadapiformes-like Purgatorius which lived only around 10 years, to the 122 years that we can live now at best, a 10 fold (1.000 %) life extension. In order to accomplish this major goal, that will free us from most of those terrible illnesses and restart our evolutionary path towards an even higher complexity, we must first understand what are the fundamental basic mechanisms of aging down to the cell level. Once we know these, the large capacity of modern molecular biology and pharmacological techniques *will finally allow accomplishing the old dream of decreasing, for the first time in history, human aging rate.*

After truly decreasing aging rate, it will be possible in the future for human beings to reach e.g. 100 years with a *biological age* equivalent to that of a young person of today of 30 years of age ("only" around 3 fold lifespan extension). That would be properly called "healthy aging", which is clearly not the case for most human centenarians nowadays. The previous approach implemented in the past century, merely to *protect many people from death but not from aging*, increased the percentage of people that reached old age, so that they are alive but they are *biologically old* and therefore weak and with a ever increasing exponential chance of suffer sooner or later degenerative diseases and death. The result is the ever increasing percentage of aged people in our societies, generating an enormous load for social security and health care systems, and strongly increasing the proportion of fragile, disabled, and long time suffering old individuals in the population. Our aged societies and their high economic burden constitute the frequently called huge socioeconomic "aging problem". All this can be solved by progressively attaining negligible senescence with the help of gerontological science. Biogerontology has the key to this solution that will help to propel humanity to a brighter future on its committed evolutionary path from simplicity to complexity, from dust to bacteria, to eukaryotic cells, to multicellular life, to better society, and then to new achievements that few can nowadays even wonder.

Therefore, what causes aging? While many different theories of aging have been proposed, the mitochondrial free radical theory of aging (MFRTA) is one of the few current main explanations of aging and longevity in mammals, birds and multicellular animals in general. Any aging theory must explain *why maximum longevity* (referred here throughout as "longevity") *varies so widely* in animals: 30 fold from mice to men, 200 fold from shrews to the longest-living whales, or more than 5,000 fold from perhaps a few days of life in some invertebrates to *Arctica islandica* mussels (longevity around 500 years). Such huge differences indicate that *longevity is markedly regulated and flexible* during species evolution. Copying only a small fraction of this natural capacity would make possible in the future to reach *negligible senescence* in humans. Animals closely related by phylogeny, like *Mus musculus* (longevity 4 years) and *Peromyscus leucopus* (longevity 8 years) mice have very different maximum lifespans, indicating that evolution of longevity is also a relatively easy and quick process. Therefore, substantially *decreasing the aging rate in mammals including humans* will be relatively easy once the underlying basic mechanisms controlling longevity at physiological, cellular and molecular levels are elucidated.

Mean lifespan and the life expectancy at birth of the individuals of a population depends more on the environment than on the genes. On the contrary (*maximum) longevity*, and its inverse -the species aging rate -, *depends more than 90 % on the genotype*, as it is also the case for any other species-specific trait. Longevity and aging rate are the parameters that matter concerning the *endogenous* process of aging.

Up to now, only two known factors have demonstrated to correlate in the right sense with animal longevity in vertebrates including mammals and birds: a) the rate of mitROSp (mitochondrial reactive oxygen species production, Refs. 1-4); and b) the degree of fatty acid unsaturation (calculated as the double bond index, DBI) of tissue cellular membranes including the mitochondrial ones (5, 6). The longer the longevity of a species, the smaller the value corresponding to these two parameters. The low mitROSp of long-lived animal species decreases their generation of endogenous (free radical) damage at mitochondria. The low fatty acid DBI and peroxidizability index (PI) decrease the sensitivity of the cellular and mitochondrial membranes to free radical attack and lipid peroxidation. *No other theory of aging* focuses on parameters like these two, that *correlate in the right sense with longevity* across animal species (and also within single species), and offers plausible mechanistic explanations for the final accumulation of damage from *endogenous* origin leading to aging. This is relevant, since the most important fact that any theory of aging *must explain is why longevity and aging rate vary so widely* among different animals.

2. ANTIOXIDANTS AND LONGEVITY

Studies about MFRTA first focused in antioxidants, mainly because they could be measured with rather simple methods. In 1993 it was found at my laboratory that both enzymatic and non-enzymatic endogenous tissue antioxidants, including superoxide dismutase, catalase, GSH-peroxidases, GSH-reductases, GSH, or ascorbate, strongly correlated with longevity across vertebrates. However, and *rather surprisingly, such correlation was negative, instead of positive* as it was until that time widely believed. Our review on the relationship between endogenous antioxidants and vertebrate longevity (7) included all the available published data on the subject obtained in vertebrates including mammals by my as well as other laboratories.

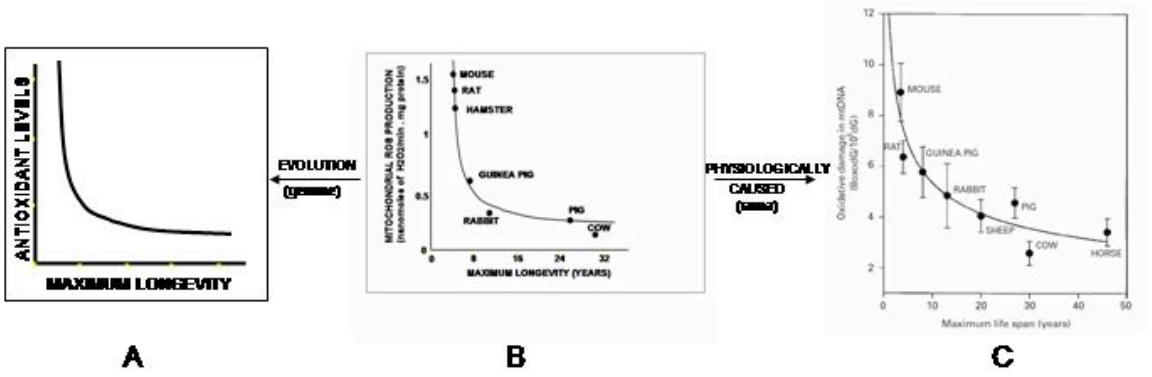

Figure 1. Production of ROS at mitochondria (A) negatively correlates with longevity across animal species. Evolutionarily, this is the cause of the very *low endogenous antioxidant* (including antioxidant enzymes) levels of long-lived animal species (B). In addition, it nowadays mechanistically causes the *low level of mtDNA oxidative damage of long-lived animals* (C). (Refs. 7,20,23,29).

All those data, coming from various different sources consistently agreed: *the longer the longevity, the lower were the levels of most endogenous tissue antioxidants and antioxidant enzymes.* A later reappraisal of the subject (8) confirmed the existence of a generally negative correlation between tissue antioxidants and longevity in all kinds of vertebrate animals (Figure 1A). It was most interesting that long-lived animals had lower instead of higher antioxidant levels. Among 27 studied correlations, 21 negatively correlated with longevity, six did not show significant differences, and not a single positive correlation with longevity was found (7). Superoxide dismutase was among the antioxidants tending to show no association with longevity. Previous believe that this enzymatic activity was positively associated with longevity was due to referring the SOD activity values (total SOD, CuZn plus Mn) to the oxygen consumption (VO_2) of the whole animal (to the weight-specific metabolic rate). Since weight-specific metabolic rate strongly decreases as body size increases, the larger SOD/VO_2 of humans compared to rats was due to the lower value of the denominator in the humans (lower weight-specific metabolic rate than rats) instead of to a higher value of the numerator (SOD). In fact when the tissue SOD values (total SOD, CuZn plus Mn, *without dividing by* VO_2) were plotted against longevity across species they showed no significant correlation with mammalian longevity in mammals in the original publication (9). In the brain and lung of vertebrate species –but not in liver- the correlation between SOD (total SOD, CuZn plus Mn) and longevity was again negative like for the other antioxidants. Further studies in different mammals including long-lived naked mole-rats, as well as ants, honey bees and marine bivalves also found a negative correlation with longevity for this antioxidant enzyme –SOD (8). In this more recent and comprehensive review of the subject, among a total of 78 correlations between endogenous tissue antioxidants and longevity, 72 were negative, six did not show significant differences, and only a single one was positive (8), corroborating global studies performed almost two decades ago (7). Therefore *endogenous antioxidant levels are clearly not the cause of the high longevity* of long-lived animal species.

3. MITOCHONDRIAL ROS GENERATION, mtDNA OXIDATIVE DAMAGE, AND LONGEVITY

3.1. Comparative studies of mitROSp

Why long-lived animals need less antioxidant levels in their vital organs? *We proposed (10) that the rate of mitROSp* could be negatively correlated with longevity and that this *would be the critical factor for aging, instead of the antioxidants*. *Long-lived animals* would not need to maintain high antioxidant enzyme levels, which is *energetically expensive*, because they would *produce mtROS at a low pace*, and they could transitorily induce them if needed. This was indeed experimentally corroborated comparing mammalian species with different longevities (3) (Figure 1B) as well as comparing short-lived rodents (rats and mice) with 8 fold longer lived birds (pigeons, parakeets and canaries) of similar body size and weight-specific metabolic rate (11, 12). A posterior investigation studying up to 12 different mammalian species confirmed these findings *even after correcting for body size* (4).

The studies in birds are especially important because the studies performed in mammals used species following the Pearl rate of living law of aging: "the lower the weight-specific metabolic rate the longer the longevity". Thus, one could not discard, in principle, the possibility that the species with longer longevity included in the comparisons between different mammals could show low rates of mitROSp simply because their rates of oxygen consumption were also lower than those of the short-lived ones. In fact mitROSp was positively correlated with mitochondrial O_2 consumption and with global metabolic rate in those studies (3). It was then important to study the problem in some of the many species that deviate from the Pearl´s rate of living law (an old theory of aging ruled out long ago). Three groups of warm-blooded vertebrates have much higher longevity than expected for their body size or weight-specific metabolic rate compared to most mammals*: birds, bats and primate*s. Birds have both a high rate of oxygen consumption per gram of tissue and a

high longevity. This makes them ideal to solve the problem mentioned above. The lower mitROSp of pigeons, canaries and parakeets, when compared to rats in the first case and to mice in the second and third case, strongly reinforces the MFRTA since it indicates that the low mitROSp of long-lived animals occurs both in comparisons between animals following Pearl´s law as well as in those not following it. *A high longevity is not a simple consequence of a slow rate of living*. It can be obtained –as the bird case shows- together with high rates of oxygen consumption and animal aerobic activity. High longevity in the studied birds is associated with a low rate of mitROSp *both* in absolute terms, and also as *percentage* of mitochondrial oxygen consumption and thus of electron flow at the electron transport chain (birds have a low percent free radical leak, FRL).

3.2. The mitROSp site at the ETC important for longevity

It was been widely believed for decades that complex III of the respiratory chain was the respiratory complex responsible for ROS production in the ETC (the mitochondrial electron transport chain) (13). Later it was found, already *working with freshly isolated and well coupled functional mitochondria*, that complex I also produces ROS in heart or brain mitochondria isolated from rats, mice, pigeons, canaries and parakeets (12, 14), which was soon confirmed in rats by other laboratories (15, 16) and soon became established knowledge in biochemistry books.

A key experiment to detect complex I ROS production was to measure mitROSp with succinate alone as well as with succinate+rotenone. In the second situation the rate of mitROSp acutely decreases because rotenone does not allow the electrons to flow back to complex I from succinate-complex II through reverse electron flow (17). But the habitual procedure of adding succinate alone, followed or not by antimycin A, and rarely using complex I-linked substrates, led to the general but erroneous believe during decades that mitROS came mainly from complex III-semiquinone.

In addition, we also found that *the lower mtROS generation* rate observed *in birds* compared to mammals of similar body size and weight-specific metabolic rate *occurred only at complex I* (12, 14, 17), not at complex III. This is most interesting since we found the same afterwards in dietary restriction (DR) rat models (see section 5). Concerning the precise site within complex I where ROS are produced three generators have been suggested, the flavin at the beginning of the electron path within the complex, the FeS clusters of the hydrophilic *matrix domain*, and the ubiquinone located in the membrane domain. Various investigators have supported the role of the flavin based on experiments with the inhibitor diphenyliodonium, which strongly decreases mitROSp. However, the site of action of diphenyliodonium, at the beginning of the electron path, also avoids electrons to reach the other two possible generators, the various FeS clusters and the ubiquinone, which therefore can not be discarded in those experiments. In other investigations, it was concluded that electron leak to oxygen occurred between the ferricyanide reduction site and the rotenone binding site of Complex I both in intact mitochondria (12, 14, 17) as well as in submitochondrial particles (18). Iron-sulphur clusters with a higher midpoint potential than FeSN1a, which could be situated in the electron path after the ferricyanide reduction site (15,18), or the unstable semiquinone known to be present in the membrane domain of Complex I and possibly functioning in H+ pumping coupled to electron transport (19), could be the complex I oxygen radical generators. However, many different Complex I FeS clusters could be responsible for complex I mitROSp because, under physiological conditions: (a) their reduced and oxidized states will not be present in equal concentrations; (b) interactions with many different factors and surrounding macromolecules could modify the final redox potential of the carriers *in vivo*; and (c) the exact position of many FeS clusters in the Complex I electron path is still unknown. Thus, the important aging-related question whether flavin, FeS clusters or ubisemiquinone, or a combination of these, are responsible for the complex I ROS generation relevant for aging was still unanswered (but see the proteomics ETC study described at section 5, Ref. 53).

3.3. Oxidative damage to mtDNA and longevity

The location where mitochondrial DNA (*mtDNA) is situated is very close to the site of mtROS generation, the ETC* at the inner mitochondrial membrane. ROS production also occurs at other cellular sites like microsomes, peroxisomes or membrane-bound NADPH-oxidases, and the rate of ROS generation at those sites can substantially exceed in various situations that coming from mitochondria. However, the ROS produced at mitochondria can be still the most important ones for longevity *due to the presence of mtDNA within the mitochondria* but not at those other organelles or parts of the cell. Since long-lived animal species have low rates of mtROS generation, it was logical to expect that this should have an effect on the steady-state level of oxidative damage in their mtDNA. Therefore we decided to measure the level of 8-oxodG (8-oxo-7,8-dihydro-2'deoxyguanosine) in the heart and brain mitochondrial and nuclear DNA of eight different mammalian species differing by up to 13-fold in longevity. The results showed that the level of *8-oxodG in the mtDNA* of both organs *is negatively correlated with longevity* (20). The longer the longevity of a species, the smaller is its mtDNA oxidative damage degree (Figure 1C). In contrast, the 8-oxodG level in nuclear DNA (nDNA) did not correlate with longevity in any organ even though mitochondrial and nuclear DNA was measured in the same samples taken from the same individual animals (20). Therefore, the different mitROSp rates of the different species seem to have a direct impact on mtDNA, and not on nDNA, concerning oxidative damage. This makes sense since the site of ROS generation at mitochondria is very close to mtDNA whereas nDNA is situated far away from it inside the nuclear compartment.

The rate of mitROSp is measured in isolated mitochondria *in vitro* due to the lack of reliable methods for direct *in vivo* mitochondrial H_2O_2 production determination. However, since the variations in the *steady-state* levels of 8-oxodG in mtDNA closely reflect the variations in the *rate* of mitROSp, both in comparative (Figure 1B,C) and in dietary restriction (DR; section 5) studies, suggests that the mitROSp *in vitro* measurements were closely indicative of the situation *in vivo*. In addition, the level of 8-oxodG in mtDNA was generally lower in the heart and brain of three long-lived birds when compared to two short-lived mammals of similar body size and specific metabolic rate, in agreement with the superior longevity of the birds, whereas again this was not the case for nDNA (21). These investigations also showed that the intensity of *oxidative damage is several fold higher in mtDNA than in nDNA* in the heart and brain of all the 11 different species of mammals and birds studied (20, 21), which is again consistent with the *close proximity between mtDNA and the sites of mtROS generation*. Studies on the longest-lived metazoan, the bivalve *A. islandica* have also shown increases with age in DNA oxidation and lower DNA oxidative damage than in other bivalve populations with much shorter (6 fold lower) longevities (22).

3.4. Increasing antioxidants does not change longevity

Initial studies about MFRTA were mainly focused on antioxidants because they were easier to measure, and because sensitive enough techniques to assay mitROSp in different species were generally not available at that time mainly due to the *generalized* use of spectrophotometry instead of fluorometry. Most of the investigations on the effect of *adding dietary antioxidants to the diet* were performed during the 1970´s and 80´s. The general result was that *antioxidants did not increase (maximum) longevity* (23, 24). In some experiments they increased only mean longevity (23-25). Interestingly, this tended to occur when the (maximum) *longevity of the control* rodents was short, usually less than 3 years. This suggests that antioxidants, when the husbandry conditions are suboptimal, could protect from causes of early death, and thus they can make more rectangular the survival curve. This is what happened in humans during the XXth century in many developing western countries when mean life expectancy increased from around 40 to 80 years without decreasing in aging rate. That is why now the old are so abundant in western populations. Antioxidants, in such cases above, were bringing back towards optimum the diminished survival of the controls reared under suboptimum environmental conditions, which is interesting but *not the goal of gerontology*. Ironically, *the poorer the survival curve of the controls, the largest is the chance of obtaining a positive result in terms of mean longevity*. A most inappropriate stimulus to not well done research indeed. Whereas, the better one performs the experiment, the less chances one has of obtaining an increase in *mean* longevity. In summary, like in the comparative inter-specific studies described above, *antioxidants clearly can not be among the factors responsible for low aging rates and high (maximum) longevities*.

When the techniques to obtain transgenic or knockout mice with *increased or lack of expression of genes codifying for antioxidant enzymes* like SODs, catalase, or GSH-peroxidases, were applied to this problem, the results were (obviously) similarly disappointing (26, 27). The *antioxidant enzyme activities increased* through modification of gene expression, like the non enzymatic dietary antioxidants added to the diet, *did not slow aging*. Independently of the way in which the antioxidants were manipulated, dietary or genetic, the result was the same: *a lack of effect of antioxidants on mammalian longevity*. This has been *interpreted by some (27) but not other (28) authors as the "death" of the MFRTA (27)*, but such conclusion (27) did not take into account that what correlates with longevity in the right sense is not the level of the antioxidants, but the mitROSp rate and the fatty acid unsaturation degree of the cellular membranes (23, 29).

Studies in simpler organisms like the fungus *Podospora anserina* have provided strong evidence for a role of mitROSp in senescence including DR-effects, and strains of this fungus deprived of mitROSp are converted to "eternal" non aging organisms (30). Recent experiments in 29 different longevity mutants, and 26 different environmental situations and drugs that increase (DR, PR, MetR, rapamycin, starvation, and others) or decrease (including 10 or 20 mg/l glucose medium, the 10μM amyloid-binding compound ThT, 1mM 4,4´-diaminodiphenylsulphone DDS, 10 mM N-acetyl cysteine) longevity used a new fluorescent probe claimed to estimate mitROSp ("free radical production and metabolic activity at the single-mitochondrion level", Ref. 31) for the first time *in vivo*. The results of these studies, performed in the nematode worm *C. elegans*, were extremely interesting. *In all the 55 cases lower "mitROSp" (mitoflash) was always observed in log-lived animals* (31). These results were quickly and heavily criticized in the same journal (Nature) by 27 other researchers from different fields related or not gerontology. The critics claimed that what the mitoflash technique estimated was not mitROSp (superoxide radical) because changes in fluorescence of the cpYFP used (the sensor circularly permuted yellow fluorescent protein in the matrix) "are due to alterations in pH, not superoxide". Thus, the critic was *exclusively methodological,* and did not consider what could be the biological significance of Shen et al. results (31). The reply of the authors of the *C. elegans* mitoflash study was also published in Nature together with the critic. The authors of the mitoflash study have continued publishing further investigations since 2014 using the same technique, leading to around a dozen further publications, some in mouse muscle, and in some studies they tried to ascertain if H^+ indeed interfered or not with their mitoflashes. The issue does not seem still resolved today. But if the critics are correct, why then low mitoflash (as claimed partial estimator of "mROSp") was always observed in long-lived animals, in full agreement with hundreds of published mitROSp-longevity studies

summarized in the present review? How a probe reacting *only* to pH could consistently vary with longevity *always in the right sense* in 55 mutant or environmental longevity models? And most importantly, what is the hypothesis relating H^+ to longevity? To the best of my knowledge none has been proposed. Perhaps proton gradient and mitochondrial membrane potential are indirectly related to longevity through changes in mitROSp, and this relationship would add to a possible reaction of some ROS with cpYFP. If this were true, both the critics and the authors of the study (31) would be right, and the impressive results would have a strong value for biogerontology. In any case, these important physiological questions have not been resolved and still wait for adequate answers. I consider most important to fully clarify this issue. The availability of a technique (mitoflash or any other better one?) that could reliably estimate how much mitochondrial ROS are produced in a mouse *in vivo* would be, no doubt, of paramount value for researchers and a most fundamental advance for gerontology.

3.5. The Contact Hypothesis of Aging. mitROSp instead of defences or repair control aging

Studies in mammals and other vertebrates clearly indicate beyond reasonable doubt that the *rate of mitROS generation importantly contributes to determine longevity, whereas antioxidants do not* (23, 24, 29, 32). This is counterintuitive only if we erroneously consider the cell as a homogeneous system without compartmentation. But cells are not like that. The *global* cellular level of oxidative stress should depend both on the rates of ROS production and ROS elimination. Both contribute to determine cell survival or cell death according to the general "balance" between them, and likely affect *mean* lifespan. However, the ROS concentration in particular compartments like mitochondria, and most especially very near to the places of mitROS generation like complex I, should be much more dependent on mitROSp than on antioxidants as the *free radical generation site* relevant for aging is approached at *micro level*. At such places, it is mitROSp what would mainly determine the local ROS concentration (Figure 2). This is especially important because the main target for aging, mtDNA, is located very close in the vicinity, perhaps even *in contact with the free radical generation source*.

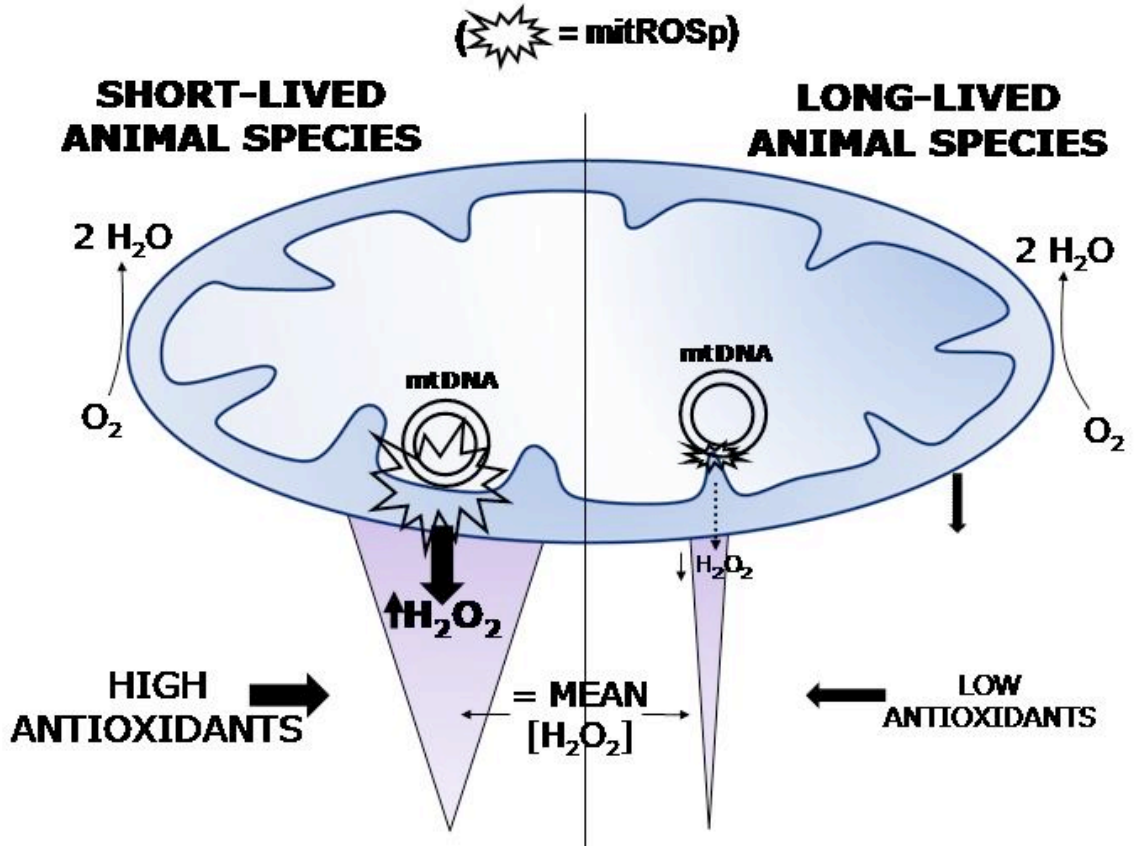

Figure 2. Low ROS local concentration near mtDNA in long-lived species. Long-lived animals show both low rates of mitROS production (Figure 1B) and low antioxidant levels (Figure 1A), and the reverse is true in short-lived-lived ones (7,10). Therefore, both kinds of animals have a *similar* and moderate level of *mean* oxidative stress (and H_2O_2 concentration) at most cellular sites. This is necessary for general cell homeostasis and survival both in animal species with high and low longevities. But the *local* concentration of ROS *at the site of ROS production* is much lower in long-lived than in short-lived species due to their low rates of mitROS production. This causes the lower oxidative damage to mtDNA (Figure 1C and Ref. 20) of long-lived species because mtDNA is situated nearby or even *in contact* with the site of ROS production at complex I relevant for aging. The model predicts the existence of a gradient of H_2O_2 concentration from mitochondria to the surrounding cytoplasm**.** The size of the stars, lower in long-lived species, represents the local ROS concentration at the sites of mitROSp at complex I, which is lower in long-lived species.

This would explain why lowering the rate of mitROSp instead of increasing antioxidants *was selected for* during the evolution of longevity in mammals, birds, and other species. And it would be consistent with the view that mitochondria are causal players in the aging process. *Contact* between mtDNA and specific mitochondrial proteins agrees with classic electron microscopic studies (33). Mitochondrial nucleoid structure has been long debated, with an estimation of about 2-10 mtDNA molecules coated by an unspecified number of proteins (34). However, using high-resolution microscopy, the structure of the nucleoids has been further clarified as single mtDNA circular molecules compacted by mitochondrial transcription factor A (TFAM, 35), which forces mtDNA to undergo a U-turn, thus collapsing the mtDNA molecule.

3.6. Low mitROSp, and then low endogenous antioxidant defences and DNA repair, in long-lived animals

In order to decrease damage to mtDNA it is much *more efficient* to lower the rate of generation of damage than to have a higher rate of generation of damage and, afterwards, try to intercept the generated ROS using antioxidants, or try to repair the damaged already inflicted on mtDNA. This last approach would not make sense if generation of damage can be controlled on the first place. This is why long-lived species have not used high (antioxidant) defence (7,8,10,11,23,24,29,32) or high repair of *endogenous* DNA damage (reviewed in 29) to increase longevity. In addition, it would be *very costly* to *continuously* maintain *high levels of antioxidant or repair enzymes* in long-lived mammals to counteract a high rate of damage generation. Instead, lowering mitROSp: i) is much *easier; ii)* is *100 % efficient*; and iii) avoids much damage at around *zero cost*. This view is fully consistent with the idea that aging is generated (evolutionarily speaking) "on purpose" by the organism itself, that aging has an *adaptive value*, likely *for the group* (2), and possibly to speed up evolutionary pace because it increases diversity and thus evolvability (2). This is why it has been *selected for* during evolution and *programmed in the genotype* to be expressed at widely different rates in agreement with the fecundity, population size, and many other traits of each particular species on its ecological niche (see section 9.2). The *association between fecundity and longevity* is not physiological, as the *Disposable Soma Theory* of aging assumes without evidence and lacking any physiologically plausible mechanism that could support it, but *genetic*. Because fecundity must be necessarily linked to longevity in the cell Aging Program (see section 9), otherwise population size would oscillate too much endangering the survival of the group.

3.7. FRL is not a constant percent, and mitROS are not simple "by-products", of the ETC

The fact that mitROSp is low in long-lived species *irrespective of the value of their specific metabolic rate* is clearly against the common belief that mitROS production is an unavoidable *"by-product"* of the respiratory chain. Mitochondria can *vary* the percentage free radical leak (FRL = % mitROSp/mitO_2 consumption) in the respiratory chain. They decrease such percentage (the FRL) in *especially long-lived* animals, like birds or dietary restricted rats. They lower the FRL to decrease mitROSp (to age slowly), while their mitochondrial oxygen consumption is not depressed to be able to continue mitochondrial ATP generation at the rates needed for the normal activity level of the animal. If ROS leakage were an "unavoidable by-product" of the mitochondrial electron transport chain, the FRL would be a rather constant % of electron flux at the ETC. Instead, the FRL is decreased especially long-lived animals like birds, that live 2-3 fold longer than mammals of the same body size and specific metabolic rate. Contrarily to the "by-product" unproven heavily repeated assumption, mitROSp is finely tuned (*regulated*) in each animal, and if necessary, it varies *independently of the rate of mitochondrial oxygen consumption and the total rate of electron flow at the ETC.* Thus, the FRL varies in many cases to contribute to determine the aging rate and the longevity of the species (birds), or the individual (during the DRs). The lazy assumption currently present in too many scientific articles that mitROS are *"unavoidable by-products of the ETC"* should be avoided because the measurements on *functional* isolated mitochondria data have demonstrated it to be false on too many occasions. The use of this "by-product" concept should be exclusively limited to particular situations on which it is known to be correct, but not as a generalization for every situation. This is important because this *wrong concept* is making *enormous damage to the scientific gerontological advance* concerning MFRTA, and therefore towards solving the aging problem.

4. LONGEVITY AND MEMBRANE FATTY ACID UNSATURATION

In addition to mitROSp, there is a second known parameter that also correlates with longevity in the right sense, the fatty acid unsaturation degree of tissue cellular (including mitochondrial) membranes. This is also well known since it has been studied many times in more than 20 well controlled different investigations and concordant results were *always* obtained. The degree of fatty acid unsaturation can be summarized as the double bond index (DBI), or alternatively, as the peroxidizability index (PI). *The longer the longevity of the species, the smaller the total number of tissue fatty acid double bonds (the smaller the DBI,* Figure 3A, and PI). A constitutively low DBI strongly decreases the sensitivity of the cellular and mitochondrial membranes to lipid peroxidation, a highly destructive process. Lipid peroxidation, in addition to membrane damage, produces mutagenic and toxic metabolites. Peroxidation of lipids quantitatively is the most intense destructive process produced in cells by ROS. Fatty acids containing a high number of double bonds (like 20:4n-6 and *especially 22:6n3*) are the cellular molecules most sensitive to lipid peroxidation, and their sensitivity to lipid peroxidation increases *exponentially* as the number of double bonds per fatty acid molecule increases. The low

DBI and PI of long-lived animals was first described in 1996 in rat compared to pigeon and human mitochondria (36) followed by many studies in mammals and birds (see 5,6 for review). A total of 23 studies extended the seminal first observation (36) to many different mammals, various bird species, and some invertebrates, without finding a single exception: low tissue DBI in long-lived animals (6). The low degree of fatty acid unsaturation occurs both in mitochondrial as well as in total cellular membranes in tissues of long-lived animals. It can therefore strongly diminish lipoxidation-derived damage in various cellular compartments, and especially in the mitochondrial one. At those organelles there is strong abundance of membranes together with a nearby and rather constant source of ROS during mitochondria respiration throughout the whole life span.

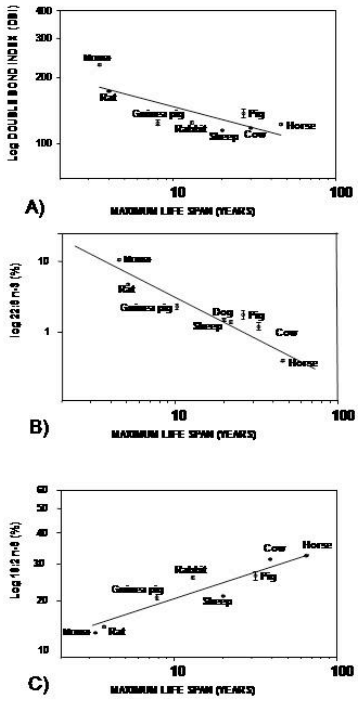

Figure 3. Low degree of membrane fatty acid unsaturation in tissues of long-lived animal species. A) Double bond index (DBI) in the heart of 8 mammals with widely different longevities**;** B) Levels of the highly unsaturated docosahexaenoic acid (22:6n-3) in liver of 8 mammals; C) Levels of the much less unsaturated linoleic acid (18:2n6) in liver of 8 mammals. DBI (and PI) (part A) and 22:6n-3 (part B) are low in long-lived animals, whereas 18:2n-6 is higher in long-lived ones (part C). In studies in some tissues or species low 20:4n-6 and high 18:1n-9 are also typical of long-lived animals. Similar results were obtained in skeletal muscle, and in mitochondria from the three mammalian tissues. Three birds also showed lower DBI than mammals of similar body size. Reviewed in Refs. 5,6, and Physiol. Reviews 87:1175-213, 2007.

Among the different fatty acids composing the different cellular membranes many are responsible for the strong decrease in DBI (and PI) as longevity increases among species. But the most important ones, both due to their content in double bonds (low or high) as well as for their larger quantitative presence and variation among species, are 18:2n-6, 18:3n-3 and 22:6n-3 in mammals (Figure 3B,C), and in some phylogenetic groups also 18:1n-9 (at least in the studied birds) and 20:4n-6**.** As longevity increases across mammalian species tissue 18:1n-9, 18:2n-6 (Figure 3C) and 18:3n-3 significantly increase, and 20:4n-6 and *especially 22:6n-3* (Figure 3B) significantly decrease in skeletal muscle, liver, and heart cellular membranes. Among them, the decrease in 22:6n-3 in long-lived animals usually is the most important to quantitatively explain their low DBI and PI values (Figure 3A,B). Interestingly, the final result is that the *total percentage of unsaturated* and saturated fatty acids *does not change* among species with different longevities. Instead *it is the unsaturation degree* of the polyunsaturated fatty acids present what decreases from short- to long-lived animals. *Long-lived animals* have fatty acids with a lower degree of unsaturation, *with less double bonds* per fatty acid molecule. With this kind of fatty acid redistribution long-lived animals obtain a strong decrease in the sensitivity of their cellular membranes to the destructive and mutagenic process of lipid peroxidation, while likely avoiding strong changes in the fluidity of their membranes, the so called homeoviscous–longevity adaptation (5). In addition, it has been recently shown that feeding 18:1n-9 reverses the increases in 20:4n-6 and 22:6n-3 and the decreases in 18:1n-9 and 18:2n-6 and complexes I and IV activities observed in the skeletal muscle of old rats (37). Recent confirmation of the low tissue fatty acid unsaturation of long-lived animals has been obtained through shotgun lipidomic analysis of mitochondrial

phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine in skeletal muscle, liver and brain of mammals with widely different longevities (38).

The low DBI of long-lived animals likely protects not only the lipids but also other kinds of cellular components. Since lipid peroxidation is a relatively massive process compared to oxidative damage to other kinds of macromolecules, long-lived animals, due to their low DBI, will produce far smaller amounts per unit time of highly toxic and mutagenic lipid peroxidation products like hydroxynonenal, malondialdehyde, and many others. These, having carbonyl groups, can modify free amino groups in proteins and DNA. Lipid peroxidation-derived protein modification seems to be involved also in aging, since comparisons among different mammalian species have found that the amount of malondialdehyde-lysine adducts in heart proteins negatively correlates with longevity (39).

What is the metabolic mechanism responsible for the negative correlation between the fatty acid unsaturation degree and species longevity? A role for acylation/deacylation of the constitutive membrane fatty acids can not be discarded. However, since the more unsaturated 20:4n-6 and 22:6n-3 are essential fatty acids synthesized from their dietary precursors 18:2n-6 and 18:3n-3 respectively, the enzymatic processes that control the n-3 and n-6 biosynthetic pathways seem to be involved. In this respect, in various comparative studies relating the degree of fatty acid unsaturation to longevity the results suggest that *desaturase and elongase enzymatic activities* in the n-3 and n-6 series (which are rate limiting for those biosynthetic pathways) *are constitutively low in long-lived animals.* In some cases decreases in peroxisomal beta-oxidation could also be involved. It is now considered that this last process is responsible for the last steps in the synthesis of the highly unsaturated 22:6n-3 in the n-3 pathway. The low delta-5 and delta-6 desaturase activities (which are rate limiting enzymes in the n-3 and n-6 fatty acid synthesis pathways) of long-lived animals will decrease the conversion of the less unsaturated 18:2n-6 and 18:3n-3 diet-derived precursors to the highly unsaturated 20:4n-6 and 22:6n-3 products. Thus, 18:2n-6 and 18:3n-3 would accumulate and 20:4n-6 and 22:6n-3 will diminish, which is just the general kind of fatty acid profile observed in long-lived animals.

In summary, the membrane fatty acid unsaturation degree is low in tissues of long-lived animals. *This is the only other known factor, in addition to mitROSp, which correlates with longevity in the right sense.* Importantly, this is true concerning the MFRTA as well as any other theory of aging. And the degree of fatty acid unsaturation suggests a plausible mechanism to contribute to the widely different aging rates of the different animal species. And what is the situation concerning experiments inducing increases in longevity in single mammalian species?

5. DIETARY RESTRICTION

It is well established that standard (40 %) dietary calorie restriction *(DR) increases not only mean but also maximum longevity* (up to 40 %) and *decreases and delays the incidence of degenerative diseases* in most animal species including rotifers, flies, spiders, worms, fish, laboratory rodents and many other mammals (40; Table 1). In a 20 years-long adult-onset study in rhesus monkeys 30 % DR strongly decreased age-related mortality (from 37 % to 13 %), the incidence of many age-related diseases including diabetes, cancer, cardiovascular disease, and brain atrophy (41). Many effects of DR have been discovered involving lowered GH and insulin/IGF-1-like signalling, modifications in nutrition and amino acid-sensing pathways, changes in sirtuins (42), apoptosis, and signalling proteins and transcription factors like mTOR,S6K, AKT, PKA, or FOXO and tissue-specific changes in gene expression profiles. Many of these changes and others are interrelated and seem to be part of an integrated cellular aging regulation system (see section 9) which includes mitochondria oxidative stress-related damage, and sensitivity to lipid peroxidation, as two of its main aging effectors (42).

In the previous sections it was described that long-lived animals have lower rates of mitROSp and lower mtDNA oxidative damage than short-lived ones (29). But what occurs in DR? If the MFRTA is correct also inside species, those two parameters should also decrease during DR. Initial studies, like in the case of the comparison between different species, focused mainly on antioxidants. They showed that DR in rodents does not lead to a generalized increase in antioxidants. Instead increases, decreases or lack of changes, depending on the particular antioxidant measured, have been reported even within the same study (43). Therefore, similarly to what happens in the inter-species comparative case, *the key to longevity again is not based on the antioxidants* levels during DR.

A different situation concerns mitochondrial ROS generation. The effect of DR on the rate of mitROSp was repeatedly investigated in mice, and especially in rats, by many different laboratories. The results of these investigations consistently agreed that long-term standard (40 %) DR, as well as short-term (e.g.: 7 weeks) *DR, significantly decrease the rate of mtROS generation* in rat organs including skeletal muscle, kidney, liver, heart and brain (44, Table 1). This agrees again with the concept that lowering mitROSp contributes to increase longevity. The decrease in mitROSp during DR was found in *freshly isolated functional mitochondria* exposed to similar incubation conditions, including the substrate concentration used to feed electrons to the ETC, in the *ad libitum* fed and DR groups. Thus, *DR mitochondria are different* from those obtained from *ad libitum* fed animals, and this difference (due to DR) is responsible for the lowered mitROSp detected *in vitro*. In addition, classic data suggest that complex I substrates like pyruvate decrease during DR in tissues (45), which has been recently confirmed by full metabolome analysis (46). The decrease in pyruvate and other mitochondrial substrates would decrease the *in vivo* matrix NADH level in DR thus

lowering electron feeding of complex I by NADH. This would in turn decrease the degree of electronic reduction of the complex I ROS generator, and then its rate of oxygen radical production. Indeed, DR also decreases the NADH concentration (45, 46), a change that is known to strongly decrease the rate of mitROSp (16). This will lead to a *further decrease* in the rate of mitROSp *in vivo* which *would add* to that due to the lowered capacity of DR mitochondria to generate ROS detected *in vitro*.

Interestingly, *the decrease in mitROSp in DR rats specifically occurred at complex I* in all the organs studied (47-49). Thus, a *low rate of mitROSp at complex I is a trait both of long-lived species and of DR mammals*. A recent study showed that a single nucleotide mutation in complex I suppresses mouse fibroblast aging (50), and inhibition of this complex by the antihyperglycemic and proposed antiaging modulators biguanides lowers its rate of mitROSp (51). Many studies have shown that mitochondrial functionality and even morphology is detrimentally altered in tissues of old animals (52). Recently, proteomic analysis of 57 out of the 96 known mouse ETC proteins (and 67 % of complex I proteins), showed that *low abundance of the "matrix domain-only" of complex I,* independent from the rest of the complex, *lowers* mitROSp and is related to increased longevity both in DR and in longer-lived mouse strains, while it increases during mouse aging (53). That is highly interesting since we and others have located the ROS generator relevant for aging precisely inside that *matrix domain of complex I*, and likely corresponds to one of the *FeS clusters* of that domain (15,18,47).

Dietary restriction, in addition to lowering mitROSp, also decreases the FRL (Table 1). This indicates that the efficiency of the mitochondrial respiratory chain in avoiding ROS generation increases in DR animals. Birds (pigeons, canaries and parakeets, Refs. 12,17) , which are especially long-lived homeothermic animals for their body size and metabolic rate, also show lower FRL values than the much shorter-lived rats or mice. This suggests that *a low FRL* can be a conserved mechanism of life span extension both *between and within species* that can be obtained without the need to decrease mitochondrial oxygen consumption. That is relevant when it is necessary to increase longevity without decreasing the general level of animal activity and thus competitiveness in the ecological niche. A decrease in FRL can be obtained through qualitative changes in the *redox mid point potential* of the complex I ROS generator related to aging. This would lead to a decrease in the degree of electronic reduction of that generator and thus in complex I mitROSp without decreasing electron flow in the ETC and thus mitochondrial oxygen consumption. *Both a low mitROSp and a low FRL at complex I* have been reported repeatedly at my laboratory in many different investigations in long-lived animals (11,12,14,17,18,29,47; Table 1). Interestingly, a recent extensive proteomic analysis of the full ETC in mice showed that partial assembly of the *"matrix domain-only" of complex I* also occurs during *ad libitum* (in addition to the whole complex I protein), but to a much lower extent during DR (53). That can help to explain both the decrease in mitROSp and the decrease in FRL at complex I in DR. Moreover *rapamycin*, the only drug known that consistently increases mouse longevity (see section 7), *also lowers mitROSp and FRL at complex I and decreases the amount of its "matrix domain-only"* in the mitochondria of mouse liver (53; Table 1). It is that matrix domain which seems to contain the subunits responsible for the decrease in mitROSp at complex I related to species longevity (15,16,18,29) and decreased both by DR (44,47) and rapamycin treatment (see below).

During *ad libitum* feeding the *extra copies* of the "matrix domain-only" complex I would take electrons from NADH but could not pass them to ubiquinone and the following complexes of the ETC. This would strongly fill up that domain with electrons and would thus strongly reduce with electrons the complex I ROS generator/s. This would increase mitROSp at this "matrix domain-only" complex. The result would be a strong increase in mitROSp without any ATP generation, because electrons in matrix domain only complex I can not reach the other separated complexes. In this way the AL animal would have a high FRL because it has both complete complex I copies, plus "matrix domain-only" complex I copies that *increase mitROSp but not oxygen consumption or ETC electron flow*. In contrast, the DR and the rapamycin-treated animals have complete fully assembled complex I copies and reduced abundance (20-50 % decrease) of "matrix domain-only" complex I copies. The DR mice would thus exhibit decreases in mitROSp *without decreases in mitochondrial oxygen consumption (lower FRL)* with a normal ATP production. Therefore, a *quantitative* decrease in the amount of the "matrix domain-only" of Complex I can lead to a *qualitative* change (decreased FRL) in DR and rapamycin-treated rodents. Lowering of the mid point redox potential of the complex I ROS generator would be another (*qualitative* in this case) mechanism than can also contribute to decrease mitROSp in long-lived animals (14,17,18,29,47).

In contrast to mitROSp, which is low both in long-lived species and in DR rodents, a low DBI occurs in long-lived species (see the previous section) and in 80 % methionine restricted (MetR) rats, but not in 40 % MetR (see section 6) or 40 % DR rats. Therefore a low DBI, like a low mitROSp, can contribute to decrease aging rate during evolution of long-lived animal species. But, in a single species, the membrane needs to decrease its sensitivity to oxidative damage by lowering fatty acid unsaturation only when the level of protein restriction in the available food is very strong, whereas the decrease in mitROSp is a response already recruited at milder (40 %) levels of DR. This makes sense, in principle, since dietary protein availability is essential for growing of the offspring. At 80 % MetR there is not enough protein for adequate growing, and the competitiveness of the offspring in the wild would be very low. Therefore it is even more important than in 40 % DR to post-pone aging to

reproduce only afterwards, when the levels of protein availability increase again in the ecological niche. That is why, at 80 % protein (methionine) restriction both a low mitROSp and a low DBI response are recruited. In relation to this, recent comparison among DR diets containing fish oil, soybean oil or lard as lipid source, showed that the lard containing diet (with less unsaturated fatty acids) was the one maximizing the beneficial effects of DR on mitROSp, proton leak, ETC, lipid peroxidation, mitochondrial structure or mitochondrial apoptotic signaling, likely due in part to increases in *monounsaturated fatty acid* content in the tissues (54). This improvement in mitochondrial functionality of diets promoting some decrease in tissue DBI is consistent again with a *healthy and pro-longevity effect of membranes containing a low number of fatty acid double bonds*.

Finally, since mitROSp is lower in DR than in the *ad libitum*-fed control animals, oxidative damage should also be lower in the nearby, or even in *contact* situated, mtDNA of the restricted animals. In agreement with this, it was found that the level of *8-oxodG in mtDNA was significantly lower* in the liver, heart and brain *of the long-term DR* rats in which mitochondrial ROS production was also diminished (reviewed in 55). Depending on the organ studied, such decrease in 8-oxodG occurred only in mtDNA, or both in mtDNA and nDNA (Table 1).

TABLE 1. Summary of changes on free radical related parameters induced by all manipulations known that consistently increase or not longevity in mammals laboratory rodents

Experimental manipulation Longevity (MLSP)	mtROSp (at CxI)	$mtVO_2$	FRL	8oxodG (at CxI)	8oxodG in mtDNA	8oxodG in nDNA	mtDNAFragments inside nDNA
DR	↓	↔	↓	↓	↔	nd	↑
PR	↓	↔	↓	↓	↔	nd	↑
LR	↔	↔	↔	↔	↔	nd	↔
CHR	↔	↔	↔	↔	↔	nd	↔
MetR	↓	↔	↓	↓	↔	nd	↑
Rapamycin	↓	↔	↓	↓(ns)	nd	↓	↑

DR= dietary restriction; PR = Protein restriction; LR = lipid restriction; CHR = Carbohydrate restriction; MetR = methionine restriction; Rapamycin (14mg/kg diet). The effects of DR,PR, and MetR on oxidative stress related parameters were obtained at a level of 40% restriction, and also at 80% MetR. The effect of MetR on longevity has been studied always restricting methionine by 80% in the diet (56,57,59). The rapamycin effect of mitochondrial oxidative stress was studied on B6D2F1 mice (139); the rest of the experiments were performed on Wistar rats. While DR, PR and MetR decreased mitROSp and 8-oxodG in mtDNA in all the vital organs studied (liver, heart and brain), rapamycin (at 14mg/Kg diet) did it in liver but not in heart of mice; mtDNA fragments inside nDNA were studied in the liver. For other Refs. see text. $mtVO_2$ = mitochondrial oxygen consumption; FRL = % free radical leak from CxI of functional mitochondria; nDNA = nuclear DNA; MLSP = maximum life span potential. DR, PR, MetR and rapamycin all increase *both* mean lifespan, and *maximum* longevity (up to 40% in DR, by 20% in PR and DR, and by 11% with rapamycin) in rats and mice.

6. PROTEIN AND METHIONINE RESTRICTION

6.1. Effect on longevity

It has been generally agreed for a long time that calorie intake *per se* would be exclusively responsible for the increase in lifespan induced by DR in rodents. However, now many studies question this classical consensus. The results of many investigations indicate that *part* of the life-extending effects on DR are due to the decreased intake of particular components of the diet, such as *proteins*, and more specifically the amino acid *methionine* (56-60). The few available studies do not support the possibility that either life-long isocaloric carbohydrate or lipid restriction increase rodent life span. Two investigations of carbohydrate restriction or supplementation reported opposite and minor changes in rat longevity (61, 62), and it was found that the longevity of Fisher 344 rats does not change after life-long lipid restriction (63). In contrast, the large majority of the investigations on the effects of *isocaloric protein restriction* (PR) in rats and mice found increases in longevity (Table 1). Ten out of eleven protein restriction (PR) investigations in rats or mice (16 out of 18 different life-long survival experiments) reported increases in longevity (58), although the mean magnitude of this increase (around 19 %) was lower than that usually found in 40 % DR (up to 40 % increase). Thus, PR would be responsible for around *half* of the life-extension effect of dietary restriction.

Among the different dietary amino acids, which is the one/s responsible for the increase in longevity induced by PR? It is consistently known that isocaloric 80 % methionine restriction (MetR) increases longevity in F344 rats (56) and mice (57-59; Table 1) to a similar extent than PR. The mean increase in (maximum) longevity in the three available MetR life-long experiments taken together was around 18 % increase. This occurred even when MetR was started as late as at 12 months of age in C6BF1 mice (59). Studies performed in *D. melanogaster* have also shown that casein restriction (64) and methionine restriction (65) extend longevity independently of the

caloric intake. Moreover, other recent studies link essential amino acids, and again especially methionine, with the positive effect of DR on longevity in yeast (66) and *D. melanogaster* (67). Interestingly, PR results in profound changes in methionine and serine metabolism (including lowering cystathionine β-synthase and cystathionine γ-lyase activities) and increases the oxidation of fatty acids in rat liver (68).

In addition to extending lifespan, 80 % MetR also decreases disease-associated markers and the incidence of age-related degenerative diseases (69, 70). The beneficial effects of this intervention in rodents include decreases in serum glucose, insulin, IGF-1, cholesterol, triglycerides and leptin. Besides, MetR protects against age-related changes in immunity, slows cataract development (57), improves colon tight junction barrier function (71) and improves metabolic flexibility, increasing respiratory uncoupling (72). MetR may be also used in the future to inhibit tumor growth, particularly in many cancers that exhibit the known phenomenon of "*methionine dependence*". These include bladder, breast, colon, glioma, kidney, melanoma, prostate and other cancers in which tumor cells have a much greater reliance on methionine than normal cells do (73). They need this amino acid for survival and proliferation and their growth seems seriously limited or inhibited in the absence of methionine (74, 75).

MetR (80 %) also decreases total adipose tissue mass and lowers visceral fat by 70 % (by more than 40 % after correcting for the decrease in body mass) in association with an improvement in insulin sensitivity (76). In addition, MetR decreases leptin and increases adiponectin in rodents in agreement with the decrease in visceral adiposity and the size of white adipose tissue depots. These beneficial effects seem to be mediated by tissue-specific responses that favor increased mitochondrial function and biogenesis, fatty acid oxidation and total energy expenditure possibly mediated by β-adrenergic receptor signaling and changes in lipid homeostasis (77). Metabolomics and genomic MetR studies found changes in the expression of a large number of genes and proteins that led the authors to conclude that MetR increases lipid metabolism in adipose tissue and muscle whereas it decreases lipid synthesis in the liver (78). These changes in lipid metabolism seem to be involved in the strong decrease in adiposity and increased insulin sensitivity observed in *isocaloric* restriction of dietary methionine.

Altered levels of sulfur-containing amino acids have been also described in MetR: serum levels of methionine, cysteine, cystathionine, and taurine decrease in MetR rats, whereas homocysteine levels (79) and glutathione (56) increase. Interestingly, adding cysteine to the MetR diet reverses most of the studied beneficial changes on adiposity and insulin resistance (79) and increases the transcription of various genes associated with inflammation and carcinogenesis (78). Therefore, the beneficial changes of the MetR diet have been attributed to the *decrease of cysteine* in serum (80) or liver (78) observed in animals subjected to MetR.

On the other hand, excessive intake of dietary methionine is toxic. This toxicity far exceeds that produced by any other amino acid (81), leading to damage in some vital organs and increases in tissue oxidative stress (81, 82) with similar negative effects to those observed in rats fed diets with a high protein content. Chronic and excessive methionine supplementation increases plasma hydroperoxides and LDL-cholesterol (83), induces vascular (84) and kidney damage with tubular hypertrophy (85), raises iron accumulation and lipid peroxidation, and leads to liver dysfunction (86), besides other alterations in other organs. In addition, methionine supplementation increases methionine and its two more nearly derived methionine cycle metabolites, S-adenosylmethyonine and S-adenosylhomocysteine in rat liver and kidney (87). Some of the harmful effects of methionine supplementation have been attributed to methionine-related metabolites like *S-adenosylmethyonine, S-adenosylhomocysteine, or homocysteine,* rather than to methionine itself, although in other investigations a direct *methionine toxic effect* has been suggested (80, 84). This last case fits well with the observation that *direct addition of methionine to isolated mitochondria in vitro increases their rate of mitROSp* in liver and kidney mitochondria (87).

Oxidation of methionine residues in proteins generates *methionine sulfoxide*, depriving them of their function as methyl donors and may lead to loss of their biological activity (88). This modification can be repaired by methionine sulfoxide reductase in a thioredoxin-dependent reaction. In this context it is interesting that over-expression of *methionine sulfoxide reductase* increases lifespan in *D. melanogaster* (89) and the opposite manipulation, knocking out the same enzyme, increases protein carbonyls and decreases longevity (90). There is evidence that this enzyme plays an important role in protection against oxidative, cold, and heat stress and seems to be involved in the regulation of aging in *D. melanogaster* (91). Also in agreement with a methionine role in aging, it has been reported that *long-lived Ames dwarf mice have an altered methionine metabolism* showing a marked increase in the transulfuration pathway compared to their wild-type siblings (92). All the above results point to *methionine as the single dietary factor* responsible for part of the *longevity extension* effect of dietary restriction.

6.2. Role of mtROS generation and oxidative damage

DR decreases oxidative stress in mitochondria. But, what is the specific dietary component responsible for the decreases in mtROS production and oxidative damage to mtDNA during DR? In agreement with their lack of effect on longevity (61-63), neither *isocaloric* 40 % lipid restriction (93) nor *isocaloric* 40 % carbohydrate restriction (94) change mitROSp or 8-oxodG in mtDNA (Table 1). However, *isocaloric 40 % PR during 7 weeks decreases mitROSp and oxidative damage to mtDNA* in rat liver (95) in a strikingly similar way, quantitatively and qualitatively to 40 % DR (Table 1). The effect of PR was

studied in rat liver without changing the amount eaten per day of the other dietary components and it was found, like in 40 % DR, that *40 % PR decreases liver mitROSp and FRL specifically at complex I, lowers 8-oxodG in mtDNA* (95, Table 1), and *decreases* five specific markers of *protein oxidative*, glycoxidative and lipoxidative modification as well as the amount of complex I protein in rat liver mitochondria and tissue (96). Strikingly, *the direction of change, the magnitude, mechanisms, and site of action exerted by PR* on mitROSp and 8-oxodG in mtDNA *are almost identical to those found in 40 % DR* (58). Taken together, those studies suggest that *proteins* are the dietary components responsible for most or all the decrease in mitROSp and oxidative damage to mtDNA that takes place in DR, as well as for *part* of the increase in longevity induced by this dietary intervention.

It was logical to suspect that dietary methionine could be involved in those PR and DR effects since it was already known that MetR, independently of energy restriction, increases rat (maximum) longevity (56) while such effect had not been described for any of the other dietary amino acids. This is why the effects of MetR on mitROSp and oxidative stress were studied at my laboratory (Table 1). The results showed that isocaloric MetR (40 % and 80 %), applied to young rats during 7 weeks, *lowers mitROSp (mainly at complex I), the FRL, the complex I content, 8-oxodG in mtDNA* (Table 1), and specific markers of *protein oxidative, glycoxidative and lipoxidative* modification in rat heart (at 40 % and 80 %MetR; 97, 98) or liver (at 40 % MetR; 99, 100) mitochondria, similarly to what occurs after 7 weeks of 40 % MetR in rat kidney and brain mitochondria (101, 102). In order to obtain these decreases it was enough to restrict methionine by 40 % (Table 1). Those decreases in mitochondrial ROSp (at complex I) and oxidative stress have recently been reproduced in liver mitochondria of pigs subjected to MetR (103). 80 % MetR leaded to similar decreases in 8-oxodG than in 40 % MetR, while the decrease in mitROSp from controls to 40 % MetR rats was more pronounced than that occurring from 40 % MetR to 80 %MetR.

Most importantly, and consistently with an important role of methionine in the DR beneficial effects, *when all the dietary amino acids -except methionine- were restricted* (also by 40 %) during 7 weeks, *neither the rate of mitROSp nor the level of 8-oxodG in mtDNA were modified* (104). In addition, it was found that 40 %MetR also decreases mitROSp, FRL and 8-oxodG in mtDNA and reverses aging-related increases in protein modification when implemented during only 7 weeks in 24 months old rats (99). All those results, taken together, indicate that *the lowered ingestion of methionine* during MetR (and PR and DR) *is responsible* for all or most of the *decreases in mitROSp* and oxidative stress observed during these three longevity extending manipulations. Such lowered methionine ingestion is most likely also responsible for all (during PR and MetR) or part (during DR) of the *life-extension* effects observed during these dietary manipulations. This extraordinary capacity of a *single dietary molecule* to induce the decrease in mitROSp is still present when the animals reach old age.

Various mechanisms can be responsible for the decrease in mtROSp during MetR. A most simple one would be based on a decrease in the content of the complex I protein in MetR that would directly lead to a decreased rate of mitROSp. This has been reported under 40 % MetR in the majority of tissues studied (Table 1), and also during DR and PR, as well as in long-lived birds (pigeons, canaries and parakeets) compared to the much short-lived mammals (rats and mice) of similar body size and weight-specific metabolic rate (105, 106). But, in principle, this would not be the full explanation because MetR also induces qualitative changes in mitochondria because it also decreases FRL. Such decrease could be due to a decrease in the mid point potential -and thus the degree of electronic reduction- of the complex I ROS generator, because the decrease in mitROSp during MetR is observed, like in DR, with partial complex I electronic reduction (with complex I-linked substrates alone) but not with full reduction (complex I-linked substrates plus rotenone). The result is that *MetR mitochondria* (from both young and old animals) *are more efficient in avoiding mtROS generation* that those of *ad libitum* fed rats. *MetR mitochondria leak less radicals per unit of electron flow* in the respiratory chain, similarly to what has been found in especially long-lived compared to short-lived animal species (birds), as well as in DR and PR rats compared to *ad libitum* fed ones (55). These quantitative and qualitative changes could be due to: i) *direct interaction of methionine*, or more likely, of a more chemically reactive methionine metabolite, *with the matrix domain* complex I polypeptide/s involved in ROS generation; ii) changes in cellular signaling molecules and the ensuing modification of *specific gene expression* of mitochondrial proteins; iii) decreases in the concentration of mitochondrial complex I substrates like pyruvate which would decrease matrix NADH and are known to occur at least during DR (see section 5).

Concerning the possible direct interaction of methionine or its metabolites with mitochondria without the information passing through the nucleus (mechanism "i"), it is known that direct *in vitro* addition of methionine to isolated functional rat mitochondria increases their rates of mitROSp (87). Therefore a rather direct and rapid effect of methionine on complex I seems to occur. A methionine metabolite could be responsible for this effect because in methionine, differing from homocysteine or cysteine, the potentially reactive sulfur is located inside the molecule and is therefore not available for direct covalent chemical reaction with protein thiols. Interestingly, the reaction of methionine with hydroxyl radicals generates *methionine* radical carbon-, nitrogen- and sulfur-centered *radicals* as intermediates in the formation of the methanetiol product, as detected by EPR spin trap techniques and GC-FID and GC-MS techniques (107). These radicals or methanetiol (CH_3SH) itself could react with complex I or some of its

subunits leading to increases in mtROS generation. Since it is known that GSSG thiolization of isolated complex I increases its rate of ROS production (108) a similar reaction of methanethiol, or cysteine (which also has a free thiol group available for direct reaction) with complex I thiol groups could be involved in the decrease in mitROSp in MetR. Methionine restriction decreases hepatic methionine and cysteine (78) and likely methanetiol levels, which can decrease thiolization of complex I subunits and then their rates of mitROSp. Alternatively, *cysteine could also interact with the protein cysteines of some of the FeS clusters* of the hydrophilic matrix domain of complex I. Interestingly, those FeS clusters have been pointed out as the ROS generator relevant for aging (15,18,47). Their reaction with cysteine would lead to iron release or availability for reaction and then to ROS generation. Therefore, the lowered cysteine levels in MetR could also decrease mitROSp through this kind of mechanism.

Changes in gene expression can be also involved in the MetR effects (mechanism "ii"). MetR studies found changes in the expression of a large number of genes and proteins involved in lipid metabolism (78). In addition, modifications of DNA methylation could be also involved (109, 110). Methionine is an essential amino acid with many key roles in mammalian metabolism including protein synthesis and function, as well as protein and DNA methylation (111). Since ageing seems to be associated with site-specific changes in DNA methylation (112-116), MetR diets could also extend longevity in rodents through modulation of DNA methylation patterns, specific changes in gene expression, and changes in translation rates, whose final effects could include decreases in mtROS generation and oxidative damage and increases in autophagy (see section 9). In agreement with that, we have recently detected that *MetR* induces a small but statistically *significant decrease in global genomic DNA methylation* in the heart of young immature rats (98), whereas when this manipulation was performed in old rats the decrease in this parameter was not statistically significant in the liver (99).

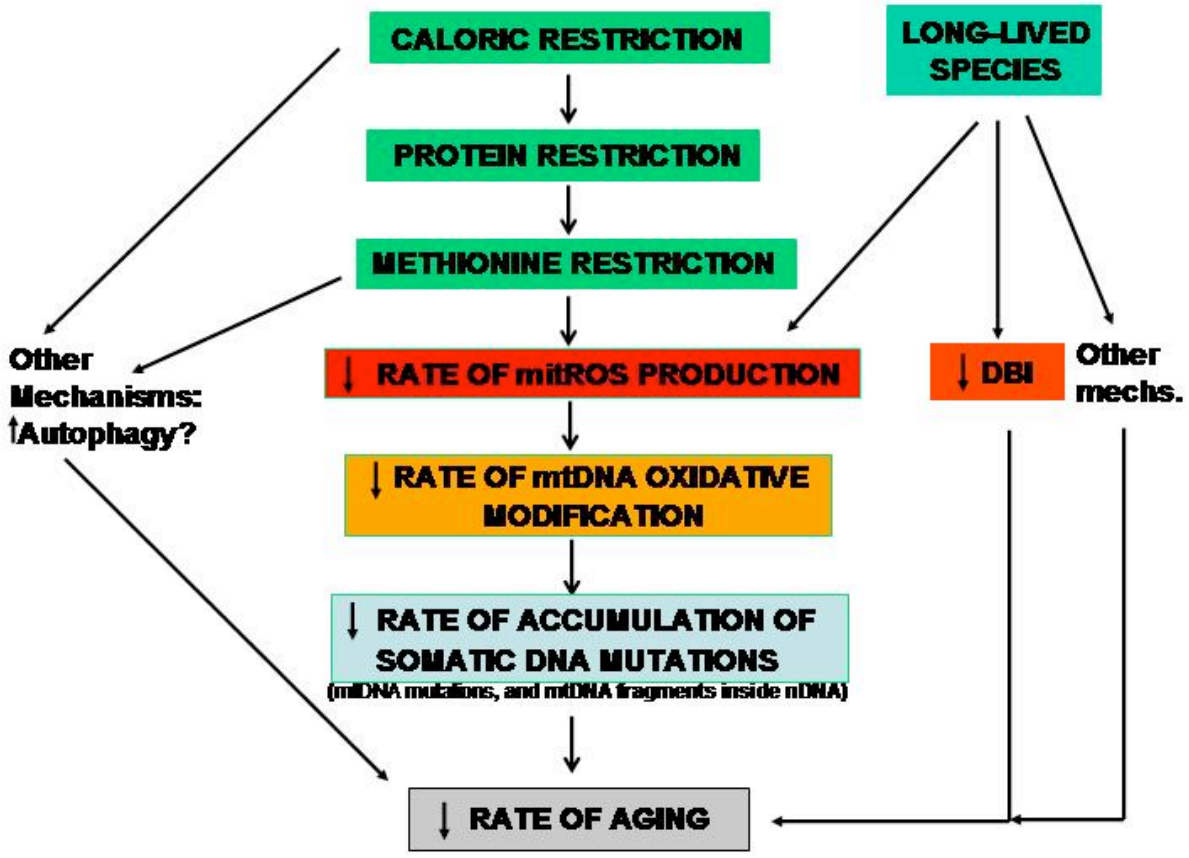

Figure 4. Low mitROSp in both long-lived species and in calorie dietary restriction (DR) as a contributor to a low aging rate. This low mitROSp decreases oxidative damage to mtDNA (see Figure 2). Oxidation of DNA bases like 8-oxodG are repaired also in mitochondria, 8-oxodG being useful as steady-state marker of rate of mtDNA oxidative modification (which is also lower in long-lived animals, see Figure 1C and Ref. 20). The final forms of damage responsible for aging (here represented as "somatic DNA mutations" should be due to mitROSp also causing DNA strand breaks leading to large *mtDNA deletions* plus the remaining *mtDNA fragments* which insert into *nuclear DNA* and accumulate during aging (139; 147-149). The low mitROSp of long-lived animals during DR is due to restriction of a single amino acid, *methionine*, and not to the other amino acids or carbohydrates or fats present in the diet (29,93-95,97,104). Low fatty acid unsaturation (low double bond index= DBI) also occurs in long-lived animals but not in 40 % DR or 40 % MetR (methionine restriction). A decrease in DBI has been observed only at 80 % MetR (which also increases rodent longevity). Since the effect of protein restriction or MetR on longevity (ca. 20 %) is around half that of DR (up to 40 %), other mechanisms, in addition to a low mitROSp, must be involved in the decrease in aging rate induced by DR. A strong candidate is an increased autophagy (Figures 6 and

8, sections 7-9).

Concerning mechanism "iii"), decreased NADH, it is more likely in DR than in MetR, due to the decreased ingestion of a large number of metabolites in DR than in (isocaloric) MetR. Substrates like pyruvate, malate, and succinate, as well as NADH and the NADH/NAD^+ ratio are indeed decreased in the tissues of rodents subjected to DR (45, 46**).** This will decrease matrix NADH and NADH/NAD^+ ratio, and therefore the degree of electronic reduction of the complex I ROS generator and its rate of mitROSp (see section 5). Treatment with a precursor of the oxidized form of the coenzyme, NAD^+, also rejuvenates the skeletal muscle, improves mitochondrial and stem cell function in aged mice, and increases their lifespan (117). This could be due to a decrease in complex I electronic reduction, or to a sirtuin-dependent effect.

In summary, *DR, PR and MetR* are nutritional interventions that *increase longevity* in rodents, although the magnitude of the longevity extension of MetR and PR is around 50 % that of DR. This lower -but significant- life extension effect in MetR than in DR would agree with the notion that various different *environmental signals target the cell* to modify its aging rate. Restriction of methionine intake can be responsible for part of the aging-delaying effects of DR by *decreasing mitROSp at complex I and oxidative damage to mtDNA* and macromolecules acting, at least in this sense, as a "DR-mimic". The information available strongly indicates that *methionine is the single dietary substance responsible for the beneficial changes of DR on mitochondrial oxidative stress*. The remaining effects of DR on aging rate could be due to decreases in other dietary components, or in the calories themselves, acting through different additional signaling mechanisms (Figure 4) that could recruit different gene clusters of the aging program in the cell nucleus (see section 9), changing their gene expression levels with different intensities (see section 9). Among those additional longevity-extending mechanisms during DR, *increased autophagy* is emerging as most important. In any case, it is interesting that not only 80 % MetR, but also 40 % MetR and 40 % PR decrease mitochondrial oxidative stress, because PR does not involve the stronger behavioral and nutritional stress of caloric restriction and therefore seems a much more feasible option for wide application to human populations. *Negative effects* such as delays in puberty and *decreases in growth rate* and final body size are shared by (40 %) DR and 80 % MetR but *do not occur at 40 % MetR*. 40 % methionine restriction (implemented through PR) could be the best kind of dietary restriction for humans because it lowers mitROSp and 8-oxodG in mtDNA to a similar extent than 80 % MetR, *without decreasing at all* body and organ weight, growth rate, maturation, and likely final body size, at variance with what occurs in 80 % MetR and 40 % DR.

Humans can obtain health benefits consuming "prudent" diets based on the intake of vegetables containing proteins rich in essential amino acids but low in the sulphur-containing amino acids methionine and cysteine (like pulses), or almost totally lacking methionine and cysteine (like many fruits and other vegetables), and avoiding the presently excessive intake of animal proteins and fats typical of western diets. The results already available after many years of PR intervention in humans seem to be positive for human health and of similar character than those found in MetR and DR rats (118). These studies also suggest that DR and PR can protect from obesity, mortality, and degenerative diseases including at least cardiovascular ones, diabetes and cancer, and can increase the human healthspan.

7. RAPAMYCIN, MITOCHONDRIAL OXIDATIVE STRESS, AND LONGEVITY

Although it is well known that DR increases longevity in many different animals including mammals, and seemingly also in monkeys (41), it is difficult to apply to large human populations due to subnutrition risks especially in children, very old people, individuals with limited cultural knowledge and education, and those with some chronic pathologies. Due to this, there is strong interest in developing *drugs than can increase longevity without the need to restrict the human diet* (119, 120). The USA National Institute of Aging Interventions Testing Program (ITP) evaluates the effects of different candidate molecules to be candidate antiaging drugs in mice (http://www.nia.nih.gov/research/dab/interventions-testing-program-itp/compounds-testing). These include known drugs, antidiabetics, antibiotics and others, like aspirin, resveratrol, simvastatin or metformin. While the rest of studied compounds have not shown reproducible positive effects on longevity (119), *rapamycin significantly increase*d *both mean lifespan, as well as maximum lifespan* (at age of 90 % mortality, mean of the three sites) by 9 % in males and by 14 % in females in heterogeneous strains of mice (121). That experiment was reproduced under the NIA ITP at three different sites. Posterior studies have *confirmed those results* and have shown that dietary rapamycin extends lifespan when initiated in young (119) or middle aged mice (122), or in mixed age mice (123). Therefore, rapamycin is widely recognized as ***the first drug known that consistently increases longevity in mammals***. Rapamycin also increase longevity *in yeast* (124), *C. elegans* (125) *and Drosophila* (126) through *inhibition of the TOR protein complex*, equivalent to mTOR (mammalian target of rapamycin). This indicates that this longevity pathway is highly conserved in evolution. The mTOR complex also shows signs of rapid evolution in amniotes and signs of *positive selection* (127).

Although it is clear that rapamycin increases mammalian longevity, the final effector mechanisms involved were unknown, although decreases in mitochondrial damage, increases in autophagy, or modifications in cell growth/proliferation could be implicated. Rapamycin also inhibits the mTOR signaling pathway in mammals (128). Interestingly, the longevity extending nutritional intervention *DR also decreases*

mTOR function (129). This indicates the existence of overlapping mechanisms of action for rapamycin and DR. Rapamycin treatment decreases body weight and food intake in mice (130) but the increase in longevity induced by the drug is independent of the decrease in food intake *per se*. However, since the longevity extension effect of DR (up to 40 %) is much larger than that of rapamycin (around 11 %), *rapamycin controls only part of the final aging effector mechanisms increasing longevity during DR* or affects them with smaller intensity than DR, or does not affect similarly all mammalian organs. Most likely DR targets also other cytosolic pre-nuclear longevity signaling molecules (see section 9) different from mTOR that are not targeted by rapamycin. Alternatively, there is evidence that DR directly modifies some of the most likely effectors of aging like mitROSp, by-passing the nuclear aging program (section 9), e.g, by decreasing mitochondrial matrix NADH due to lower concentrations of complex I-linked substrates like pyruvate (see sections 5 and 6).

In addition to increasing longevity, there is a general consensus that rapamycin *attenuates age-associated declines* in some measures of cardiac, immune, muscular, and cognitive function (122, 131). The increase in longevity induced by rapamycin was initially unexpected, because rapamycin was used, together with other drugs, in post-transplant therapy (132). Strikingly, increases (instead of decreases) in various immune activities have been also observed at low rapamycin doses (129, 133). It is now known that mTOR inhibition in mammals has positive functional effects in the majority of the physiological systems (129, 131), and protection from degenerative diseases, solid tumors, cardiovascular metabolic diseases and obesity have been observed even in humans. Rapamycin also prevented from degenerative brain changes in an Alzheimer disease mouse model and improved anxiety and depression in normal mice (134). Although some detrimental effects of the drug have been described, the positive effects are much more diverse and extensive. In any case *the global effect of rapamycin is positive since both mean and maximum longevity are increased*.

DR increases longevity and lowers mitROSp, FRL, and oxidative stress (29), as well as mTOR function (59). And *intermittent rapamycin administration*, like intermittent DR, also extends lifespan of female mice (135). Decreased mTOR downstream activity could constitute one of the various signaling mechanisms through which DR decreases aging rate (129, 136, 137). In agreement with this, knocking out the gene coding the ribosome protein kinase S6K (a main target of mTOR signaling) slowed aging of bone, immune, and motor functions, and led to a larger than normal longevity (138). In many cases the nuclear gene response to these signals (see section 9) slows the aging rate. Among them, the decrease in mitROSp produced by both DR and rapamycin can be important. In agreement with this concept, *rapamycin* dietary treatment, at the same dose that increased longevity (121), *decreased hepatic mitROSp (Figure 5A) and FRL at complex I* both in mice subjected to a diet that induced fatty liver (53) as well as in middle aged mice receiving standard (LabDiet 5LG6 w) diets (139; Table 1).

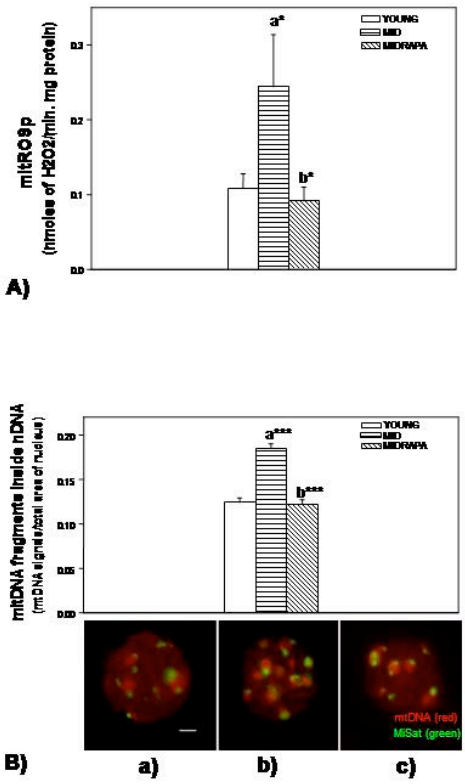

Figure 5. Rapamycin fully reverses the age-related increases in mitROSp and mtDNA fragments inside nuclear DNA. Rapamycin dietary treatment, at the same dose that increases mouse longevity (14 mg/Kg diet, Ref. 121), totally abolished the A) age-related increase in mitROSp, and B) the accumulation of mtDNA fragments inside nDNA in the liver of middle-aged mice (16 months of age). In part B the pictures show single representative hepatocyte nuclei corresponding to: a) YOUNG; b) MID; c) MIDRAPA; red signals come from mtDNA; green signals from minor satellite mouse centromeric sequence (MiSat) were used as controls; white bar is equal to 2µm. MID = Middle aged mice. MIDRAPA = middle aged mice treated with rapamycin during 7 weeks. a: significantly different from Young group; b: significantly different from Middle group; * P<0.05, *** P<0.001. The qualitative and quantitative similarity of the changes induced by rapamycin in both parameters suggest a cause-effect relationship between them. Modified from ref. 139.

Moreover, this drug *completely reversed age-related increases in mitROSp (*Figure 5A), and the age-related increase in the *insertion of mtDNA fragments inside nDNA*, in the liver of middle aged mice (139; Figure 5B; see also section 8). In the same investigation, it also completely *reversed* age-related *decreases in the autophagocytosis* marker ATG13 (Figure 6A), and *partially reversed* the accumulation of the best tissue marker of aging, *lipofuscin*, in the liver (Figure 6B). Rapamycin also increases mouse longevity when the treatment is started at middle age, with an effect similar to that observed starting at 9 months (119, 121), agreeing again with the decreases observed in mitROSp also in *middle aged* mice (139). All those results, taken together, are most remarkable since there is currently strong interest in finding drugs with positive effects on longevity *in middle aged humans*.

In summary, it is most interesting that *all the (four) experimental manipulations* which consistently and reproducibly increase both mean and *maximum longevity* in mammals, ***DR, PR, MetR and rapamycin, decrease mitROSp and FRL at complex I and mtDNA damage*** (Table 1). This strongly supports the *antioxidant-independent version of MFRTA* exposed in this review that focuses on: *i) the rates of ROS production* (initiation) at mitochondria; and *ii) cellular components highly resistant* to (oxidative) modifications like less unsaturated membrane fatty acids (low DBI). These two traits of the updated MFRTA could be accompanied by a third related factor in long-lived animals, *iii) increased autophagy* (see section 9).

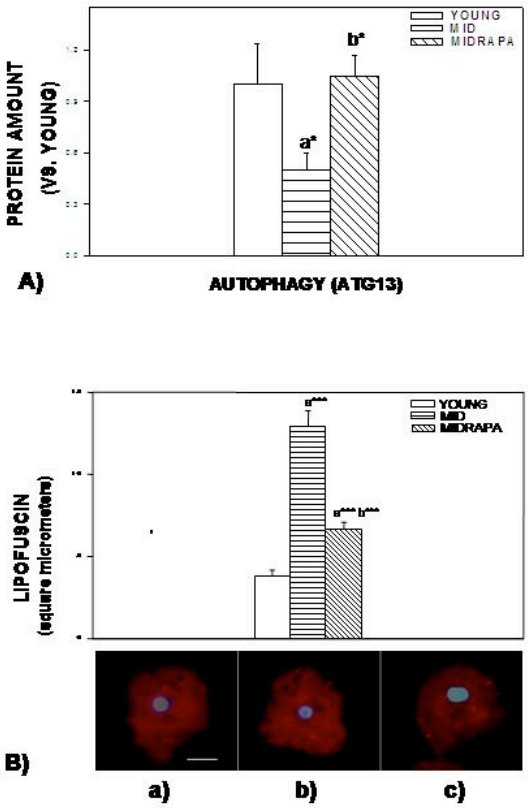

Figure 6. Rapamycin totally reverses the age-related decrease in autophagy and partially reverses the increase in lipofuscin accumulation. Rapamycin dietary treatment of middle aged mice (16 months of age), at the same dose that increases mouse longevity (14 mg/Kg diet, Ref. 121) totally abolished A) the age-related decrease in the autophagocytosis marker protein ATG13, and B) partially reversed the age-related accumulation of lipofuscin (B) in the liver of middle-aged mice (16 months of age). In part B the pictures show single representative hepatocytes corresponding to: a) YOUNG; b) MID; c) MIDRAPA; bright red signals come from lipofuscin; the nucleus is seen in blue colour; white bar is equal to 10µm. MID = Middle aged mice. MIDRAPA = middle aged mice treated with rapamycin during 7 weeks. a: significantly different from Young group; b: significantly different from Middle group; * P<0.05; *** P<0.001.

The inverse nature of the changes induced by rapamycin in both parameters suggest a cause-effect relationship between the increase in autophagy and the partial reversal of lipofuscin accumulation. Modified from ref. 139.

8. mtDNA FRAGMENTS INSIDE NUCLEAR DNA AND AGING

A further complication is the possibility that mitochondrial ROS-derived damage affects aging genes back in the nucleus through the *insertion of mtDNA fragments inside nDNA* (Figures 5B and 7).

Oxidative damage to mtDNA bases, like 8-oxodG, is also repaired in the mitochondria. But mitROS, in addition to DNA base and sugar oxidative modifications, have the capacity to produce double *strand breaks* in DNA in general, and with more reason in the very *nearby situated mtDNA*. *Fragmentation of mtDNA* through double strand breaks by the nearby generated mitROS can be one cause of the well known accumulation of mtDNA mutations, including large mtDNA deletions, with age (140). Recently, it has been proposed that mtDNA mutations can also be due to errors during DNA replication and repair, rather than to mitROSp. However, while those random errors can contribute to accumulated damage during the lifespan of a single individual, they can not be responsible for the strongly different longevity of the different species nor for the change in longevity induced by the different kinds o DRs, since these longevities are *genetically, instead of randomly*, controlled. In other words, *there is no plausible mechanisms that would lead rats to commit 30 fold more errors than humans during mtDNA replication or repair*. Replication and repair as source of mtDNA mutations suffers the same limitation that many other wrong proposals based on random processes (e.g. wear and tear theories of aging). Instead, the longevity of a species, or fine tuning of longevity to a new level in DR, is *determined by the genotype*. Then, it must necessarily be due to the existence of *genetically programmed processes* residing in the cell nucleus which can respond to environmental nutrient availability (during DRs) with appropriate changes in longevity (see section 9).

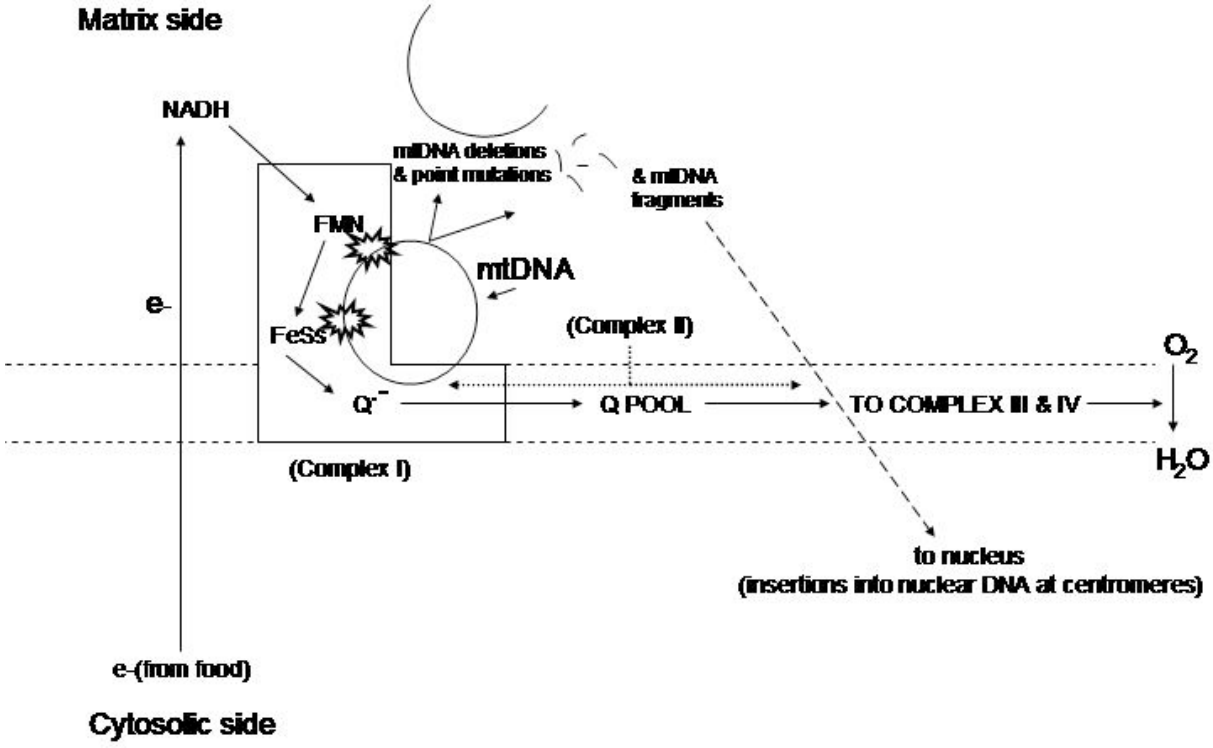

Figure 7 mtROSp and mtDNA fragments inside nuclear DNA. mtROS produced at Cx I generators (stars) cause damage in the mtDNA situated nearby or even *in contact* with the complex I site of ROS production. This causes, in addition to oxidized bases, double strand breaks leading to large deletions and, most importantly, also mtDNA fragments. These exit the mitochondria and insert into nuclear DNA at the centromeres during aging in yeast, rats and mice, and contribute to aging. This is reminiscent of what happened during evolution after the symbiogenesis of the eukaryotic cell from α-proteobacteria and *Archea* around 2.000 million years ago. Rapamycin treatment decreases such mtDNA fragment accumulation in the liver of middle aged mice (Figure 5B; Refs. 139,143-149).

The occurrence of large mtDNA deletions, which increase with age in mammalian tissues, has been proposed as one final detrimental effect causing aging. Since mtDNA is highly compacted, without introns, the large deletions detected in old tissues would lead to the lack of many genes coding for electron transport chain or mitochondrial ribosome subunits in a single mtDNA circle molecule. However it is now clear that, with the exception of a few tissues, the level of these deletions does not reach the threshold needed, in homoplasmia, to be of negative

functional consequences in most tissues of old animals. The high level of heteroplasmia of mtDNA, due to the presence of thousands of mitochondria per cell, and various mtDNA copies per mitochondrion, strongly protects against direct functional effects of mtDNA mutations. Many copies of mtDNA are present in each cell. Only if a large majority of these mtDNA copies are mutated, mitochondrial ATP production would be compromised. Cells essentially homoplasmic for deleted mtDNA are abundant (between 43 and 60 %) in a few areas like substantia nigra in humans (141, 142), but in the brain in general and in other vital tissues of old individuals their percentage is too low (0.5-2 %) to cause damage during aging.

However, *double strand breaks in mtDNA not only can generate mtDNA deletions. At the same time* than they generate deletions, *they produce mtDNA fragments*, the segments of deleted mtDNA. In agreement with earlier proposals (143,144) those fragments can escape from mitochondria (145, 146) and *are also present inside the nucleus* (Figures 5B and 7). It was proposed that this could randomly change nuclear gene information and thereby contribute to cause cancer and aging (143). It has been simultaneously demonstrated that these mtDNA fragments *accumulate inside nDNA with age both in yeast (147) and in rat liver and brain (148*), and that such accumulation causes damage and *contributes to aging in yeast* (149). Mouse liver also accumulates mtDNA fragments inserted inside nDNA with age (139). And the dietary treatment with *rapamycin*, in addition to decreasing mitROSp, *totally reversed mtDNA fragments accumulation in nDNA (139; Figure 5A,B) and decreased lipofuscin* (materials non-autophagocytosed; Figure 6B) in middle-aged mice. This last change can be due to a decrease in the presence of damaged mitochondria, and/or to the *increase in autophagy* induced by the drug (139, Figure 6A). The changes observed for mitROSp and for mtDNA fragments inserted in nDNA are strikingly similar since *full(100 %) reversion of age related increases* occurred in both cases (Figure 5A,B). This could be due to a *cause-effect* relationship between these two parameters, since ROS have strong capacity to produce double strand breaks and then DNA fragmentation. The increase in autophagy and the decrease in lipofuscin could also represent a cause-effect relationship (Figure 6A,B) although in the case of lipofuscin reversion to young levels was only partial.

In summary, part of the *increase in longevity of rapamycin* treated mice can be *due to*: *(i) a decrease in mitROSp*; *(ii) a decrease in the insertion of mtDNA fragments inside nDNA*; and *(iii) an increase in autophagy*. Interestingly, in a parallel experiment we did not detect significant changes in mitROSp in the heart after the rapamycin treatment. Therefore, the reason why rapamycin increases life span to a much lower extent (11 % increase in maximum lifespan) than DR (up to 40 % increase) can be due to the impact of DR on more longevity signaling pathways than those involving mTOR (Figure 8). But it can also be due to the possibility that rapamycin decreases mitROSp, or increases autophagy, in some but not in all organs at least at the dose used of 14 mg of rapamycin/Kg of diet (121, 139).

The decrease in mitROSp and FRL induced by rapamycin treatment in mouse liver occurred together with *decreases in the amount of complex I*, which contains the ROS generator relevant for aging (see previous sections). That was associated with an *increase* (instead of decrease) in *mitochondrial biogenesis* (139; PGC1α). A possible explanation of this apparently paradoxical result is that *rapamycin could selectively induce mitochondrial biogenesis from the more youthful pool of liver mitochondria*. These are expected to show lowered FRL than the more damaged ones. That selection could be another reason why rapamycin decreased mitROSp. It has been observed that *rapamycin increases autophagy and mitochondrial biogenesis* in mouse heart suggesting that damaged mitochondria are replaced by newly synthesized ones to *rejuvenate mitochondrial homeostasis.* Likely related to this possibility, it has been observed that *DR* (which also inhibits mTOR) *and rapamycin* both lower mitROSp and FRL and *decrease the amount of the "matrix domain-only" of complex I* in the mitochondria of mouse liver (53). That matrix domain contains the mitROS generator responsible for the decrease in mitROSp during DR (15-18,47,53) and during rapamycin treatment (139).

The mtDNA fragments exit from the mitochondria towards the nucleus during the lifetime of the individual and they insert into nDNA. *They are visualized heavily concentrated at the centromeres* (Figure 5B, a-c 139). Thus, the *centromeres seem to be the "entry doors"* for the access of mtDNA fragments *into chromosomes*, perhaps to be distributed afterwards to other specific chromosome locations at regulatory regions of the master genes controlling the "Gene Clusters of Aging" (constituting the pro-aging program) likely lying in the cell nucleus (see section 9). The final result would be to promote aging and degenerative diseases including cancer. If that phenomenon finally were a regulated one, the initial proposal (143) would only be wrong concerning the suggested *randomness* of the process. Random insertion of mtDNA fragments inside nDNA, even if it were restricted to the structural genes (around 1 % of total nDNA) could cause cancer (e.g., inserting in, and randomly inactivating tumour suppressor genes) but not aging, because species longevity is a tightly regulated species-specific property. Further studies are needed to ascertain the random or regulated character of mtDNA fragments insertion into nDNA starting at the centromeres.

Migration of mtDNA fragments from mitochondria to nucleus is strongly reminiscent of what happened during millions of years of evolution after the symbiogenesis event that created the Eukaryotic cell around 2.000 million years ago. During such evolution most genes of the initially free living Rickettsia-like α-proteobacteria were transferred to what is now the nuclear genome of the eukaryotic cell. This would constitute a further example of the old observation that in various cases "ontogeny

recapitulates phylogeny" (e.g. presence of gills or tail in human embryo or foetus), in this case applied to programmed aging as a continuation of development.

The lack of increase to phenotypic threshold (minimum of around 60 %-80 % deleted) of mtDNA deletions due to the very high copy number of mtDNA in heteroplasmia per cell was a strong problem for the validity of MFRTA, similarly to the main origin of mtDNA point mutations from replication/repair instead of from mitROSp as deduced from the relative frequency of base transversions vs. transitions. However, now there is evidence that mtDNA fragments accumulate inside nDNA with age in yeast and mammals, and that this promotes aging (147). Furthermore, the longevity increasing drug rapamycin reverses such age-related accumulation in mouse liver strongly paralleling what happens for complex I mitROSp (Figure 5A,B; Ref.139). All that means that ***MFRTA can not be considered "dead" any more*** except by those not aware of the recent breakthrough discoveries of mtDNA transfer from mitochondria to the nucleus during the lifetime of the individual. On the contrary MFRTA, like the *bird phoenix*, emerges again well alive and all pristine from its ashes. Perhaps the failure to detect mtDNA mutations relevant for aging has been due to looking at the wrong place. The ROS related to aging should be looked at mitochondria. However, what could be more important for aging would not be the (*remaining*) deleted mtDNA, but *what is lacking* at mtDNA after the deletion: *the mtDNA fragments*. The consequences likely relevant for aging, the mtDNA fragments, have been found inserted into nDNA inside the nucleus. Therefore, **we should look *both* at the mitochondria and to the nucleus to understand aging**, as much as we should look at the mitochondria and at the nucleus to understand *the creation and further evolution of the eukaryotic cell.*

9. THE CELLULAR AGING REGULATION SYSTEM (CARS)

9.1. Signaling, the nuclear Aging Program, and aging Effectors

The integrated *Cellular Aging Regulation System*, for convenience called *CARS* here for the first time, is composed of three main parts (Figure 8):

A) Cytoplasmic Pre-nuclear **Signaling** (mostly signaling proteins)

B) The nuclear genetic Pro Aging Program (**PAP**) including the Gene Clusters of Aging (153); and

C) Post-nuclear **Effectors** (executors) of Aging.

This CARS occurs *both in post-mitotic and in mitotic* tissue cells. Mitotic cells could harbour, in addition, other additional hypothetical aging effectors specific for such kinds of tissues, like *telomere shortening* or perhaps *apoptosis*, but these are not expected to be relevant in cells that do not divide. Especially, *if the cell does not divide, the telomeres would not shorten*. And the most im**p**ortant tissues for mammalian aging are those mainly composed of post-mitotic cells, skeletal muscle, heart and brain.

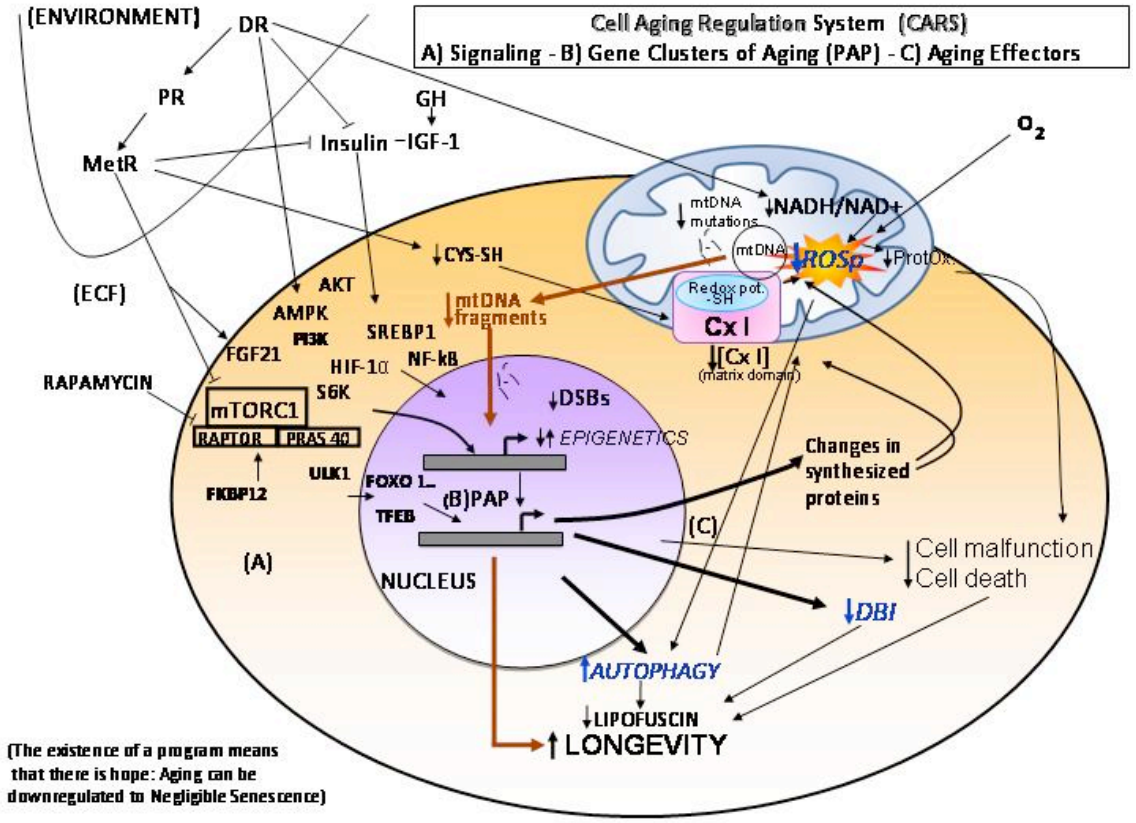

Figure 8. Cell Aging Regulation System (CARS). The working model of the CARS (including the *Pro Aging Program*-PAP lying inside the

nucleus) broadly depicts known mechanisms of control of longevity at cell level. Different kinds of dietary restrictions (DR, PR, MetR) and rapamycin (signals coming from the environment) alter humoral, endocrine, and finally cytosolic pre-PAP signaling proteins like mTOR, AMPK, and many others (part A, left of figure). In many cases this modifies the expression (through transcription factors like FOXOs, TEFB and others) of nuclear genes likely organized as Gene Clusters of Aging (153) of the PAP (part B center). PAP output (solid arrows leaving the nucleus on the right of the figure), in turn, modifies the activity of at least three *Aging Effectors* (shown in italics, Part C, right): a) *Mitochondria* (including mitROSp), b) fatty acid double bond index (*DBI*), and c) and likely *Autophagy*. The integrated responses of the aging effectors to the DRs and rapamycin, or to knocking out GH or IGF-1/insulin-like signaling genes increases longevity. *Across species* the same (plus additional) genes forming the PAP are constitutively active at different levels in species with different longevities. In some cases the signals can reach the mitochondria directly by-passing the nuclear PAP, as it is the case for MetR possibly lowering cysteine thiol groups at complex I (Ref. 159). The action of other possible effectors of aging like *telomere shortening or apoptosis* is likely limited to mitotic tissue cells and evidences for their validity are still lacking. mtDNA deletions, and mtDNA fragments inserted in the nucleus increase with age (139,147-49) and can contribute to aging (139,149). *Mitochondrial ROSp* (and updated MFRTA) *are not alternative to the humoral (e.g. insulin-IGF-1 like) or cytosolic proteins* (e.g. mTOR or S6K) part of CARS and signaling to PAP. Instead, the three main kinds of cellular processes (A, B and C) work together integrated into a single CARS to control longevity in each species and to finely adjust changes in longevity in response to DRs or pro-longevity drugs like rapamycin. During 40 % DR *lowered mitROSp and increased autophagy* execute the PAP response. At 80 % (but not 40 %) MetR, *decreasing the DBI* is also a recruited response. These *three aging effectors* can be involved in the change in longevity between species. Epigenetics can be also involved in changing the PAP gene expression level. DBI (double bond index) indicates the number of double bonds of membrane fatty acids (DBI). ECF = extracellular fluids; MFRTA= mitochondrial free radical theory of aging.

Intensive aging research during the present century, including studies with gene knockout and transgenic animals, has clarified a series of important facts concerning the CARS:

a) Inactivation of many single genes leads to an increase in mouse longevity (up to, ca., 40 %). Most importantly, those genes are *highly conserved* in evolution which agrees with the concept that aging is an adaptation that increases biological fitness at group or higher level. Thus, aging constitutes a good example of an adaptation covered under the umbrella of the modern concept of Multilevel Selection. Many of those genes are the homologous in organisms so different as yeast, nematodes like *C. elegans*, insects like *Droshophila*, and many vertebrates including mammals and humans. Therefore, *aging is a very old adaptation* of most multicellular and even at least the unicellular eukaryotic life.

b) Although DR (and MetR) can control aging in part by *directly modifying* the aging effectors, they also bring about *changes in expression of a large number of nuclear genes* (78,150,151). The modified expression of those genes, in turn, modifies the *activity of the three known aging effectors* too.

c) The different kinds of DRs increase mouse longevity by modifying the *expression levels of hundreds of genes*, as shown by many microarray-based studies. Those changes are *species- and tissue-specific*. This, together with the increase in longevity after *inactivation of single genes* codifying for pre-nuclear hormones, their receptors, cytoplasmic signaling proteins or transcription factors (part A of the CARS), constitutes *robust evidence* that a *Pro-Aging Program (PAP)* exists in the cell nucleus.

d) DR and MetR *signal* the abundance of food or proteins available for feeding in the external world to the cell inside the body using humoral factors like insulin, GH/IGF-1, or blood amino acids like methionine. These in turn modify the activity of many cytoplasmic signaling proteins like mTOR, S6K, AMPK, AKT, PI3K, FGF21, ULK1, NFkB, HIF and many others. Many of those signals enter the nucleus, where, through the action of transcription factors like FOXOs or TFEB (152), modify the expression of PAP genes involved in the control of longevity. These have been proposed to be organized in different but interrelated *Gene Clusters of Aging*, analogously to the Hox genes located in four clusters and involved in the control of development from embryo to adult (153).

e) Most long-lived *mouse mutants live up to 40 % longer* than the wild type animals. This, most interestingly, *coincides with the amount of longevity extension elicited by DR* (also up to 40 %). Many of those known genes mainly codify for proteins involved in GH/ or insulin/IGF-1-like Pre-nuclear Signaling, or cytoplasmic pre-PAP cytosolic signaling proteins, or nuclear transcription factors like FOXOs or TFEB.

f) The cytosolic signaling proteins work in many cases by modification of nuclear PAP activity. The induced changes in gene expression modify the levels of *specific proteins* corresponding to PAP output. The final result is the variation in activity level of the systems finally controlling the rate of aging, the cellular Post-nuclear *Aging Effectors*. Three of them have been already identified:

(i) The *Mitochondria*, and their rate of *mitROSp* at complex I

(ii) The degree of *Unsaturation of Cell Membrane Fatty Acids* (DBI); and

(iii) Likely, the *Macro-Autophagocytosis* (Autophagy) system.

These three main effectors are operative in aerobic vital tissues, both mitotic and post-mitotic. The action of other possible effectors like *telomere shortening* or apoptosis would be *restricted to mitotic cells*, of smaller relevance for aging. Therefore increased longevity, both during DRs and between different animal species, is obtained through *decreased mitROSp at complex I, and increased autophagy*. *A decrease in cell membrane DBI* is involved also in increasing longevity *across species*.

The first two aging effectors (mitROSp and DBI) were identified in the 1990´s. With these two the key to long life is to *decrease the rate of generation of (oxidative) damage at mitochondria; and to have cellular membranes*,

including those of mitochondria, composed of fatty acid constituents *more resistant* (with less double bonds) to ROS induced damage. Now *autophagy is emergin*g as a third possible aging effector. Macro-autophagy (154) can eliminate *heavily cross-linked, oxidized*, and aggregated mixtures of peroxidized lipids and proteins (155) and even sometimes whole heavily damaged mitochondria (156). Thus animals like us seem to age slowly at least because they "purposely" (in evolutionary sense) *produce less toxic substances (ROS)* per unit time, their *membrane fatty acids are more resistant* to them, and most of *the still remaining molecular damage is eliminated* by the macro-autophagocytosis machinery. This shows the strong interrelationship between the three aging effectors. The materials that can not be digested and eliminated by autophagy accumulate in the cytosol as *lipofuscin* granules, the *best known marker of aging* at tissue level.

Many different mutant mice with single autophagy genes knocked out have a decreased life span (reviewed in 154). However, we still *lack rigorous evidence that over-expression of macro-autophagy genes increases longevity*. The same problem affects DBI except for successful knocking out of desaturase/elongase genes that increased longevity in *C. elegans* nematodes. It is known, hoever, that *mitROSp* at complex I, consistently *decreases in all the four* known experimental manipulations that increase mammalian longevity: ***DR, PR, MetR, and rapamycin* feeding**. Some positive evidence suggesting that increasing autophagy increases longevity has been published, although it was limited to a *short-lived* mice strain in which overexpression of the essential autophagosomal protein ATG5 increased maximum lifespan from *only 781 to 900 days* (157). In contrast, in the first three experiments taken together in which rapamycin increased longevity the *control* mice of both sexes reached 1,086 days and the *rapamycin* treated ones reached *1,212 days of age at 90 % mortality* (121), a capacity surpassed by MetR and DR.

The husbandry conditions and the strain used clearly affect the longevity attained by the animals in aging experiments. Further aging experiments using longer lived control mice, approaching *1,000-1,200 days at least*, are needed to identify other drugs that could increase longevity. *It is necessary to use long-lived instead of short-lived strains as controls, and to implement husbandry conditions that maximize longevity* and produce more rectangular and smooth survival curves.

Another key requisite for acceptation of any theory of aging is that it must be able to explain why different animals age at so different rates. It is still *unknown whether autophagy is higher or not* in tissues of *long-lived* animals compared to those of short-lived ones. However, *telomere length is higher in mice than in men, the opposite of what is expected* if telomere shortening were another causal factor involved in aging. Consistent and reproducible evidence concerning these aspects is urgently needed before definitively considering macro-autophagy a third aging effector. In addition, when those comparative studies are performed, care must be taken *not to correct the real values by body size* because this will erase most of the real causes of aging responsible for the correlations between the studied hypothetical mechanism of aging and longevity. If the authors of a study still doubt whether to correct or not correct for body size, the best option would be to *show* in the publications the correlations with longevity for *both the corrected and the non corrected values*. That is most important. Otherwise, the high potential of comparative gerontology to facilitate the cheap and fast possible identification or elimination of hypothetical causes of aging will be almost fully erased, which will be a too great loss for gerontology.

Although correlation does not imply causality, *the central parameters of any valid theory of aging must correlate with the longevity of different species* ***in the right sense***. Thus, mitROSp is low in tissues of long-lived mammals and birds, which fully fits with the modern version of MFRTA. On the contrary, antioxidant enzymes also correlate with longevity but in the *wrong sense*: they are up to more than one order of magnitude lower (instead of higher) in long-lived than in short-lived mammals or vertebrates (7). The same is true for the repair of DNA damage of *endogenous* origin (reviewed in 29, section 3E1). Therefore, both defence (antioxidants) and DNA repair are discarded as causes of aging. *High "maintenance"* of the animal (leading to high longevity) *is not due to defence plus repair* contrarily to what the three mainstream evolutionary theories of aging (mutation accumulation, antagonist pleiotropy, and disposable soma theory) wrongly predicted. On the contrary *high maintenance is due to a low rate of generation of endogenous damage* (low mitROSp and low DBI) and, likely, also *to a high rate of elimination of damaged components* (high autophagy). MitROSp and autophagy are strongly complementary mechanisms, helped also by a third factor: *high resistance* of tissue cellular membranes to lipid peroxidation obtained through a low DBI and PI. In short**,** longevity is obtained through ***a low rate of production of "damage" (mitROS,), a high rate of cleaning their consequences (high autophagy), and a high constitutive resistance to oxidative modification*** *(e.g., low DBI)***.** The identification of the rate of mitROSp with the concept of "rate of generation of endogenous damage" comes from the fact that, *after more than one century* of intensive gerontological research, *ROS continue to be the only know highly damaging substances endogenously produced by the healthy animal organism throughout their lives that have the capacity to break covalent bonds.* If any other different family of substances having that property is discovered in the future, it could be tested to be added or not to the list of aging effectors. Meanwhile mitROS continue to be the only toxic highly damaging substances that the body "*purposely*" produces to cause and increase its aging rate.

The Pre-nuclear Signals, the PAP, and the Aging Effectors, are *integrated working together* to constitute the CARS. Therefore, *it is illogic to consider MFRTA*

incorrect while believing at the same time that proteins like mTOR, AMPK or S6K, et cetera, control longevity. Looking at Figure 8 it is evident that the *three parts of the CARS work together* in a highly *integrated manner to determine species longevity*.

On the other hand, it is logical to suspect that the same gene clusters of aging central to the PAP and reacting to the DRs or rapamycin, plus others additional ones, are also involved in the control of longevity *between different species*. It is already known that different genes can change their expression at different tissues in response to the different kinds of dietary restrictions (DR, PR, MetR, or IF-intermittent feeding) or pro-longevity drugs. These responses include both *quantitative and qualitative* changes concerning the genes involved in each case as well as their levels of gene expression. The same is expected between species with much bigger differences in longevity than in the DRs. *Between species, the same genes* involved in response to the DRs would be involved, plus *additional inter-species genes*. The degree of expression of the longevity genes involved is expected to vary more intensely in different species than in the DRs. Since the number of genes involved in the response to DR is already large (up to 1.000) in relation to the total structural genes (around 20.000 genes), it would be a prohibited luxury for evolution to use totally different genes to control aging rate in the case of the different species than in the DRs. Instead, the same genes would be used, plus additional inter-specific ones. One well known example is the case of oxidative stress. While both mitROSp and cell membrane DBI (global fatty acid unsaturation) varies between species, in the case of (40 %) DR and (40 %) MetR mitROSp at complex I is decreased while the DBI does not change (see section 5). However, at 80 % MetR, the DBI is also lowered (97). Thus, the implication of a given effector in a response depends on whether: a) the longevity difference occurs at the individual or at the species level; and b) the intensity of the signal reaching the PAP. In addition, there can be also inter-individual differences involved. Transcriptions factors like FOXO1 reacting to DR show polymorphisms in relation to differences in longevity in human populations (158).

Although a large part of the change in aging rate is controlled by the flow of information through the PAP in the nucleus, at least in the case of mitochondria part of the control can directly flow from the dietary substances and derived substrates to modify mitROSp bypassing the PAP. This occurs in DR, that decreases metabolites like pyruvate and others, which in turn lower matrix NADH and then mitROSp at complex I (Figure 8 top). Or the case of MetR, in which direct control of mitROSp by post-translational modification of complex I could occur. Reaction with complex I thiol cysteine groups could decrease the degree of electronic reduction of the complex I ROS generator - like the lowered NADH in DR- and then its rate of mitROSp (159). On the other hand, components of CARS are interrelated in more complex ways than delineated in Figure 8. Thus, the mitochondrial uncoupler 2,4-dinitrophenol lowers mitROSp, which is expected since it accelerates electron flow at ETC, like during the state 4 (without ADP) to state 3 (with ADP) energy transition. However, it also lowers mTOR activity and insulin-PI3K-MAPK signaling, and increases autophagy (160).

Another example is the recent description that lowering mitROSp decreases the amount of damaged mitochondria and the cellular level of lipofuscin (161), again relating two main cellular effectors of longevity of post-mitotic cells, mitochondria and autophagy. Mitochondria have been repeatedly observed half digested inside secondary lysosomes under the electron microscope, these vesicles contributing afterwards to form lipofuscin granules Strikingly, many genes controlling autophagy are the same involved in the increase in longevity induced by lowering mitochondrial respiration (154).

9.2. Intermittent food restriction (IF): is the Aging Program functioning gradual or not?

It would be most interesting to know whether the PAP works in a *graded form* in response to DR *or* like an *ON-OFF switch*. The last possibility is supported by the fact that DR can also be performed by *intermittent fasting* (IF) instead of decreasing the calories or methionine eaten per day by the DR animals. It is known that IF rodents increase longevity even in strains that gorge the days that they receive food. In this last case, even though the total number of calories eaten is roughly the same in the IF animals and the controls, longevity still increases in IF. This apparently paradoxical result would be explained if the PAP genes react to DR (Figure 8), at least to a certain extent, in an ON-OFF fashion, instead of responding in strict proportion to the amount of calories eaten. If this were the case, when the animal eats a certain amount of food and a threshold of calories is reached, the PAP would increase its activity output (increase its aging effectors) from a low (called “OFF” for simplicity) to a higher (“ON”) level, irrespective of the amount of calories eaten above the threshold. *The PAP will never be at zero activity* in mammals because a minimum activity (here called “OFF”) is needed to give a PAP output that generates the mean rate of aging of each species. This “OFF” aging rate would be maintained when there is no food available. Shortly after eating, food is digested and absorbed and tissue PAP activity would jump in a *qualitative leap* to the “ON” higher output level. When the animal reaches the postprandial state and the substrates ingested have been fully metabolized, PAP activity will go back to the lower "OFF" resting level of aging effector (PAP output) activity.

Further research is sorely needed to clarify whether the PAP output is graded or “all-or-none”, because this could have important implications concerning which is the *healthiest way of eating* to be recommended for human beings. If PAP works qualitatively (ON/OFF) rather than gradually, the classical recommendation of the last decade, eating 5 times per day, would not be the best. On the

contrary, putting all the food in a single early meal, or perhaps, in two big meals one at around 15:00 and the other at around 20:30, together with a very frugal breakfast -as it is typical in Mediterranean countries which are among the most long-lived worldwide- would be an adequate option. In this way, people would expend most of the day (19 hours) fasting (the PAP working in OFF position), and their PAP will be turned ON for a few hours only once or twice per day. If this were correct, the habitude of eating small amounts of food, so typical of some western countries, but with high frequency at almost every time during the day should be avoided. Food that strongly signals to PAP, like *simple carbohydrates* (sugar) or sweetened beverages generating high insulin peaks should be specially avoided, while *complex carbohydrates* that are slowly absorbed would lead to much lower insulin peaks. High amounts of *methionin*e, an amino acid that specifically signals the PAP, could be prevented avoiding excessive consumption of meat and restricting the intake of total protein to around 0.6 g of protein/Kg of body weight per day (0.8 is the current RDA at USA and many other countries).

9.3. Epigenetics, aging and PAP

A further complication involves interactions within the cell nucleus functionally affecting the nuclear gene clusters of aging PAP genes. In addition to varying their expression in response to cytoplasmic, hormonal, and environmental signals, there can be nuclear feed-back among these genes due to epigenetic modifications (Figure 8). Epigenetic changes like DNA methylation, acetylation or phosphorylation and histone modifications seem to be important factors in aging. Epigenetic marks establish changes in gene expression in response to environmental stimuli (like the different DRs) and drugs (like rapamycin) and can be even part of the PAP itself (162). Some authors even support the existence of an "epigenetic clock"(163, 164). This would be essentially different from the "epigenetic drift" during aging which would correspond to stochastic changes that will be of small or not interest for the control of longevity (164). There is a decrease in global DNA methylation during aging, whereas there is an increase with aging in local methylation at CpG islands and specific promoters (164-166).

A recent analysis of human body epigenome has found widespread tissue-specific differential CG methylation, allele-specific methylation and transcription, and the unexpected presence of non-CG methylation in almost all human tissues which correlates with tissue-specific functions (167). Changes in specific histones have been also described during aging, including global increases in H4K20 trimethylation and H3S10 phosphorylation, and decreases in H3K9 and H3K27 trimethylation and H3K9 acetylation (168), which would contribute to modulate aging rate. Such DNA modifications modulate gene expression through *regulation of chromatin structure*, which is known to change during aging. Many age-dependent histone methylations are reversed by both DR and rapamycin treatments in mouse brain (169). Histone deacetylases like SIRT1, and the mitochondria-specific SIRT3 are involved in the decrease in oxidative damage and antiaging effects induced by DR in mice (170). Interestingly, SIRT3 deacetylates various Complex I subunits (171) which could modulate the nearby placed sites of mitROSp. It is also of interest that epigenetics seems to influence two main effectors of PAP, the mitochondria (172-174) and the autophagy system (154).

10. CONCLUSIONS

1. Long-lived mammals and birds have species-specific low mitochondrial ROS generation rates at complex I, low levels of mtDNA oxidative damage, and low fatty acid unsaturation degrees in their cellular and mitochondrial membranes. After almost two decades of research, ***the rate of mitROSp and the fatty acid unsaturati****on* of cellular membranes continue to be ***the only two known traits correlating with animal longevity in the right sense*** and offering a *plausible mechanism* to cause aging. This is true not only concerning MFRTA but also *all theories* of aging in general so far proposed. The close vicinity or even ***contact*** between the site of ROS generation and mtDNA avoids antioxidants to interfere with the ROS produced at the mitROS generator relevant for aging, which is *situated at complex I*. This is likely why antioxidants do not modify longevity. The ROS-dependent final forms of mtDNA damage most relevant for aging –mtDNA deletions, and mtDNA fragments inside nDNA- seem finely tuned and controlled by the rate of mitROS production of each animal species. This significantly contributes to determine the species-specific aging rate and longevity.

2. It is well known that *DR also decreases mitROSp and FRL at complex I and oxidative damage to mtDNA*. This is exclusively *due to the lower methionine intake* (MetR) of the animals subjected to DR. Around 50 % of the longevity extension effect of DR and PR is due to MetR, and seems to increase longevity in part through decreases in mitROSp at complex I; the other 50 % effect of DR on longevity would act through other mechanisms like, perhaps, increased macro-autophagy. *Rapamycin, the only known drug* that consistently *increases longevity* in mammals *also decreases mitROSp at complex I and FRL*, like all the different types of DRs. Interestingly, *all the four known experimental manipulations that have proven to increase mammalian longevity decrease mitROSp at complex I, FRL, and mtDNA damage* (either 8-oxodG in mtDNA, or mtDNA fragments insertion inside nDNA).

3. *The constantly produced mtROS throughout life at a different rate in each species* leads to the generation of oxidative damage in mtDNA (e.g. 8-oxodG) which is repaired and can lead to point mutations in the process. But *mitROS*, substances produced by the organism that have capacity to break covalent bonds, *also generate single and double strand breaks in mtDNA*. This leads to *irreversible forms of damag*e (mutations) like mtDNA deletions, and *mtDNA fragments which enter the chromosomes through the centromeres and accumulate in nDNA during aging.* The steady-state level of 8-oxodG in mtDNA is a marker of the *flow* of ROS-dependent damage

generation and repair through mtDNA. Its measurement is a useful *marker* of the rate of generation of mtDNA deletions and mtDNA fragments. Mutations can also arise due to processes unrelated to oxidative stress like mtDNA replication and repair. However, it is highly unlikely that these last mechanisms of damage generation are related to longevity, because their random nature can not explain the determination of longevity during DRs and in different animal species. It has been argued that the types of base mutations (transitions or transversions) mainly present in mtDNA indicate that they mainly come from mtDNA replication and repair. This has been taken as evidence against MFRTA. But this applies only to base substitutions and does not concern to mitROS-induced DNA strand breaks leading to mtDNA large deletions, and to mtDNA fragments insertion inside nDNA. When irreversibly damaged mtDNA reaches a high threshold level in a cell, approaching homoplasmy of mutated mtDNA, mitochondrial ATP generation through oxidative phosphorylation is decreased to levels great enough to contribute to aging. There is no consensus if this classical concept of MFRTA (176) can contribute to explain aging and longevity.

It is now known beyond reasonable doubt that mtDNA fragments accumulate during aging inside nDNA in yeast, rat liver and brain, and mouse liver (139,147-149), causing an increase in chronological aging at least in yeast (147). Recent investigations show that *such accumulation, as well as the increase in complex I mitROSp and FRL with age, are fully (100 %) reversed by rapamycin* dietary treatment in the liver of middle age mice (139). This is accompanied by rapamycin-induced strong *increases in autophagy* fully reverting to young levels, and by *partial reversion of lipofuscin* accumulation with age (139). Interestingly, recent studies indicate that the mtDNA fragments do not enter nDNA randomly. Instead they are directed to the centromeres as a main "*entry door*" to the nucleus. From there, they can potentially disseminate to other chromosome regions, perhaps being specifically directed to nDNA regulatory regions controlling the gene clusters of aging (PAP) residing in the cell nucleus (153), thus contributing to aging and to accelerate the mortality rate at old age. Further research is urgently needed to clarify this possibility. Alternatively, and more simply, the mtDNA fragments can potentially alter the information coded in nDNA. They could be directed specifically to structural genes, thus promoting cell malfunction, cell death, or cellular malignant transformation, and thus aging and cancer.

4. The *low fatty acid unsaturation degree* of cellular and mitochondrial membranes of *long-lived animals* leads to relatively *low rates of endogenous lipid peroxidation in vivo*, which is, quantitatively, *the most destructive oxidative stress process* to the main different types of cellular macromolecules. A low rate of membrane lipid peroxidation in long-lived animals also leads to decreases in the generation of highly toxic and mutagenic lipid peroxidation products like malondialdehyde, hydroxynonenal and many others, which can diffuse from membrane peroxidized lipids to the cell nucleus. Some of these products have the potential to modify DNA, e.g., through direct interaction of the carbonyl group of the aldehydes with free amino groups in mtDNA or nDNA. This would add secondary DNA damage to that primarily coming from the complex I mtROS generator relevant for aging. There is a paucity of studies concerning lipid peroxidation-dependent damage to mtDNA and nDNA (175), especially due to technical limitations. They are however potentially interesting and should be studied.

5. DR and MetR also decrease protein oxidation, glycoxidation and lipoxidation, perhaps due to the decrease in mitROSp at complex I or to an increase in protein catabolism during the DRs. Mitochondrial protein oxidation-derived modification can also contribute to the accumulation of mtDNA mutations, although this possibility has also been poorly investigated.

6. Many different kinds of evidence converge in the concept that aerobic tissue cells (both the post-mitotic and the mitotic ones) have a *Pro-Aging Program (PAP)* lying inside the cell nucleus, likely composed of *hierarquically interrelated gene clusters of aging* analogously to the Hox genes controlling development (153). The *PAP* is a main central part of the *Cellular Aging Regulatory System (CARS)*. The large amount of *pro-aging genes* already discovered during the last two decades *are highly conserved* during evolution from yeast and nematodes to *Drosophila*, and mammals, like in the case of the Hox genes. The PAP reacts to GH/ insulin/IGF-1 like signaling and to *cytoplasmic signals* like those from AMPK, mTOR, and many others, in response to the different *environmental* types of dietary restrictions or drugs like rapamycin. The signals entering the *nucleus* would modify different PAP *master genes* through *transcription factors* like FOXO, TFEB and many others. These master genes would then modify the expression of different *gene clusters of aging* mainly constituting the PAP. These clusters must be organized in a *hierarchical cascade* of genes interrelated trough transcription factors, enhancers, promoters, et cetera. The *target genes* situated at the lower level in such hierarchy would modify the synthesis of *specific proteins* which change the *activity level of the aging effectors* (executors of aging).

Among PAP target genes some have been already identified. These are the ones controlling: i) the synthesis of matrix-only complex I domain; ii) delta 5 and 6 desaturases and elongases in the n-6 and n-3 pathways of fatty acid synthesis; iii) many autophagy genes. Some signals coming from the environment (the DRs) can by-pass the nuclear PAP and *directly modify the mitochondria* (or likely other aging effectors) changing their rate of mitROSp at complex I.

7. Among candidate aging effectors in aerobic tissues (both mitotic and post-mitotic), three emerge at present: 1) *Mitochondria* (including *mitROSp*); 2) The *degree of fatty acid unsaturation (DBI)* of cellular membranes; and 3) A*utophagy*. Decreases in the first two effectors slow aging

and increase longevity. Clarifying if over-expression of autophagy genes increases o not mean and maximum longevity over that of normal controls in mice, and if long-lived animals have higher autophagy activity than short-lived ones is urgently needed. The rate of *mitROSp at complex I and the DBI are low in long-lived animals*, thus correlating with longevity in the right sense. In addition, *mtROSp at complex I and autophagy* respond to the environmental pro-longevity signals of the different kinds of DRs or rapamycin: *mitROSp at complex I is decreased and, autophagy is increased,* both collaborating in a integrated manner to *increase longevity*.

8. With so few aging effectors reasonably well known up to now (only three) it seems strongly inappropriate the pretension of some authors that "MFRTA is dead". Such conclusion, heavily repeated by some authors through titles of recent articles is largely based either on: a) *low quality measurements* like the use in of *unspecific kits* to estimate oxidative stress in naked mole rats (177,178), and lack of stating if the naked mole rats used for the measurements were *queens* (30 years of longevity) *or soldiers* (few years of longevity) (177,178); in the second case a high oxidative stress in naked mole rats would confirm MFRTA instead of being contradictory with it; or b) technically well done experiments but based on hypothesis impossible to be correct, especially those testing whether over expression of antioxidant enzymes could increase longevity (for a, and b see more detailed comments on ref. 29). It was well know from the decade of 1990 on that antioxidants, for various different reasons described in sections 2 and *3.4*, do not control longevity (2,7,10-12,14,17,23,24,29,32,55). The wrong hypothesis that "antioxidant enzyme overexpression could increase longevity" was necessarily destined to falsification, because it was wrongly formulated from the beginning, ignoring dozens of previous investigations on the subject. Avoiding such overexpression experiments would have saved a lot of time and resources as well as reaching wrong conclusions highly damaging for gerontology like erroneously considering the mitochondrial free radical theory "dead". Such mistake would "eliminate" from current models one of the two aging effectors changing longevity in the DRs or rapamycin treatment, mitROSp, leaving autophagy alone to respond to DRs or rapamycin. It would "eliminate" precisely the aging effector about which more positive evidences are available, including the differences in longevity between mammalian and bird species. Such pernicious situation for biogerontology should be avoided.

11. CONFLICT OF INTEREST

None declared.

12. ACKNOWLEDGEMENTS

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