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Pharmacological Approaches to Improve Ageing

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1. Introduction

Aging is generally considered as a progressive and irreversible set of structural and functional changes, due both to the genetic background of the individual and the oxidative damage and modifications of intracellular signaling mechanisms. Although the anatomical and physiological alterations associated to aging (e.g. sarcopenia, cognitive and sensorial decline, functional loss in cardiovascular system…) are not a disease, they reduce the functional reserve of the organism, ultimately leading to pathological alterations and death.

Improvements of nutrition, hygiene and public health, and medical diagnosis and treatments have dramatically extended life expectancy in the last decades. However, the rate of human aging is to the moment an elusive target in biomedical interventions. The achievement of a slowing of human age is not necessarily linked to an increase of morbid, unhealthy population, but is likely to postpone the onset of age-related pathologies (Blagosklonny, 2010). Pharmacological intervention to decelerate aging and age-related diseases is highly attractive because it would target all the population during many years. If successful, antiaging therapy will be more efficient in reducing mortality than to fight separately each age-related disease (Olshansky *et al.*, 2007). Research on anti-aging interventions has evolved along the main theories of aging. We describe here the available explanations for aging before presenting the updated status of each approach.

2. Oxidative stress, aging and antioxidant treatments

2.1 Mitochondrial free radicals theory of aging

After formulation of the "rate of living" hypothesis at the beginning of the last century, proposing that longevity is determined by the metabolic rate (Pearl, 1928), the main explanation for aging has been oxidative damage due to free radicals, especially when comparative studies made clear that metabolic rate alone could not explain longevity (see Speakman & Selman, 2011). The oxidative theory of aging (Harman, 1956) proposes that aging is driven by the damage inflicted to cellular components by reactive oxygen species (ROS) produced by mitochondria in the course of respiration, and has evolved into the mitochondrial free radicals theory of aging pointing to this organelle as a key factor in aging (Harman, 1972;Miquel *et al.*, 1980), including some refinement (de Grey, 2004). Briefly, endogenous ROS (and some nitrogen reactive species derived from them) modify lipids, DNA and proteins,

leading to functional and structural alterations of the cell, both directly and by modifications of the nuclear and mitochondrial genomic material (Pak *et al.*, 2003), the later especially exposed to ROS due to proximity and to the lack of histones (Yakes & Van, 1997).

ROS are formed in the inner mitochondrial membrane by transfer of electrons to molecular oxygen from complex I and III of the electron transport system (ETS) during the flow of electrons from reduced NADH and FADH generated by metabolism. At resting, about 0.1% of consumed oxygen (in spite of the erroneous figure of about 2%) (Fridovich, 2004) produces the highly reactive superoxide anion, enzymatically mutated to H_2O_2 and then to H_2O. It is important to note that the rate of ROS formation is not determined by the level of O_2 consumption, but by electrochemical potential of the inner membrane (generated by the H^+ gradient created by the ETS) and by the amount, efficiency and reduction level of complex I and III (Skulachev, 2004). This led to the "uncoupling to survive" theory of aging: H^+-permeating proteins at the inner mitochondrial membrane inhibit ROS production and are correspondingly enhanced in more long-lived species and in some life-extending manipulations (Brand, 2000).

To limit oxidative damage cells have developed enzymatic (superoxide dismutase, catalase,…) and non-enzymatic (glutathione) antioxidants to scavenge and metabolize radicals and have reduced the most ROS-sensitive components of proteins and lipids, i.e. methionine and cysteine contents of mitochondrial proteins and the number of double bonds of unsaturated fatty acids (lipid oxidation forms long lasting reactive carbonyl species which attack lipids, proteins and DNA) (Pamplona & Barja, 2011).

Although a causal link for mitochondrial radical production in aging has been generally accepted in the last three decades, the actual status is rather controversial. This view was supported by correlative studies between longevity and mitochondrial ROS production (Ku *et al.*, 1993;Sohal *et al.*, 1990). These reports did not controlled for phylogeny, body mass and metabolic rate level (Speakman, 2005), but posterior controlled studies confirmed the correlation and extended it to mitochondrial DNA oxidation (see Pamplona & Barja, 2011). On the other hand, initial studies with transgenic mice showed that inhibition or enhancement of endogenous antioxidant enzymes respectively shortens (Yamamoto *et al.*, 2005) or extends (Hu *et al.*, 2007;Schriner *et al.*, 2005) lifespan, but more recent studies did not reproduce this (Lapointe & Hekimi, 2010;Page *et al.*, 2010) or were ambiguous (Perez *et al.*, 2009). In addition, although mice with defective mitochondrial DNA repair enzymes show normal ROS production they age faster (Trifunovic *et al.*, 2005), supporting the idea that mitochondria alterations drive senescence even with normal oxidative damage.

The conflicting results of genetic experiments and the poor effects of antioxidants therapy in longevity (see 2.2) have been used to refute the free radical theory of aging (e.g., Lapointe & Hekimi, 2010;Perez *et al.*, 2009). However, it is likely that ROS production and not antioxidant defenses is the main factor determining longevity, as indicated by comparative and phylogenic studies on the correlation between longevity and antioxidants (Pamplona & Barja, 2011). This is supported by the finding that caloric restriction (CR) (the most successful life-extending manipulation) decreases mitochondrial ROS output and DNA oxidation (Migliaccio *et al.*, 1999), and by life extension in mice with genetic ablation of the protein p66shc (which produces mitochondrial ROS in response to insulin/IGF-1 signaling and stress factors) (e.g. Vendelbo & Nair, 2011).

It is clear that even if mitochondrial ROS were not the only cause of aging, is unlikely that oxidative stress and mitochondria do not participate in the aging process. Several modifications of the theory have focused on the mitochondrial DNA alterations induced by radicals (de Grey, 2004;Pamplona, 2011). Other authors have proposed that although high ROS concentration are detrimental, physiological levels protect from aging by increasing stress defense systems, so that non physiological increases of antioxidant activity can paradoxically accelerate aging (mitochondrial hormesis or mitohormesis, Tapia, 2006).

2.2 Antioxidative therapies

Given the large evidence linking oxidative stress with aging, the use of antioxidants has been a repeated approach in anti-aging research for decades. Even if aging itself is not due to oxidative damage, this approach could extend average life by reducing the mortality of a number of pathological conditions associated to oxidation.

The most frequently assayed antioxidants are present in vegetables and fruits, not only vitamins E (tocopherols), A (carotenes) and C, but also flavonoids (from tea and *Ginkgo biloba),* phenolic compounds (e.g. resveratrol in grapes), catechins and others. A number of artificial antioxidants have also been assayed (deprenyl, NDGA, PBN, thioproline,…). It must be noted that efficiency not only depends on their oxidant scavenging activity, but also in humans bioavailability factors (absorption, lifetime,…) so that animal studies are a requisite even for initial evaluation of the potential utility of an antioxidant.

Part of the initial studies in rodent models showed that some antioxidants could extend average and/or maximum lifespan (see Meydani *et al.*, 1998;Spindler, 2011). Unfortunately no measurements of the oxidative stress were performed in the initial reports, a requisite to confirm that a treatment lowers oxidative stress (Knasmuller *et al.*, 2008). Also, the effects could be due to a decrease in caloric intake of the animals and not by direct antioxidant effects, but when other authors controlled this variable still found an increase in mice lifespan (Bezlepkin *et al.*, 1996;Miquel & Economos, 1979). Recently, an extensive meta-analysis of the rodent lifespan studies reveals that a range of antioxidants (from chemicals such as deprenyl to naturally occurring compounds such as polyphenols) extend lifespan independently of the CR effect observed in other studies (Spindler, 2011).

In human, the available information is epidemiological or observational, including transversal studies about alimentary habits. Vitamins C, E and A operate synergistically against lipid peroxidation (see a review in Fusco *et al.*, 2007), and vitamin C can also regenerate vitamin E levels (Niki *et al.*, 1995). There is a negative correlation between plasmatic levels of antioxidants, mainly vitamin E, and incidence of cardiovascular diseases and some types of cancer (see Fusco *et al.*, 2007 and Hercberg *et al.*, 2009). This correlation is also present for fruits and vegetables intake (Genkinger *et al.*, 2004) and flavonoids and polyphenols (Manach *et al.*, 2005) and for other age-related diseases such as Alzheimer disease (Viña *et al.*, 2004) or diabetes (Czernichow *et al.*, 2006).

The life-extending effects of antioxidants in humans must be inferred from trials assessing the mortality of age-related pathologies. Contrary to the observational studies, randomized trials have not confirmed the expected decrease of mortality after long-term antioxidant treatment. In the trial SUVIMAX, with low doses of antioxidants, a reduction in mortality after 7.5 years of treatment was observed only in men (Hercberg *et al.*, 2004), which could be

explained by a possible lower level of endogenous antioxidants compared to women, similar to a trial in a Chinese population with poor nutritive status (Blot *et al.*, 1993). Other large scale trials have not found beneficial effects of vitamin E supplementation (alone or with other antioxidants) on the incidence of cardiovascular and cancer mortality (Jacobs *et al.*, 2003;Lee *et al.*, 2005;Lonn *et al.*, 2005). It has been suggested that only individuals with low levels of antioxidant would benefice from these treatments, as found in lung cancer rates and selenium supplement (Reid *et al.*, 2002).

A concerning outcome of the controlled trials is the finding that supplementation can have detrimental effects in some groups (Hercberg *et al.*, 2009;Pham & Plakogiannis, 2005). Therefore, the official recommendation is an adequate intake of antioxidant-enriched aliments until more evidence makes clear if supplementation is safe (Fusco et al., 2007).

The discrepancy between the epidemiological and interventional studies could be due to limitations in the design of the studies. As pointed above, the "shotgun" approach of "flooding" the tissues with an antioxidant is likely inefficient or even detrimental *per se* (see 2.1) and it depends critically on the dosage (supplementation does not guarantee redox normalization (Knasmuller et al., 2008)) and the moment of application (in rodent models lifespan effects require initiation at late (PBN) or early age (vitamins mixture, NDGA) (Bezlepkin *et al.*, 1996;Spindler, 2011)). Also, it is likely that only certain combinations of antioxidants can block the redox network of multiple endogenous radicals (as shown in experimental models, (Rebrin *et al.*, 2005)). Last, the plasma measurements commonly used in human studies are not an unequivocal account of systemic redox (Knasmuller et al., 2008). On the other hand, the epidemiological results could be due to differences in lifestyles and genetic and environmental influences, all of them factors cancelled in randomized controlled trials.

A great interest has been raised by resveratrol, a polyphenol found in grapes and red wine. Resveratrol extends longevity in mice fed a high calorie diet (Baur *et al.*, 2006), but not under normal diet (Pearson *et al.*, 2008) and, relevant for human studies, improves in rodent models several markers for senescence and oxidative stress, mimicking caloric restriction (see Minor *et al.*, 2010a). Moreover, resveratrol also improves endothelial function in human patients with coronary heart disease (Lekakis *et al.*, 2005) (see also section 4).

The mechanism of action of resveratrol is however different to other antioxidants. Its main targets seem to be activation of sirtuins, deacetylases that activate transcription of antioxidant enzymes and promote mitogenesis (Vendelbo & Nair, 2011), although recent data indicate that its action on Sirt1 is indirect. It is noteworthy that Sirt1 and Sirt3 interact with metabolic pathways related to aging (see 4), working as sensors of energy availability (Guarente, 2000): upon low energy levels, increased NAD+ concentration activates Sirt1, which in turn operates on FOXO3, a transcription factor correlated with longevity in humans (Willcox *et al.*, 2008) that increases transcription of antioxidants in response to caloric restriction. Sirt3 is genetically linked to longevity in humans (Rose *et al.*, 2003), declines with age (Lanza *et al.*, 2008) and also activates FOXO3 (Sundaresan *et al.*, 2009).

A special mention is deserved by melatonin, the hormone released during the night by the pineal gland (see section 3). In addition to its chronobiological function it is one of the most potent antioxidants known. Melatonin not only acts as a direct antioxidant and inductor of the antioxidant enzymes, but it also generates, after oxidative cleavage, a series of

derivatives with potent antioxidant activity (see Hardeland *et al.*, 2009). Melatonin accumulates in nuclei and mitochondria, protecting against oxidation of genetic material and it has been repeatedly shown to be an excellent antioxidant in conditions of oxidative stress, both in animals and humans (for recent reviews see Anisimov *et al.*, 2006 or Pozo *et al.*, 2010). Melatonin has also been shown in animal models to slow functional changes associated to aging in a number of systems (Camello-Almaraz *et al.*, 2008;Gomez-Pinilla *et al.*, 2008;Pascua *et al.*, 2011). More important, melatonin extends lifespan in more than 50% of rodent studies and has well established anticarcinogenic properties for mammary and colon cancer in animal models (Anisimov et al., 2006). Although to date there are no human mortality data in healthy individuals treated with melatonin, the results from clinical assays are promising. For example, a meta-analysis shows in human patients of solid tumors a decrease in risk of death at 1 year (Mills *et al.*, 2005), and numerous animal and human (clinical) studies support the potential of this hormone to limit cancer development (see a review in Karasek, 2004). Additionally, controlled trials in humans have shown the absence of toxicity and significant side effects (Singer *et al.*, 2003).

3. Hormonal replacement as antiaging therapy

The observation that several endocrine secretions decay with aging (sexual hormones, growth hormone (GH), melatonin and others (Pandi-Perumal *et al.*, 2008), laid the basis for attempts for hormonal replacement as antiaging therapy.

3.1 Melatonin

Melatonin, discovered 50 years ago, is a hormone synthesized by the pineal gland, retina, gastrointestinal tract and immune cells. Melatonin plasma levels follow a circadian rhythm: it is secreted by the pineal gland during the dark phase of the day, because light input into retinal cells activates nerve impulses to the suprachiasmatic nuclei of hypothalamus (SCN), which in turn suppresses the excitatory sympathetic input to the pineal gland and the release of melatonin. Thus, melatonin monitors the onset and duration of the dark phase, synchronizing the central circadian oscillator (SCN) and the peripheral organs with the environmental light-dark cycle, but is also involved in vasomotor control, sleep initiation,… (Pandi-Perumal et al., 2008). In humans the rhythmic secretion starts around the 6th month of age, peak levels are achieved at 4 - 7th years, melatonin concentration drops at puberty and diminishes gradually in old people (Karasek, 1999). Melatonin acts through plasma membrane and nuclear receptors and by interaction with intracellular signalling proteins and it has potent antioxidant properties (this aspect has been treated above).

The decline in melatonin secretion with age is accompanied by a progressive deterioration of the central circadian oscillator (Hofman & Swaab, 1994) and by sleep disruption, a feature of aging in humans (Neubauer, 1999). Although a meta-analysis did not find conclusive evidence that melatonin was effective to improve sleep parameters in patients with insomnia due to great discrepancies in pharmacological preparation, dose and time of treatment and measurements of melatonin and circadian parameters (Buscemi *et al.*, 2005), a more recent meta-analysis supports the effectiveness of exogenous melatonin in patients with delayed sleep phase disorder (van Geijlswijk *et al.*, 2010). The study found three requisites for optimal melatonin therapy: adequate dose (too low is inefficient, too high is hypnotic), administration 3-6 hours before the so-called dim light melatonin onset and choice of appropriate patients (with a delayed biological timing).

A recent improvement is a formulation that releases melatonin slowly in the gut after oral administration and increases its plasma concentration over the following 8-10 h (Circadin®, Neurim Pharmaceuticals, Israel), which has been approved by the European Medicines Evaluation Agency in June 2007 for the short-term treatment of primary insomnia. Several studies have shown its efficiency and safety for short-term treatment (3 weeks) of adults and old people (Luthringer *et al.*, 2009), including a double-blind, placebo-controlled randomized trial evaluating the short and long-term effects of Circadin (Wade *et al.*, 2010). Circadin also seems to improve blood pressure rhythms (Grossman *et al.*, 2006).

The effects of melatonin on sleep rhythm are due to its plasma membrane receptors (MT1 and MT2) in the suprachiasmatic nucleus. MT1 receptors inhibit firing of suprachiasmatic neurons and MT2 receptors entrain circadian rhythms and have phase-shifting effects (Hunt *et al.*, 2001). This finding lead to the design of specific agonists ramelteon (Rozerem®, Takeda Pharmaceuticals, Japan) and agomelatine (Valdoxan®, Servier and Novartis). Ramelteon, approved by the FDA (July 2005) for the treatment of insomnia, has been assayed for the treatment of primary insomnia in humans (Erman *et al.*, 2006). Although these studies found it effective and safe, the European Medicines Evaluation Agentcy found the efficacy of ramelteon insufficient for marketing authorization. Agomelatine binds to melatonin receptors but is also an antagonist of serotonin 5-HT2C receptors used to decrease anxiety and promote sleep (Lemoine *et al.*, 2007). Its efficacy, tolerability and safety have been assessed by several randomized, placebo and active-controlled studies (Kennedy & Emsley, 2006) and improves the disrupted sleep of depressed patients (Lemoine et al., 2007).

3.2 GH

The growth hormone (GH) and its key mediator insulin-like growth factor-I (IGF-I) regulate somatic growth and development, metabolism and body composition, but seems to be also related to aging. The pulsatile GH secretion shows an age-related decay after the high amplitude pulses of the postnatal and puberty stages (Finkelstein *et al.*, 1972), and correlates to aging-related changes in body composition (sarcopenia, osteopenia, increase in fat content,...) (Veldhuis *et al.*, 1995). This correlation, together with the fact that replacement therapy in GH deficient adults and elderly improves body composition, lipoprotein profile, exercise capacity and bone density (Rudman, 1985), elicited interest in the possible use of GH as antiaging therapy. However, a higher mortality has been found in patients critically ill treated with GH (Takala *et al.*, 1999) and in rodents and humans suffering high levels of GH (acromegaly) (Sheppard, 2005). This is in keeping with the increased lifespan of mutant mice with defects in GH/IGF-1 secretion/pathways (Bartke, 2003) and by some data on human lifespan (Suh *et al.*, 2008). Therefore, the clinical use of GH is only approved in US for treatment of GH deficiency, idiopathic short stature and HIV/AIDS.

3.3 Vitamin D

In addition to its key role in calcium homeostasis, there are evidences that vitamin D can influence longevity by decreasing the morbidity of age-related diseases, such as cancer or cardiovascular diseases, in addition to osteoporosis (not treated in this review). The active form of vitamin D3 (1,25(OH)2-cholecalciferol or calcitriol) binds to nuclear receptors (VDRs) to modulate the transcription of genes involved in systemic and intracellular Ca^{2+}

homeostasis and in cellular proliferation. The later are also due to fast, non genomic effects mediated by plasma membrane VDRs (Dusso *et al.*, 2005).

The main actions of vitamin D of interest as antiaging therapy are its anti-inflammatory and anti-cytokine effects in humans (shown in controlled trials (Schleithoff *et al.*, 2006)), and its ability to promote neuronal survival in different experimental models (Regulska *et al.*, 2006). In fact, human observational studies show a negative correlation of levels of vitamin D3 with cardiovascular disease (Zittermann *et al.*, 2005) (an inflammatory process), and with cognitive performance in elderly (Llewellyn *et al.*, 2009;Oudshoorn *et al.*, 2008). Additional support for vitamin D3 as antiaging treatment comes up from evidences that serum concentration of vitamin D decreases with age (Utiger, 1998) and its role in the control of cell cycle and apoptosis, which are altered in aging: calcitriol reduces proliferation of normal and cancer cells (Ylikomi *et al.*, 2002) and up-regulates apoptosis of cells damaged by redox stress and DNA alteration (Higami & Shimokawa, 2000).

The beneficial effects of vitamin D in age-associated diseases is expected to result in a prolongation of average lifespan. A study showed that vitamin D deprivation decreased the lifespan of male, but not female, rats (Thomas *et al.*, 1984). In humans, a recent meta-analysis of randomized trial showed a clear reduction in all-cause mortality in old individuals under vitamin D supplementation (Autier & Gandini, 2007). However, some reports have raised concerns with the safety of calcitriol supplementation (Stolzenberg-Solomon, 2009), although limitations in the design of the studies avoid definitive conclusions.

4. Caloric restriction

CR is the most robust non-genetic nutritional experimental intervention for slowing aging, and maintaining health and vitality in organisms ranging from budding yeast (*Sacharomyces cerevisiae*) to humans (Fontana *et al.*, 2010b). It is defined as a reduction of total macronutrient intake without causing malnutrition, with food intake reduced by 30-40% compared to *ad libitum* levels. Experiments involving CR in rodents in 1935 provided the first promise for modulation of lifespan (McCay *et al.*, 1935). Since then CR has been repeatedly proved to be effective in extending average and maximum lifespan and delaying the onset of age-associated pathologies in diverse species (for review see (Minor *et al.*, 2010a;Omodei & Fontana, 2011)). It was not until the 1990s that CR became widely viewed as a scientific model that could provide insights into the underlying mechanisms of aging and lifespan extension. The fact that CR significantly increased the average and maximum lifespan in many simpler eukaryotes, including the common model organisms used in aging research, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae* pointed out that CR represents an evolutionarily conserved mechanism for modulating longevity and opened the possibility of using genetic tools in these models that helped to unveil intracellular pathways related to pro-longevity.

Alternative approaches to CR are a controlled reduction of a particular macronutrient of the diet (dietary restriction, DR) or temporal variations of food intake (intermittent fasting, IF). Particularly, protein restriction, PR, where a percentage of calories derived from protein is replaced by fat or carbohydrate, has been investigated in rodents and decreases in mitochondrial reactive oxygen species production and DNA and protein oxidative modifications have been reported (Ayala *et al.*, 2007), which could explain the increase in

lifespan previously reported for PR (Leto *et al.*, 1976). Similar effects were obtained with reduction of the amino acid methionine (Naudi *et al.*, 2007), but they could not be replicated by restricting lipid intake alone (Sanz *et al.*, 2006a) or carbohydrate intake alone (Sanz *et al.*, 2006b). IF, a regimen of either alternate day fasting or fasting for a day after 2 days of feeding, both increases lifespan and delays or prevents some age-related diseases (reviewed in Mattson & Wan, 2005). However, CR is by now the most powerful nutritional intervention to prolong life.

4.1 Effects of caloric restriction

It is totally accepted that the effects of CR on lifespan and mortality in rodents increase linearly with the extent of the restriction until reaching approximately a 50-60% of restriction at which lifespan is negatively affected. In addition, the effect of CR on lifespan is stronger when initiated at weaning and weaker later in life (reviewed in (Fontana, 2009b;Speakman & Hambly, 2007;Speakman & Mitchell, 2011)). In fact, CR increases rather than decreases mortality if initiated in advanced age (Forster *et al.*, 2003). CR inhibits growth and body size after maturation and reduces body weight, as consequence of changes in the endocrine profile as discussed below. CR also inhibits fertility, especially in females, but there is an increase in their reproductive performance when they are subsequently returned to *ad libitum* feeding (Selesniemi *et al.*, 2008)

CR induces transcriptional alterations that are indicative of metabolic reprogramming, a change in how energy is generated and how fuel is utilized. A key metabolic change during CR is a shift from fat storage to fat utilization impacting stress signaling pathways and ROS production (Anderson & Weindruch, 2010). Immediately following food intake there was a period of endogenous fatty acid synthesis that was then followed by a period of prolonged fatty acid oxidation, which induces large changes in the respiratory quotient (RQ) (Speakman & Mitchell, 2011). In addition, during CR there is an increase in the AMP/ATP ratio which leads to the activation of the AMP-activated protein kinase (AMPK) that promotes fat oxidation increasing the transport of fatty acids into the mitochondrion. In fact, marked phosphorylation of AMPK has been found after long term CR (Edwards *et al.*, 2010). Because fatty acid substrates enter the electron transport chain predominantly via complex II rather than complex I, the main ROS generator is bypassed when the metabolism is switched predominantly to fatty acid oxidation. This might represent a mechanism minimizing oxidative stress under CR.

The hormonal profile of long-term CR is characterized by a suppression of the gonadal, thyroid and GH-insulin-like growth factor I (GH-IGF-I) axes, an increase in the insulin sensitivity and an increase in the daily peak levels of plasma corticosterone that takes part in successfully coping with stressors (Xiang & He, 2011). CR also results in decreased levels of leptin and increased blood concentration of ghrelin and adiponectin, a modulator of a number of metabolic processes appearing to have anti-inflammatory, anti-diabetic, and anti-atherogenic properties that seem to play an important role in life extension effect of CR (Chiba *et al.*, 2002;Lago *et al.*, 2007).

The reductions in IGF-I and insulin signaling that occur under CR have been suggested to be causally linked to the lifespan enhancing effects of CR. This is in part based on the observation that several rodent models that present mutations that modified

insulin/GH/IGF-I signals, live longer than controls and there is considerable phenotypic overlap between long-lived mutant mice and normal mice on chronic CR. These models include, amongst others, *Prop-1* (Ames mice) and *pit-1* (Snell mice) mutant dwarf mice, GH receptor/binding protein homozygous knockout mice (*GHR/BP*$^{-/-}$ or GHRKO), insulin receptor substrate 1 knockout mice (*Irs1*$^{-/-}$)... Most of these mice have a body weight smaller than their normal siblings and present decreased levels of IGF-I , and increased sensibility to insulin (Chiba *et al*., 2007), except *Irs1*$^{-/-}$ mice whose IGF-I levels are unchanged and show a mild but lifelong insulin resistance having increased lifespan and reduced markers of aging (Selman *et al*., 2008). In GHRKO mice CR increased lifespan only in females and failed to further enhance the remarkable insulin sensitivity and the insulin signaling cascade in GHRKO mutants (Bonkowski *et al*., 2006;Bonkowski *et al*., 2009). These data imply that somatotropic signaling is critically important in mediating the effects of CR on lifespan and also support the notion that enhanced sensitivity to insulin plays a prominent role in the actions of CR and GH resistance on longevity. It was originally reported that long-term severe CR did not reduce serum IGF-I concentration or the IGF-I/IGF binding protein ratio in humans but total and free IGF-I concentrations were significantly lower in moderately protein-restricted individuals (Fontana *et al*., 2008). In addition, it has been recently shown that CR for 4 years leads to reduced IGF-I serum levels in formerly obese women relative to normal-weight women eating *ad libitum* (Mitterberger *et al*., 2011), which suggests that growth hormone/IGF-I axis is also important in the effects of CR in humans.

4.2 Caloric restriction in non-human primates

Most CR research on longevity in mammals has been performed in rodents, mainly in mice. However, studies designed to evaluate the effects of CR on species closer to humans are of great interest in order to translate the knowledge to humans. Two prospective investigations of the effects of CR on long-lived nonhuman primate species began nearly 25 years ago and are still under way. These studies (randomized controlled trials) revealed beneficial effects of CR on physiological functions and the retardation of disease. In the study conducted in the Wisconsin National Primate Research Center a recent report showed that animals on 30% of CR appeared subjectively younger than controls, the body weight was reduced and the age-related sarcopenia attenuated. Improvements in metabolic function (improved insulin sensitivity and glucose tolerance) and preservation of grey matter volume in subcortical regions were reported. In addition, there was a lower incidence of neoplasia, cardiovascular disease and type 2 diabetes mellitus. Survival analysis considering only age-related deaths revealed a significant effect of CR in increasing survival, but when assessing "all-cause" mortality CR did not provide a statistically significant lifespan increase (Colman *et al*., 2009). In any case, the reduction of age-related diseases and the potential increase in longevity are promising. Data regarding CR-induced longevity from the National Institute of Aging's are not yet available, although a decrease in age-related diseases and beneficial effects on other physiological parameters have been provided (Mattison *et al*., 2007).

4.3 Caloric restriction in humans

The studies about human responses to CR have some limitations that should be taken into account when interpreting the results. An important amount of data come from members of the CR Society International (www.calorierestriction.org) which has the mission to

promote the use of CR in humans. In agreement with the research results from animal studies, voluntary CR in humans results in sustained beneficial effects on the major atherosclerosis risk factors, and has protective effect against obesity and insulin resistance. In addition, the CR society members have reduced circulating levels of insulin, PDGF, TGF-β and pro-inflammatory cytokines (reviewed in Fontana, 2009a). Nonetheless, despite high serum adiponectin and low inflammation, approximately 40% of CR individuals exhibited an exaggerated hyperglycemic response to a glucose load. This impaired glucose tolerance is associated with lower circulating levels of IGF-1, total testosterone, and triiodothyronine, which are typical adaptations to life-extending CR in rodents (Fontana *et al.*, 2010a). Assuming the importance of these findings, it should be noted that these volunteers are clearly a self selected population and this is not a randomized controlled trial.

There is a randomized controlled trial for the effects of CR on humans, and that is the CALERIE (Comprehensive Assessment of the Long term Effects of Reducing Intake of Energy) trial sponsored by the NIA in the USA. In the phase 1 of the trial all the studies have been performed in non-obese healthy but overweight subjects, therefore it is difficult to separate beneficial effects due to the weight loss or to CR. The most relevant findings of phase 1 trials were the reduced body weight and total fat mass, the reduced fasting levels of insulin, leptin and T3 and the increased insulin sensitivity. Activity energy expenditure and core body temperature were decreased in response to the CR. In addition, CR decreased cardiovascular risk, increased some antioxidant defenses and reduced markers of inflammation (reviewed in (Speakman & Hambly, 2007)). Interestingly, "in vitro" studies utilizing CR human serum to examine effects on markers of health and longevity in cultured cells resulted in increased stress resistance and an up-regulation of genes (sirt1 and PGC1α) proposed to be indicators of increased longevity (Allard *et al.*, 2008). In the phase 2 trial of the study, a two-year CR period was selected to attempt to provide for a sustained period of weight stability following weight loss that would more accurately unveil the effects of CR in humans (Rickman *et al.*, 2011). Whether CR extends life in humans and the magnitude of this potential effect also remain unclear and far for been resolved

4.4 Intracellular pathways mediating CR effects

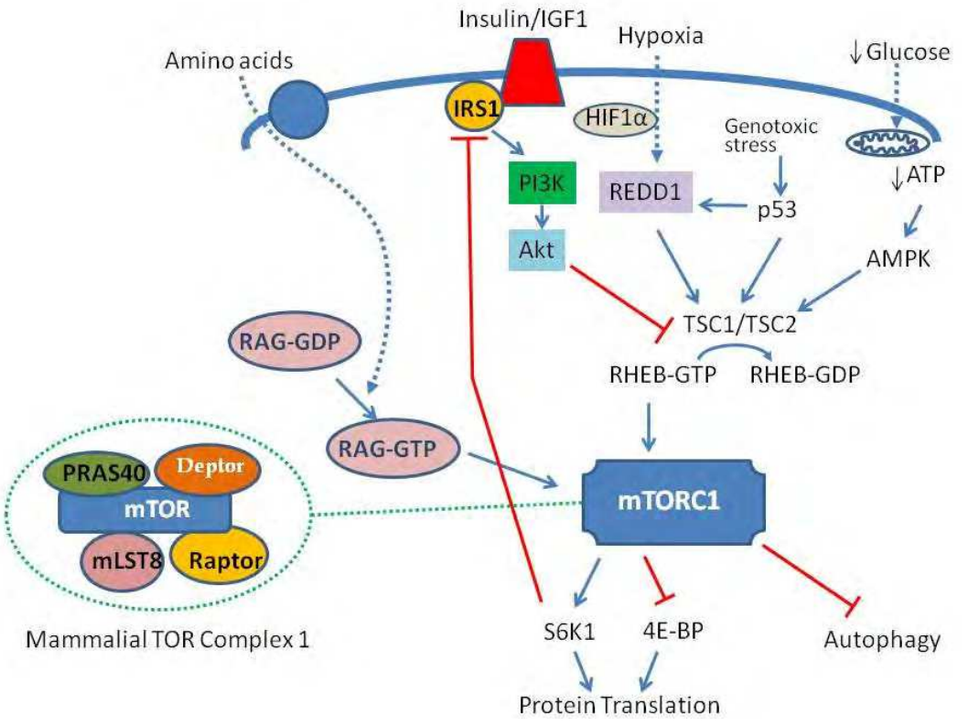
For many individuals, the hardships of maintaining a CR lifestyle are too great to justify the improved health profile and potential life-extension benefits. Nevertheless, identifying the genetic and physiological mediators of CR could aid in the discovery of compounds/treatments that would act on those pathways, thereby mimicking the positive aspects of CR without imposed food restriction.

TOR, a serine/threonine protein kinase that belongs to the family of phosphoinositide-3-kinase (PI3K)-related kinases (PIKK), is the primary candidate involved in the regulation of lifespan in animals under CR. Its name, Target Of Rapamycin, indicates that it mediates the effects of rapamycin, an antifungal and immunosuppressant agent that inhibits TOR. In mammalian cells, TOR participates in the mammalian complex 1 (mTORC1) that is sensitive to rapamycin and controls cell size, proliferation and lifespan via a variety of downstream pathways. mTORC1 is a homodimer that has four components in addition to

the Serine/Threonine kinase mTOR: Raptor, mLST8, PRAS40 and Deptor. Raptor binds mTOR and recruits the downstream kinase substrates (S6K and 4E-BP) in a manner that enables their phosphorylation by the mTOR catalytic domain. Other proteins that participate in TORC1 regulation are Tuberous Sclerosis Complex proteins TSC1 and TSC2, with a GTPase-activating (GAP) domain, and the Ras-like small GTPase RHEb, the preferred substrate of the TSC2 GAP activity. TSC complex inhibits TOR signaling as the result of its ability to deactivate Rheb (reduced GTP/GDP) (reviewed in Kapahi *et al.*, 2010).

TORC1 integrates responses to growth factor stimulation, changes in energy status, nutrients, oxygen levels and various types of stress. Thus growth factors like IGF and insulin, via Akt, directly phosphorylate several sites on TSC2, which decrease the inhibitory activity of TSC and the increase TORC1 activity. A drop in the cell energy content, as that induced by glucose deprivation, is reflected in the rise of the AMP/ATP ratio that triggers AMP-dependent activation of AMPK. In turn, AMPK reduces the activity of TORC1 by direct phosphorylation and stimulation of Tsc2 activity and inhibition of Raptor. Amino-acid regulation is exerted predominantly through Rag GTPases (RagA, RagB, RagC, and RagD) that sense amino acid levels. In the presence of amino acids the complex interacts with raptor and promotes TORC1 through relocalization to RHEB rich cellular compartments. Conversely, deprivation of amino acid inhibits mTORC1 with leucine or arginine withdrawal mimicking total amino acid deprivation. Amongst the environmental stresses to which cells are exposed, TORC1 also senses hypoxia. Low levels of oxygen, through stabilization of HIF1α induce the transcription of hypoxic response genes, mostly REDD1 that inhibits TORC1 activity by a TSC2-dependent mechanism. In addition, hypoxia reduces ATP levels, and then it controls TORC1 through AMPK. Genotoxic stress represses TORC1 activity through p53-mediated increased expression of PTEN, TSC2 and REDD1 (reviewed in Kapahi *et al.*, 2010;Ma & Blenis, 2009;Speakman & Mitchell, 2011).

TORC1 controls cell growth maintaining the adequate balance between anabolic processes such as protein synthesis and catabolic processes like autophagy. As commented before, S6K and 4E-BP1 are the best-known substrates of TORC1 and through them TORC1 regulates protein synthesis by regulating the activity of the translational machinery and also specifically controlling the translation of subset of mRNAs that are thought to promote cell growth and proliferation (Ma & Blenis, 2009). The limiting step of protein synthesis is translation initiation. The recruitment of the small ribosomal subunit to mRNA requires the participation of the translation initiation factor 4F (eIF4F) complex. 4E-BP binds eIF4E, a component of this complex, and prevents translation initiation. When hyperphosphorylated by mTORC1, 4E-BP1 dissociates from eIF4E, allowing the initiation of translation. Evidence suggests that S6Ks modulate the functions of translation initiation factors during protein synthesis and also coordinate the regulation of ribosome biogenesis, which in turn drives efficient translation (see for review (Ma & Blenis, 2009)). S6K1 interacts back with the insulin signaling pathway by phosphorylating the insulin receptor substrates IRS1 and IRS2, which seems related to insulin resistance. Consistent with the important role of S6K on mediating mTOR induced lifespan extension, $S6K1^{-/-}$ mice have gene expression profiles similar to those of CR mice, and females have extended longevity with evidence of fewer age-related diseases (Selman *et al.*, 2009).



In mammalial cells mTORC1 receives positive inputs from RHEB-GTP, but it is inhibited when RHEB is bound mainly to GDP. TSC1/TSC2 complex, through its GTPase-activating domain favors GDP-bound RHEB and then it mediates mTORC1 inhibition. Several extracellular and intracellular pathways activate (by phosphorylation) TSC complex, such as the ATP sensor AMPK that is stimulated when cellular energy decreases, the hypoxic response gene REDD1 that senses hypoxia, and p53 that senses genotoxic stress. TSC complex is inhibited by Akt-mediated phosphorylation, which results in mTORC1 activation. An important pathway for Akt activation is the insulin/IGF signaling. The amino acid level in the cell controls the state of Rag GTPases. The presence of amino acids enhances Rag-GTP and activates mTORC1 through relocalization to RHEB rich cellular compartments. Activation of mTORC1 increases mRNA translation and protein synthesis through phosphorylation of S6K1 and 4E-BP and inhibits autophagy, which results in cell growth and also in senescence. Inhibition of mTORC1 increases autophagy, reduces protein synthesis and cell grothw/differentiation decreasing senescence and extending lifespan.

Fig. 1. mTORC1 and insulin/IGF1 signalling and lifespan.

Accumulating evidence demonstrates that longevity pathways, including mTOR, interact with the macroautophagic process. Autophagy is a lysosome-mediated degradative process of eukaryotic cells to digest their own constituents during development or starvation. Macroautophagy (hereafter referred as autophagy) is a type of autophagy that involves the formation of subcellular double membrane-bound structures called autophagosomes to sequester cytoplasmatic materials and deliver them into lysosomes for breakdown by acid hydrolases. According to the current knowledge, the first signalling component downstream of mTOR in the autophagy pathway in mammals is ULK1, a serine/threonine kinase. ULK1 plays a key role at the nucleation (the early event when membrane structures are initiated) and formation of the preautophagosome structures. mTOR-induced ULK1 phosphorylation avoids the recruitment of proteins to the autophagosome membranes inhibiting downstream events essential for autophagy (reviewed in (Jung *et al.*, 2010)).

Sirtuins are a family of NAD+-dependent protein deacetylases that exert multiple cellular functions by interacting with, and deacetylating a wide range of signaling molecules, transcription factors, histones and enzymes (Yamamoto *et al.*, 2007). In mammals, the family is represented by seven members (SIRT1-7) with different cellular locations. Several studies have demonstrated that CR regulates sirtuin system and that a functional sirtuin system is required for lifespan extension to occur (Bamps *et al.*, 2009;Cohen *et al.*, 2004). Thus, CR does not have any effects of lifespan extension in SIRT1 deficient mice (Boily *et al.*, 2008). By contrast, elevation of SIRT1 expression results in a phenotype resembling that of caloric restriction (Bordone *et al.*, 2007). In humans SIRT1 gene expression also appears to be responsive to caloric restriction (reviewed in (Kelly, 2010)). SIRT1 elicits anti-senescence activity by targeting a wide range of protein substrates that are critically involved in regulating key cellular processes, such as oxidative stress, DNA damage, mitochondrial biogenesis and autophagy. Targets for SIRT1-mediated deacetylation include p53, NFkB, PGC-1a (peroxisome proliferator-activated receptor-c coactivator 1a], eNOS, mTOR and FoxOs. It is also of great importance the interaction of SIRT1 with LKB/AMPK. While acute activation of the LKB1/AMPK pathway confers adaption to stress, sustained stimulation of this pathway leads to irreversible senescence. SIRT1-mediated deacetilation of LKB, and consequent ubiquintination and degradation serves to prevent persistent AMPK signaling, reinforcing the anti-age effects of SIRT1 (reviewed in (Wang *et al.*, 2011)).

4.5 Therapies based on caloric restriction

The knowledge of the intracellular pathways related to aging led to the development of drugs, named generically caloric restriction mimetics (CRM) that replicate the effects of CR. These drugs targets the main pathways affected by CR: insulin/IGF1, mTOR, and sirtuins.

4.5.1 Insulin/IGF1 pathway

The firts CRM used was 2-deoxy-D-glucose (2DG), a compound that inhibits glycolisis. In keeping with the effects of CR, 2DG in rodents reduced body temperature, body weight and circulating glucose and insulin and increased glucocorticoids and heat-shock proteins. In addition, reduced tumors and increased stress resistance to neurotoxins and cold shock (Le Couteur *et al.*, 2011;Minor *et al.*, 2010a). Despite of these findings, long-term administration of 0.4% 2DG did not enhanced lifespan but increased mortality due to cardiac toxicity and adrenal tumors (Minor *et al.*, 2010b), which indicates that this drug could have therapeutic value for short-term treatment but it would not be indicated for aging interventions.

The biguanide antidiabetic drug metformin has been shown to molecularly recapitulate most of the pro-longevity effects occurring upon CR (Dhahbi *et al.*, 2005) and to suppress S6K1 activity in cultured proliferating epithelial cells (Vazquez-Martin *et al.*, 2009). In keeping with these effects, it has been reported that chronic metformin treatment of mice from different strains predisposed to high incidence of mammary tumors decreased body temperature, increased mean and maximal lifespan and postponed tumors and age-related switch-off of estrous function. These effects were dependent on the gender and the strain of mice (reviewed in (Anisimov, 2010)). In humans a retrospective study has reported an impressive 56% decrease in breast cancer risk among diabetic receiving metformin (Bodmer *et al.*, 2010), which together with the animal studies suggest that metformin could increase

lifespan in humans. Metformin treatment phenocopies the effects of amino acid-deprivation on mTORC1, suggesting that this drug may inhibit mTORC1 via modulation of Rag signaling (Kalender *et al.*, 2010). The effects of metformin on mTOR and its effector S6K1 can also be due to the well recognized activation of AMPK by the drug (Hardie, 2011), which as described above, inhibits mTORC1. Other bioguanides such as buformin and phenformin have shown promising results in rodent tumor suppression, but they have to be withdrawn from the clinical practice due to association with lactic acidosis (Minor *et al.*, 2010a).

4.5.2 mTOR pathway

Rapamycin, a macrolide antibiotic with antitumor and immunosuppressant actions, selectively and effectively inhibits mTORC1 as CR does, as discussed above. The inhibitory action of rapamycin on TOR signaling requires the formation of the rapamycin/FKBP12 complex, which interferes with the proper interaction between raptor and mTOR, rather than or in addition to a more direct inhibition of mTOR catalytic activity. Many roles of mTORC1 on cell growth and survival have been unveiled by the use of rapamycin. Rapamycin is, by now, the pharmacological treatment that more resembles CR-induced lifespan extension. One of the most important contributions to this field has been the report of the National Institute on Aging Intervention Testing Program (ITP) showing that rapamycin supplementation late in life (20 months of age) induced a significant mean lifespan extension in both male and female mice fed a standard diet. This study was conducted in three different sites in the USA and used genetically heterogenous mice to avoid genotype-specific effects on disease susceptibility. According to this study, rapamycin may extend lifespan by postponing death from cancer, by retarding mechanisms of ageing, or both (Harrison *et al.*, 2009). Rapamycin treatment increases autophagy, reduces cell senescence and have anti-inflammatory as well as antitumor effects. In addition rapamycin and rapamycin analogs (rapalogs) ameliorate age-related diseases such as cancer, metabolic syndrome, neurodegenerative and cardiovascular diseases (reviewed in (Sharp & Richardson, 2011)).

4.5.3 Sirtuin pathway

Resveratrol, in addition to its antioxidant properties, has been reported to mediate yeast lifespan extension through the activation of the sirtuin family of deacetylases (Howitz *et al.*, 2003). This led to the idea that resveratrol might act as a CRM, and was supported by results on rodents showing impressive protection against age-related diseases including neurodegeneration, cancer, cardiovascular diseases and obesity (Markus & Morris, 2008). Regarding the targets of resveratrol, biochemical studies indicate that resveratrol may not activate sirtuins directly but thorugh activation of AMPK (Hwang *et al.*, 2009). Although in mice fed with a high fat diet resveratrol induces lifespan extension and its commonly associated features (increased insulin sensitivity and AMPK activity, reduced IGF-I levels, …) (Baur *et al.*, 2006), there are no reports of increased lifespan in healthy mammals. This may indicate that resveratrol induces effects by targeting intracellular pathways activated by CR without slowing aging. The effects of resveratrol have led to development of more potent SIRT1 activators. These newly synthesized compounds (by a pharmaceutical biotechnology company called Sirtris Pharmaceuticals) are potent small-molecule activators of SIRT1 that are structurally unrelated to natural polyphenols. There are promising data

regarding the effects of these compounds in mouse models and some of them are in phase II clinical trials for type 2 diabetes, opening an approach for other age-related diseases (reviewed in Camins *et al.*, 2010).

5. Conclusion

The evolution of our understanding of the biological basis of aging has focused antiaging research on antioxidant, metabolic and hormonal replacement therapies. Although beneficial effects of antioxidant therapies are not in doubt, controlled trials have revealed poor efficiency for hormonal and antioxidant treatments. Thus, more controlled and properly designed trials are needed to determine the potential of these approaches. The recent advances in knowledge about metabolic signalling pathways involved in the aging process especially in mTOR/Insulin/IGF-1 pathway that mediate the beneficial effects of CR, have opened new venues for the development of effective antiaging or CRM treatments. The results reported for rapamycin treatment starting later in life are of great interest in terms of the potential use of this inhibitor of mTORC1 for slowing aging and probably its combination with resveratrol, an stimulator of Sirt1 that improves age-related diseases without increasing lifespan in mammals, will render a more potent and efficient treatment. In addition, future research in how the different pathways integrate and interact to mediate CR effects will provide us with new pharmacological interventions that can slow the process of aging. An important issue in the prescription of these drugs to healthy humans is that aging is not recognized as a condition to be treated. Thus, the anti-aging drugs should be introduced in human if they affect disease, and later on, when showed effective the day will come when they become anti-aging drugs.

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7. References

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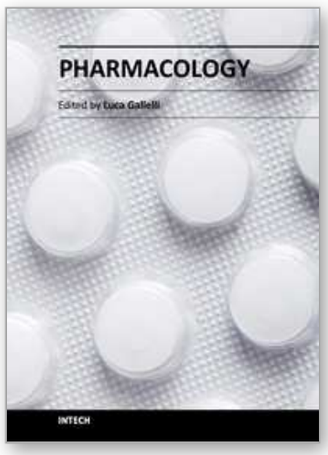

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The history of pharmacology travels together to history of scientific method and the latest frontiers of pharmacology open a new world in the search of drugs. New technologies and continuing progress in the field of pharmacology has also changed radically the way of designing a new drug. In fact, modern drug discovery is based on deep knowledge of the disease and of both cellular and molecular mechanisms involved in its development. The purpose of this book was to give a new idea from the beginning of the pharmacology, starting from pharmacodynamic and reaching the new field of pharmacogenetic and ethnopharmacology.

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