

# Caloric Restriction

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Caloric restriction (CR) is a traditional but scientifically verified approach to promoting health and increasing lifespan. CR exerts its effects through multiple molecular pathways that trigger major metabolic adaptations. It influences key nutrient and energy-sensing pathways including mammalian target of rapamycin, Sirtuin 1, AMP-activated protein kinase, and insulin signaling, ultimately resulting in reductions in basic metabolic rate, inflammation, and oxidative stress, as well as increased autophagy and mitochondrial efficiency.

Keywords: Caloric restriction ; Nutrition ; Diet

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## 1. Introduction

Caloric restriction (CR) is one of the primary interventions for weight loss and health maintenance. As early as the 16<sup>th</sup> century, Luigi Cornaro (1484–1566) described the beneficial effects of this approach in his “Discorsa della vita sobria.” Later, at the beginning of the 20<sup>th</sup> century, the first experimental evidence emerged when Osborne *et al.* reported that CR slowed the growth of rats but prolonged their lifespan [1]. In rats, a CR of 40% applied from weaning onward has been linked to a lifespan extension of almost two fold [2]. In fact, CR has been associated with increases in mean and maximum life span, regardless of sex, in multiple species, including various rat and mouse strains, yeasts, worms, fruit flies, fishes, hamsters, dogs, cows, and owls [3]. The effects of CR in these organisms include reduced neurodegenerative disease incidence, diminished rates of age-specific mortality, and a lower incidence of cancer, diabetes, atherosclerosis, and cardiovascular disease. CR also is linked to delayed onset of age-related processes, such as immunosenescence, sarcopenia, and atrophy of the brain grey matter [3-7]. In monkeys, CR leads to diabetes suppression and a reduced incidence of neoplasia and cardiovascular diseases by up to 50% [6]. These effects have been attributed to a reduction in major risk factors, including cholesterol, C-reactive protein, blood pressure, and intima–media thickness of the carotid arteries [7-9]. Beneficial outcomes of CR have been consistently reported, which supports this approach considering that distinct CR protocols are used in different publications. CR applied in diverse studies ranges from 10% to up to 50% of daily caloric intake. Furthermore, the length of CR varies from a few weeks to life-long treatment. Additionally, some protocols restrict all nutrients, whereas others limit macronutrients only and supplement micronutrients in order to investigate selectively the impact of calorie reduction and prevent malnutrition making a distinction between “dietary restriction” and “energy restriction” [10]. As expected, the type of CR protocol influences the magnitude of outcomes [11,12]. Moreover, the results obtained for experimental models cannot be directly translated to humans [13]. Therefore, it is important to compile the results of multiple studies to identify common patterns of responses regardless of the type of CR. A comparison of the responses from different species may help to draw a more comprehensive picture of the outcomes of CR.

CR has been tied to a complex network of pathways implicating insulin-like growth factor 1 (IGF-1), sirtuins (SIRTs), adenosine monophosphate (AMP) activated protein kinase (AMPK), and target of rapamycin (TOR). The sympathetic and neuroendocrine systems, as well as thyroid hormones, adipokines, and ghrelin, also have been associated with the beneficial outcomes of CR [4]. This ensemble of processes associated with CR affects the whole body, manifesting in reduced inflammation, body fat mass, resting metabolic rate, and body temperature and improved insulin sensitivity [14]. As a result of the variety of outcomes related to CR and the complexity of the contributing pathways, the exact mechanisms underlying these health benefits are still not well understood.

## 2. Major pathways affected by CR

### 2.1. mTOR

The mammalian (m)TOR pathway is a major nutrient sensor signaling pathway known to regulate longevity. TOR is a well-conserved Ser/Thr protein kinase that belongs to the family of phosphatidylinositol 3 (PI3) kinase–related kinases [15,16]. It functions as an essential part of two complexes, mTORC1 and mTORC2, which have some proteins in common and

some different proteins between them [15]. mTORC1 comprises the following core subunits: mTOR, mLST8 (mammalian lethal with sec-13 or G $\beta$ L), DEPTOR (DEP domain-containing mTOR-interacting protein Tti1/Tel2 complex), PRAS40 (proline-rich Akt substrate of 40 kDa), and Raptor (regulatory-associated protein of mammalian target of rapamycin). mTORC2 is composed of mTOR, mLST8, DEPTOR, the Tti1/Tel2 complex, Rictor (rapamycin-insensitive companion of mTOR), mSin1 (mammalian stress-activated MAP kinase-interacting protein 1 or MAPKAP1), and protor1/2 (protein observed with Rictor 1 and 2) [17-20]. The configuration of each of these two complexes is conserved from yeast to mammals [21]. mTORC1 is sensitive to inhibition by rapamycin and plays essential roles in the regulation of mRNA translation and autophagy. Cellular energy and nutrient status regulate it directly, whereas mTORC2, which is not rapamycin sensitive, functions mainly as an important regulator of the cellular actin cytoskeleton [22,23].

Rheb (Ras homolog enriched in the brain) is a GTPase that in its GTP-bound form directly binds to and activates mTOR [24-27]. Rheb activity is inhibited by the heterodimer complex of tuberous sclerosis proteins 1 and 2 (TSC1 and TSC2) [28-33]. TSC1/2 mediates for mTORC1 many of the upstream signals from growth factors, such as insulin and IGF-1, which stimulate the PI3K and Ras pathways. The effector kinases of these pathways, including Akt (or protein kinase B or PKB), extracellular-signal-regulated kinase 1/2 (ERK1/2), and S6K1, directly phosphorylate and inactivate the TSC1/TSC2 complex, leading to activation of mTORC1 [27,30,34-40]. Rheb also can transmit upstream signals from the p38 $\beta$ -PRAK pathway, which is activated upon glucose starvation [41]. Finally, as a core component of mTORC2, mTOR functions as a tyrosine-protein kinase that promotes activation of the insulin receptor and IGF-1 receptors [42]. These interactions illustrate the tightly interconnected signaling between mTOR and insulin.

The mTOR pathway integrates inputs from major intracellular and extracellular physiological stimuli (growth factors, stress, energy balance, oxygen, amino acids) and controls many major downstream processes, including macromolecule synthesis, autophagy, cell cycle, growth, and metabolism [15,16,43]. For example, the canonical Wnt pathway, AMPK, some proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), and the hypoxia-inducible proteins REDD1 and REDD2 modulate mTORC1 activity via TSC1/2 [44-49]. In addition to phosphorylating TSC1/2, AMPK phosphorylates Raptor, leading to the allosteric inhibition of mTOR [50]. mTORC1 activity is further regulated by lipid-derived signaling molecules (phosphatidic acid) [51], the redox status of the cell [52], and amino acids, particularly leucine and arginine [53,54]. DNA damage also signals to mTORC1 through multiple mechanisms, all of which require p53-dependent transcription, induction of the expression of TSC2 and phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and AMPK activation [55-57].

Downstream signaling of mTORC1 controls autophagy and energy metabolism, including the glycolytic flux, lipid synthesis [58-61], and cholesterol synthesis via activation of sterol regulatory element-binding protein (SREBP) 1/2 [58,62,63]. mTORC1 also promotes anabolism in the fed state by controlling lipid metabolism in the liver through modulation of *Srebp1c* expression, a regulator of lipogenesis and lipid storage [64,65].

Under mTORC1 regulation, mitochondrial DNA content and the expression of genes involved in oxidative metabolism increase. mTORC1 exerts this effect in part by mediating the nuclear association between PPARy coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) and the transcription factor Yin-Yang 1, which positively regulate mitochondrial biogenesis and oxidative function [66].

Activation of mTOR also leads to the phosphorylation of many target proteins related to the translational machinery and ribosome biogenesis, such as p70 ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP) [43,67-72]. Regulation of protein metabolism also is a much-recognized function of mTOR. Amino acid activation of mTORC1 promotes protein synthesis via activation of S6K and/or inhibition of 4E-BP, whereas inactivation of mTORC1 promotes degradation of damaged proteins and intracellular organelles via autophagy [73,74].

mTORC2 functions mainly as an important regulator of the actin cytoskeleton through its stimulation of F-actin stress fibers, paxillin, RhoA, Rac1, Cdc42, and protein kinase C (PKC)  $\alpha$  [19]. mTORC2 phosphorylates Akt [75,76] and thus affects metabolism and cell survival. mTORC2 also directly activates SGK1, a kinase controlling ion transport and growth [77]. Both Akt and SGK1 phosphorylate FoxO1/3a [78-80].

Because of its role as an amino acid sensor, the TOR pathway has been proposed as a mediator of CR. High activity of mTORC1 is a major driving force of aging, whereas suppression of mTOR is tied to many of the benefits associated with CR, including lifespan extension [81-84], as has been demonstrated in yeast [81,85], worms [82], and flies [83]. Rapamycin treatment slightly extends lifespan in flies subjected to CR [86]. In yeast, CR does not further extend the lifespan in the absence of *TOR1*, one of the two *TOR* genes in yeast, suggesting that TOR inhibition and CR promote lifespan via a common mechanism [81]. Similarly, in *C. elegans*, using RNA interference against TOR or autophagy genes in eat-2 mutant worms, which have impaired feeding behavior and are used as a genetic model for CR, does not extend the lifespan [87,88]. Furthermore, inhibition of one of the principal targets of TOR signaling, S6K, extends the lifespan of

*eat-2* *C. elegans* [89]. Of note, mTOR activation in the rat's brain results in reduced food intake by promoting expression of the orexigenic neuropeptide Y and agouti-related peptide in the hypothalamus [90,91]. These data suggest that CR and TOR inhibition promote lifespan via overlapping pathways.

## 2.2. AMPK

CR decreases energy input, which leads to activation of a signaling cascade to generate fuel and increase longevity. Decreased glucose intake reduces carbon flow through the glycolytic pathway and slows the conversion of ADP to ATP. As a principal cellular energy sensor, AMPK monitors the AMP:ATP and ADP:ATP ratios. Functionally, AMPK is a serine/threonine kinase comprising one catalytic subunit,  $\alpha$ , and two regulatory subunits,  $\beta$  and  $\gamma$ . Each of the subunits occurs as different isoforms ( $\alpha 1$ ,  $\alpha 2$ ,  $\beta 1$ ,  $\beta 2$ ,  $\gamma 1$ ,  $\gamma 2$ ,  $\gamma 3$ ) allowing for different versions of AMPK in various tissues [92,93]. From nematodes to humans, the kinase activity of AMPK is rapidly increased by the binding of AMP or ADP to the AMPK $\gamma$  subunit [94]. This binding promotes allosteric activation and phosphorylation of AMPK by the upstream AMPK kinase and thus also inhibits its dephosphorylation [95]. An alternative activating pathway triggers AMPK in response to increases in cellular  $\text{Ca}^{2+}$  and involves the  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase  $\beta$  [96]. Once activated, AMPK promotes ATP preservation by repressing energy-consuming biosynthetic pathways while enhancing the expression or activity of proteins involved in catabolism. This process results in the mobilization of deposited energy to restore the ATP supply[97]. Several downstream factors including CREB-regulated transcriptional coactivator-2 (CRTC2) [98], TBC1D1/AS160 [99,100], PGC-1 $\alpha$  [101], and histone deacetylase (HDAC) 5 [102] mediate the impact of AMPK on metabolism. Functionally, AMPK phosphorylates acetyl-CoA carboxylase 1 (ACC1) and ACC2 [103,104], SREBP1c [105], glycerol phosphate acyl-transferase, [106] and HMG-CoA reductase [107], resulting in inhibition of FA, cholesterol, and TG synthesis while activating FA uptake and  $\beta$ -oxidation. Additionally, AMPK prevents protein biosynthesis by inhibiting mTOR and TIF-IA/RRN3, a transcription factor for RNA polymerase I that is responsible for ribosomal RNA synthesis [108]. AMPK also influences glucose metabolism by stimulating both nutrient-induced insulin secretion from pancreatic  $\beta$ -cells [109] and glucose uptake by phosphorylating Rab-GTPase-activating protein TBC1D1, which ultimately induces fusion of glucose transporter (GLUT)4 vesicles with the plasma membrane in skeletal muscle [110]. AMPK stimulates glycolysis by phosphorylation of 6-phosphofructo-2-kinase (fructose-2,6-bisphosphatase 2) [111] and, in parallel, inhibits glycogen synthesis through phosphorylation of glycogen synthase [112]. In the liver, AMPK inhibits gluconeogenesis by inhibiting transcription factors including hepatocyte nuclear factor 4 and CRTC2 [113-115]. AMPK also affects the energy balance by regulating circadian metabolic activities and promoting feeding through its action in the hypothalamus [116,117]. It promotes mitochondrial biogenesis via PGC-1 $\alpha$  [101] and activates antioxidant defenses. AMPK plays a major role in metabolism but is also involved in inflammation, cell growth, autophagy, and apoptosis [118]. Therefore, reducing AMPK signaling exerts a cytostatic and tumor-suppressing effect [119,120].

In *C. elegans*, the lifespan extension effect of CR depends on AMPK [121,122]. Similarly, in *Drosophila*, pathways mediating increased lifespan include AMPK activation [123]. In addition, tissue-specific overexpression of AMPK in muscle and body fat extends lifespan in *Drosophila*, whereas AMPK RNA interference shortens the lifespan [124].

## 2.3. Insulin signaling

Increased glucose levels in serum after food intake promote insulin secretion from pancreatic  $\beta$ -cells, which in turn activates insulin receptors on the surface of target cells. The tyrosine kinase activity of the insulin receptor triggers a signaling cascade starting with the activation of insulin receptor substrates (IRS 1–4) followed by phosphorylation of PI3K, which is responsible for metabolic actions including PDK1 and Akt activation. Akt occurs in three isoforms (1-3) with Akt2 being essential for glucose homeostasis, whereas Akt1 is important for growth and Akt3 for brain development [125]. The Akt-driven inhibition of AS160 phosphorylation induces GLUT4 to translocate to the cell membrane, which promotes glucose transport into the intracellular compartment. Akt also phosphorylates and deactivates glycogen synthase (GS) kinase 3 (GSK3) which stimulates GS and glycogen production. In parallel, it disrupts the CBP/Torc2/CREB complex and consequently inhibits gluconeogenesis. Moreover, Akt activates mTOR, which facilitates protein synthesis, whereas mTORC2 is a critical regulator of Akt [126]. Another Akt regulator, tumor suppressor PTEN, previously mentioned in the context of mTOR, prevents Akt activation, and reduces mTOR activity. In line with the above, inhibition of IGF-1/PI3K/Akt signaling participates in the anti-cancer and DNA-repair activity of CR [127-129]. Further, Akt activation leads to inhibitory phosphorylation of FOXO1 resulting in its nuclear exclusion [130]. Therefore, Akt functions at the crossroads of several pathways responding to CR.

Among other pathways affected by insulin signaling, the most important include mitogen-activated protein kinase (MAPK), which regulates growth; SREBP-1, which promotes lipid and cholesterol synthesis; and the family of FoxO transcriptional regulators, which regulate metabolism and autophagy. In general, insulin signals an abundance of fuels and thus promotes storage and prevents further production of energy molecules [131-134].

The beneficial effects of CR have been associated with changes in metabolism, modification of the activity of the insulin/IGF-1 pathways, reduction in fat mass, and increased stress resistance because of FoxO activation [135-137]. Insulin release and insulin action seem to play a major role in the control of aging. Modulation of longevity by insulin signaling is supported by the extended lifespan associated with mutations in the insulin/IRS/growth hormone (GH)/IGF-1/FOXO signaling pathways in humans, mice, *C. elegans*, and *Drosophila* [138-144]. Female, but not male, *Igf1r<sup>+/−</sup>* mice live on average 33% longer than their wild-type counterparts [142], and fat-specific deletion of *Igf1r* results in an 18% increased longevity in both sexes [138]. Accordingly, GH receptor/binding protein knockout (GHR/BP-KO) mice are characterized by markedly extended lifespan and show severely reduced plasma IGF-1 and insulin levels, as well as low glucose levels [145,146]. Transgenic Klotho mice, which also have an increased lifespan, are insulin resistant. These findings collectively suggest that aging can be delayed by reducing insulin signaling [147]. It has even been hypothesized that insulin resistance is a physiological protective mechanism against aging and age-related disorders [148].

## 2.4. Sirtuins

A CR-related decrease in energy levels leads to activation of several signaling cascades. Decreased glucose intake reduces the flow of carbon through the glycolytic pathway and regeneration of ATP from ADP, which eventually alters the NAD<sup>+</sup>:NADH ratio. This shift activates SIRTs, which serve as both energy sensors and transcriptional effectors by acting as NAD<sup>+</sup>-dependent HDACs. In addition to CR and fasting, exercise activates SIRTs [149,150], which are remarkably conserved and can even be found in archaeabacteria [151]. Originally categorized as class III HDACs, SIRTs are involved in the proper functioning of nucleic acids including DNA repair, homologous recombination, and DNA deacetylation, and promote transcriptional gene silencing [152,153].

The seven subtypes of SIRTs (SIRT1–7) in mice and humans vary in their cellular distribution and function. SIRT1–SIRT3, SIRT5, and SIRT6 catalyze deacetylation, whereas SIRT4 and SIRT6 have ADP-ribosylation capacity. In addition to histones, SIRT substrates include several transcriptional regulators, such as the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), p53, FOXO, and PGC-1α, but also enzymes, including acetyl coenzyme A (CoA) synthetase 2 (AceCS2), long-chain acyl-coenzyme A dehydrogenase (LCAD), HMGCS2, superoxide dismutase 2, and structural proteins, such as α-tubulin [154-158]. Therefore, SIRTs influence a wide range of cellular processes including circadian clocks, cell cycle, mitochondrial biogenesis, and energy homeostasis, and on the whole-body level regulate aging, apoptosis, inflammation, and stress resistance [159,160].

SIRT1 is the most thoroughly investigated mammalian SIRT and is closely involved in metabolism. Studies in *S. cerevisiae* have shown that an extra copy of the *Sir2* gene, a yeast homolog of mammalian *Sirt1*, increases lifespan in a dose-dependent manner [161,162] and deletion of this gene shortens lifespan [161]. In yeast and *Drosophila*, lack of *Sir2* and *dSir2*, respectively, prevents CR-associated life extension [163-165]. *SIR2*, a yeast analog of *Sirt1*, assists in DNA repair and regulates genes that change expression with age [166].

The most important metabolic regulator affected by SIRT1 is PGC-1α, which is activated by SIRT1-mediated deacetylation[167,168]. Deacetylated PGC-1α increases hepatic gluconeogenic activity [167], whereas in muscle and BAT, PGC-1α enhances mitochondrial activity. The activity of PGC-1α translates into increased exercise capacity and thermogenesis, leading to protection against the onset of obesity and associated metabolic dysfunction [169]. Deacetylation of PGC-1α by SIRT1 depends on cellular NAD<sup>+</sup>levels, so the status of cellular energy affects PGC-1α activity, which adapts cellular energy production through mitochondrial biogenesis and function. Furthermore, among the SIRT1 substrates are factors that control cell proliferation and apoptosis, including the tumor suppressor protein p53 [170]. Overexpression of SIRT1 hinders p53 transcriptional activity and p53-dependent apoptosis triggered by DNA damage and oxidative stress, whereas overexpression of dominant-negative SIRT1 can enhance cellular stress responses [170,171].

SIRTs also regulate the activity of the FOXO family of transcription factors [172,173], which affects cell differentiation, transformation, and metabolism as well as play an important role in CR and longevity regulation [174-176]. SIRT1-mediated deacetylation FOXO1 affects its shuttling between the nucleus and cytoplasm, influencing the expression of FOXO1 target genes and promoting gluconeogenesis and glucose release from hepatocytes [177]. Deacetylation of FOXO3a by SIRT1 increases its translocation from the cytoplasm to the nucleus [178] and its DNA-binding activity. In the nucleus, SIRT1 and FOXO3a form a complex that induces cell-cycle arrest and resistance to oxidative stress, also inhibiting the ability of FOXO3a to induce apoptosis [177]. SIRT1 directly suppresses expression of UCP2, leading to improved coupling of mitochondrial respiration and ATP synthesis, which induces insulin secretion in β-cells [179]. Confirming the role of SIRT1 in the pancreas, SIRT1<sup>−/−</sup> mice are characterized by impaired insulin secretion in response to glucose compared with wild-type littermates [179,180]. Conversely, β-cell-specific SIRT1-overexpressing mice exhibit improved glucose tolerance and an enhanced glucose-stimulated insulin secretion [180]. In contrast, SIRT4 has an

inhibitory effect on amino acid-stimulated insulin secretion. It represses the activity of glutamate dehydrogenase, reducing  $\alpha$ -ketoglutarate production and ATP generation, which are known to activate insulin secretion in pancreatic  $\beta$ -cells [181]. To avoid amino acid-stimulated insulin secretion during CR, when amino acid turnover increases, CR decreases SIRT4 activity, which is opposite to the induction of SIRT1 activity during CR [181]. Considering that NAD<sup>+</sup> controls the activities of both SIRT4 and SIRT1, their opposing effects on insulin secretion are surprising, and the full implications remain to be understood.

The role of other SIRT family members has been less investigated, and their function thus is less well known. SIRT2 is localized mainly in the cytoplasm, where it deacetylates tubulin filaments, HOXA10, and FOXO [182-185]. It takes part in multiple processes including cell cycle regulation [186], lifespan extension [161,187], and glucose and lipid metabolism [155,188]. SIRT3 plays an important role in mitochondria maintenance by acting as a deacetylase for a number of mitochondrial matrix proteins [189,190]. During a prolonged fast, SIRT3 activates FA breakdown by deacetylation of LCAD [157] and stimulates the production of ketone bodies by activating HMGCS2 [156]. Of note, SIRT3 is genetically linked to lifespan in the elderly [191].

SIRT4 has ADP-ribosylation activity and in addition to blocking amino acid-induced insulin secretion [181], it regulates FA oxidation in hepatocytes and myocytes [192]. Both SIRT4 and SIRT5 show mitochondrial localization [181,193]. SIRT6 resides in the nucleus and is involved in genomic DNA stability and promotes the repair of DNA double-strand breaks [194]. SIRT6-deficient mice present a shortened lifespan and a degenerative aging-like phenotype [195]. In contrast, transgenic male mice overexpressing SIRT6 display lower serum levels of IGF-1, higher levels of IGF-1-binding protein, and modified phosphorylation patterns of different components of the IGF-1 signaling pathway, possibly contributing to about a 15% increase in lifespan when compared to wild-type animals [196].

SIRT1 and SIRT6 are both connected with CR-triggered extension of ovarian lifespan, mediated by inhibition of the transition from primordial to developing follicles and by a delay in the growth phase of follicles to preserve the supply of germ cells [197]. SIRT7 is associated with nucleoli and is implicated in activation of transcription by RNA polymerase I [198] as well as repair of double-strand breaks by non-homologous end-joining [199]. SIRT7 knockout mice display features of premature aging [199]. SIRT1, SIRT6, and SIRT7 facilitate DNA repair, and this repair slows the aging process. During CR, except for SIRT4, the expression and activity of SIRTs are increased in many tissues, including adipose and brain [200-202], heart [203,204], and liver [205]. SIRT1 mediates a broad array of physiological effects of CR. The overexpression of SIRT in worms and flies increases their lifespan [164,165], and accordingly, mutants of SIRT do not show lifespan extension by CR [163,206]. Moreover, transgenic mice overexpressing SIRT1 show phenotypes similar to those of CR mice [207]. The previously mentioned role of Sir2 in lifespan is particularly critical in the context of CR.

Resveratrol, a polyphenolic compound present in, for example, red grapes and wine, stimulates SIRT1 expression, resulting in extended lifespan and health span in treated animals [208]. SIRT1 activation by resveratrol mimics CR and delays aging in a wide range of organisms, from *S. cerevisiae* [209] to *C. elegans* to *Drosophila* [210] and mice [211]. Resveratrol is considered one of the mimetics not only of CR but also of exercise [208,212]. In mice, resveratrol inhibits gene expression profiles associated with muscle aging and age-related cardiac dysfunction [213]. The compound protects mice against diet-induced obesity and the associated insulin resistance through enhanced mitochondrial function mediated by PGC-1 $\alpha$  [169].

### 3. Major outcomes of CR

#### 3.1. Oxidative stress reduction

ROS are generated as a byproduct of cellular respiration, contributing to the accumulation of oxidative damage and the formation of a range of oxidation products of different macromolecules including lipids, proteins, and nucleic acids [214]. A small amount of ROS is normally beneficial because it plays an important role in cellular processes such as cell cycle progression, regulation of signaling pathways in response to intra- and extracellular stimuli, and inflammation [215]. However, high uncontrolled levels of ROS are detrimental.

During oxidative stress, the sustained production of ROS and reactive nitrogen species leads to a perturbed equilibrium between pro-oxidants and antioxidants. Consequently, macromolecules, organelles, and cells are altered, and if much damage accumulates, necrotic or apoptotic cell death occurs. The “free radical theory” of aging [216] proposes that the generation of oxidative stress is a major factor contributing to the onset of the aging process and age-related diseases. Therefore, the mammalian life span is reduced in relation to the mitochondrial production of oxidizing free radicals [215]. CR likely exerts its diverse benefits through reducing ROS levels and suppressing age-related oxidative stress while

supporting the antioxidant defense system [217-219]. CR diminishes the impact of ROS through three processes: reduction of oxygen free-radical generation by slowing metabolism, acceleration of ROS neutralization, and stimulation of the repair of ROS-damaged molecules [220-224].

### 3.2. Mitochondrial function

One of the several theories tightly connected with the effects of ROS is the “mitochondrial theory of aging”, which proposes that mitochondria are the critical component in the aging process. In fact, mitochondrial DNA damage and dysfunction increase with aging and are associated with a vast number of pathologies. Defective mitochondria determine the turnover not only of the organelles themselves but also whole cells, resulting in the acceleration of aging [215,225,226]. Aging has been linked to a reduced capacity for oxidative phosphorylation in the muscle and heart, most likely because of a decline in mitochondrial content and/or function [227-229]. Accordingly, young individuals have higher respiratory function compared to the elderly [230-232]. Disturbed mitochondrial electron transfer increases the likelihood of electron leakage and ROS production. Consequently, components of the electron transport chain and mitochondrial DNA become damaged, leading to further increases in intracellular ROS levels and a decline in mitochondrial function. Because mitochondrial DNA is spatially close to the source of ROS production, it is thought to be particularly vulnerable to ROS-mediated lesions [216,233].

An interesting feature of CR, one associated with ROS and changes in metabolism, is mitochondria biogenesis, which is relatively high in various tissues such as in the brain, heart, liver, and particularly the BAT of mice [202,234]. It is associated with activation of the master regulator of mitochondrial biogenesis, PGC-1 $\alpha$  [235-237]. PGC-1 $\alpha$  is expressed at a high level in BAT, heart, skeletal muscle, brain, and kidney, whereas its expression is low in the liver and very low in WAT [238]. Various physiological stimuli highly induce PGC-1 $\alpha$  in different organs. It is increased in BAT by cold exposure and in skeletal muscle by exercise and decreased ATP level, whereas in the liver, it is mostly affected by CR [239]. When ectopically expressed in fat or muscle cells, PGC-1 $\alpha$  strongly increases mitochondrial biogenesis and oxidative metabolism, which correlates with an increase in mitochondrial DNA and the expression of multiple mitochondrial genes [239,240]. To prevent a mitochondrial biogenesis-associated increase in ROS levels, PGC-1 $\alpha$  also induces expression of the antioxidant genes *GPx1* and *MnSOD* [241]. One hypothesis regarding the beneficial outcomes of CR proposes is that CR preserves mitochondrial function by maintaining protein and DNA integrity through decreasing mitochondrial oxidant emission and increasing endogenous antioxidant activity [242,243]. Its impact on mitochondria biogenesis remains a matter of discussion [244,245].

In addition to affecting mitochondria biogenesis, PGC-1 $\alpha$  also influences metabolism. It mediates a fasting-induced increase in FA metabolism and downregulation of pyruvate dehydrogenase, which is part of the mitochondrial pyruvate dehydrogenase complex that catalyzes the reaction representing pyruvate entry into the tricarboxylic acid cycle. In PGC-1 $\alpha$  knockout mice, pyruvate dehydrogenase fails to adapt to CR, and the ability of the mice to endure prolonged starvation is decreased [246]. PGC-1 $\alpha$  knockout mice also show a reduced content of mitochondrial electron transport chain proteins in skeletal muscle [247,248]. The activity of PGC-1 $\alpha$  is directly regulated by the energy sensors SIRT1 and AMPK [101,167]. Functionally, the transcriptional activity of PGC-1 $\alpha$  relies on its interactions with transcriptional factors for controlling FA metabolism. Of note, all three PPAR isotypes are subject to transcriptional coactivation by PGC-1 $\alpha$  and are major executors of PGC-1 $\alpha$ -induced regulation [238,249-251].

### 3.3. Reduction of inflammation

The “inflammation hypothesis of aging” posits a molecular mechanism of aging based on inflammation. Inflammation is a complex defense reaction to insult and both physiological and nonphysiological stress, induced by agents such as chemicals, drugs, or microbial entities. Inflammation responses are activated by well-coordinated, sequential events controlled by humoral and cellular reactions. Elevated tissue levels of TNF $\alpha$ , IL-1, and IL-6, among other proinflammatory mediators, have been observed in experimental animal models of inflammation. With aging, inflammatory responses may be overactive or even cause damage, resulting in pathological conditions [14].

During aging, a shift occurs in the ratio of naive to memory T cells, with associated changes in the cytokine profile in favor of inflammatory cytokines such as TNF $\alpha$ , IL-1, IL-6, INF $\gamma$ , and transforming growth factor  $\beta$  [252-255]. There is also a progressively higher dysregulation of immune cells and proinflammatory responses. Macrophages from old mice produce more prostaglandin E2 than those from young mice because of higher COX-2 activity [256]. One major causative factor in tissue inflammation is the uncontrolled overproduction ROS/reactive nitrogen species. The transcriptional regulator NF- $\kappa$ B is an inflammatory reaction factor of major importance that is extremely sensitive to oxidants [257-262]. Enhanced IL-6 production by activated NF- $\kappa$ B has been implicated in many pathophysiological dysfunctions of aging ranging, from Alzheimer’s disease to atherosclerosis [263]. CR exhibits a broad and effective anti-inflammatory effect. It blunts age-

triggered increases in COX-2 levels and activity through the modulation of NF- $\kappa$ B and I $\kappa$ B, in which COX-2-derived ROS generation decreases. Also, the production of iNOS, IL- $\beta$ , IL-6, TNF $\alpha$ , and prostanooids such as TXA 2, prostacyclin 2, and prostaglandin E2 is suppressed [14,219]. The prevention of the age-related decline triggered by CR correlates with dampening the reduction of PPAR expression and activity seen during aging. Therefore, under CR conditions, higher PPAR expression may play a role in the suppression of the age-induced increase in inflammation [264]. PPARs are implicated in inflammation at the transcriptional level by interfering with proinflammatory mediators such as NF- $\kappa$ B, STAT-1, and activating protein-1, leading to downregulation of the gene targets of these factors [265-268]. In this way, PPAR $\alpha$  and PPAR $\gamma$  inhibit the expression of inflammatory genes, such as COX-2, iNOS, cytokines, metalloproteases, and acute-phase proteins [266,269]. Inflammatory eicosanoids serve as ligands for PPARs, and levels of these signaling molecules, including prostaglandins and leukotrienes, increase with age [270].

### 3.3.1. Metabolic adaptation

The shortage of energy during CR leads to a sequence of metabolic changes. Following depletion of dietary glucose, glycogen is mobilized as an energy supply and, upon prolonged CR, hepatic metabolism shifts to gluconeogenesis to prevent hypoglycemia. Further energy restriction, carbohydrate depletion triggers a shift to fat recruitment and ketone body production.

### 3.3.2. Physical exercise

Exercise, like CR, yields multiple beneficial effects. Research outcomes point towards the effectiveness of regular moderate exercise in preventing and delaying several metabolic disorders, chronic diseases, and premature death. Increased physical activity reduces mortality risk from many age-related diseases, including cardiovascular disease, stroke, T2D, certain cancers, hypertension, obesity, depression, and osteoporosis [271-275]. However, in rodents, exercise improves the mean lifespan without increasing maximum longevity [276,277]. Similarly, high physical activity fails to extend maximum lifespan in humans [278]. Compared to exercise, long-term CR in humans improves several biomarkers related to aging [279,280]. Accordingly, exercise has been deemed as unable to fully mimic the beneficial hormonal and/or metabolic changes associated with CR [281]. Therefore, despite a mutual influence with CR on similar molecular pathways and providing multiple advantages, physical activity has been recognized as yielding inferior benefits compared to CR.

## 3.4. Longevity and aging

Both genetic and environmental factors control the progression of aging. Aging is associated with immunosenescence, increased oxidative stress, decreased hormonal secretion, changes in metabolic rate, mitochondrial function, insulin resistance, and dysregulated lipid metabolism [282-284]. Preservation of insulin sensitivity by reducing levels of blood glucose and insulin without compromising glucose fuel may prevent age-related metabolic phenotype [140]. Glucose metabolism maintenance is a key feature of the anti-aging actions of CR [3]. In fact, genes connected with the insulin/IGF-1 signaling pathway have been proposed as longevity candidate markers [143,144,285]. Paradoxically, impaired insulin signaling through the insulin receptor or its substrates increases rather than decreases lifespan in a number of mouse models [138,143,144].

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