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Summary

Aging is the leading risk factor for neoplastic disease. However, the mechanisms underlying this age-associated increase in cancer are poorly understood.

It is increasingly appreciated that cancer cells and their precursors are not absolute entities, but they are deeply embedded in the tissue context where they reside, which can profoundly affect their phenotype. However, addressing the impact of aging on the tissue microenvironment and its possible relevance to the pathogenesis of cancer has revealed a difficult task to pursue. This is largely due to the fact that aging can potentially alter both initiated/pre-neoplastic cells and the surrounding “healthy” tissue, making it particularly challenging to dissect the specific role of either effect in the origin of neoplastic disease.

Most recently, results obtained by our research group have clearly indicated that aging promotes carcinogenesis at least in part by altering the tissue context and providing a more permissive soil for the selective growth of pre-neoplastic cell populations.

While biological aging is a process that cannot be averted altogether, there are various strategies that can modulate its evolution. Among the latter, caloric restriction (CR) is considered as one of the most effective. In fact, CR delays aging and age-associated diseases, including cancer.

The aim of the studies presented in this dissertation is to probe into the mechanisms involved in the anti-neoplastic effect of CR. More specifically, the hypothesis was tested that long-term CR may delay carcinogenesis via effects exerted on the microenvironment. An orthotopic cell transplantation model was used to address this issue. Pre-neoplastic hepatocytes expressing the marker protein

dipeptidyl-peptidase type 4 ($DPPIV^+$) were transplanted into host animals lacking the enzyme ($DPPIV^-$). Recipient groups were fed either ad libitum or a 30% CR diet. The transplantation model was selected because it provides two advantages: (i) it allows to follow the fate of transplanted cells ($DPPIV^+$) in the recipient liver ($DPPIV^-$); and (ii) it enables to discern the effects of CR mediated by the tissue microenvironment from those exerted directly on altered/pre-neoplastic cells.

Our results showed that pre-neoplastic transplanted cells grew more in the liver of animals fed *ad libitum* compared to long-term CR animals, suggesting that long term CR exerts a modulatory effect on the emergence of the neoplastic-prone tissue microenvironment associated with aging. Animals fed long-term CR also showed a reduced incidence of “spontaneous” endogenous nodules.

Possible molecular/biochemical mechanisms mediating the modulatory effect of CR on the tissue microenvironment were considered. Cellular senescence seems not to be the key mediator of the effects of CR on the tissue microenvironment. In fact, long-term CR did not appear to affect the senescent phenotype expressed in the liver.

On the other hand, we confirmed and extended several findings related to the effect of CR on critical metabolic, nutrient-sensing pathways. Long-term CR increased the expression of some putative mediators of CR effect, such as the nuclear sirtuin SIRT1 and the forkhead-box O transcription factor FOXO1, which regulate the expression of genes that may be important in the context of the tissue microenvironment, such as NF-kB, involved in the inflammatory response. Thus, the increased expression of SIRT1 and FOXO1 may mediate the protective effect of CR on the tissue microenvironment by reducing inflammation.

Furthermore, long-term CR may also improve the ability of the old liver to regenerate, since our results showed a reduced expression of some negative regulators of hepatic regeneration, such as HDAC1 and C/EBPβ, in the liver of animal fed long-term CR.

In the last section of this thesis, we performed a metabolomics analysis of phenotypic changes associated with long-term exposure to CR using a GC-MS-based method. Our results evidenced that CR animals underwent a metabolic reprogramming, shifting metabolism toward gluconeogenesis and lipid oxidation. Moreover, some metabolites that have been suggested to play a role in fueling the carcinogenic process were downregulated in CR-fed animals.

These results suggest that the delaying effect of CR on the carcinogenic process is mediated at least in part through effects on the tissue microenvironment. The overall effect translates in a delayed emergence of the age-associated, neoplastic-prone tissue landscape. Our findings support the notion that strategies aimed at delaying biological aging, such as CR, are also effective in decreasing the risk of neoplastic disease.

1. Introduction

1.1 Aging

Aging is the leading risk factor for many among the major chronic diseases and it coincides with an overall decline of physical and mental performance [1,2,3]. Many mechanisms have been proposed to explain the aging process, including decline in stem cell number and/or function, chronic inflammation, oxidative stress, cellular senescence and genetic drift.

Tissue-level changes in cell number and composition are hallmarks of age-associated tissue degeneration and the loss of tissue during aging has been attributed to stem cell depletion [3]. In many organs of mature vertebrates, resident stem cells participate in tissue maintenance and regeneration after injury. Although stem cells have characteristics that may protect them from age-associated insults, data indicate that they deteriorate with aging. One of the possible explanation to this phenomenon is the influence of the local and systemic environment on stem cell function, as shown in some studies in which young stem cells were transplanted into an old donor. By exposing young cells to an aged milieu, they exhibited a functional decline that resembled accelerated aging. This means that the microenvironment may be responsible at least in part of the age associated-decline in stem cell functionality [4]. Furthermore, decline of stem cells with aging has been linked to the appearance of mitochondrial dysfunction which can result from depletion of NAD+. Indeed, it has been shown that the repletion of NAD+ improves muscle SC number and function in aged mice [5].

Aging is characterized by a chronic low-grade inflammation due to deregulation of the immune response and an altered redox status [6]. The reduced capability to cope with a variety of stressors is well exemplified by the word “inflammaging” that is

usually adopted to describe the pro-inflammatory status typical of older ages, provoked by a continuous antigenic load and stress [7]. It is possible that the chronic inflammation typical of the older ages is due to an accretion of adipose tissue, increasing the release of pro-inflammatory cytokines such as IL1b, TNFa and IL6 [8]. Indeed, it is now widely accepted that adipose tissue is an endocrine organ that expresses and secretes factors such as cytokines, leptin, adiponectin, complement components, plasminogen activator inhibitor-1, among others, with important endocrine functions[9].

Another hypothesis of aging postulates that the loss of functional capacity is due to the accumulation of molecular oxidative damage [10]. Reactive oxygen species (ROS) collectively describes a number of reactive molecules, such as superoxide ($O_2^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), which can be generated endogenously by natural biological processes, or generated upon exposure to external stimuli. Once produced, ROS react with lipids, proteins, and nucleic acids causing oxidative damage to these macromolecules [11,12]. The altered redox status associated with aging is partly due to a decline in the efficiency of the mitochondrial respiratory chain, leading to increased generation of ROS, which is also fueled by increased activity of the prostaglandin synthetic pathway [13].

Senescent cells have been shown to accumulate in some tissues of aged mice and primates. In particular, age-dependent accumulation of senescent cells in tissue stem and progenitor cell compartments suggests that senescence could contribute to aging by limiting the regenerative capacity of the tissue [14]. Senescent cells exert important protective roles such as secreting proteases, growth factors, cytokines and chemokines (SASP) to clear senescent cells. However, senescence is a

“double-edged” program. Indeed, unregulated inflammation induced by SASP may lead to development of cancer [15,16].

On the DNA level, damage accumulates during aging and inserts mutations into the genome causing genomic instability [3]. DNA damage has long been considered as a causal factor in aging [17]. Thousands of DNA lesions are introduced into the genome of a somatic cell each day from diverse sources like ultraviolet light (UV), ionizing radiation (IR), as well as many genotoxic chemicals. DNA can be affected in different ways, varying from single-strand breaks (SSBs) to highly toxic lesions such as adducts and double-strand breaks (DSBs) [18]. DNA damage levels may gradually increase during normal aging, as a consequence of increased genotoxic stress, or when genome maintenance is defective or suboptimal. In fact, multiple symptoms of premature aging has been observed in response to defective DNA repair in both humans and mice [19,20]. For example, telomerase knockout mice, which are incapable of preventing telomere shortening, show an age-associated phenotype, such as shortened life-span and increased incidence of malignancies [21]. A growing body of research has reported associations between age and the state of the epigenome. In particular, chronological age has been linked to changes in DNA methylation, and changes in methylation have been linked to age-associated diseases such as metabolic syndrome and cancer [22, 23]. In addition to changes in DNA methylation, altered histone acetylation patterns have been observed in aging tissues and are associated with cancer and neurodegeneration [24]. Other sources of DNA damage are nucleic acids that derive from necrotic cells. These degraded DNA fragments, known as circulating free DNA, become increasingly frequent as we age. They behave as mobile genetic elements that integrate in the chromosomal DNA of healthy cells in its own host, thereby contributing to genome instability and possibly causing age-associated functional decline and age-related diseases [25].

1.1.1 Aging and cancer

The incidence of cancer exponentially increases with age, and aging represents the single biggest risk factor for most cancers. However, the reasons for this aging associated increase in cancer are poorly understood.

According to a widely held view, cancer is largely driven by genome dysfunction. In line with such interpretation, a progressive decline in genetic integrity of tissue stem cells is thought to represent a major risk factor in cancer formation during aging [26, 27]. In fact, the most entertained explanation which attempts to account for the relationship between cancer and aging stems from the “multi-hit” hypothesis, which states that cancer occurs more frequently as we age because cells need time to accumulate sufficient genetic mutations [28, 29]. Indeed, cancer and aging are both fuelled by accumulation of cellular damage.

An alternative or complimentary hypothesis to explain the link between cancer and aging related to the role of the immune system. Immune surveillance is the mechanism by which the immune system can potentially target and destroy developing malignancies. Aging is characterized by a substantial diminution in immune responsiveness, from hematopoietic stem cells to mature lymphocytes in secondary lymphoid organs[30]. Thus, one of the explanation to the relationship between aging and cancer could be found in the weakened immune response. Recent works have shown that cytotoxic T-cells have a central role in immune-mediated control of cancer [31,32,33]. T-cells are able to recognize and clear cancer cells following T-cell receptor-mediated recognition of tumor-derived peptides bound to MHC proteins[32]. Cellular senescence program also plays a crucial role in the induction of immune response against pre-cancerous cells [34,35]. In this work from Zender’s research group, oncogenic Nras was delivered into mouse liver inducing

the senescent phenotype. Nras-expressing hepatocytes were shown to express a myriad of cytokines and chemokines leading to the attraction of immune cells that, in turn, mediated the clearance of pre-malignant senescent cells. The clearance of pre-malignant Nras expressing hepatocytes was abrogated in severe combined immunodeficient mice (SCID) and CD4 knockout mice. In addition to this, the impaired immune-surveillance of pre-cancerous senescent cells resulted in development of hepatocellular carcinoma (HCC) [34,35].

As already mentioned, aging is characterized by a chronic low-grade inflammation, which in turn has been shown to predispose to different forms of cancer. Two pathways have been proposed to explain the link between inflammation and cancer. According to the intrinsic pathway, genetic events causing neoplasia initiate the expression of inflammation-related programs that guide the construction of an inflammatory microenvironment. On the other hand, the extrinsic pathway states that inflammatory conditions facilitate cancer development [36]. In the panoply of molecules involved in cancer-related inflammation, key endogenous factors can be identified. These include transcription factors such as NF-kB and STAT3, and the major inflammatory cytokines, such as IL-6, IL-1β, IL-23 and TNF-α. NF-kB is a key coordinator of the innate immunity and inflammation. NF-kB is activated in response to microbe and tissue damage via toll-like-receptor (TLR), and by inflammatory cytokines. In addition to this, NF-kB has been shown to be activated following cell-autonomous genetic alterations in cancer cells. NF-kB induces the expression of inflammatory cytokines, adhesion molecules, and angiogenic factors. Moreover, by inducing antiapoptotic genes such as Bcl2, it promotes survival in tumor cells and in epithelial cells targeted by carcinogens. NF-kB has been shown to be involved in tumor initiation and progression where cancer-related inflammation occurs, such as gastrointestinal tract and liver [36,37]. Along with NF-kB, STAT3 is a point of

convergence for numerous oncogenic signaling pathways. STAT3 is a major controller of cell proliferation and survival, regulating the expression of c-Myc, Cyclin D and Bcl2. A major effector molecule of NF-kB activation and also linked to STAT3 is IL-6, a cytokine with growth-promoting and antiapoptotic activity. IL-6 is a critical tumor promoter. In fact, it protects normal and premalignant cells from apoptosis and promotes the proliferation of tumor-initiating cells. IL-6 has been shown to be involved in liver cancer. Indeed, it promotes liver inflammation, injury, compensatory cell proliferation and carcinogenesis [38].

While the role of genetic alterations in the pathogenesis of cancer is undisputable, it is also increasingly appreciated that cancer cells and their precursor are not absolute entities, but are dependent on their environment in order to express their phenotype. The influence of the tissue microenvironment on cell behavior had been already proposed in 1890 by Paget in the “seed and soil theory”. According to this theory, cells, be they normal or fully neoplastic, are affected by the environment where they reside in order to express their potential phenotypical properties [39]. Later, the classical studies on teratocarcinoma cells clearly exemplified the profound influence that the microenvironment could exert on the fate of potentially neoplastic cells. In fact, when placed in the mouse blastocysts, teratocarcinoma cells were shown to normally integrate in the embryonic microenvironment and to deliver a normal and tumor-free progeny [40]. In another example, Rous sarcoma virus, one of the most important oncogenic viruses, was shown to be unable to cause sarcomas when injected into the wings of early chicken embryos, suggesting that the embryonic microenvironment could override the ability of even potent oncogenes to cause malignant transformation [41]. A transplantation system, developed by our research group several years ago, has provided direct and unequivocal evidence supporting the role of the microenvironment in the

carcinogenic process [42]. Altered hepatocytes isolated from hepatic nodules were transplanted into the liver of syngenic animals pre-treated with restrorsine, an agent that causes a long lasting block of resident hepatocyte cell cycle. As a result, transplanted nodular cells could grow selectively, forming hepatic nodules in the host retrorsine-treated liver and rapidly progressing to HCC. The same nodular cells transplanted into the liver of untreated recipients failed to form hepatic nodules. This result suggests that the growth-inhibitory environment induced by retrorsine provides the driving force for the growth of transplanted altered cells setting the stage for cancer progression [42].

Aging, which is the strongest risk factor for cancer disease, has been shown to be associated with changes in the tissue microenvironment. Several years ago, McCullogh et al pointed out that the aged tissue microenvironment has a crucial role in the relationship between aging and cancer [43]. Taking advantage of a transplantation system that allowed to address directly the effect of age on the ability of neoplastic cells to form tumors, they transplanted neoplastically transformed rat liver epithelial cell lines into the livers of both old and young rats. The same cells did not form tumors or were weakly tumorigenic when transplanted into young livers, while their tumorigenicity increased in livers of increasingly aged rats. This work evidenced that aging is accompanied by progressive alterations in the liver microenvironment, which in turn provide a less suppressive microenvironment for the expression of the tumorigenic phenotype [43]. Recently, our research group confirmed this possibility using a similar approach [44]. Pre-neoplastic hepatocytes freshly isolated from chemically-induced liver nodules were orthotopically injected into either young or old syngeneic rats, resulting in increased growth of pre-neoplastic transplanted cells in old recipients while very limited growth was observed in the young recipients at 3, 8 and 12 months after transplantation [44].

The result of the experiment is clear: the microenvironment of aged liver provides a promoting soil for the seeded pre-neoplastic hepatocytes. Thus, it is reasonable that the age-associated changes in the tissue microenvironment may also favor the emergence of rare pre-existing altered cells that may reside in such altered milieu, giving rise to cancer.

1.2 Caloric restriction

Interventions that slow down the aging process can delay or prevent multiple chronic diseases and improve the quality of life in older ages [1].

Caloric restriction (CR) is the most studied and reproducible non-genetic intervention known to extend healthspan and/or lifespan in organisms ranging from unicellular yeast to monkeys [1]. CR is a dietary intervention whereby caloric intake is reduced but malnutrition is avoided. CR applied at 10-30% reduced caloric intake extends lifespan in rodents in an inverse linear manner. The lower the intake of calories, the greater the extension in lifespan [45]. There is a limit of course, as the extension of life does not continue indefinitely as restriction is increased. The maximum effect seems to come about with 55-60% restriction [46]. Indeed, beneficial effects are lost if animals approach malnutrition.

Initiating CR later in life has significant effects on lifespan as well but these are reduced compared to the effects observed when restriction is started at weaning and it may be negative if started very late [45]. The later the restriction starts, the lower the benefit relative to the same restriction in the same strain and conditions started at weaning [47]. Because there is a natural decline in intake late in life which is linked to the onset of many terminal illnesses, it is possible that late-life restriction

might serve to bring forward this terminal weight loss, thereby shortening lifespan, consistent with the rodents studies that indicate that very late onset CR brings no benefits [45].

In 1935 Crowell and McCay demonstrated that simply reducing caloric intake without causing malnutrition nearly doubled the lifespan of rats, providing an experimental model to begin to demonstrate that aging can be slowed down [48]. Since then, chronic restriction of food intake has become the most common environmental intervention to extend lifespan. Nearly half a century later, Walford and Weindurch reported that CR started at 12 months of age not only increased lifespan but also reduced the incidence of spontaneous cancer by more than 50% in rats [49]. The effect of caloric restriction on healthspan and lifespan has since been confirmed in model organisms ranging from yeast, worms, flies, rodents to primates [1]. However, some investigators propose that lifespan extension by CR is an artifact of overfeeding in laboratory animals. According to this interpretation, CR would bring back the *ad libitum* overfed animals to the "wild". Thus, typical laboratory studies, instead of comparing normal controls with caloric restricted animals, are comparing overfed animals with adequately fed ones and not surprisingly the overfed ones develop a series of pathologies and die younger [50].

1.2.1 CR in non-human primates

The Rhesus monkeys are the closest model organism to humans in which CR has been experimentally tested in a controlled environment [51]. Over the past twenty years, substantial data have been generated supporting the concept that CR improves health in Rhesus monkeys as well.

In the study conducted at the Wisconsin National Primate Research Center (WNPRC), 76 rhesus monkeys were exposed to adult-onset CR. A 2.9-fold increased risk of age-associated pathologies, such as diabetes, cancer, cardiovascular disease and brain atrophy, and 3.0-fold increased risk of death was observed in AL-fed controls compared to CR animals [52]. Animals (male and females) were adults when introduced into the study (7-14 years old), although the classic rodent CR experiment, in which lifespan was extended by about 40%, involved animals starting the diet just after weaning. However, careful studies have shown that, with gradual reduction in food intake, adult-initiated CR is almost as effective as early-onset in extending lifespan in rodents [49].

In contrast to the WNPRC, a parallel study involving 120 rhesus monkeys conducted at the National Institute on Aging (NIA) did not result a significant difference in survival between control and CR animals. The NIA-CR study began in 1987 and included both males and females of different ages. Control monkeys were fed a meal twice a day based on their age and body weight. They were considered approximately *ad libitum* since they often left few uneaten biscuits at each meal that were subsequently removed. CR monkeys received 30% less food than their matched controls. Compared to WNPRC, the NIA study investigated both the effect of early- and late-onset CR. Late-onset CR was beneficial on several parameters of metabolic health and overall function. However, no changes in survival were observed. In monkeys exposed to young-onset CR, no significant effects were noted in survival compared to AL group. The incidence of cancer was dramatically reduced in young-onset CR monkeys, while the incidence was similar between AL and late-onset CR group, indicating that an early intervention may have a more significant impact on cancer development [53].

Important details in study design could account for the discrepancies in the findings of WNPRC and NIA studies. For example, implementation of the diet was different between the two studies. The WNPRC controls were effectively fed *ad libitum* (food was always available) while NIA controls received food according to regulated portioning, as mentioned above [51,52,53]. This latter approach may provide a slight restriction, preventing obesity in the control monkeys, which may therefore experience survival benefits compared to truly AL-fed animals [54]. In line with the latter interpretation, final body weights in control groups were higher in WNPRC monkeys compare to NIA counterparts. Importantly, CR effectively decreased body weight in both studies. Furthermore, composition of the diet in the NIA study was based on natural ingredients, while the WNPRC diet was semipurified. Other notable differences were in the source of nutrients: in the NIA diet proteins were from wheat, corn, soybean while the only protein source in the WNPRC study was lactoalbumin. Also the content of carbohydrates was highly different: although both diets had 57-61% of carbohydrates, they differed substantially for the content of sucrose, which was 28,5% in the WNPRC study and only 3.9% in the one carried out at NIA. Finally, genetic origin of the monkeys was different: Indian origin in the WNPRC study, Chinese and Indian background in the NIA-based investigation [51–53].

The contrasting results in survival obtained in the two reports described above exemplify difficulties inherent to studies on dietary manipulation in long-lived animals. Thus, the relative impact of CR on lifespan may be more prominent in short-lived species than in long-lived ones such as primates [2].

1.2.2 CR in humans

In humans, there is some indication that a decrease in caloric intake of around 15% may delay mortality associated with aging [2,55]. Decades of studies have shown that the Okinawans, one of the world's longest-lived population, enjoy an 80% reduction in mortality from cardiovascular diseases and a 40% decrease in cancer mortality compared to the US population. Interestingly, older Okinawans consumed a diet corresponding to mild caloric restriction and rich in foods that may have a caloric restriction mimicking effect [56]. Recently, a 2-years randomized trial known by the eponym of CALERIE, involving non-obese men and women between 21 and 50 years, was conducted to assess CR's feasibility, safety and improvements in terms of quality of life and disease risk. The degree of CR achieved in the study (theoretically fixed at 25%, it was effectively at 19.5% during the first 6 months and at 9.1% for the remainder of the study) was safe and well tolerated, with no adverse effects on the quality of life. CR participants lost weight significantly compared to AL and they experienced improvements in some potential modulators of longevity and in cardio-metabolic risk factors, such as decrease in triglycerides and total cholesterol, increase in HDL and reduction in LDL, decrease in both systolic and diastolic blood pressure [57].

1.2.3 Anti-cancer effects of CR

CR has a long-established beneficial effect on the incidence and progression of cancer, although the mechanisms underlying such effect are poorly understood.

The relationship between cancer and metabolism has been known since 1930s, when Otto Warburg found out that cancer cells showed sustained glycolytic

metabolism. The fact that cancer cells differ metabolically from normal cells makes them extremely susceptible to nutrient deprivation. Conditions such as obesity are linked to high risk of cancer development, while diets characterized by low-fat and low-sugar consumption are associated to a low risk of cancer [58]. Due to the overlapping pathways involved in metabolism and cancer, utilization of CR may bring benefits for cancer patients [59].

The protective effect of CR on the incidence of cancer has been observed in the early 1900s. Indeed, Moreschi showed that the growth of transplanted tumors was significantly reduced in mice under CR, where the weight of transplanted tumor was lower (1.3 ± 0.2 g) compared to that of tumors transpanted in AL fed animals (7.6 ± 0.8 g) [60]. Later, other reports indicated that CR was effective in preventing the growth of both spontaneous and chemically induced tumors in rodents [58]. Indeed, CR has been shown to reduce the incidence of 7,12-dimethylbenzantracene (DMBA)-induced mammary tumors, the incidence of intestinal tumors induced by methylazoxymethanol (MAM), and to delay the formation of hepatocellular adenomas in mice treated with diethylnitrosamine (DEN) [61]. With reference to spontaneous tumors, McCay et al. demonstrated that simply reducing caloric intake caused both a near doubling of the lifespan of rats and a sizeable reduction in tumor incidence [48]. Later, Weindurch and Walford demonstrated a 40% CR lowered cancer incidence by 50% in rats [49]. The antineoplastic effect of CR has been observed also in monkeys. In both studies carried out in non-human primates, the WNPRC and NIA studies, CR has been shown to reduce markedly the incidence of neoplasia [52,53]. CR seems to affect tumor development also in humans. Indeed, a retrospective cohort study in women with history of anorexia nervosa and low BMI demonstrated a 50% reduction of breast cancer, suggesting that severe CR in humans may protect from invasive breast cancer [59].

More recently, CR is being increasingly investigated as a novel complement to standard cancer therapies and as an aid to help reduce treatment-related morbidities [59]. For example, in a murine model, CR has been shown to potentiate the effectiveness of radiation therapy in metastatic breast cancer, retarding the development of metastasis and their number, possibly through downregulation of the IIS pathway in CR treated mice, as suggested by a decreased expression of IGF-1R (IGF-1 receptor) compared to AL fed group [62]. There are a number of clinical trials investigating the benefits of associating CR with standard anti-cancer treatments. One is the CAREFOR trial at the Jefferson University, where in breast cancer patient at stage 0-I, radiation therapy has been coupled with CR, with the aim to assess tumor recurrence and metastasis. CR may be useful also in mitigating the side effects related to chemotherapy by inducing a differential stress resistance response in normal tissues but preserving toxicity in cancer cells [59]. Indeed, in mammalian cells and in mice, starvation for 48h or longer protects normal cells but not cancer cells from chemotherapy, through a shift to a protective mode called differential stress resistance, which is not induced in cancer cells [63]. Furthermore, this protocol has been applied also in humans. In fact, ten patients who fasted in combination with chemotherapy reported the feasibility of fasting in combination with anticancer drugs and reduced treatment-related side effects, suggesting that fasting before chemotherapy could be beneficial [63].

CR alone as anticancer treatment would not be feasible for several reasons. First, CR is expected to delay cancer development but it does not stop it. Moreover, not all kinds of neoplasia responds to CR. Second, 20 % CR reduces body weight at about 20% and this weight loss could be tolerated only by a small portion of cancer patients. Moreover, one of the side effects of CR is to delay wound healing, which may impose serious risks on cancer patients [63].

1.3 Mechanisms of action of CR

The biochemical/molecular pathways involved in the modulatory effect of CR on lifespan and aging process represent an area of active investigation. Several possibilities are being considered.

1.3.1 Anti-inflammatory properties of CR

There are data to indicate that CR attenuates age-associated increase in ROS, thus reducing oxidative damage to DNA, proteins and lipids [64]. NFkB is the master regulator of genes involved in the inflammatory response. CR appears to exert its anti-inflammatory effect in part by repressing NFkB and the synthesis of downstream effectors such as the major pro-inflammatory cytokines IL-6, IL-1 and TNFa [13,7]. Furthermore, CR reduces circulating levels of C-reactive protein, an inflammatory biomarker predicting cardiovascular disease, whose expression is also regulated by transcription factors such as NFkB and CCAAT/enhancer binding protein (C-EBP) β and δ [65].

1.3.2 Nutrient sensing-signaling pathways

There is increasing evidence that the nutrient-sensing signaling pathways play a role in mediating the beneficial effects of CR. It is unlikely that a single pathway mediates the effects of CR in any organism, as different organisms grow and reproduce at different rates and experience different degrees of food shortage in nature [66]. Indeed, the beneficial effects of CR may represent an adaptive evolutionary response acquired during evolution to overcome short-term

unavailability of nutrients [2]. Thus, responses of organisms to CR may differ in mechanisms and extent [66].

1.3.2.1 *Insulin/ igf-1 signaling pathway (IIS)*

The reduced activity of IIS has been implicated in CR-dependent extension of lifespan in *C. elegans* and other multicellular organisms [66]. As shown by several studies, mutations in GH and IGF-1 signaling pathways increase life span in different species. For example, GH deficient mice are not only extremely long-lived [67] but they also show a delayed occurrence of age related cancer [68]. The effect of mutations in the IIS pathway has been studied also in humans, in particular in an Ecuadorian cohort with GHR deficiency (GHRD), and consequently lack of IGF-1. This study showed the lack of incidence of cancer in GHDR subjects, while it accounted for 20% of deaths in the unaffected age-matched controls. Furthermore, *in vitro* studies showed that supplementation of mammalian cells with serum from GHDR subjects protected cells from DNA damage induced by oxidative stress, suggesting that the lack of pro-growth stimulus from IGF-1 protects cells from DNA mutations, and this may in part explain the lack of cancer incidence in GHDR subjects [69]. IGF-1 pathway is involved in several human cancers, with a potential role for IGF-1 pathway in promoting age-dependent mutations that may increase the risk of neoplastic disease [70,71]. Since one of the major effects of CR is a reduction in release of GH hormone and IGF-1 [67,70], CR may reduce cancer incidence in different species by reducing the activity of the IIS signaling pathway. However, studies in humans are in contrast with findings in rodents, where CR has been shown to decrease IGF-1 levels by 40% and protect against cancer [70]. In humans, CR alone is not able to reduce IGF-1 levels, which are reduced only when CR is

accompanied to a reduction in protein intake, suggesting that reduction of protein consumption may become an important component of anti-aging and anti-cancer interventions [72]. Indeed, protein restriction and restriction of some AA (Ser, Thr, Val) can affect lifespan through IGF1 and mTOR signaling pathways in a similar way to CR [68].

1.3.2.2 *Sirtuins as mediators of CR's effects*

Another mechanism proposed to explain some of the beneficial effects of CR relies in the ability to increase NAD+ availability through a shift toward oxidative metabolism, and consequently to activate the nuclear sirtuin SIRT1. The latter mediates some of the CR effects on processes such as mitochondrial biogenesis, metabolism, body weight and longevity [73].

The growing interest in sirtuins stems from previous studies of yeast silent information regulator 2 (Sir2) which encodes a histone deacetylase, showing that in lower organisms increased Sir2 gene dosage is sufficient to extend lifespan, and that Sir2 is a nexus between CR and longevity [74].

Sirtuins are a family of nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylases [75]. Mammals have seven sirtuins, SIRT1-7, but SIRT1 is the closest homologue to the Sir2 protein present in lower organisms [76]. SIRT1 is the nutrient-sensing deacetylase whose levels and activity increase with CR to preserve euglycemia and promote efficient energy utilization [77]. The biological roles of SIRT1 are implemented through deacetylation of several non-histone substrates involved in a wide range of cellular functions, such as metabolic and oxidative/genotoxic response. These substrates can be classified as: 1) transcriptional factors such as p53, FOXO1, FOXO3a, NFkB, c-MYC, HIF-1a, which regulate cell cycle

progression and cell survival, and reactive oxygen species generation by increasing p27kip1 and manganese superoxide dismutase (MnSOD). 2) DNA repair machinery for improving DNA damage repair. 3) Nuclear receptors such as PGC1a and PPAR for regulating respectively glucose and lipid metabolism [74],[78].

In the last decade, sirtuins have gained increasing interest in the field of antiaging research due to their remarkable effect on lifespan. In fact, in yeast, worms, and flies an extra copy of the sir2 gene increases lifespan by up to 30%, whereas its deletion, in yeast, shortens lifespan by about 50% [79,80,81]. In addition to its ability to extend lifespan, SIRT1 has been attributed a tumor-suppressor role as well. First, SIRT1 is a positive regulator of telomere length. In fact, SIRT1 attenuates telomere shortening associated with aging, contributing to protect DNA from damage [82]. Overexpression of SIRT1 reduces intestinal tumor formation in a beta-catenin driven mouse model of colon cancer [83] and transgenic mice overexpressing Sirt1 are less susceptible to spontaneous cancer and to liver cancer associated with metabolic syndrome [84]. Its tumor suppression action may be due to several mechanisms, for example the ability to improve genetic stability and to suppress the NFkB pathway that promotes inflammation, survival and metastasis of cancer cells [74]. As already mentioned, SIRT1 is involved in the metabolic shift away from glycolysis toward mitochondria [73]. This action may be important in the context of the Warburg effect, in which cancer cells show a massive up-regulation of glycolysis, thereby suggesting tumor-suppressing functions of sirtuins [85]. Despite the role of SIRT1 as tumor suppressor, some studies demonstrated that SIRT1 has a role in cancer evolution as well. For example, SIRT1 overexpression promotes thyroid cancer and lung metastasis. A possible explanation to this finding, SIRT1 seems to be involved in the genomic stability maintenance both in normal and cancer cells, but with different outcomes [74].

SIRT1 plays a central role in energy metabolism. In fact, SIRT1 activity is dependent on NAD+ availability suggesting the link to the energy state of the cell. Several metabolic processes are under SIRT1 control, for example, insulin expression, adiponectin secretion, glucose and fatty acid metabolism [86,87]. During states of prolonged CR, a key function of the liver is to produce glucose through gluconeogenesis and glycogenolysis, in order to maintain blood glucose levels within a narrow range. SIRT1, which senses the metabolic state of the cell and is induced by signals mediated by pyruvate, intercedes in these processes through deacetylation and activation of some downstream targets such as PGC-1a and FOXO1, increasing transcription of gluconeogenic enzymes and inhibition of glycolytic genes [87]. Moreover, SIRT1 has also been shown to regulate hepatic metabolism facilitating lipid oxidation during CR [77]. All these processes, i.e. the control of glucose homeostasis and fat metabolism, are decreased in the aged liver. In fact, SIRT1 expression decreases in the aged tissues including liver, causing low levels of glucose and triglycerides to sustain regeneration after partial hepatectomy. SIRT1 loss in old liver seems to be mediated by a complex formed by CCAAT/Enhancer binding protein beta (C-EBPβ) and histone deacetylase 1 (HDCA1), which binds to the SIRT1 promoter and blocks activation of SIRT1, reducing the ability of the liver to regenerate after injury [88].

The SIRT1 deacetylase is one of the best-studied putative mediators of some of the anti-aging effects of CR [66]. Indeed, CR has been shown to increase SIRT1 expression in many tissues, from brain to liver in mice [87,89]. One of the most important effects of CR mediated by SIRT1 is the extension of lifespan. Indeed, limiting glucose availability in the growing medium of the budding yeast *S. cerevisiae* leads to Sir2 activation and to extension of replicative lifespan [90], likely through shifts from fermentation to respiration resulting in increased availability of

NAD+ required for Sir2 activity [75]. Importantly, the extension was abolished when the gene Sir2 was knocked out [90]. Regarding mammals, studies carried out in mice subjected to CR, demonstrated that SIRT1 is required for CR in order to exert lifespan extension and effects on health span [86,91]. Yet, SIRT1 is an important regulator of metabolic activity during CR. In fact, Sirt1-null mice do not adapt normally to CR. They are smaller and lethargic compared to normal controls, hyperphagic and their liver mitochondria are less efficient in producing ATP [86].

Adiposity is inversely correlated to longevity and fat storage is readily affected by caloric intake. Thus, another mechanism whereby CR may extend lifespan is by reducing fat mass. Again, SIRT1 is though to mediate CR reduction of fat mass through inhibition of PPARγ necessary for adipogenesis [64]. Adipose tissue is a source of inflammatory factors. Thus, by lowering fat deposition, CR decreases inflammation, which is thought to be crucial for the aging process. SIRT1 is also involved in the inflammatory response by deacetylating NFkB, which controls transcription of inflammatory genes. Thus, SIRT1 might mediate in part the anti-inflammatory effect of CR. This is consistent with its role to regulate the availability of nutrients during restriction of energy, diverting calories away from immune system, whose activation is energetically expensive, and preserving them for survival [77].

1.3.2.3 *CR and FOXOs*

As already mentioned, CR exerts its beneficial effects through several mechanisms, and Forkhead box O (FOXO) transcription factor also appears to be involved [92]. FOXO proteins represent a subfamily of transcription factors conserved from *C. Elegans* to mammals. They act as key regulators of longevity and are downstream

targets of insulin and IGF-1 signalling pathway [93]. In mammals, there are four FOXO family members (FOXO1, FOXO3, FOXO4 and FOXO6), whose distribution is tissue-specific [94]. FOXOs play an important role in the maintenance of metabolic homeostasis and in protecting from oxidative stress during aging [95]. The most well defined FOXO-dependent target genes include cell cycle inhibitors, such as p27 and p21, the stress response genes MnSOD and Gadd45a, the pro-apoptotic factors Bim and Fas ligand, and the glycogenolytic gene glucose-6-phosphatase [94]. In fact, Foxo acts at different levels to increase systemic levels of glucose [95]. FOXO factors, in particular FOXO3, are also involved in differentiation processes. In fact, FOXO3 has been shown to be essential for the maintenance of the self-renewal capacity of hematopoietic stem cells; in fact, FOXO3 deficiency leads to abnormalities in the cell cycle of HSCs via dysregulation of ROS production [96].

FOXO factors are downstream targets of IIS, which triggers an intracellular pathway mediated by PI3K/Akt. In response to growth factors and insulin stimulation, Akt phosphorylates FOXO promoting its export from the nucleus to the cytoplasm, thereby repressing FOXO transcriptional function. CR, which reduces insulin and IGF-1 levels, reverses this process. In fact, in the absence of the growth factor (or upon cellular stress) FOXO translocates into the nucleus and activates FOXO-dependent genes [93,94]. In addition to the PI3K/Akt pathway, other major signalling pathways that modulate the activity of the FOXOs are the energy sensors AMPK and the deacetylase SIRT1 [94,95]. In mammalian cells, SIRT1 deacetylates FOXO3 in response to oxidative stress, potentiating FOXO's effects on cell cycle arrest and DNA repair target genes, meanwhile attenuating the activation of apoptosis. SIRT1 potentiates FOXO3's ability to induce cell cycle arrest, allowing more time for cells to detoxify ROS and to repair damaged DNA, drifting away from cell death toward stress resistance [97]. FOXO factors, in particular FOXO1 and FOXO3, regulate

proliferation and migration of endothelial cells, limiting blood vessel formation. Thus, through the two mechanisms just mentioned, FOXOs may have a tumour-suppressor potential both through the tight control of the cell cycle and limiting angiogenesis in developing tumors [94].

As already anticipated, FOXOs mediate some of the effects of CR. In particular, FOXO1 is involved in the CR-specific response to oxidative stress and seems to play a role in its antineoplastic effects, since CR-mice lacking FOXO1 develop tumors as wild type (WT) mice fed ad libitum, while WT-CR mice are protected against tumor formation [98]. FOXO3, as suggested by studies in centenarians, is a mediator of longevity. Indeed, genetic variation in FOXO3 is associated with the ability to reach exceptional ages [93]. FOXO3 may be also one of the key mediators in the life-extending effect of CR in mice. In fact, CR-mice lacking FOXO3 are not as long-lived as wild type CR-mice, suggesting that FOXO3 may be involved in mediating the effects of CR on lifespan in mice [92]. FOXO1 seems to be also involved in the antioxidant effect of CR by regulating NFkB activation. It has been shown that in aged rats fed ad libitum, in which FOXO1 is phosphorylated and sequestered into the cytoplasm through PI3K/Akt pathway, NFkB activation is increased, compared to old rats under CR. This suggests that the phosphorylation of FOXO1, mediated by ISS, regulates NFkB nuclear translocation during aging, which can be repressed by the hypoinsulinemic action of CR [99].

1.3.2.4 *mTOR (Mammalian Target of Rapamycin*)

TOR (target of rapamycin) is an evolutionarily conserved nutrient sensing protein kinase at the nexus of eukaryotic cellular responses to nutrients, growth factors, and

energy status, mostly involved in the promotion of mRNA translation and protein synthesis under conditions favouring growth [100].

The story of TOR began in the 1970s, when a new antifungal activity was discovered in soil samples from the Polynesian island of Rapa Nui (Easter island). The compound isolated, which was the product of the bacterium Streptomyces hygroscopicus, was Rapamycin, widely studied as immunosuppressant before discovering its mechanism of action [101].

Studies in S. cerevisiae firstly identified TOR1 and TOR2 genes as target of rapamycin. Subsequently, mTOR (mammalian target of rapamycin) was isolated from mammalian cells demonstrating to be the physical target of rapamycin [101]. mTOR exists in two distinct complexes with different functions, mTORC1 and mTORC2. mTORC1 is rapamycin-sensitive and is the central effector of the mTOR signalling pathway. It controls and integrates several intra- and extracellular parameters, proliferation and lifespan. mTORC2 is not directly affected by rapamycin, although chronic exposure to this chemical sequesters mTOR from mTORC2, inhibiting the complex formation and resulting in metabolic complications, including glucose intolerance and abnormal lipid profiles, associated with prolonged treatment with rapamycin [100,101]. mTORC2 acts as negative regulator of autophagy and is involved in the phosphorylation and activation of Akt/PKB and protein kinase C [102].

mTOR and the insulin/IGF pathways crosstalk to coordinate overall cell and organismal growth. Indeed, mTOR pathway is activated by insulin and other growth factors through PI3K/Akt. The latter phosphorylates the Tuberous Sclerosis Complex protein 2 (TSC2) of the complex TSC1/TSC2, thereby resulting in inhibition of the complex and, in turn, activation of mTORC1 whose downstream

targets are S6K (p70 S6 kinase) and 4E-BP1 (eukaryotic initiation factor 4E binding protein 1), thus, leading to protein synthesis and ribosome biogenesis [103]. In addition to this mechanism controlling mTORC1 activity, it has been suggested that AMPK, the conserved sensor of energy status activated in response to low ATP levels, negatively regulates mTOR. In fact, AMPK inhibits mTORC1 through phosphorylation of TCS2, resulting in stabilization of the TSC1/TSC2 complex and inhibition of mTOR activity [101,103].

Of all the nutrient-sensing pathways, the TOR pathway has been most consistently linked to dietary restriction. In fact, when nutrient levels fall, TOR activity falls and consequently translational levels are reduced. CR has been shown to decrease TOR activity in several experimental models, from worms to mice. Moreover, genetic deletion of TOR is sufficient to extend lifespan in both invertebrate and in mice under non-CR conditions [101], while the lifespan extension obtained with TOR inhibition is not further increased with CR [104].

Two of the major pathways involved in mediating the beneficial effects of CR, namely insulin/IGF1 and AMPK, converge to mTOR. CR could reduce mTORC1 activity partly through increasing AMPK, which impairs mTOR signalling. Alternatively, CR may downregulate mTOR pathway through a decrease in the insulin/IGF-1 signalling and consequently PI3K/Akt, which it has been already reported to be an upstream modulator of mTORC1.

mTOR is a key regulator of protein homeostasis. Aging is accompained by an increase in damaged proteins caused by misfolding, translation errors, and post-translational modifications such as oxidative damage. Increasing evidence suggests that CR, through inhibition of mTOR, delays aging by reducing protein synthesis and oxidative damage, and increasing the quality of the synthetized proteins, at

least in mouse liver. This is consistent with the hypothesis that CR switches the cells to an energy conservation state, decreasing the expensive process of protein synthesis [105]. mTOR pathway has been shown to be modulated also by another key player in CR's beneficial effects, SIRT1. Unlike most cell types in which CR downregulates mTOR pathway, CR does not directly affect such pathway in the intestinal stem cells (ISCs). CR affects the neighbouring Paneth cells, where, by downregulating mTORC1, stimulates the production of the paracrine factor cyclic ADP ribose favouring the self-renewal of the ISC pool. The paracrine signal from Paneth cells upregulates SIRT in ISCs that in turn deacetylates S6K1 rendering it a better substrate for phosphorylation by mTOR and resulting in increased protein synthesis and expansion of stem cell number [106]. Others have demonstrated that SIRT1 is a negative regulator of mTOR. Indeed, mice under CR showed increased SIRT1 and a decrease in mTOR and S6K expression, while controls, where the levels of SIRT1 were lower compared to CR mice, showed an increase in mTOR and its downstream target, suggesting that SIRT1 may be a negative regulator of mTOR [107]. SIRT1 negatively regulates mTOR through an interaction with the mTOR-inhibitory complex TSC1-TSC2, in particular through its association with TSC2 [108].

Another key process regulated by mTORC1 is the activation of autophagy, a cellular starvation response likely to have a central role in promoting longevity mediated by CR. Inhibition of TOR activity in response to nutrient deprivation is a crucial step for autophagy induction in all eukaryotes. In fact, autophagy is the major degradation pathway mobilizing intracellular nutrient resources such as damaged organelles, macromolecules and amino acids during periods of starvation, to optimize the usage of limited energy supplies. This process is tightly coupled to regulation of cell

growth, where TOR plays a key role. Furthermore, autophagy is induced also by reduced growth factor signalling [101,102,109].

1.3.2.5 *AMPK (AMP-activated protein kinase)*

The AMP-activated protein kinase (AMPK) is a key energy sensor able to reprogram cellular metabolism in response to external cues, and it seems to have a crucial role in mediating the beneficial effects of CR on health and lifespan [110].

AMPK is activated following an increase in AMP/ATP ratio, which reflects the energy status of the cell, and manifests during hypoxia, low nutrient availability, exercise or starvation. Upon activation, AMPK triggers pathways involved in restoring ATP levels, firstly promoting glycolysis and fatty acid oxidation and later increasing mitochondrial respiration [111]. In this way, AMPK activation stimulates ATP synthesis and inhibits ATP-consuming processes not necessary for the immediate survival of the cell [110,112].

The fact that AMPK may be a mediator of CR effects has been shown in several organisms. For example, in the absence of glucose, the yeast homologue of AMPK, Snf1, potentiates the respiratory metabolism of non-fermentable carbon sources, thus promoting the beneficial effect of glucose restriction in yeast and suggesting that Snf1 could be the key mediator for the effect of CR [113]. Furthermore, it has been shown that in *C. Elegans*, several CR regimes require aak-2, which is the homolog of AMPK in worms, to extend lifespan [114]. In flies, inhibition of AMPK decreases lifespan and reduces lifespan extension under starvation [115]. In mammals, AMPK deficiency leads to the development of age-associated diseases such as metabolic syndrome and cardiovascular diseases [111].

AMPK is linked to relevant pathways mediating the effects of CR. First, AMPK allows higher levels of SIRT1 activity because it promotes intracellular increases in NAD+, the substrate limiting the activity of SIRT1. AMPK can also phosphorylate different FOXO family members such as FOXO3, favouring its deacetylation by SIRT1 and the consequent activation [116]. Furthermore, AMPK controls also mTOR activity. Indeed, AMPK activation decreases mTOR-signalling pathway through phosphorylation of the components mTORC1 and TCS2. Thus, by inhibiting mTOR and its downstream target S6K, AMPK negatively regulates the translation process [100]. AMPK has been shown to regulate also the inflammatory response through the inhibition of the NFkB signalling, which is linked to the chronic low-level inflammation typical of aging [111].

1.4 CR mimetics

Over the last few years, increasing interest is being raised by caloric restriction mimetics. Indeed, taking drugs that confer the same benefits of CR would be more feasible than chronically restricting calorie intake in humans, since the duration and the severity of dietary regimen required for optimal benefits is not feasible for most people and is likely to be associated with undesirable side effects. In fact some studies have shown that prolonged CR may decrease fertility and libido, cause wound healing problems, amenorrhea, osteoporosis and decreased potential to combat infections [117].

Among the most studied caloric restriction mimetics are resveratrol, rapamycin and metformin.

1.4.1 Resveratrol

Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenol commonly found in grapes and red wine. This compound became popular because it was proposed to be the main responsible for the so-called French paradox. In fact, the presence of resveratrol in red wine has been linked to a decreased incidence of cardiovascular diseases in the French population, despite the fact that they eat a high fat diet [118]. Resveratrol has been shown to extend lifespan in a wide range of organisms [119,120,121]. In mammals, resveratrol supplementation has been shown to prolong lifespan of middle-aged mice under high-fat diet or fed every-other-day [122]. In contrast, mice fed a standard diet did not live longer under resveratrol treatment [123]. Resveratrol administration has been shown to be beneficial also in obese rhesus monkeys fed high-fat and high-sugar diet. Indeed, a two-year treatment with this compound reduced adipocyte size, inflammatory response and increased insulin sensitivity in white adipose tissue from obese monkeys [124].

Resveratrol has been documented to have beneficial effects on neoplastic process. Specifically, it has been suggested that it can modulate all three stages of carcinogenesis, i.e. initiation, promotion and progression. For example, resveratrol was shown to inhibit tumorigenesis in a mouse skin tumor model and to extend the latent period of tumor induction by DMBA in a mammary carcinogenesis protocol in rats [125]. Moreover, it causes reduction in proliferation in a number of cancer-cell lines, such as prostate, ovarian and breast [126,127].

The mechanisms by which resveratrol exerts these effects rely on the fact that it mirrors some of the metabolic actions of CR. Like others polyphenolic compounds, it possesses intrinsic anti-oxidant activity, but it is also suggested to induce the expression of a number of antioxidant enzymes. Furthermore, exposure to

resveratrol has been associated to increased SIRT1 activity. Other studies have indicated that the first target of resveratrol is AMPK. Indeed, in the absence of AMPK resveratrol could not activate SIRT1 [128]. Thus, AMPK activation by resveratrol would increase NAD+ levels resulting in SIRT1 activation. Resveratrol probably mimics CR by SIRT-mediated deacetylation of pro-inflammatory complexes, such as NFkB, which has been shown to be a target of resveratrol [85,126,129].

1.4.2 Rapamycin

In all model organisms tested so far, CR has been shown to downregulate mTOR pathway, decreasing protein translation and increasing autophagy. The most interesting aspect of mTOR pathway as a target for anti-aging interventions is the availability of rapamycin, which is a specific inhibitor of mTOR [130].

Rapamycin is a macrocyclic lactone produced by the bacterium *Streptomyces hygroscopicus* isolated from soil samples from the Rapa Nui Island, which gives the name to this compound. Also known as sarolimus, rapamycin was studied as an immunosuppressant before its mechanism of action was understood and in 1999 it was approved for use in post-transplantation therapy. Since then, rapamycin has been approved for a variety of uses, including as a treatment for some cancers such as advanced renal carcinoma [101,131].

Several studies have shown that rapamycin extends lifespan in a wide spectrum of organisms, from yeast to rodents [132,133]. Whether rapamycin increases lifespan through a broad protection against age-related diseases or through a specific anti-aging action is still under debate. Late-in-life administration of rapamycin retards multiple aspects of aging, such as liver dysfunction, alterations in heart and adrenal

and endometrial lesions in mice [134]. According to a different point of view, the life extension obtained upon rapamycin treatment would be due to its anti-cancer action rather than its anti-aging effect. In fact, rapamycin has been shown to have important anti-cancer effects, since it has been approved as an anticancer drug, due to its anti-proliferative properties. Indeed, blocking mTOR pathway in turn blocks translation of proteins required for the cell cycle progression, in particular from G1 to S phase [135]. In addition to this, rapamycin treatment has been shown to reduce significantly the proportion of mice (16months old) developing cancers and pre-cancerous lesions compared to untreated mice of the same age. However, rapamycin-treated 25 and 34 month-old mice showed no differences in terms of tumor development. This suggests that cancer may be retarded by rapamycin treatment during the course of normal aging [136]. Moreover, in mice lacking one copy of p53, which are prone to develop cancer, rapamycin extends both lifespan and reduces the incidence of spontaneous tumors [137].

The mechanism by which rapamycin inhibits TOR signaling is not well understood. According to the most accepted theory, rapamycin is not an active site inhibitor, but rather creates a complex with the other components forming mTORC1. It specifically acts on FK-binding protein 12 (FKBP12). In turn, the rapamycin-FKBP12 complex binds to mTOR. This action leads to disruption of the mTORC1 complex and the consequent inhibition of the pathway [58,130].

Despite its beneficial effects, rapamycin treatment has important side effects that limit its usage as anti-aging intervention. The most important adverse effects are hyperglycemia, hyperinsulinemia and insulin resistance. Furthermore, it has immunosuppressive properties that make it not suitable for life extension in humans. The side effects seem to be due to the inhibitory effect of rapamycin on mTORC2,

suggesting that increasing the specificity for mTORC1 would enhance the efficacy-to-adverse effect ratios [58,130]. Thus, rapamycin has been considered a CR mimetic even if it causes some side effects that are instead prevented by CR. This fact led to investigate whether CR and rapamycin work through the same mechanisms in mice, and the most important finding was that the two mechanisms have overlapping and distinct effects on many pathways. For example, while CR improves glucose tolerance, treatment with rapamycin failed to show improved glucose tolerance. Furthermore, the two treatment showed differences in terms of lipid metabolism. Both inhibited fat storage and lipogenesis, but only CR induced β-oxidation and production of ketone bodies [138]. These findings render questionable the notion that rapamycin is a faithful CR mimetic.

1.4.3 Metformin

Metformin is a biguanide commonly used in the treatment of type 2 diabetes and metabolic syndrome since the 1960s. It has anti-hypeglycemic function in diabetic patients by reducing hepatic gluconeogenesis, increasing insulin sensitivity and increasing glucose utilization by muscles and adipocytes [58]. Patients with metabolic syndrome exhibit manifestation of accelerated aging, such as cardiovascular disease and cancer, all of which reduce lifespan. The fact that patients treated with metformin are less susceptible to cancer and cardiovascular diseases raises the possibility that metformin could exert beneficial effect in age-related diseases [139].

The specific mechanism by which metformin acts at molecular level is still under investigation, even if there is evidence that it likely affects AMPK activity by modulating ATP production by mitochondria. Indeed, metformin has been shown to inhibit complex I of the electron transport chain, compromising ATP production in

mitochondria and resulting in an increased AMP/ATP ratio, which finally leads to AMPK activation and consequent downregulation of mTOR and IGF-1/Akt pathways [126, 139]. Furthermore, upon metformin treatment, the transcription factor SKN-1/Nrf2 is activated, resulting in increased expression of antioxidant genes and subsequent protection from oxidative damage [126]. This metformin-mediated action may mirror CR. However, in contrast with CR, metformin does not decrease glycaemia and insulinemia in normal subjects [140]. Microarray analysis have shown that metformin induces a gene expression profile that is similar to CR, and like CR, it improves also survival in middle-age mice when administered at low doses [139].

Metformin has been shown to have a potential anticancer effect. There are several studies reporting the ability of biguanides to inhibit both chemically induced and spontaneous cancers in rodents [141]. Moreover, retrospective studies reported a potential to prevent cancer also in humans. Indeed, among diabetic patients, those treated with metformin have a low incidence of and mortality from cancer [142]. However, to consider metformin as a possible anti-aging intervention, it has to be tested for long-term use in a wide range of subjects, particularly those who are healthy, since metformin, being a drug, could cause side effects in the healthy population [130].

1.5 Cellular senescence

Cellular senescence, often considered as the cellular counterpart of tissue and organismal aging, has been defined as the specific phenomenon wherein a proliferation-competent cell undergoes permanent growth arrest in response to various cellular stresses/stimuli [143]. It is an irreversible state, in the sense that known physiological stimuli cannot force senescent cells to re-enter the cell cycle

[144]. Senescence has been considered a hallmark of aging since senescent cells accumulate with age in many tissues of vertebrate organisms.

The concept of cellular senescence was first described by Hayflick and colleagues, as a mechanism that halts the proliferation of normal cells in vitro. In fact, they observed that, after many cell doublings, cell proliferation declined, although cells remained metabolically active [145]. Decades later, it was hypothesized that senescence may represent a potent tumor-suppressive mechanism that arrests proliferation of premalignant cells. In fact, mice deficient in p53, one of the effectors of the senescence process, contain cells that are unable to stop in response to appropriate stimuli and they are inevitably cancer-prone [146]. Ironically, the accumulation of senescent cells over time can also promote tumorigenesis, through the secretion of a myriad of bioactive molecules, described as senescence-associated phenotype (SASP), which is the result of the high metabolic activity of senescent cells. Although SASP can give rise to a pro-tumorigenic microenvironment, recently it has been shown to promote also tissue remodeling [147]. Thus, what is now clear is that senescence is a double-edged sword, in the sense that it can be both beneficial and deleterious depending on the context.

1.5.1 Triggers of senescence

Senescence can be induced by many different stimuli.

1.5.1.1 *Telomere-dependent senescence*

Telomeres are stretches of repetitive DNA that cap the ends of linear chromosomes and protect them from degradation or fusion during the DNA-repair processes. Because DNA polymerase cannot completely replicate DNA ends, cells undergo

loss of hundreds of bases each replication cycle [148], resulting in progressively shorter telomeres. Telomere shortening does not occur in cells expressing telomerase, the enzyme that can replenish the repetitive DNA de novo, which is expressed by many cancer cells and germ-line but not by normal somatic cells [144]. In humans, telomere dysfunction has been linked to early onset of some aspects of aging, such as pulmonary fibrosis, bone marrow failure and cirrhosis [143]. Shorter and dysfunctional telomeres trigger the classical DNA damage response (DDR), which results in the permanent exit of the cell from the cell cycle (senescence) [146]. The proteins involved in DDR are protein kinases, such as ataxia telangiectasia mutated (ATM), adaptor proteins such as 53-binding protein 1 (53BP1) and chromatin modifiers such as H2AX. These proteins, which localize in the DNA-damage foci, are often detected in senescent cells. Indeed, they have been used as senescence markers [15].

1.5.1.2 *Genomic damage*

Many cells undergo senescence in response to severe damage to DNA, such as that induced by ionizing radiation, chemotherapeutic drugs and other genotoxic agents. Damage that results in double strand breaks (DSBs) is specifically associated to senescence and it depends strongly on p53 and consequently on p21 recruitment [15,144]. Other DNA lesions, such as those induced by ROS, may also drive cells into senescence, for example accelerating telomere shortening [144,149].

1.5.1.3 *Oncogene-induced senescence (OIS)*

Oncogenes are mutant version of normal genes having the potential to induce cell transformation. Oncogene-induced senescence (OIS) was first described in cultured cells that underwent senescence upon the activation of an oncogenic mutant RAS homolog [150]. Different oncogenes can induce senescence by several mechanisms:

activation of RAS leads to increased proliferation rates, then increasing the chance for DNA damage, while activation of BRAF induces senescence involving p16 induction, upregulation of IL-6 and IL-8, and activation of pyruvate dehydrogenase, which increases mitochondrial metabolism with increased ROS generation [150]. Because oncogenes that induce senescence stimulate cell growth, cell senescence may be viewed as a process contrasting the excessive mitogenic stimulation that puts cells at risk of oncogenic transformation [15]. OIS is often accompanied by increased expression of CDKI, such as p21 and p16 [143].

1.5.1.4 *Mitochondrial dysfunction-associated senescence (MiDAS)*

The importance of mitochondria in cellular senescence has been linked to their ability to generate ROS that induce DDR. In addition to this, the mitochondrial content was linked to the senescent response, showing that increased DNA damage in aged hepatocytes correlates with higher mitochondrial content [151]. The role of mitochondria in aging has been associated with the accumulation of dysfunctional mitochondria. Mitochondrial dysfunction, mediated by several stimuli, results in a distinct senescent phenotype termed mitochondrial dysfunction-associated senescence (MiDAS). MiDAS results from a reduced NAD+/NADH ratio, AMPK activation and p53 phosphorylation, which causes cell cycle arrest [146]. Moreover, MiDAS has been shown to be characterized by a distinct SASP, due to the p53-mediated inhibition of the IL-1 arm of the SASP [152], which has been shown to amplify the pro-inflammatory network of SASP [153].

1.5.2 Markers of senescence

Identification of senescent cells in vivo relies on few markers thought to be commonly expressed by all senescent cells. Unfortunately, the majority of these markers were identified via *in vitro* studies in fibroblast, raising the possibility that they could not be relevant in *in vivo* studies. Moreover, features of senescence may differ from tissue to tissue, and depending on the trigger [150]. Thus, no single marker can be considered exclusive to the senescent state. Rather, a combination of phenotypic feature is considered the best approach to identify senescent cells [143].

1.5.2.1 *Cell enlargement*

Senescent cells undergo morphological alterations such as flattening, vacuolization and enlarged cell size. This continuous cell enlargement is likely due to the absence of cell division and it may reflect a continuation of anabolic processes such as protein synthesis during the senescent state [143].

1.5.2.2 *SA-beta-gal activity*

SA-beta-gal is the most commonly used biomarker of senescence, due to its ease of detection in tissues by histochemical staining. It is measured at pH 6.0 using the artificial substrate X-gal [154], [155]. Endogenous beta-galactosidase in humans is a lysosomal enzyme active at pH 4.0-4.5, so its detection at suboptimal pH 6.0 appears to be selective for senescent cells, which express very high levels of beta-galactosidase, possibly due to the increased biogenesis of lysosome in senescent cells. SA-beta-gal activity has been detected in organs of old individuals and animals, suggesting that cellular senescence is an aging trait and accumulates in aged tissues. Unfortunately, SA-beta-gal activity at pH 6.0 can be detected not only in aged tissues, but also in cells with exuberant lysosomal synthesis and activity, such as phagocytes and macrophages, as well as in resident Kupffer cells in the

liver, and in confluent cell cultures. Thus, its use as senescence marker in vivo should be interpreted with caution, particularly in tumors or in other inflammatory conditions, where the immune response may predominate [15,143].

1.5.2.3 *Expression of inhibitors of cell cycle progression*

Senescent cells show striking changes in gene expression, including changes in known cell cycle inhibitors. Two cell-cycle inhibitors frequently expressed by senescent cells are p21 and p16. They are components of tumor-suppressor pathways governed by p53 and retinoblastoma (RB) proteins, which are often mutated in tumors. $p16^{INK4A}$, a selective inhibitor of cyclin D-dependent CDK4 and CDK6 [156] and a tumor suppressor in its own right, induces cell cycle arrest by acting upstream of retinoblastoma (RB) and its expression is highly dynamic: it is generally absent in healthy tissues at young age, but highly expressed during tumorigenesis, wounding and aging [157]. The cyclin-dependent kinase inhibitor (CDKI) p21 is a down-stream target of p53 transactivation, in response to genomic damage and it is crucial for maintaining the senescence growth arrest mediated by p53. Both p21 and p16 maintain RB in a hypophosporylated and active state, thereby preventing E2F from transcribing genes that are needed for proliferation. However, their activities are not equivalent. Even if p16 is considered an important regulator of senescence, it is expressed by many but not all senescent cells and in tumors that have lost RB function. Moreover p16-RB pathway is crucial for generating senescence-associated heterochromatin foci (SAHFs), needed for silencing genes involved in cell cycle progression [143,144]. P53, the tumor suppressor highly mutated in many human cancers, is critical in inducing cell death and cell cycle arrest in response to stress factors. It is involved in apoptosis, but also in cell senescence and quiescence. P53 has been shown to regulate different response programs

depending on the stimulus. For example, upon acute induction of p53, such as the classical DDR, it has been shown to stimulate genes involved in RNA metabolism and processing, while chronic conditions, such as OIS, are mostly associated to the typical p53 functions, such as cell cycle arrest, DNA damage response and apoptosis [158].

1.5.2.4 *DNA damage foci*

DNA damage is associated with senescence both in vitro and in vivo. It can be due to replication errors during S phase, but also to endogenous or exogenous genotoxic insults such as oxidative stress and telomere shortening, or UV and ionizing radiations. Both double strand breaks and telomere uncapping are able to induce the DNA damage response (DDR). In this process, ataxia telangiectasia mutated (ATM) is recruited to the site of damage, causing the phosphorylation at Ser-139 of histone H2AX, which consequently facilitate the focal assembly of checkpoint and DNA repair factors such as p53-binding protein 1 (53BP1). These markers of DDR have been used as in vitro and in vivo markers of senescence [159]. Although staining of H2AX or 53BP1 are often visible in senescent cells, they are not invariant, as some compounds such as chloroquine can induce the ATM cascade without overt DNA damage [143].

1.5.2.5 *Senescence-associated heterochromatin foci (SAHF)*

Alterations to chromatin state during senescence has long been observed. In fact, senescent cells show a characteristic heterochromatin condensation structure involving the formation of heterochromatin foci, referred to SAHF. These regions are readily visible by fluorescent microscopy as DAPI dense foci inside the nucleus and are enriched of many markers of heterochromatin such as HP1 proteins. SAHFs are thought to contribute to the irreversibility of the senescent phenotype by packaging

proliferative genes into heterochomatic domains [14]. A key component of the nuclear lamina, lamin B1 (LMNB1), which is implicated in the integrity of the nuclear structure, chromatin positioning and gene expression, was reported to be downregulated during senescence in human diploid fibroblasts [160]. Moreover, LMNB1 knocking down, in particular in the central regions of lamina-associated domains, has been shown to promote SAHF formation [161].

1.5.2.6 *Senescence-associated secretory phenotype (SASP)*

Many senescent cells overexpress genes that encode secreted proteins that can alter the tissue microenvironment. As already mentioned, senescent cells are metabolically very active, and SASP is the result of this activity. SASP consists of several proteins with different functions, including cytokines (mainly IL-6 and IL-8 that are considered the major SASP components), proteases and growth factors that are secreted in the extracellular environment, having a profound impact on the tissue microenvironment. SASP influences the surrounding through four main mechanisms: an autocrine function, reinforcing the senescent phenotype within the secreting cell; a pro-oncogenic effect on surrounding pre-malignant and transformed cells; an inflammatory effect driving infiltration of immune components; a paracrine function driving senescence within the normal cells nearby the secretory cell [162]. Among the effects induced by SASP are recruitment of inflammatory cells, alteration of tissue composition and architecture, invasion, angiogenesis and cell proliferation.

SASP is thought to be aging-promoting and either tumor-promoting and tumor-inhibitor. Regarding to its tumor-promoting effect, SASP has been shown to support cell invasion and metastasis by disrupting and remodeling tissue structure and promoting the proliferation and assembly of endothelial cells for neo-angiogenesis [146]. Paracrine effects of SASP also provoke anti-tumor immunity. In particular,

SASP factors recruit natural killer cells to eliminate tumor cells [163]. In relation to aging, SASP has been shown to reinforce the senescent phenotype in secretory cells through an autocrine manner, but also to spread senescence to normal neighboring cells, mainly though IL-1β [164]. In addition, SASP has been shown to be involved also in physiological processes such as embryonic development, for fine-tuning the morphogenesis of certain structures and wound healing [165]. In response to cutaneous wounds, fibroblasts and endothelial cells undergo senescence accelerating wound closure through secretion of the SASP component PDGF-AA [147]. Moreover, SASP has been shown to play an important role in liver injuries, in particular limiting liver fibrosis through secretion of the SASP component metalloproteinases [146,166]. SASP composition has been suggested to vary among different tissues and within the same tissue, depending on the type and/or the intensity of the stimuli inducing senescence. In particular, when SASP is the result of genomic or epigenetic damage and cells enter senescence by p21 and p16 activation, they may not exhibit the secretory phenotype [167].

SASP activation seems to be partly mediated by NF-kB and C-EBPβ while the basis for the regulation of its different components is unclear. One of the candidates that may control the SASP is autophagy, and the link between autophagy and senescence is thought to be the transcription factor GATA4. GATA4 stabilization (and in turn activation) through inhibition of autophagy leads to activation of the transcription factor NFkB to initiate the SASP and facilitate senescence [168,169]**.** Recently, it has been shown that OIS is accompanied by a fluctuation of NOTCH activity, which has been suggested to be a possible regulator of SASP composition through a temporal and functional switch between two distinct secretomes, one characterized by TGF-β and, when NOTCH is downregulated, by increased levels

of pro-inflammatory cytokines [170]. Epithelial cells stimulated to senesce by loss of PTEN secrete a SASP enriched of immunosuppressive cytokines resulting in more infiltrating tumors [171]. Increasing evidence suggests that the senescence arrest and the SASP are linked to the metabolic state of the cell [172]. Indeed, it has been reported that SASP is regulated by mTOR kinase, which senses the nutrient state of the cell. Inhibiting mTORC1 by using rapamycin, the secretion of the major SASP components such as IL6 has been observed to be mTOR dependent [153,173].

1.5.3 Metabolic features of senescent cells

Senescent cells have been shown to shift towards a more glycolytic state, which is accompanied by a less energetic state [172,174,175]. Indeed, senescence was linked to increased levels of ADP and AMP relative to ATP. One of the links between metabolism and cell senescence has been suggested to be AMPK, which behaves as a sensor of the energetic state of the cell. AMPK is activated by increased AMP:ATP and ADP:ATP ratios. In addition to its role in triggering fatty acid oxidation, mitochondrial biogenesis and glucose uptake [176], AMPK activation can also induce cell-cycle arrest and senescence through two main mechanisms. First, AMPK can directly phosphorylate and activate p53, allowing cells undergo arrest via upregulation of p21. Second, AMPK inhibits degradation of the mRNA encoding p21 and p16, resulting in a senescence arrest of cell proliferation. Thus, AMPK acts as a metabolic checkpoint during energy stress that can result in senescence [172].

Among metabolites thought to play a role in senescence are pyruvate and malate. Pyruvate is at the crossroads of glycolysis and mitochondrial respiration. It is the final product of glycolysis together with NADH, and the primary carbon source for acetyl-CoA, which enters to the tricarboxylic acid cycle. Pyruvate has been shown

to induce senescence likely through increased mitochondrial ROS production. Whereas malate has been shown to antagonize senescence, increasing cellular antioxidant defenses [172].

Low levels of NAD+/NADH ratios promote cellular senescence. Indeed, NAD+ is the primary source of ADP-ribose for poly-ADP-ribose polymerase (PARP), one of the major enzymes involved in DNA repair in response to genotoxic stress. Thus, low levels of NAD+ promotes senescence through inhibition of PARP. Moreover, NAD+ is the key cofactor for sirtuins, and its depletion is followed by decreased sirtuin activity. Sirtuin loss has been shown to induce senescence both in human and mouse cells [172]. For example, SIRT1 protects against cigarette smoke/oxidative stress-induced lung cellular senescence by deacetylation and repression of p53, while loss of SIRT1 leads to accumulation of p53 acetylation, thereby enhancing oxidative-stress induced senescence. Yet, SIRT1 has been shown to prevent senescence also by maintaining telomere length [177]. Furthermore, SIRT1 is involved also in cellular senescence mediated by IGF-1, which levels increase with aging. Prolonged IGF-1 exposure induces premature senescence through inhibition of SIRT1, resulting in p53 acetylation as well as stabilization and activation [178].

1.5.4 Caloric restriction and cellular senescence

Because cellular senescence has been shown to be a process strongly correlated with aging, and this latter has been shown to be delayed by CR in several models [66], one would expect that CR might also modulate cellular senescence, which has been proposed to parallel the aging process at cellular level [179].

Several studies have suggested that CR might in fact influences cell senescence. Short-term CR (26%) started late in life has been shown to reduce the expression of

some senescence markers, such as SA-beta-gal and H2AX, to improve telomere maintenance and to reduce oxidative stress markers, both in liver and small intestine of mice [180]. Similar results were obtained testing the effect of short-term CR in kidneys of aged rats. CR was shown to improve renal function and to decrease the expression of SA-beta-gal and p16, while it increased SIRT1 expression and reduced mTOR levels [181]. Furthermore, long-term exposure to 40% CR in rats led to a decrease in SA-beta-gal and lipofuscin accumulation in heart muscle cells [182]. Lipofuscin levels increase with aging and is being used as senescence marker [183]. This CR-mediated reduction in cellular senescence was accompanied by an improved organ function, namely diastolic function. It has been proposed that CR may mediate a reduction in cellular senescence by downregulating mTOR and its effector S6K1, which, among its functions, binds to MDM2 and inhibits p53 ubiquitination, thereby increasing its stability and potentially inducing senescence. Thus, suppression of mTOR-S6K1 signaling, as it occurs during CR, would lead to MDM2 nuclear transduction and degradation of p53, resulting in reduction of senescence [184].

While most of the studies aiming to gain insights into the link between CR and cellular senescence involve protocols of short-term CR, very few studies have tried to address this issue over a long period of observation. Thus, the results we may obtain in long-term experiments may be different and/or the differences may be less evident, due to possible adaptation processes the organism may undergo during long-term diet. Indeed, as suggested by studies carried out in non-human primates, a period of 9-12 years of CR did not decrease the expression of SA-beta-gal and did not increase the replicative lifespan of fibroblasts from CR rhesus monkeys [185].

2. Aim

The overall aim of the studies discussed in this dissertation is to explore possible mechanisms mediating the delaying effect long-term CR on carcinogenesis. More specifically, we investigated the possibility that CR may affect the evolution of neoplastic disease via modulation of the tissue microenvironment, i.e. by delaying the emergence of the neoplastic-prone milieu associated with aging. If indeed an aged microenvironmnet represents a favourable landscape for the emergence of pre-neoplastic cells, delaying age-associated tissue changes may be one of the mechanisms whereby CR protects against cancer.

3. Materials and methods

3.1 Animals and diet

A colony of DPPIV$^-$ Fischer 344 rats has been established in our laboratory, at the Department of Biomedical Sciences, University of Cagliari. Animals were maintained on an alternating 12hr light/dark cycle with water available ad libitum and housed in double cages.

In a first series of experiments, eight 8-week-old male F344 rats were randomly divided into two groups: 4 animals were included into the control group receiving food *ad libitum* (AL) and 4 animals were included into the caloric restriction group (CR) receiving 70% of food consumed by AL-fed rats. Both group received the same standard rodent laboratory chow (Mucedola, Italy; composition is reported in Table 1). After 18 months under AL or CR diet, animals were transplanted with normal hepatocytes, using an orthotopic system of transplantation (see below).

In a second series of experiments, ten 8-week-old male F344 rats were similarly divided into two groups (5 AL and 5 CR), and were given either AL or CR diet. After 18 months, animals in the CR group were transferred to AL diet until the end of the experiment. Three weeks after the dietary shift, all animals were transplanted with nodular (pre-neoplastic) hepatocytes, using the orthotopic transplantation model referred to above.

Body weight and food consumption were monitored weekly. The 70% amount of CR diet was calculated weekly based on the average of food consumption in AL rats over the preceding week. The level of 30% restriction in calorie intake was chosen in order to achieve a beneficial effect without causing malnutrition, and was based on numerous reports in literature [45,46,51]. All experiments were approved by the

University of Cagliari Ethical Committee for Animal Experimentation; all animals received human care in accordance with NIH Guidelines for the care and use of animals. Animals were killed at 24 months of age. Blood samples were collected and serum was separated within 1h of blood collection after spinning at 1500g. Livers were excised and samples from each lobe were snap frozen (for cryostat sections, protein and gene expression analysis) or fixed in 10% buffered formalin and embedded in paraffin (for histology and immunohistochemistry).

3.2 The dipeptidyl-peptidase-type IV-deficient rat model for hepatocyte transplantation

Normal F344 rats express a specific exopeptidase, dipeptidyl peptidase IV (DPPIV), in a characteristic pattern in the liver, restricted to the apical domain of the plasma membrane [186]. To follow the fate of donor hepatocytes into the recipient liver, the dipeptidyl peptidase type IV-deficient ($DPPIV^-$) rat model can be used. In this system, hepatocytes freshly isolated from the liver of a Fischer 344 rat expressing the enzyme (DPPIV+) are transplanted into a syngeneic $DPPIV^-$ host, such that donor-derived cells can be detected in the recipient liver through simple histochemical and/or immunohistochemical techniques.

3.3 Generation of hepatic nodules and isolation of hepatocytes

Hepatocyte nodules were induced according to a well characterized experimental model in the rat [187]. Briefly, two-month old male Fischer 344 rats, expressing DPPIV enzyme activity, were injected with a single dose of diethylnitrosamine (DENA, 200

mg/kg. b.w., i.p., Sigma-Aldrich Chemical Co., St. Louis, MO) followed 3 weeks later, by exposure to a modified version of the Solt and Faber protocol [42,188], to stimulate the growth of hepatocyte foci and nodules. Such protocol consisted of three consecutive daily doses of 2-acetylaminofluorene (20 mg/kg b.w., given by gavage tube, from Sigma-Aldrich) followed, on the fourth day, by a single administration of CCl_4 (0.2 ml/kg b.w., by gavage, mixed in corn oil, 1:1 v:v). Six months after the initial treatment livers were perfused according to a standard 2-step collagenase perfusion technique [189,190]. Typically, 3 to 5 large (5-10mm in size) persistent nodules are present in the liver at this time point using the above experimental protocol. Large (>5 mm) nodules were physically separated from surrounding tissue and isolated cells were suspended in PBS and prepared for transplantation experiments. Prior to transplantation, cell suspension was filtered through a nylon mesh with a pore diameter of 100μ, in order to eliminate any large cell clumps. Cell viability, determined by trypan blue dye exclusion, was 80-85% in the nodular cell preparation. Normal hepatocytes were isolated from a liver of a male F344 rat expressing DPPIV enzyme activity, perfused as reported above. Cell viability for normal hepatocytes was 85-90%.

3.4 Hepatocyte transplantation

AL or long-term CR male Fischer 344 rats of the $DPPIV^-$ strain were used as recipients. In a first series of experiments, rats were injected with $2x10^6$ normal hepatocytes freshly isolated from a $DPPIV^+$ syngeneic donor. In a second series of experiments, animals were transplanted with $1.7x10^6$ cells freshly isolated from hepatic nodules induced in a $DPPIV^+$ syngeneic donor. In both studies, cells were transplanted into the liver via a mesenteric vein.

3.5 Histochemical determination of DPPIV enzyme activity

Histochemical determination of DPPIV enzyme activity was performed on 5-µM thick cryostat sections from frozen tissue [191]. Fixation was for 5 min in 95% ethanol/5% glacial acetic acid (99:1 vol/vol) at 0°C to −10°C, followed by a 5-min wash in 95% ethanol at 4°C. Air-dried slides were incubated for 10–20 min at RT with the substrate reagent: 2.5 mg Gly-Pro-4-methoxy-β-naphthylamide (Sigma) dissolved in 150 ml of dimethylformamide and mixed with a 5 ml solution of Fast blue BB salt (Sigma) in PBS.

3.6 Staining for SA-beta-gal activity

Staining for SA-β-gal was performed according to published procedures [154]. Immediately before staining, X-Gal stock solution was prepared by dissolving 20mg/ml X-Gal (Invitrogen, Carlsbed, CA) in dimetylformamide. SA-β-Gal staining solution was prepared as follows: 1 mg/ml of X-Gal stock solution were dissolved in 40 mM citric acid in sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM MgCl2. Frozen sections of 10-µm thickness were fixed for 5’ in 4% formaldehyde/0.5% glutaraldehyde at 4°C, washed in PBS and incubated in fresh SA-β-Gal staining solution for 16h at 37°C. Sections were counterstained with Hematoxylin.

3.7 Histology, immunohistochemistry and immunofluorescence

Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin (H&E) according to standard procedures. Immunohistochemical staining for

p21, p27 and GSTP was performed on paraffin embedded sections, following de-wax and antigen retrieval with sodium citrate buffer (pH 6, 0.01M). Slides were blocked for 30', incubated with primary antibodies (diluted in PBS) overnight at 4°C, and then incubated with AP-conjugated secondary antibodies. Detection of specific signal was accomplished using the avidin/biotin alkaline phosphatase system (Vectastain ABC kit; Vector Lab, Burlingame, CA) or using a HRP/AEC detection kit (Abcam). Double staining of DPPIV and GSTP was performed on frozen sections fixed in acetic alcohol/ethylic alcohol, first stained for DPPIV (as reported above), then blocked with goat serum, incubated with anti-GSTP overnight at 4°C and detected with the same protocol for paraffin sections. Immunoflorescent staining for H2AX was performed on frozen sections, following fixation in cold acetone. Slides were blocked for 20' with goat serum, incubated with primary antibody for 1 h at RT, followed by incubation (45') with fluorescent-conjugated secondary antibody. Slides were counterstained with DAPI and images were acquired with an IX71 fluorescence microscope with CCD camera (Olympus, Tokyo, Japan). Antibodies are listed in Table 2.

3.8 Cell imaging analysis

Three dimensional analysis of $DPPIV^+$ cluster was performed on 10 consecutive serial sections by scanning slides with Hammamatsu NanoZoomer 2.0 rs. Acquired images were overlaid and analyzed using NDP 2.0 view software.

GSTP positive clusters analysis was performed in at least 2 random sections from each sample by scanning slides with Pathscan Enabler IV scanner (Meyer Instruments, Houston, TX, USA). Acquired images were analyzed using Image-Pro Premier Software (Media Cybernetics, Rockville, MD, USA).

3.9 Western blot

Western blot analysis was performed either on nuclear or cytoplasmic proteins extracted from liver samples by using a commercially available kit (CelLytic nuclear extraction kit, Sigma) according to manufacturers' protocol. Protein concentration in supernatants was measured using the BCA method [192]. Absorbance was read at 562nm with a microplate reader (Infinite F200 Pro, TECAN).

Samples (80µg and 30µg for nuclear and cytoplasmic proteins, respectively) were prepared in Laemmli buffer, boiled at 95°C for 5' then loaded into SDS-PAGE precast gels (Bio-Rad, Hercules, CA) and run under denaturing conditions. Proteins were transferred onto nitrocellulose membranes (Amersham, UK), blocked with 5% non-fat milk for 45', followed by incubation with primary antibodies overnight at 4°C. Antibodies are listed in Table 1. Membranes were washed and incubated for 2 h with the appropriate secondary antibody conjugated with HRP. Protein bands were detected using a chemiluminescent substrate (Bio-Rad) and imaged onto Kodak film.

3.10 RNA isolation, RT-PCR and Real-Time qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA integrity and purity were confirmed by 1% agarose gel electrophoresis and OD260/OD280 nm absorption ratio >1.8. Two grams of DNase-I treated RNA of each sample were reverse-transcribed by PCR using Promega reagents. The resulting cDNA was analysed by quantitative real-time PCR using specific TaqMan assays and TaqMan Gene Expression Master Mix on an StepOne System (all from Applied Biosystems, Carlbad, CA). The rat specific

assays were: IL-6 (Rn01410330_m1); β2-microglobulin (Rn00560865_m1). For both assays the thermal profile was as follows: 50˚C for 2 minutes, 95˚C for 10 minutes, 45 cycles at 95˚C for 15 seconds and 60˚C for 1 minute. Fold change was calculated by the 2-ΔΔCT method [193].

3.11 Enzyme-linked immunosorbent assay (ELISA)

ELISA for quantitative measurement of IL-6 was performed on rat serum. Serum IL-6 concentration was determined using the ELISA kit (high sensitive) supplied by Cloud Clone Corp., following the manufacturer's protocol. The assay employs the quantitative sandwich immunoassay technique using IL-6 antibody raised against rat IL-6 protein. The IL-6 ELISA assay has a performance sensitivity of less than 100pg/mL and the minimum detectable was 1.56pg/mL. Absorbance was read with a microplate reader at 450nm (Infinite F200 pro, TECAN). All samples were assayed in duplicate.

3.12 Lipid profile

Total cholesterol, low-density lipoproteins (LDL), high-density lipoproteins (HDL) and triglycerides (TG) were measured in rat serum collected at different time points during the study: 2 months, 10 months and 22 months of AL or CR diet. Serum lipids and lipoprotein concentrations were measured by using commercial enzymatic kits according to manufacturer's procedures (Architect cSystems, Abbot, Germany).

3.13 Graphical representation of results and statistical analysis

All results and statistical analysis were computed using Graph Pad Prism 5. Student "t" test analysis was performed to compare two groups of data. For cluster size distribution, statistical analysis of frequency distribution was performed via Chi-square for contingency table using Graph Pad Prism 5.

3.14 Metabolomics analysis

Chemicals and reagents. Methoxamine hydrochloride, N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), and all analytical standards were purchased from Sigma Aldrich (Milano, Italy). Bidistilled water was obtained from a MilliQ purification system (Millipore, Milan, Italy).

Samples preparation. Following ice thawing, 100µL of plasma were transferred into an Eppendorf tube and mixed with 250 µL of methanol and 120 µL of chloroform. After 1h, 380 µL of chloroform and 90 µL of aqueous potassium chloride 0.2 M were added, samples were then vortexed and centrifuged at 14000rpm for 10 min at 4°C. Finally, 200 µL of the aqueous layer were transferred into a glass vial and dried in nitrogen stream. 100 µL of the homogenized liver tissue (10x PBS, 1:5 ratio, w/v) was processed following a similar procedure. All dried samples were derivatized with 50 µL of pyridine containing methoxamine hydrochloride at 10 mg/mL. After 17 h, 0.05 mL of MSTFA were added and 1 hour later samples were re-suspended in 0.1 mL (for serum-derived extracts) or 0.4 mL (for liver-derived extracts) of hexane containing 2-dodecanone as internal standard. Three quality

control samples for each biological matrices were prepared mixing 10 μL of each sample into a unique Eppendorf tube and treating these such as normal samples.

GC-MS analysis. After silylation samples were analyzed using a 6850 gas chromatograph equipped with a 30 m DB-5MS column (0.25 μm × 0.25 mm i.d) (J&W scientific, Folsom, CA, USA) and a 5973 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The injector was operated at 200 °C in the splitless mode and the injected volume was 1μl. Helium was used as carrier gas at the flow rate of 1 mL/min. The column temperature was set at 50°C for 10 min then increased to 300 °C at 10 °C/min and kept at 300 °C for 4 min. For GC-MS detection an electron ionization system was used with an ionization energy of 70 eV and produced ions were recorded at 1.6 scan/sec over the mass range m/z 50-550. GC-MS data analysis was conducted by integrating each resolved chromatogram peak. Identification of metabolites was performed by mass spectra searching using the NIST08 and the Golm (http://gmd.mpimp-golm.mpg.de/) libraries and, when available, by comparison with analytical standards [194]. GC–MS spectra deconvolution was performed by the AMDIS tool in the NIST08 library. For each metabolites retention indices were calculated according to Kovats, for the alkanes series C9-C24. The peak area relative to the most abundant mass fragment was calculated and the resulting data were analyzed through MVA.

Statistical multivariate analysis. For each sample, GC-MS peak intensities of metabolites (variables) were normalized to 100. Each variable was mean centered and unit variance scaled over all samples and, when necessary, log10-transformed to correct deviation from normality. The unsupervised Principal Component Analysis (PCA) was performed to overview information contained in the data set, to highlight any relationship between samples and variables, to check for deviating features and

for tendency of samples to cluster. PCA is a hypothesis-free approach, and it is suitable to build new hypothesis to be tested. Moreover, to find discriminant metabolites between two predefined classes, the orthogonal variant of pair-wise Partial Least Squares-Discriminant Analysis (OPLS-DA) was performed. The quality of the OPLS-DA models and the optimum number of principal components were evaluated based on the cumulative parameters R2Y and the analogue in cross validation Q2Y. Discriminant metabolites were obtained using an S-plot, which combines covariance and correlation loading profiles [195]. Furthermore, a Student's t-test analysis was performed with the aim to confirm the statistical importance of metabolites found after the S-plot analysis. S-plot discriminant metabolites with a p value lower than 0.01 were considered significant for the analysis. All MVA were performed with SIMCA-P+ program (Version 14.1, Umetrics, Sweden).

Table 1. Composition of the standard rodent laboratory chow Mucedola 4RF21.	
Water	12.00%
Protein	18.50%
Fat	3.00%
Fiber	6.00%
Ash	7.00%
NFE (nitrogen free extract)	53.50%

Table 2. Primary and secondary antibodies.		
Primary antibody	**Brand**	**Cat. No.**
p16	Santa Cruz	sc1207
p21 (M-19)	Santa Cruz	sc-471
p27 (C-19)	Santa Cruz	sc-528
p53	Santa Cruz	sc1311
53BP1	Abcam	ab87097
H2AX (phosphor S139)	Abcam	ab2893
SIRT1	Santa Cruz	sc15404
FOXO1	Cell signaling	C29H4
HDAC1	Santa Cruz	sc7872
C/EBPβ	Santa Cruz	sc150
mTOR	Abcam	ab2732
IL-6	Abcam	ab6672
GSTP	LS BIO	c179188
β-actin	Abcam	ab8227
actin	Santa Cruz	sc1616
Secondary antibody	**Brand**	**Cat. No.**
Anti-rabbit IgG HRP-conj.	Abcam	ab205722
Anti-goat IgG HRP-conj.	Santa Cruz	sc2020
Anti-rabbit IgG 568-conj.	Abcam	ab175692

4. Results

4.1 The growth of transplanted normal hepatocytes in the liver of rats exposed to long term CR.

Previous investigations from our research group revealed that isolated hepatocytes form larger clusters of daughter cells when transplanted into the liver of old recipients as compared to those formed in young animals, suggesting that the aged tissue microenvironment is stimulatory for the growth of normal cells[196]. Since it has been shown by several reports that CR delays the aging process[1,48,49], we tested whether long-term CR exerted any effect on the growth promoting ability of the aged liver microenvironment. To this end, we used a similar approach to the one in the study mentioned above, i.e. a syngeneic, orthotopic transplantation system based on the DPPIV marker enzyme[196]. Briefly, normal hepatocytes isolated from a $DPPIV^+$ donor were injected into the liver of old $DPPIV^-$ recipients fed either AL or 30% CR diet throughout life. Transplanted cells expressing the DPPIV marker were detected through a histochemical reaction that generates an orange precipitate in cells expressing the DPPIV enzyme activity (figure 2).

The growth of donor-derived cells was evaluated following transplantation (Tx) into 22 month-old recipients fed either AL or a CR diet. Clusters of DPPIV-expressing cells were evaluated 3 months post-Tx. Each cluster was reconstructed in 3D through the analysis of serial sections.

Figure 1 shows the percentage distribution of clusters size in the two groups. The number of single and double cell clusters did not differ between AL and CR-fed animals (figure 1A). However, differences became apparent when clusters of larger size were computed. As reported in figure 1B, AL fed rats displayed a higher

percentage of clusters ranging from 5 to 10 cells than rats exposed to CR diet (10.0% vs. 4.4%). Furthermore, clusters comprising more than 10 cells were 8 times more represented in AL vs. CR groups (3.2% vs. 0.4%). Clusters originating from transplanted cells in AL and CR group are reported in figure 2.

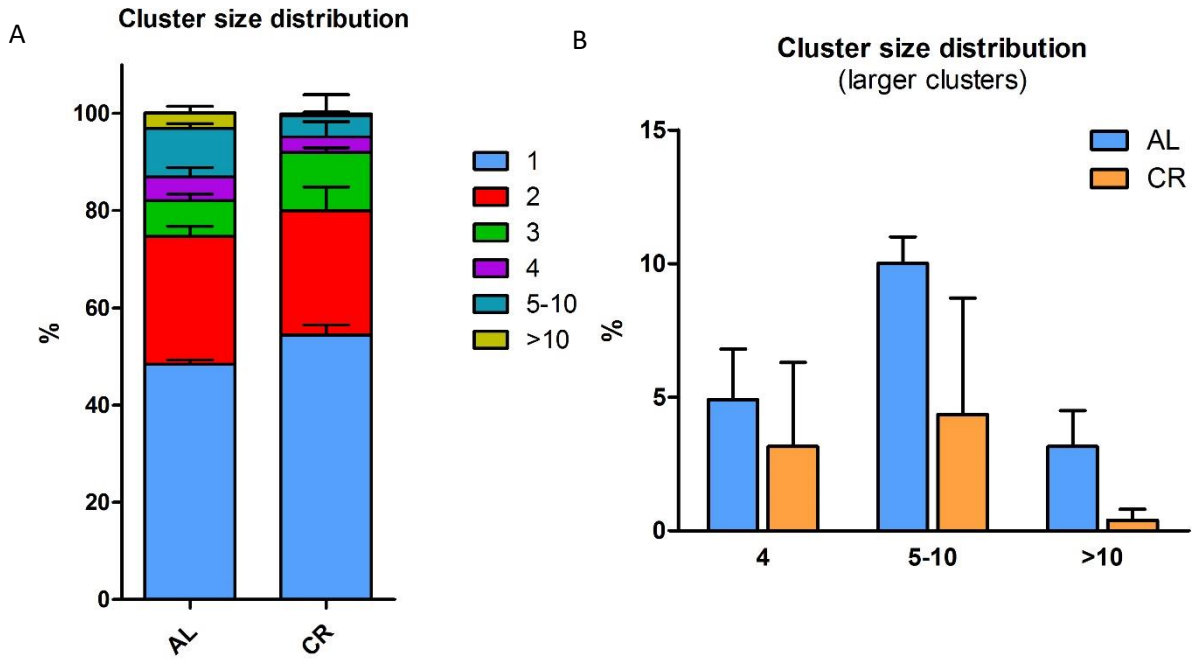

Figure 1. A and B show the percentage of clusters originating from DPPIV$^+$ transplanted cells according their size in AL and CR animals. Each cluster was 3D reconstructed (see Material and methods for details) and number of hepatocytes counted. Bars of the histogram report the frequency of each class size expressed as percent of the total number of clusters. Data are mean ± SE.

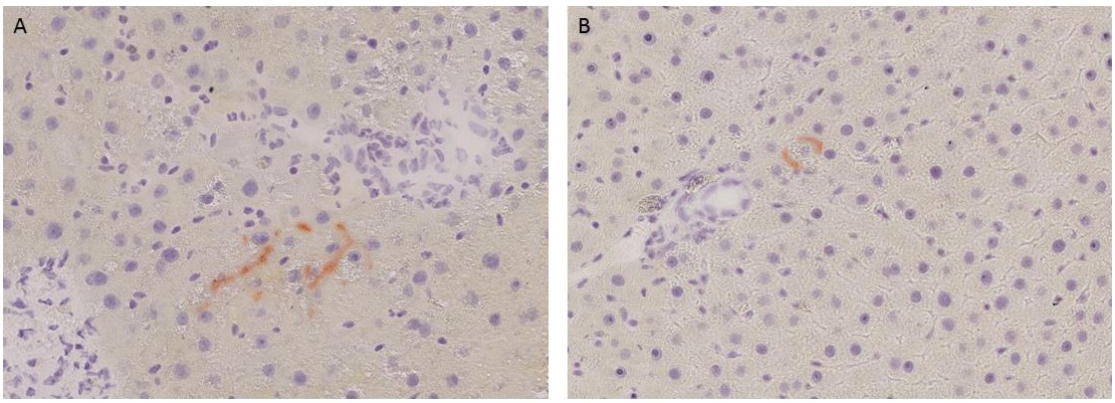

Figure 2. Clusters originating from transplanted normal DPPIV$^+$ hepatocytes (red) in AL (A) and in CR (B) rats.

4.2 Body weight and food consumption after long-term feeding of CR diet.

Rats exposed to CR were given an amount of food corresponding to 70% of daily dietary intake of the AL fed (control) group. The CR diet was started at 8 weeks of age. Food consumption was monitored weekly in the AL fed group and this value served as a reference to calculate the 70% level for the CR group. The average food consumption throughout the experiment in AL and CR groups is reported in figure 3C. Growth curves of AL and CR animals were very well separated after one month from the beginning of the experiment (figure 3A). Details of growth during the first 8 weeks of the experiment are reported in figure 3B indicating that the body weight (BW) of CR animals was significantly different from AL group after the first week of diet. AL group showed a fast BW increase until month 5 (figure 3A). They continued to gain weight until month 13. Afterwards their BW was quite stable with little variation until the end of the experiment. In contrast, CR rats showed a slower growth compared to AL group. They showed a BW gain until month 5. Then, their BW became quite stable until the end of the experiment. The average final BW was 433±27 and 304±17 in AL and CR group, respectively. Food intake/BW ratios were significantly different between two groups during the first 6 months of experiment (figure 3D). Afterwards, as BW started to stabilize, the ratios became identical.

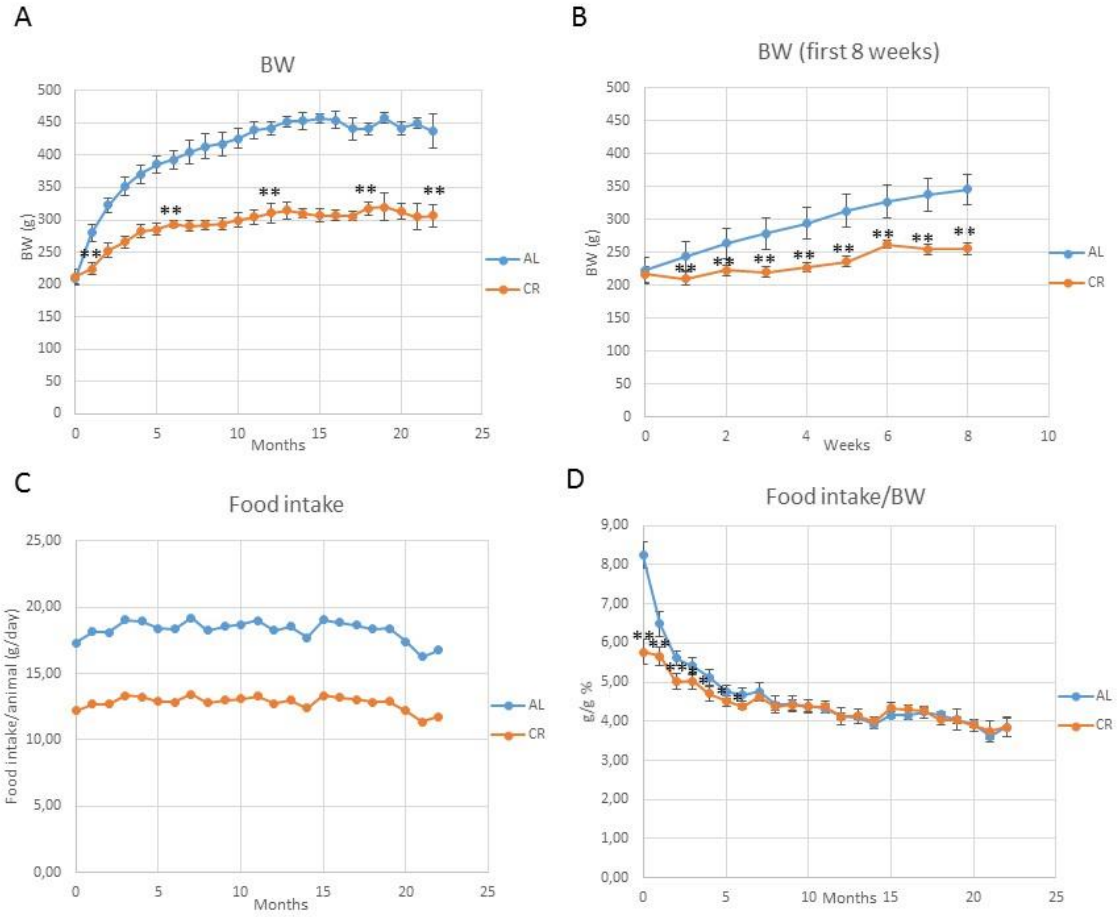

Figure 3. Growth curves throughout the experiment (panel A), growth curves during the first 2 months of experiment (panel B), food consumption (panel C) and food intake/BW (panel D) in rats fed AL or CR diet. * and ** Values are significantly different from controls (AL), *p <0.05 and **p < 0.001

4.3 The growth of transplanted nodular hepatocytes in the liver of rats exposed to long term CR.

Previous results from our laboratory have indicated that the aging process fuels carcinogenesis via alterations imposed on the tissue microenvironment. In fact, it was found that upon Tx of pre-neoplastic hepatocytes in both young and old recipients, the size of donor-derived cell clusters was significantly greater in old rats; furthermore, only old animals displayed visible hepatic nodules and tumors 8 months post-Tx [44]. In light of this observations and based on results presented in the preceding paragraphs, we then addressed the core aim of this thesis. The hypothesis was tested that the delaying effect of CR on carcinogenesis might be exerted, at least in part, via modulation of the neoplastic-prone microenvironment associated with aging. To this end, nodular (pre-neoplastic) hepatocytes isolated from a $DPPIV^+$ donor were transplanted into old rats lacking DPPIV and fed either AL or a 70% CR diet for 18 months (detailed experimental protocol is reported in figure 4). Importantly, both groups were under AL feeding at the time of Tx and they were continued on this dietary regimen until the end of the experiment. All animals were killed 3 months after Tx.

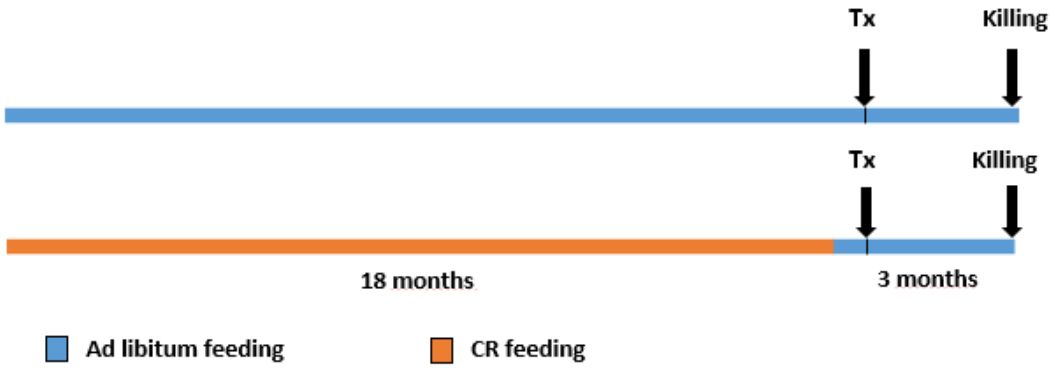

Figure 4. Experimental protocol. CR diet was fed for 18 months and then rats in this group were shifted to AL feeding.

Livers were removed and macroscopically examined. Small lesions ranging from 0.5 to 2.5 mm in diameter were seen in both groups. However, the total number of nodules was higher in AL group (table 3). Larger lesions expressed the DPPIV marker enzyme (figure 5A), indicating that they originated from transplanted cells. Dual histochemical/immunohistochemical staining for DPPIV and glutathione-S-transferase, placental form (GSTP, figure 5C) confirmed the pre-neoplastic nature of donor-derived focal lesions.

Table 3. Incidence of hepatic lesions in old animals transplanted with pre-neoplastic hepatocytes following administration of AL or CR diet.

Diet	Animals with lesions	Total No. of nodules	Size range
AL	4/5	11	1-2 mm
CR	2/5	2	0.5,2.5 mm

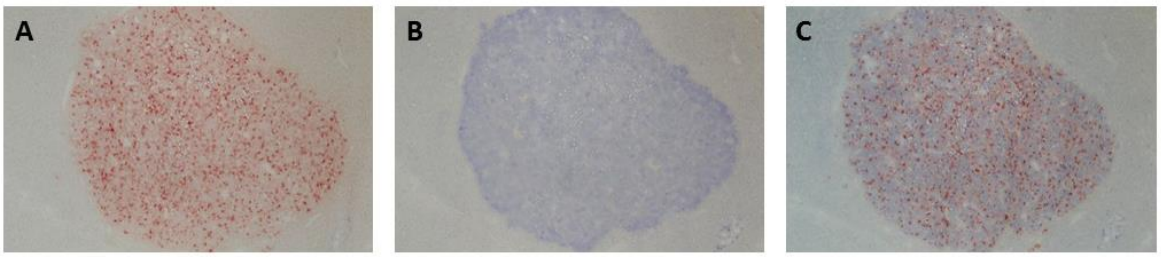

Figure 5. Large hepatocyte cluster of donor origin expressing DPPIV (orange-rust, panel A). Serial sections was stained for GSTP (blue) (panel B) confirming the pre-neoplastic nature of the lesion. Panel C shows the double-staining with DPPIV and GSTP. Magnification: 40X.

Figure 6 shows the size distribution of donor-derived clusters in AL and CR fed rats. Each cluster was reconstructed in 3D through the analysis of serial sections. AL group displayed a higher number of cluster larger than 5 cells (64 vs. 18 clusters). Clusters ranging from 5 to 10 cells represented 11.1% of total clusters in AL vs. 4.6% in CR animals. Clusters ranging from 10 to 50 cells represented 7.7% in AL vs. 0.8% in CR, and finally those comprising more than 50 cells represented 1.3%

of total clusters in AL vs. 0.4% in CR animals (figure 6B). Figure 7 shows a representative donor derived clusters from transplanted nodular hepatocytes in AL (A) and in CR (B) animals.

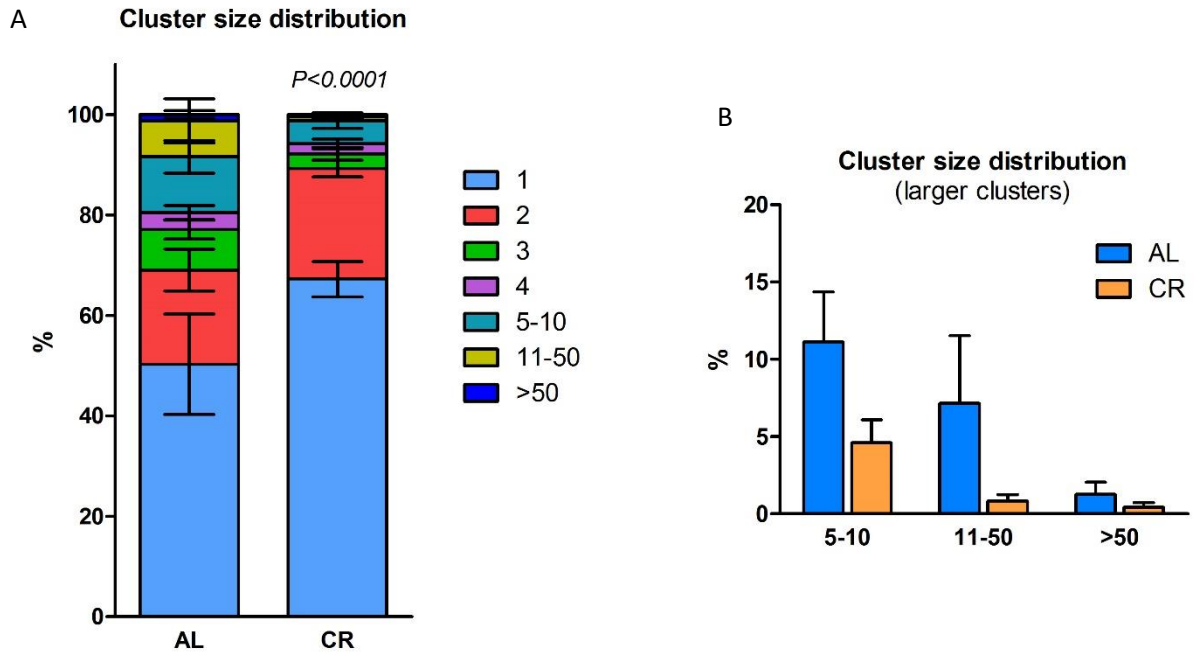

Figure 6. Size distribution of clusters originating from nodular hepatocytes transplanted in animals fed AL or CR diet. Panel A: clusters of any size; panel B: clusters of larger size (from 5 cells to bigger than 50 cells). Each cluster was 3D reconstructed and number of hepatocytes was counted. Bars of the histogram report the frequency of each class size expressed as percent of the total number of clusters. Data are mean ± SE. Frequency distribution, calculated via Chi square for contingency table, was significantly different from control (AL), $p<0.0001$.

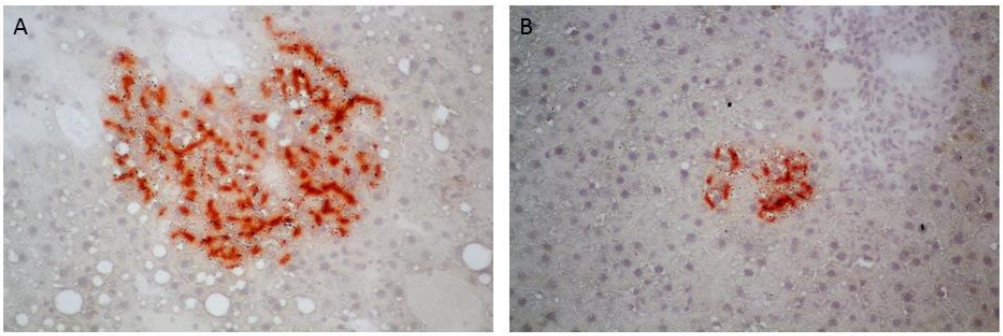

Figure 7. Representative example of donor derived hepatocyte clusters expressing DPPIV (orange-rust) in animals from AL (A) or CR (B) fed groups. Magnification: 100X.

4.4 CR and “spontaneous” endogenous nodules.

It is noteworthy that the beneficial effect of CR on carcinogenesis was also confirmed by analyzing the frequency of “spontaneous” endogenous GSTP-positive lesions, which is a common finding in the Fischer 344 strain of rats [197]. While all animals in both groups displayed GSTP-positive foci, the size of these lesions (which were negative for the DPPIV marker) was significantly lower in animals given the CR diet (figure 8 and 9). These findings are in line with previous reports showing that long-term CR delays the development of spontaneous cancers in several models, from rodents to monkeys [49,51,53].

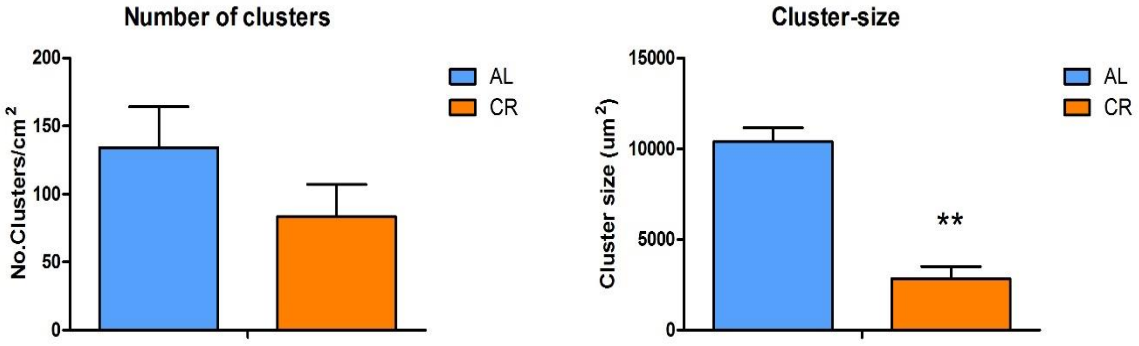

Figure 8. The incidence of endogenous GSTP-positive foci in animals fed AL or the CR diet. Panel A: number of GSTP positive foci in AL and CR animals; panel B: size of GSTP positive clusters in the two groups. Data are mean ± SE. ** Significantly different from control (AL), p<0.0001.

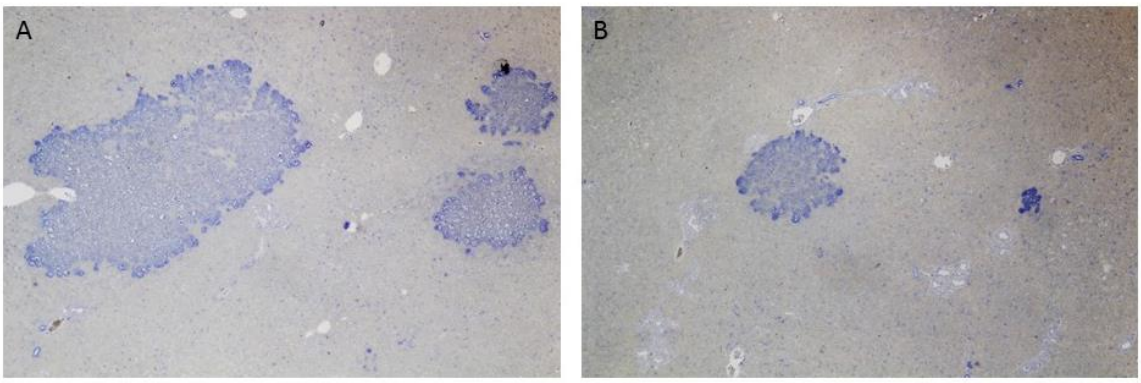

Figure 9. Immunohistochemical staining for GSTP on paraffin embedded liver sections. Panel A: AL group; panel B: CR group. Magnification: 40X.

4.5 Food consumption, body weight and liver weight after long-term feeding of CR diet.

As in the previous study, food consumption and growth curves were recorded throughout the experiment and are reported in the following figures. Data were similar to those presented above.

After 18 months on CR diet, rats were transferred to AL feeding. As a result their body weight increased till the end of the study, although it was significantly lower than that of control group (Figure 10A). The average final BW was 473±28g and 364±36g in AL and CR group, respectively.

We also calculated the food intake/BW ratio in both groups. We observed that during the first 3 months of experiment the ratio was significantly decreased in CR animals. Afterwards, as the BW started to stabilize the ratio became similar to that in AL animals. The ratio became again significantly different after 18 month of CR, when CR animals were transferred to AL diet. At this point, the ratio raised significantly in CR rats (Figure 10D).

Liver weight (LW) was significantly reduced in CR animals even if they were re-feed ad libitum diet in the last three months of the experiment. LW/BW ratio was significantly lower in CR (3.0±0.2% vs. 3.5±0.3% in CR and AL, respectively) (figure 11).

Figure 12 shows the H&E staining of liver sections from AL and CR animals (panel A and B, respectively). Only AL fed animals presented pre-neoplastic lesions, known as ground glass foci (panel C and D, at higher magnification).

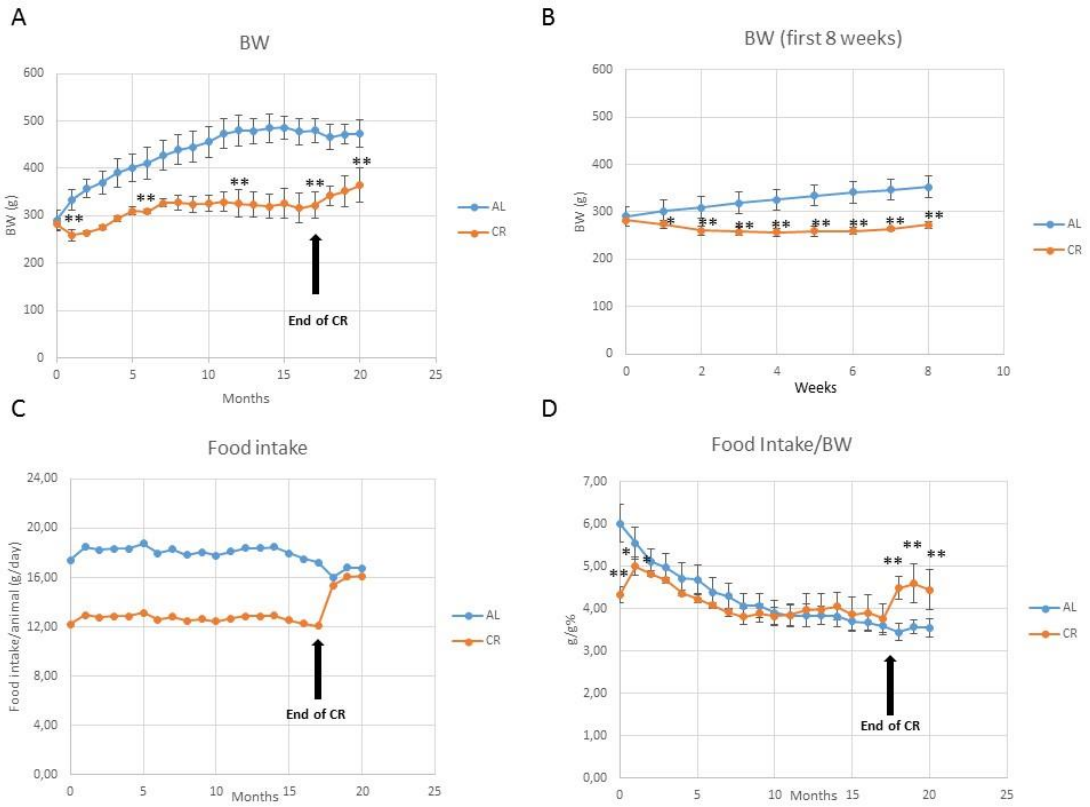

Figure 10. Growth curves throughout the experiment (panel A), detail of growth curves during the first 2 months of experiment (panel B), food consumption (panel C) and food intake/BW ratio (panel D) in rats fed AL or CR diet. From 18mo to the end of the experiment, CR animals were transferred to AL diet (black arrow). * and ** significantly different from control (AL), * $p < 0.05$, ** $p < 0.001$.

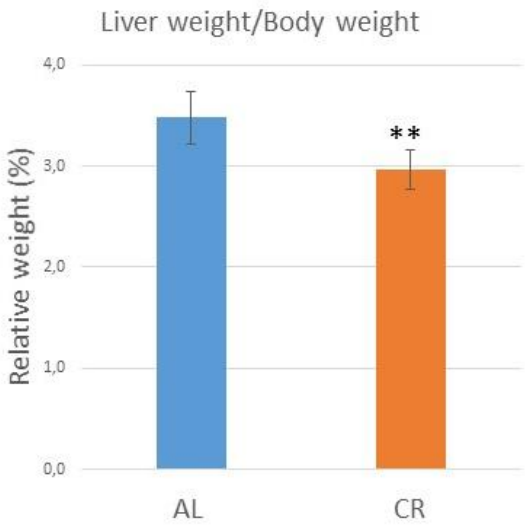

Figure 11. LW/BW in rats fed AL or CR diet. **Values are significantly different from control, $p<0.01$.

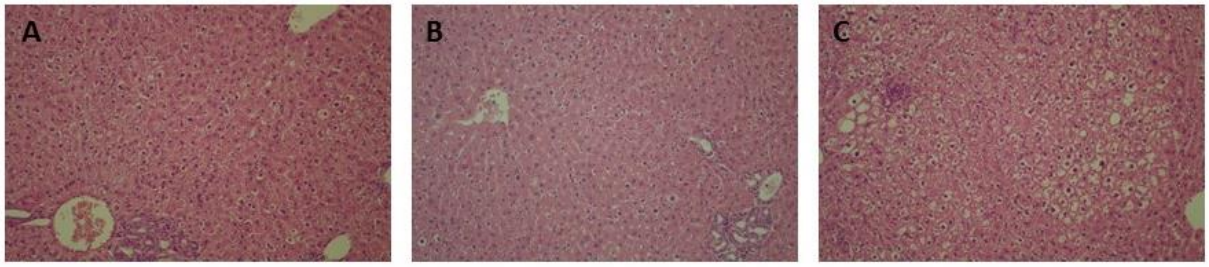

Figure 12. H&E staining of liver sections from AL (panel A) and CR (panel B) rats. Panel C shows a large “ground-glass” hepatocyte nodule, a common finding in AL fed animals.

4.6 Possible mechanisms mediating the modulatory effect of CR on the age-associated, neoplastic-prone tissue microenvironment.

4.6.1 Cell senescence

As referred to in the Introduction, senescence has been defined as the irreversible cell-cycle arrest that manifests in proliferative-competent cells in response to various cellular stresses/stimuli, such as DNA damage, telomere shortening, mitochondrial dysfunction [143,144]. It is now well established that senescence may exert two opposing roles in carcinogenesis. In fact, it has been regarded as a fail-safe mechanism to limit the risk of neoplastic transformation following genotoxic insult. At the same time, cellular senescence has been shown to fuel neoplastic process, possibly via SASP, which includes growth factors, pro-inflammatory cytokines and matrix-remodeling enzymes [15,146]. Cell senescence is considered as one of the hallmarks of aging. Since CR is known to delay the aging process, we sought to determine whether long-term CR has any effect on the emergence of cellular senescence in liver, given the potential role of this phenotype in the context of the tissue microenvironment. Previous reports have shown that short-term CR reduces the expression of some senescence markers in liver, small intestine and

kidney in mice. A similar result was obtained in rat heart after long-term CR started at 8months of age until 30months [180–182]. Thus, we analyzed the expression of some senescence markers in liver of rats after long-term exposure to CR.

SA-beta-gal

Senescence-associated beta galactosidase is among the most commonly used senescence markers. SA-beta-gal is a lysosomal enzyme normally active at pH 4. Its detection at suboptimal pH 6 is selective for senescent cells, reflecting the increased biogenesis of lysosomes associated with senescence [154,155]. Histochemical staining revealed the expression of SA-beta-gal in both groups. This is not surprising, since at the time of killing animals were about 2 years old. However, we did not detect any significant difference in SA-beta-gal expression between AL and CR animals (figure 13).

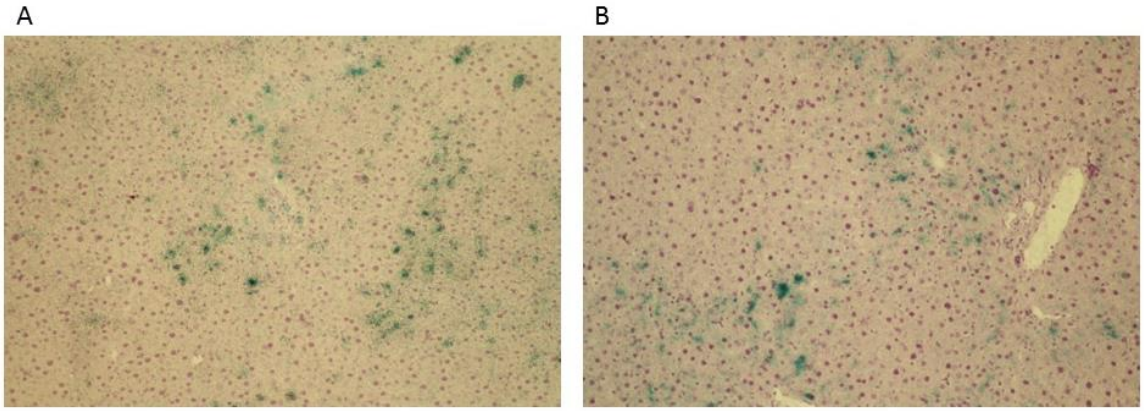

Figure 13. SA-beta-gal staining (blue) in liver from AL (A) and CR (B) animals.

DNA damage foci

DNA damage is often associated with cellular senescence. Replication errors, telomeres shortening, genotoxic stress, ionizing radiation induce the DNA damage

response (DDR), recruiting on the site of the damage ATM, which causes the phosphorylation of histone H2AX; this in turn facilitates the assembly of checkpoints and DNA repair factors such as 53BP1 (DNA damage foci) [143,159]. Accordingly, we tested if long-term CR exerted any effect on the appearance of such foci. However, no significant differences were detected in the expression of DDR markers between AL and CR groups, as showed by immunofluorescence staining for H2AX (figure 14) and by western blot (WB) analysis on 53BP1 (figure 15).

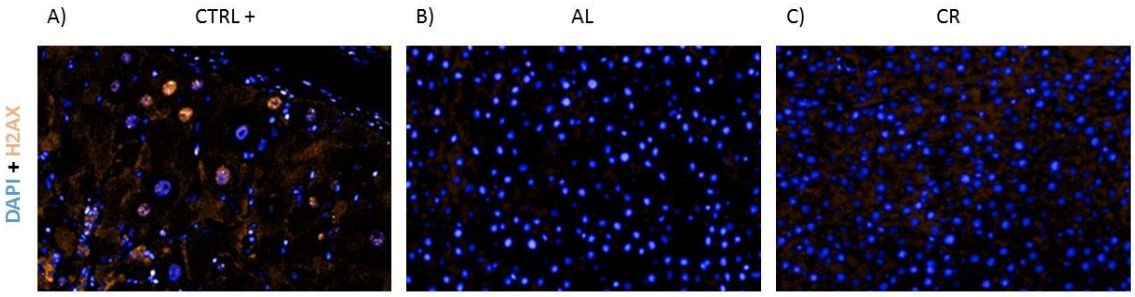

Figure 14. Immunofluorescence staining for H2AX. Panel A represents a positive control liver section (orange nuclei) from a rat liver treated with retrorsine [198] while panels B and C represent liver samples obtained from AL and CR rats, respectively. No positive staining was observed in either group.

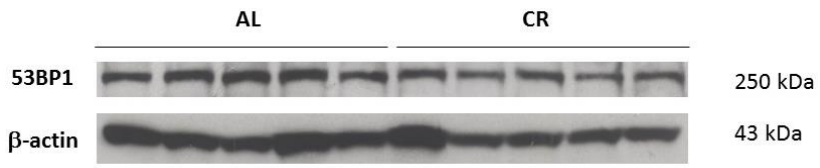

Figure 15. WB analysis of 53BP1 in AL and CR rats. WB was performed on nuclear proteins and nuclear βactin was used as control.

Cell-cycle inhibitors

Cell senescence is often associated with up-regulation of cell-cycle inhibitors such as p16, p21, p27 and p53, which regulate progression through cell-cycle. Most

frequently, senescent cells over-express p21 and p16, which are components of the tumor-suppressor pathway governed by p53 and RB [15,143,156]. When liver samples obtained from both AL and long-term CR groups were analyzed, no significant differences could be detected in the expression of any of these putative markers of cell senescence. Thus, immunohistochemistry on paraffin-embedded liver sections showed no sizeable differences between AL and CR groups in the number of p21 (figure 16) or p27 (figure 17) positive hepatocyte nuclei. Results were also confirmed by WB analysis (figure 18 and 19). Similar findings were obtained for p16 and p53, i.e. no changes in their expression between AL and CR animals (figure 20).

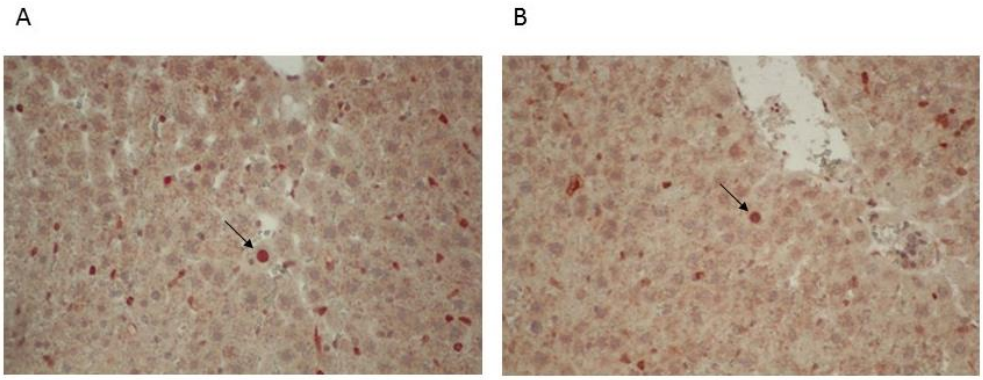

Figure 16. Immunostaining for p21 (nuclear staining, red) in liver from AL (A) and CR (B) animals.

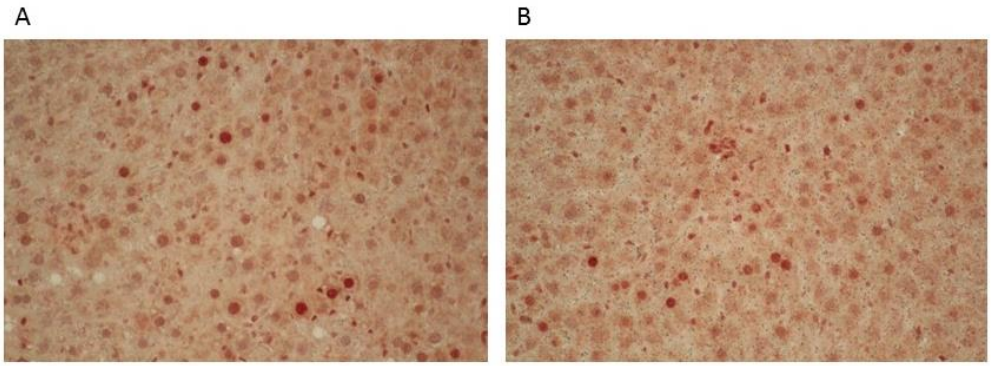

Figure 17. Immunostaining for p27 (nuclear staining, red) in liver from AL (A) and CR (B) animals.

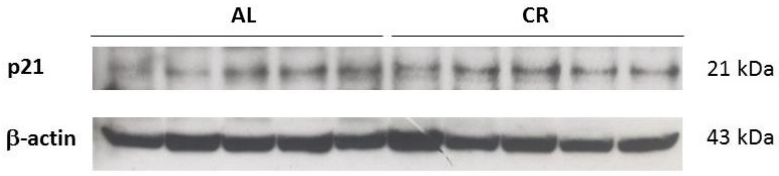

Figure 18. WB analysis of p21 in AL and CR rats. WB was performed on nuclear proteins and nuclear β-actin was used as control.

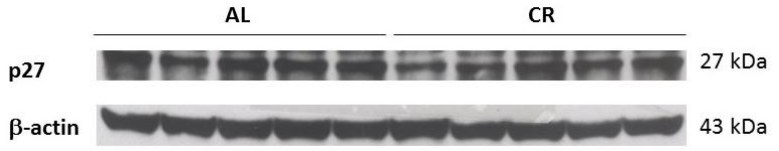

Figure 19. WB analysis of p27 in AL and CR rats. WB was performed on nuclear proteins and nuclear β-actin was used as control.

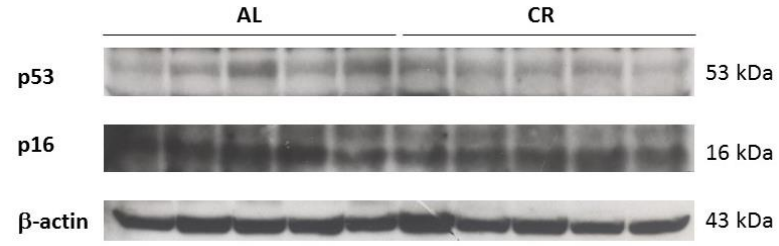

Figure 20. WB analysis of p53 and p16 in AL and CR rats. WB was performed on nuclear proteins and nuclear β-actin was used as control.

The senescence-associated secretory phenotype (SASP)

Among the features of senescent cells is the high metabolic activity, and SASP is one facet of this phenotype. SASP comprises numerous cytokines, growth factors and proteases that influence the tissue microenvironment allowing to spread the effects of senescence to the neghboring cells and to promote/inhibit tumorigenesis depending on the context [162]. IL-6 is the most well characterized and the major component of SASP. IL-6 is a cytokine associated with obesity and insulin resistance, and circulates in multiple glycosilated forms ranging from 22 to 27 kDa in size. IL-6 circulates at high levels in the bloodstream, and as much as one third of IL-6 originates from the adipose tissue. Both expression and circulating levels of IL-

6 have been shown to decrease with weight loss [9]. Previous results from our reasearch group have shown that both mRNA and IL-6 protein were increased in the liver of aged rats compared to young controls, suggesting that IL-6 may in part play a role in driving the carcinogenesis process in the aged liver microenvironment [44]. Accordingly, in the present work we sougth to determine whether the modulatory effect of CR on the emergence of the neoplastic-prone microenvironment associated with aging is exerted, at least in part, via changes in SASP expression. We focused mainly on IL-6, which is one of the best characterized SASP components. To this end, we analysed IL-6 mRNA expression in liver from AL and CR rats. qRT-PCR revealed no significant changes in terms of mRNA expression between the two groups, although a trend towards reduced levels in CR animals was observed (figure 21A). However, WB analysis showed that IL-6 expression was significantly reduced in the liver of CR rats compared to AL group (figure 22A and B). Because IL-6 circulates mainly in the bloodstream, we performed also ELISA in serum from AL and CR animals. No significant differences were seen in IL-6 in serum between two groups; once again, a trend towards a decrease in long-term CR group was seen (figure 21B).

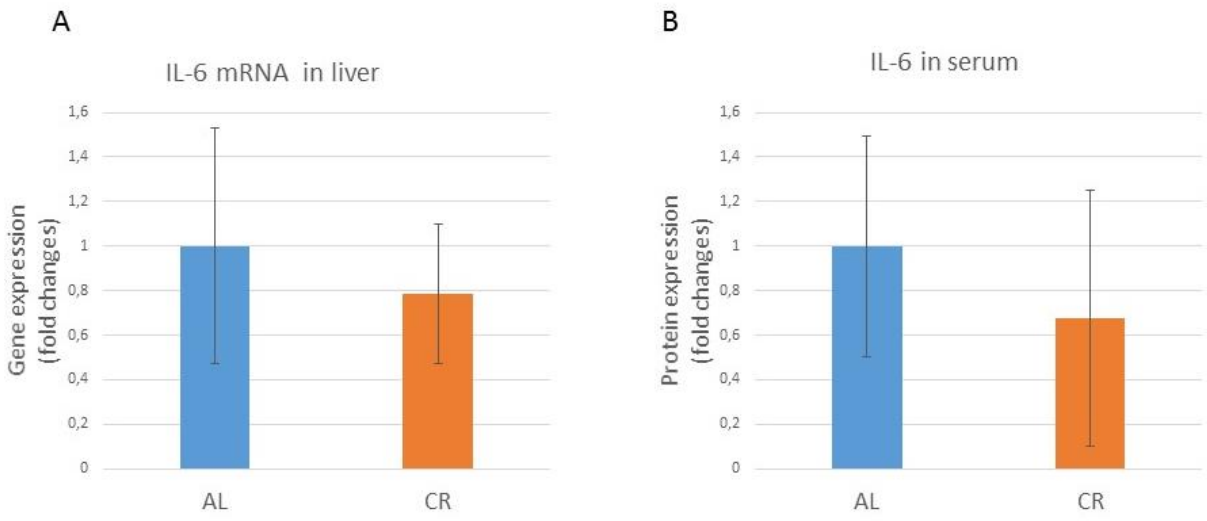

Figure 21. IL-6 mRNA expression in liver (A) and IL-6 expression in serum (B) in AL and CR rats. Both mRNA and protein expression were normalized to AL group. No significant differences were seen between two groups in both parameters analyzed.

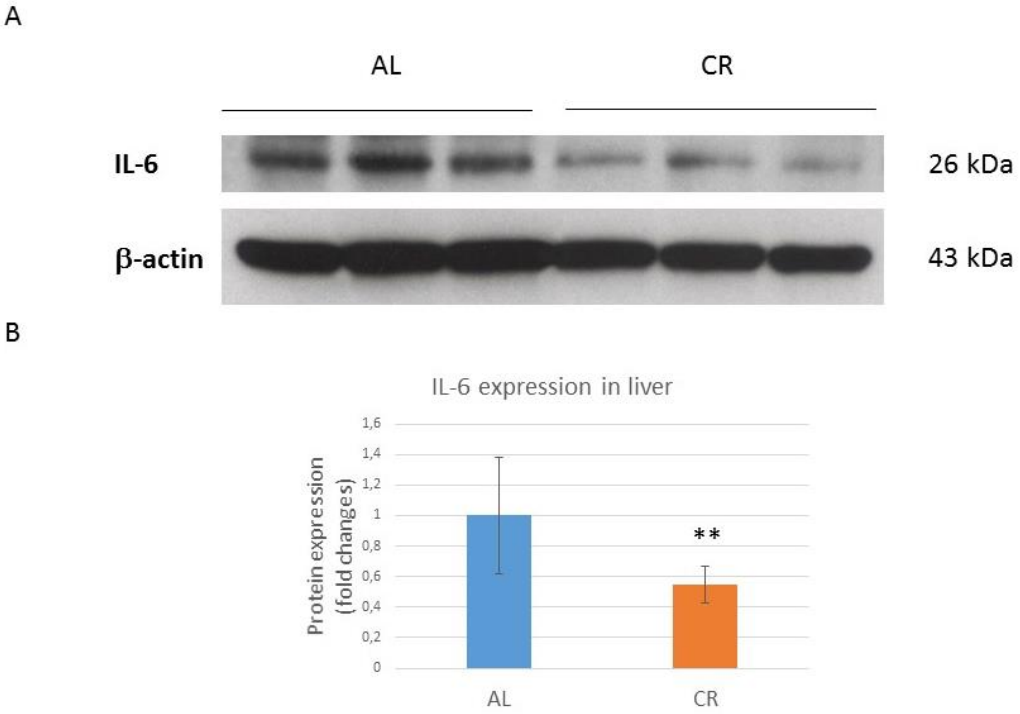

Figure 22. WB analysis of IL-6 in liver from AL and CR rats was performed on cytoplasmic proteins and β-actin was used as control. IL-6 was significantly reduced in CR animals, $p < 0.01$.

4.6.2 Nutrient-sensing pathways

Long-term CR increases SIRT1 expression in liver

The nuclear sirtuin SIRT1 is considered one of the putative mediators of the anti-aging effect of CR [66]. Indeed, CR has been shown to increase SIRT1 levels in several tissues, from brain to liver [87,89]. SIRT1 plays several roles in cellular biology. First, it senses the nutritional state of the cell, since its deacetylase activity depends on NAD^+ availability. During CR, it contributes to maintain euglycemia and promote efficient energy utilization [77]. Second, SIRT1 is involved in the oxidative/genotoxic response through deacetylation of substrates such as FOXO1 and FOXO3 that in turn promote transcription of genes involved in the antioxidant response such as MnSOD. Thus, SIRT1 contributes to maintain genomic stability. In addition to this, it also suppresses NFkB pathway that promotes inflammation. Moreover, SIRT1 has been shown to decrease in old tissues including liver, where one of the most important age-associated alterations is the reduced ability to regenerate after partial hepatectomy. SIRT1 seems to play a role also in liver regeneration after injury/partial hepatectomy. Indeed, it sustains the levels of glucose and triglycerides needed for the regenerative response of the liver. SIRT1 loss in old liver seems to be mediated by a complex formed by HDAC1 and C/EBPb, whose levels have been shown to be increased in old liver [88].

Due to the central role of SIRT1 in mediating some of the effects of CR and in controlling some pathways that may affect the tissue microenvironment, such as inflammation, it appeared reasonable to ask whether long-term CR might affect SIRT1 levels in rat liver under the experimental conditions employed in these studies. Since SIRT1 localizes mainly in the nucleus, we performed WB analysis on

nuclear proteins. As shown in figure 23, it was found that long-term CR increased significantly SIRT1 levels in liver, compared to the very low levels detected in AL fed animals (figure 23).

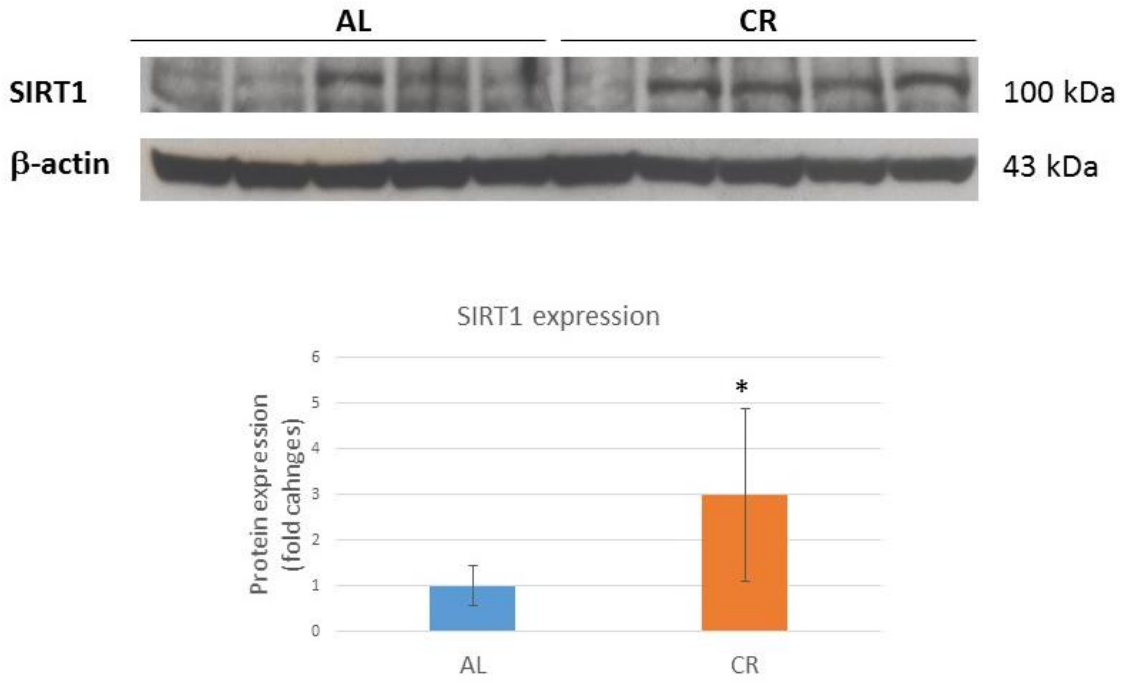

Figure 23. WB analysis of SIRT1. WB was performed on nuclear proteins and SIRT1 expression was normalized to β-actin. * Significantly different from control (AL), * $p < 0.05$.

Long-term CR increases FOXO1 expression in liver

CR exerts its beneficial effects through several mechanisms, and FOXO factors appear to be involved in these pathways [92]. FOXOs play an important role in the maintenance of the metabolic homeostasis and in protecting from oxidative stress during aging. FOXOs are downstream targets of IIS and their transcriptional activity is regulated by PI3K/Akt, which in response to insulin phosphorylates FOXOs promoting their export from the nucleus to the cytoplasm resulting in repression of their transcriptional activity [93-93]. FOXOs are also downstream targets of SIRT1. Indeed SIRT1 deacetylates FOXOs in response to oxidative stress, potentiating FOXOs ability to detoxify ROS and protect cells from oxidative damage. It has been shown that FOXO1 mediates the antioxidant effects of CR, through repression of NFkB, and it seems to be involved in its antineoplastic effects as well [97,98]. In light of this information, it was important to determine whether long-term CR affected FOXO1 expression in rat liver, as part of its modulatory effect on the aged tissue microenvironment. Once again, we tested FOXO1 expression on nuclear proteins, since the active form of FOXO1 is present in the nucleus. WB analysis revealed that long-term CR significantly increased FOXO1 expression in rat liver compared to AL fed animals, where FOXO1 was expressed at very low levels (figure 24). This result was consistent with the increased levels of SIRT1, given the known connections between these two pathways.

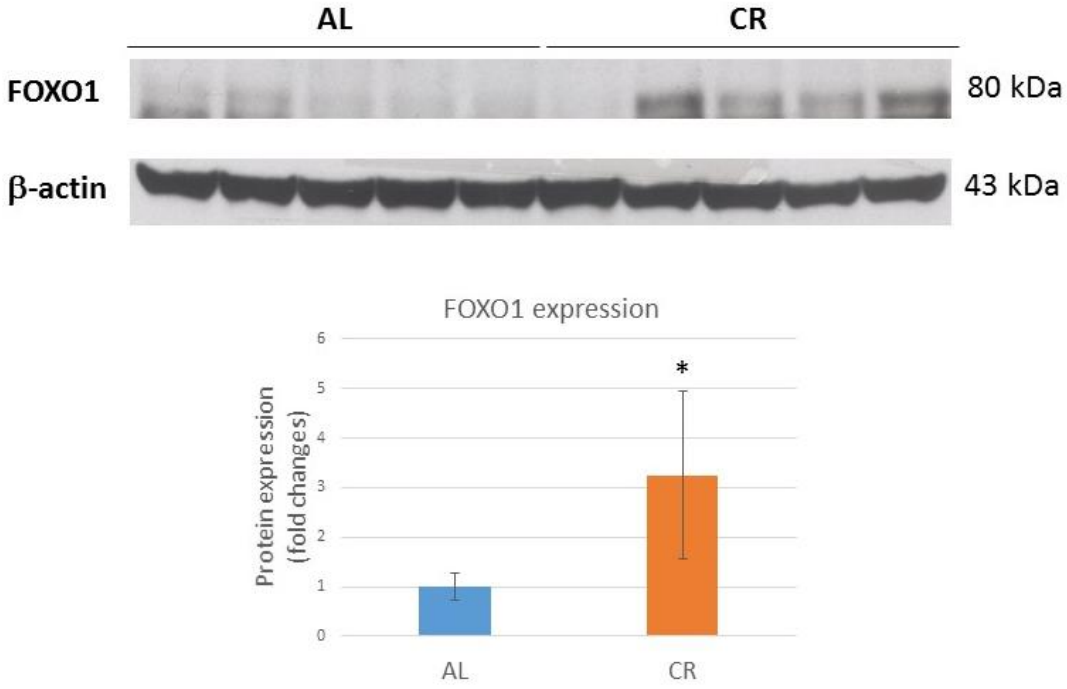

Figure 24. WB analysis of FOXO1 performed on nuclear proteins. FOXO1 expression was normalized to nuclear β-actin and showed to be significantly increased in CR rats ($p < 0.05$).

Long-term CR reduces HDAC1 and C/EBPβ expression in liver

The ability of the liver to regenerate after injury and surgical resection decreases in old age [199]. SIRT1, whose levels are deceased in old liver, seems to play a crucial role in maintaining the regenerative capacity of liver. Two factors, the histone deacetylase 1 (HDAC1) and C/EBPβ have been shown to increase in liver of old mice and to repress the ability to reconstitute the liver mass after PH. Indeed, it has been shown that HDAC1, in addition to forming a complex with C/EBPα which represses liver regeneration, also associates with C/EBPβ [88,199]. This complex binds to SIRT1 promoter region suppressing its role in sustaining glucose and triglycerides supply after PH, and this results in an impaired regenerative capacity in old liver [88]. It was therefore important to test if long-term CR may affect the expression of HDCA1 and C/EBPβ, which have already been shown to be up-regulated in old liver. We performed WB on nuclear proteins, as reported previously by Timcenko et al. [88]. Our results showed a decrease of both HDAC1 and C/EBPβ in livers of CR animals compared to AL fed group (figure 25), suggesting that long-term CR may improve the regenerative capacity of old liver, whose hepatocytes would preserve their functional proficiency and proliferative competitiveness in presence of altered/pre-neoplastic cells.

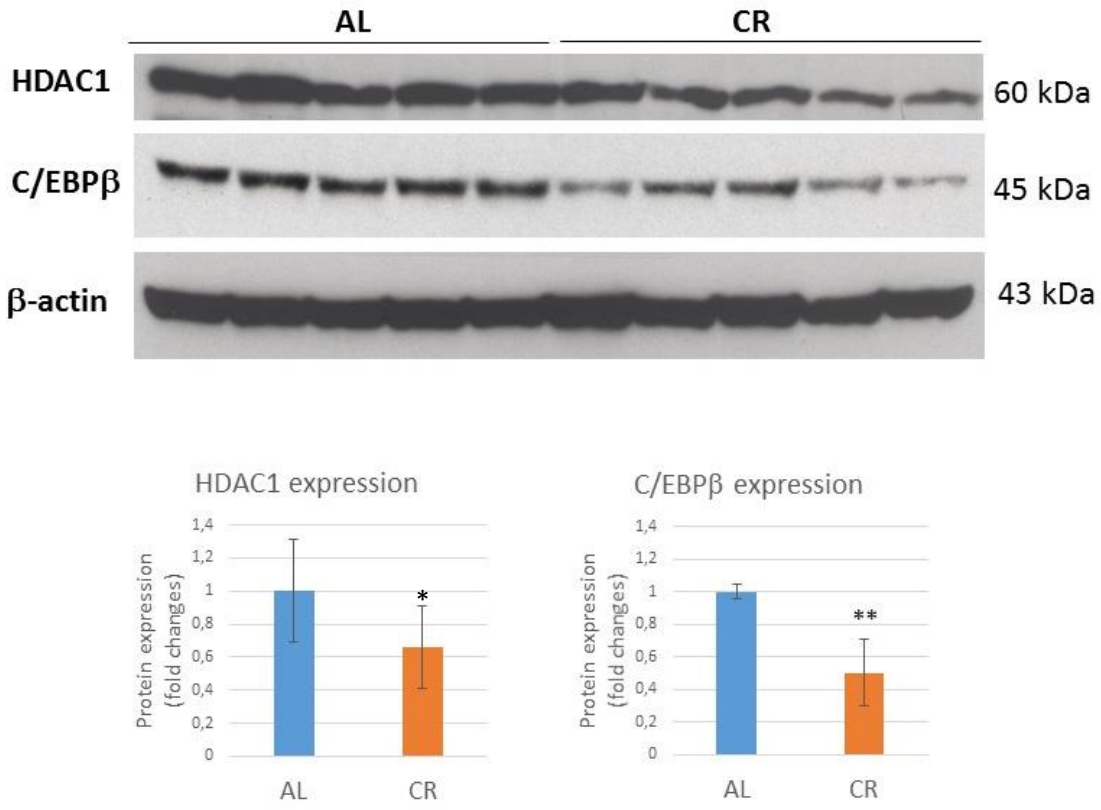

Figure 25. WB analysis of HDAC1 and C/EBPβ. WB was performed on nuclear proteins. Either HDAC1 and C/EBPβ expression was normalized to nuclear β-actin. * and ** Significantly different from control, *$p < 0.05$ and **$p < 0.001$.

mTOR pathway

The mammalian target of rapamycin (mTOR) pathway has been consistently linked to CR. In fact, when nutrient levels fall, mTOR activity falls and consequently translational levels are reduced. CR has been shown to decrease TOR activity in several experimental models, from worms to mice [100,104]. CR can suppress mTOR activity through different mechanisms. First, CR increases AMPK activity, which in turn is a negative regulator of mTOR pathway. Second, CR decreases insulin/IGF-1 signalling pathway, which, through PI3K/Akt, has been shown to be an upstream regulator of mTOR [105]. In our study, we investigated whether long term-CR might affect mTOR pathway in liver. However, western blot analysis revealed variable but no significant differences in the levels of this protein between AL and CR fed groups (figure 26).

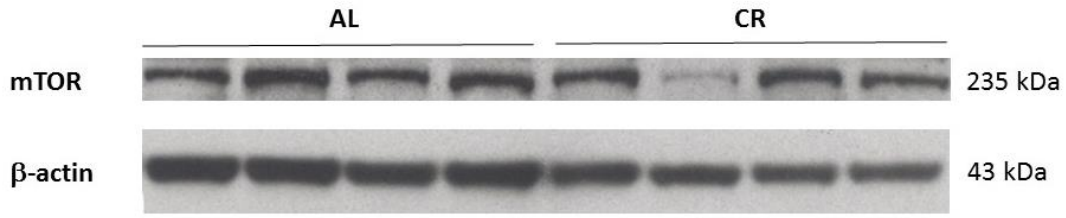

Figure 26. WB analysis of mTOR. WB was performed on cytoplasmatic proteins and β-actin was used as control.

4.6.3 Metabolomic analyses in liver and serum of rats exposed to long-term CR.

In order to gain further insights into possible metabolic mediators of CR effects on liver microenvironment, we performed a metabolomic analysis of phenotypic changes associated with long-term exposure to CR using GC-MS on both serum and liver samples collected at different time points during the experiment: 10 months of CR, 22 months of CR and 18 months of CR followed by AL re-feeding. The chemical silylation procedure used in this study for the determination of low molecular weight polar metabolites allows the improvement of metabolite volatility, thermal stability, detectability while on the other hand affecting the coverage of the detected metabolome. Biological samples were regularly interconnected and only the multivariate approach is able to define these systems, taking into consideration, at the same time, variables and their relationship. Two data matrices were generated through the GC-MS data processing. The plasma matrix included 26 samples (10 samples at 10 months of CR, 6 samples at 22 months of CR and 10 samples at 18 months of CR followed by AL feeding) and 46 metabolites, while liver matrix was composed of 20 samples (7 samples at 10 months of CR, 6 samples at 22 months of CR and 7samples at 18 months of CR followed by AL feeding) and 76 metabolites. Both matrices were normalized using the total area normalization. At first, an unsupervised statistical analysis (PCA) was performed without showing clusterization. In order to study the most discriminant metabolites for different membership classes, supervised analysis OPLS-DA was performed for both serum and liver samples at each time point. Analyses reported a good classificatory power with a high confidence degree. OPLS-DA validation parameters for each time point and matrices are reported in table 4. Upregulated and downregulated metabolites in serum of CR animals at different time points are reported in table 5, while score

plots are shown in figure 27. Upregulated and downregulated metabolites in liver of CR animals at different time points are reported in table 6, while S-plot are reported in figure 28. S-plots show a good separation of two groups according the diet at each time point considered, both in serum (figure 27) and in liver (figure 28).

Table 4. OPLS-DA validation parameters in serum and liver.

	SERUM			LIVER		
Time	**R2X**	**R2Y**	**Q2Y**	**R2X**	**R2Y**	**Q2Y**
10 mo CR	0,719	0,997	0,933	0,878	0,999	0,837
22 mo CR	0,546	0,960	0,608	0,687	0,999	0,935
18mo CR+AL	0,752	0,997	0,620	0,631	0,987	0,623

Table 5. Upregulated and downregulated metabolites in serum from CR vs AL animals at different time points.

10 mo CR		22 mo CR		18mo CR+AL	
Upregulated	**Downregulated**	**Upregulated**	**Downregulated**	**Upregulated**	**Downregulated**
Serine	Urea	Unknown 4	Palmitic acid	Glucose	Stearin
Threonine	Unknown 3		Glycine	Unknown 3	Ornithine
Stearic Acid			Inositol		
Unknown 1			Glucose		
Unknown 2					

Table 6. Upregulated and downregulated metabolites in liver from CR vs AL animals at different time points.

10 mo CR		22 mo CR		18mo CR + AL	
Upregulated	**Downregulated**	**Upregulated**	**Downregulated**	**Upregulated**	**Downregulated**
D-gluconic acid	Palmitic acid	L-serine	Palmitic acid	D-gluconic acid	Malic acid
Lactic acid	D-mannose	D-gluconic acid	Oleic acid	Unknown 6	Stearic acid
L-serine	Unknown 1			Unknown 7	Palmitic acid
Unknown 5	Unknown 6				Oleic acid

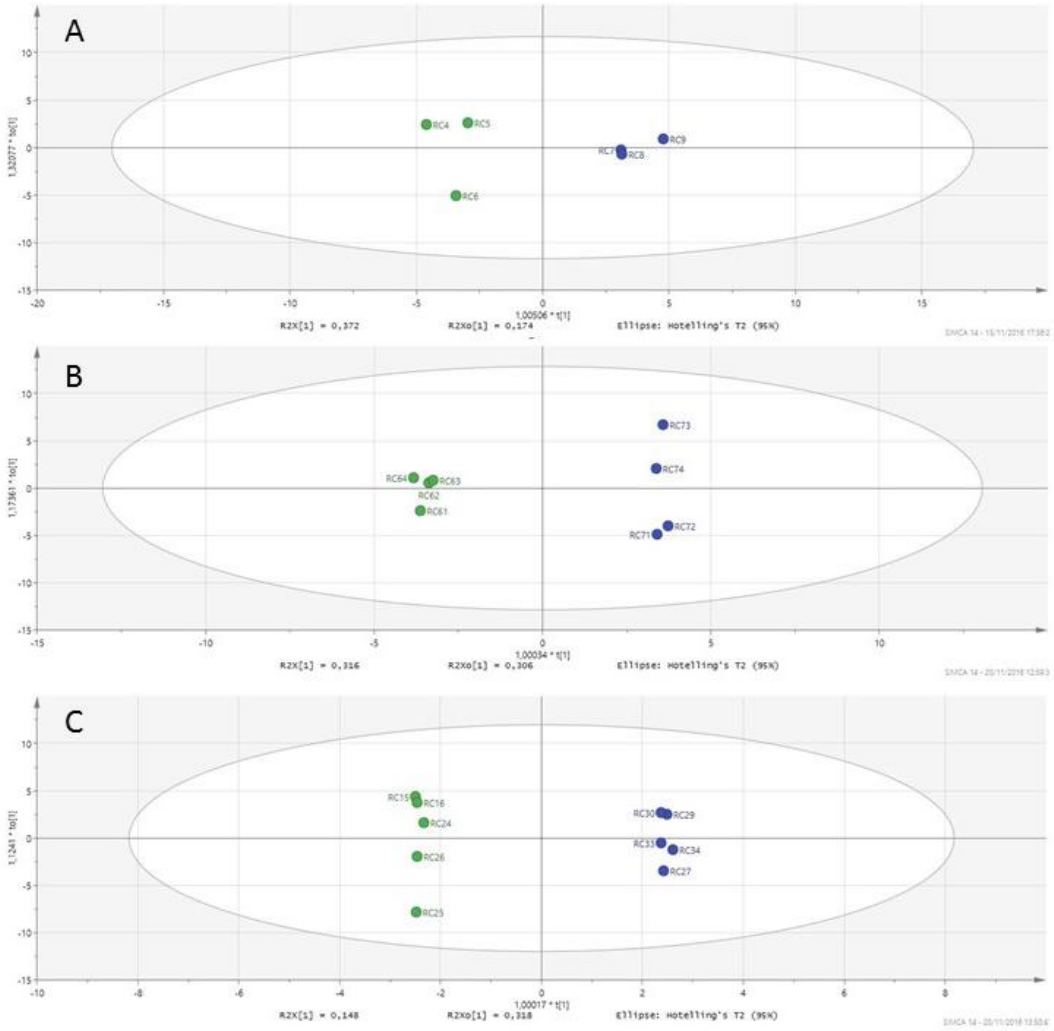

Figure 27. S-plot analyses of serum samples at different time points: 10mo CR (A), 22mo CR (B) and 18mo CR+AL (C). Green dots corresponds to AL animals, while blue dots are CR animals.

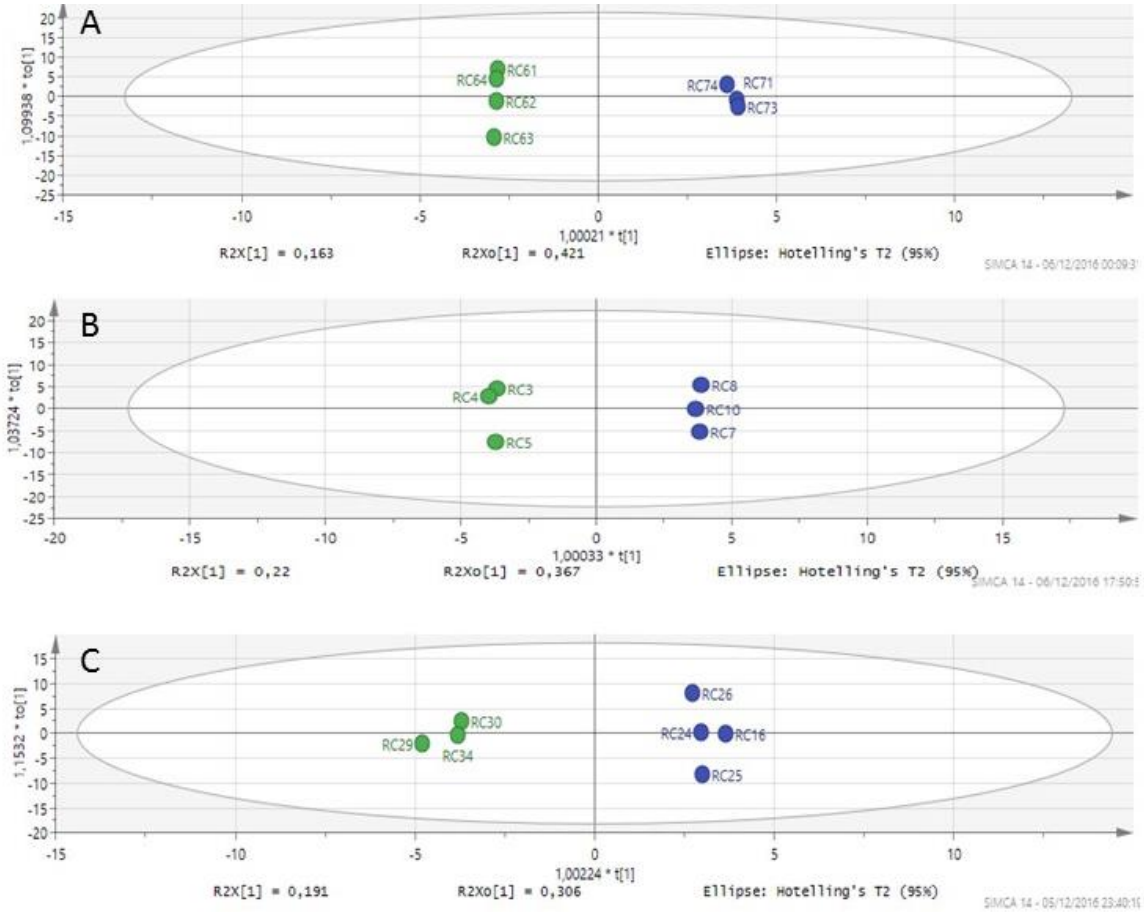

Figure 28. S-plot analyses of liver samples at different time points: 10mo CR (A), 22mo CR (B) and 18mo CR+AL (C). Green dots corresponds to AL animals, while blue dots are CR animals.

4.6.4 CR alters lipid profile in serum

Lipids are the major cell membrane components essential for various biological functions including cell growth and division of normal and malignant cells [200]. Increased total plasma cholesterol is a major risk factor for cardiovascular disease (CVD) and the majority of cholesterol is transported by LDL, which play a central role in CVD [201]. Indeed, elevated plasma LDL represent a widely accepted risk factor for CVD. Low HDL levels predispose to CVD, and high HDL levels protect against development of atherosclerosis. Moreover, there is also evidence that high serum triglycerides (TG) may be atherogenic [202]. Although their primary role in the pathogenesis of coronary heart disease, it has been reported the association of plasma/serum lipids and lipoproteins with different cancers, such as breast cancer and colorectal cancer [200]. In the present study, we tested the impact of CR on serum total cholesterol, LDL, HDL and TG at different time points: 2 months, 10 months and 22 months of CR. Our model of CR was shown to be effective in reducing total cholesterol and TG immediately after 2 months of CR. This effect was maintained after 10 months of diet, and continued to be marked after 22 months. Concerning LDL, we observed a tendency towards decreasing both at 2 and 10 months of CR that became significant at 22 months. However, the levels of LDL increase in both AL and CR animals as the animals got older, suggesting that CR may help to delay the accumulation of LDL, which high levels indicate an increased risk of cardiovascular risk. HDL levels did not change upon dietary intervention (figure 29). Our results are in accordance with those reported in long-term calorie restricted humans, wherein a period of CR ranging from 3 to 15 years produced a significant decrease in cardiometabolic risk factors such as TG, total cholesterol and LDL, while increasing HDL levels in plasma [203]. Long-term CR was shown to reduce plasma TG and LDL levels also in rhesus monkeys [204], and recently CR has been

shown to revert hepatic steatosis in obese mice, reducing total cholesterol and TG both in liver and in serum [205]. In our model, CR was shown to decrease efficiently total cholesterol and TG already after 2 months of diet, as shown by others in short-term CR in mice [206].

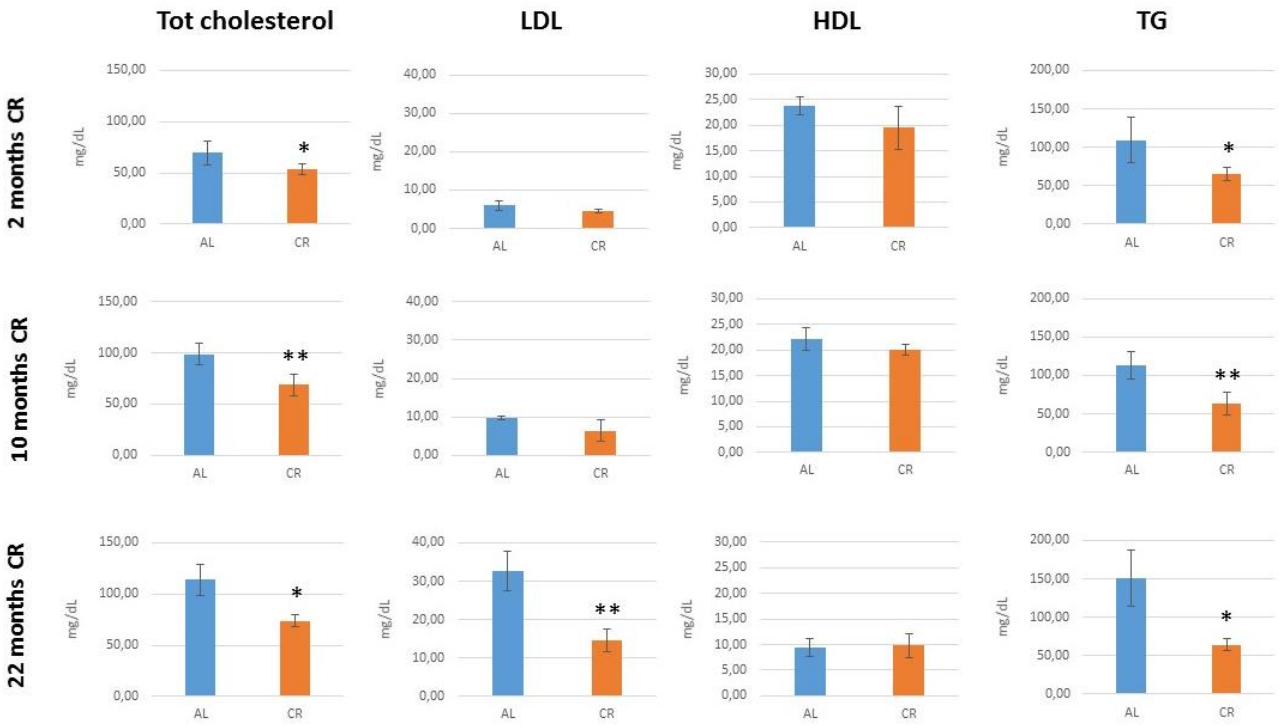

Figure 29. Levels of total cholesterol, LDL, HDL and TG in serum of AL and CR animals after 2, 10 and 22 months of ad libitum diet or caloric restriction. (* $p < 0.05$, ** $p < 0.01$)

5. Discussion

Caloric restriction (CR), the non-genetic intervention known to delay aging in several species from yeast to monkeys, has been shown to retard both spontaneous and chemically-induced neoplastic disease in experimental animals [1,48,53,61]. Several mechanism have been proposed in the literature to explain the delaying effect of CR on cancer development [64,66,74,92,110]. The studies reported in this dissertation present evidence to suggest that long term CR exerts a modulatory effect on the emergence of the neoplastic-prone tissue microenvironment associated with aging, thereby decreasing the risk of cancer. To our knowledge this is the first study focusing on the effect of long-term CR on the tissue microenvironment in relation to neoplastic disease.

We have initially performed a study involving transplantation of normal hepatocytes in rats of 22 months of age that were fed either AL or a CR diet throughout life. There were 4 animals in each dietary group and results gave a clear indication that clusters of donor-derived hepatocytes were larger in animals that had free access to food compared to those exposed to CR. Indeed, clusters ranging from 5 to 10 cells were more represented in rats fed AL that in those under CR (over two-fold more frequent). Furthermore, clusters comprising more than 10 cells were 8 times more represented in AL vs. CR groups. These results suggest that CR helps to preserve the young phenotypic features of the liver tissue microenvironment, which make it less permissive for the clonal expansion of transplanted normal cells compared to its aged counterpart. Such interpretation stems from previous findings indicating that the growth of normal hepatocytes orthotopically transplanted in both young and old recipients is more prominent in the old liver [196].

Given these encouraging results, we then addressed the core aim of this project, i.e. testing the hypothesis that CR may impact on the evolution of the neoplastic process via effects exerted on the tissue microenvironment. This hypothesis was also grounded on our recent report describing the promoting capability of an aged tissue-microenvironment on the growth of pre-neoplastic hepatocytes [44]. Accordingly, male F344 rats were fed either AL or a CR diet for 18 months starting at 8 weeks of age and then transplanted with pre-neoplastic hepatocytes, using the orthotopic model of Tx previously described. As detailed in the preceding section, results were clear-cut: larger nodular cell clusters were far more common in AL compared to CR fed group, i.e. feeding a CR diet for 18 months was able to reduce the tumor promoting potential of the aged liver microenvironment. It is important to point out that exposure to CR was halted prior to transplantation of nodular hepatocytes, ruling out the possibility that the dietary regimen exerted its effect directly on the pre-neoplastic cell population.

Pertinent to the latter consideration, we also observed a decreased incidence of endogenous hepatic nodules in animals fed the CR diet. The Fischer 344 rat strain is known to be prone to develop various types of “spontaneous” neoplasms, including liver lesions, with advancing age [197]. Interestingly, the decrease in size of endogenous nodules in the CR group was of a similar order of magnitude to that observed for donor-derived lesions. This suggests that the delaying effect exerted by CR on “spontaneous” neoplastic disease may be in fact largely mediated by alterations imposed on the tissue microenvironment, i.e. through a modulatory effect on the emergence of the age-associated, neoplastic-prone tissue landscape.

The biological determinants contributing to the increased proneness of the aged tissue environment to carcinogenesis are yet to be defined. It is noteworthy that

both normal and pre-neoplastic hepatocytes appear to be more susceptible to clonal expansion when injected into the liver of old animals compared to that of young counterparts [44,196] or to animals of the same age exposed to CR, as shown in the present studies. This suggests that similar biological forces drive the growth of both cell types, raising the possibility that a common denominator might be at play.

If this is the case, one may address the issue by exploring the mechanisms sustaining the growth of normal hepatocytes transplanted in the aged host liver [196]. It is well established that the regenerative capacity of the liver declines with age [199,207]. Moreover, work for our research group has indicated that a cell-autonomous decrease in proliferative proficiency is in fact present in hepatocytes isolated from aged animals [208]. It is therefore conceivable that a decreased proliferative competiveness of the aged hepatocyte may provide a growth advantage for transplanted cells, favouring their selective expansion. In contrast, the same cells do not emerge following infusion into the liver of young animals [208]. Interestingly, it has also been reported that CR (60% of AL diet for 14 months) was able to improve the regenerative response of old Fischer 344 rat liver to partial surgical hepatectomy [209], suggesting that CR may recover, at least in part, the proliferative competiveness of the aged hepatocytes. Thus, the liver microenvironment of the CR-exposed aged rat appears to maintain a higher functional competiveness, which is less permissive for the clonal emergence of either normal or putative altered/pre-neoplastic cells.

In the next series of studies, we examined the possible contribution of cells senescence to the delaying effect of CR on carcinogenesis. Senescent cells are considered a hallmark of aging since they accumulate in many tissues of old vertebrate organisms [143,144]. While it was initially interpreted as a fail-safe

mechanism to limit the risk of neoplastic transformation following genotoxic insult, there is now increasing evidence to indicate that cellular senescence can fuel carcinogenesis, possibly via SASP components [15,146]. In light of this possibility, we tested if long-term CR has any effect on the emergence of cell senescence in liver. Livers from AL and CR animals were screened for a range of senescence markers, such as SA-beta-gal, DDR-associated foci and cell-cycle inhibitors; however, no significant differences were found between the two groups. Our results differ with previous findings in mouse heart, liver, small intestine and kidney, where both short-term and long-term CR were shown to decrease the expression of some senescence markers such as SA-beta-gal, H2AX, p16 and lipofuscin [180,181,182]. The reasons for these discrepancies are difficult to evaluate at this point; however, species differences (mouse vs. rats) might be involved. For example, studies in rhesus monkeys, where CR was implemented for 9-12 years, showed no reduction in the expression of senescence markers [185].

On the other hand, a reduced expression of IL6, an important SASP component, was found in the liver of animals exposed to CR diet. This might be important, because IL 6 plays a role in liver regeneration and has also been proposed to be involved in the development of HCC. IL-6 appears to be implicated both at early phases of HCC development and at a later stage, when pre-neoplastic lesions are fed by an autocrine IL-6 loop but are still dependent on additional signals from the tumor microenvironment to develop into overt HCC [210,211]. Thus, a decrease in IL-6 expression in liver may render the tissue microenvironment less favorable for the growth and the expansion of pre-neoplastic cells. On the other hand, upregulated levels of IL-6 in AL fed animals may be linked to an excess of adipose tissue. In fact, obesity leads to the development of a low-grade systemic chronic inflammatory state. Macrophages make up to 40% of adipose tissue cells in obese mice

compared to 10% in lean mice probably due to increased amounts of several factors, including free fatty acids, cholesterol and lipopolysaccharide. Obesity triggers the accumulation of classically activated macrophages M1, which are characterized by the expression of high levels of proinflammatory cytokines (IL-6, TNF-α, IL-1β, IL-12 and IL-23), high production of reactive nitrogen and oxygen intermediates. Adipose tissue inflammation induced by pro-inflammatory macrophages M1 leads to obesity-associated insulin resistance, diabetes and metabolic syndrome, all conditions that are sustained by chronic subclinical inflammation [212,213]. Chronic inflammation predispose also to different form of cancer; key features of cancer-related inflammation include the infiltration of white blood cells, tumor-associated macrophages, the presence of polypeptides messengers of inflammation (cytokines such as TNF, IL-1, IL-6 and chemokines). Thus, chronic inflammation may facilitate cancer development through the construction of an inflammatory microenvironment that may contribute to the genetic instability of cancer cells [36,37].

At the biochemical and metabolic level, we confirmed and extended several findings related to the effect CR. Thus, levels of SIRT1, which were shown to increase during CR in several tissues in mice [87,89] were higher in the liver of CR rats compared to AL fed controls. Since SIRT1 deacetylates several targets whose action may be important in the context of the tissue microenvironment, such as NF-kB and MnSOD, the high expression of SIRT1 in CR animals may render the tissue microenvironment less favorable to the growth of pre-neoplastic cells, both reducing inflammation and limiting the availability of metabolic precursor necessary for pre-neoplastic cells to grow. FOXO1, which is another important mediator of the anti-aging and, more specifically, the anti-neoplastic effects of CR, represents one of the targets of SIRT1. In fact, in response to oxidative stress, SIRT1 deacetylates

FOXO1 favoring its translocation to the nucleus where it activates genes involved in ROS detoxification protecting cells from oxidative damage. Like SIRT1, FOXO1 mediates the antioxidant effects of CR through NF-kB inhibition. Interestingly, FOXO1 was significantly increased in livers of the group of rats exposed to CR, in agreement with results obtained in mice [98,99]. Once again, the increased expression of FOXO1 in CR liver may mediate the protective effect of CR on the tissue microenvironment.

It has long been known that aging decreases the ability of the liver to regenerate [199,214]. The molecular bases of such functional decline have been extensively investigated [215]. HDAC1 appears to play a role in the epigenetic attenuation of liver regeneration in rodents, via formation of a multiprotein complex with C/EBPα. The latter occupies and silences elongation factor 2 (E2F)-dependent promoters, and this appears to be mechanistically linked to the reduced regenerative capacity of the liver in old mice [215]. In addition, HDAC1 has been shown to form a complex also with C/EBPβ, whose levels have been found increase in old liver as well. On the other hand, SIRT1 is downregulated in the aged liver and it has been suggested as a key protein linking aging and liver dysfunction. Old mice, which have low expression of SIRT1, showed reduced supply of triglycerides and glucose following PH, resulting in impaired liver regeneration. HDAC1 and C/EBPβ control SIRT1 expression. Indeed, they have been shown to form HDAC1/C-EBPβ complex that repress SIRT1 promoter in old liver, resulting in impaired regeneration [88]. In our studies, we observed a decrease in both HDAC1 and C/EBPβ expression in CR livers, indicating that long-term CR may improve the regenerative capacity of old liver. As referred to above, there is direct experimental evidence that CR ameliorates the proliferative response of the liver to partial hepatectomy [209]. Thus,

metabolic changes associated with long-term exposure to CR are consistent with the hypothesis proposed above: the CR diet appears to delay the emergence of decreased tissue fitness associated with aging, thereby reducing the competitive advantage of transplanted normal and/or pre-neoplastic cells.

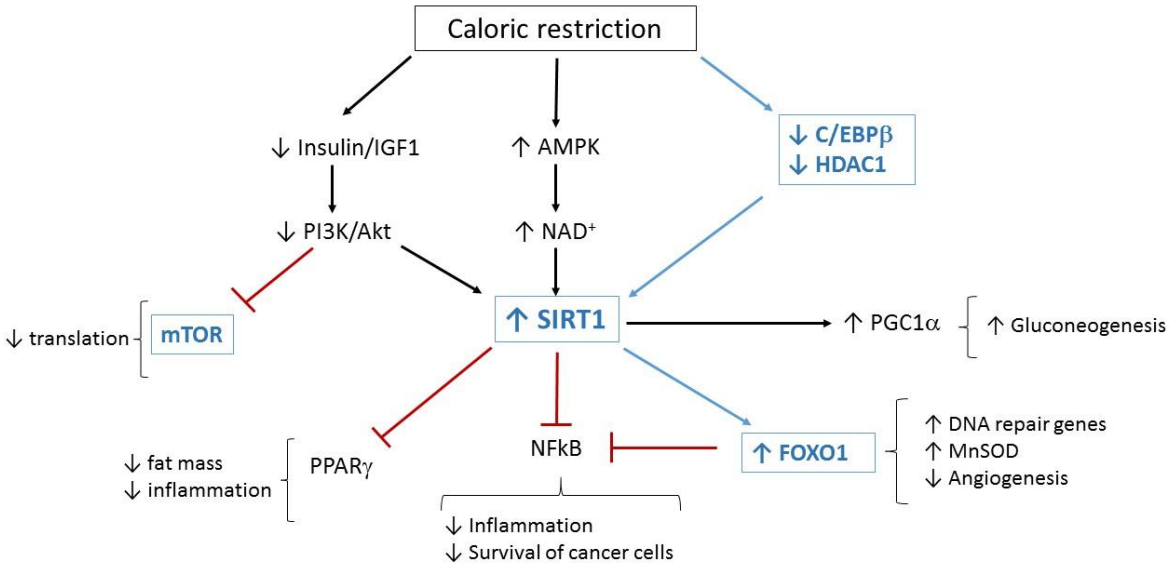

Figure 30. Schematic representation of molecular changes observed after long-term CR. Pathways analyzed in the present work are indicated in blue.

In order to gain further insights into possible metabolic mediators of CR effects on liver microenvironment, we performed a metabolomic analysis of phenotypic changes associated with long-term exposure to CR using a GC-MS-based method. Using an untargeted approach, we screened both serum and liver samples collected at different time points for hydrophylic metabolite profiles. Comparing each CR group with their age-matched controls fed AL diet, we observed that each time point considered has a peculiar metabolic profile, both in serum and liver. The only metabolite consistently increased in the liver of CR animals at each time point was D-gluconic acid. Experiments employing gluconic acid uniformly labeled with C^{14} showed that gluconic acid is converted to glucose in rats, since a high concentration

of C^{14} was found in liver glycogen [216]. Our result suggests that CR induces a switch in energy metabolism towarsd gluconeogenesis. Indeed, gluconate is a key metabolite of the pentose phosphate pathway (PPP), which is involved in the biosyntesis of NADPH, essential for various reductive processes and the syntesis of ribose-5-phosphate for nucleotide production [204]. In addition to this, it has been shown that gluconic acid may affect also the gut microbiota composition increasing the levels of bifidobacteria and reducing levels of the harmful bacteria *Clostridium perfrigens,* whose toxins may contribute to diarrhoea, cancer, hypertension and aging. In contrast, bifidobacteria have been shown to inhibit the growth of harmful bacteria, and stimulate immune function [217]. Thus, the presence of higher levels of gluconic acid in CR livers may reflect its seemingly beneficial effect also in the gut microbiota through the gut-liver axis. Consistent with a metabolic switch towards energy conservation and gluconeogenesis during CR, we observed also up-regulation of lactate and of some gluconeogenic aminoacids, such as Thr, in both serum and liver of CR animals after 10 months of diet, and this is in accordance with previous results both in serum and in liver of CR non-human primates and mice [204,218]. Another aminoacid we found increased in both serum and liver of 10 months CR rats was serine. Increased levels of this aminoacid were found also by Rezzi et. al in serum of CR-rhesus monkeys, and it has been interpreted as an index of efficient protein turnover during aging [204]. Moreover, the presence of plasma serine with other metabolites such as glutamate and choline, may preserve the neurological function often observed with CR [204,219]**.** The aminoacid glycine was found to be downregulated in serum from 22 months CR group. Glycine provides the carbon units to fuel the one-carbon metabolism, which represents a complex metabolic network based on the chemical reactions of folate compounds. This pathway provides one-carbon units required for synthesis of proteins, lipids and

nucleic acids. Recent studies have focused on the role of some non-essential aminoacids, such as glycine and serine, in supporting tumor growth. In fact, maintaining adequate flux through the one carbon cycle and supporting nucleotide synthesis is emerging as as a critical pathway in malignant transformation. Their depletion, which is possible through dietary manipulation, may inhibit tumor growth without impairing health [220,221]. Yet, in serum from 10 months CR rats we observed also decreased levels of urea. Increase in blood urea nitrogen, along with creatinine and urinry proteins, are the most commonly used markers of kidney function [222]. The reduction of urea observed in our study may suggest that CR improves also renal function, as previously reported [222]. We observed also a decrease in ornithine levels in serum, in particular in older animals after 18 months of CR and subsequently re-fed AL diet. Ornithine is a non-essential aminoacid produced as an intermediate molecule in the urea cycle. Moreover, it is a key substrate for the synthesis of polyamines (such as spermine and spermidine), which are involved in diverse functions, such as cell growth and differentiation, DNA synthesis and stability. Numerous studies have shown that polyamines synthetized from ornithine are often increased in cancer patients. In fact, polyamine biosynthesis is up-regulated in actively growing cells, including cancer cells. Thus, the decreased availability of ornithine observed in our study in CR group may lead to a reduction of polyamine synthesis, which may hamper proliferative processes, including carcinogenesis [223,224]. We found also lower levels of serum glucose and inositol in 22 months CR rats, and this is in agreement with several reports showing the hypoglycemic effect of CR and its ability to increase insulin sensitivity. However, levels of glucose were found increased in animals fed CR for 18 months followed by AL diet.

The method used to monitor small water soluble molecules was also able to extract a few saturated and monounsaturated fatty acids. We detected a tendency towards a decrease in free fatty acid levels (palmitic acid, stearic acid and oleic acid) both in liver and serum of CR-fed animals in most of the time points considered, suggesting that once they were released from triglycerides they were immediately oxidized. In support of this observation, data from the literature showed an increased mitochondrial complex II content in animals subjected to CR, reinforcing the idea of enhanced fatty acid oxidation rather than their export to the systemic circulation [225]. Finally, we detected lower levels of malic acid in livers of animals fed CR 18 months followed by AL diet compared to animals fed AL diet throughout life. Malic acid, or its anion malate, is a key metabolite that regulates senescence. Malate is produced from fumarate by fumarase, and it is decarboxylated to piruvate and CO_2 using NAD+ and NADP+ as cofactors by malic enzymes (MEs). MEs are a major source of NADPH for the antioxidant system, but they have also been involved in increased glutamine consumption in tumors. Thus MEs can divert the TCA cycle intermediate, malic acid, to the synthesis of macromolecules to form building blocks. MEs activity has been shown to decrease with aging, and this leads to lower NADPH levels and reduced NADPH-dependent functions such as antioxidant defenses. Thus, malate metabolism can antagonize senescence by bolstering cellular antioxidant defenses [172,16].

We assayed also serum lipid and lipoprotein profiles in both AL and CR dietary groups. Our data indicate a significant decrease of TG and total cholesterol immediately after 2 months of diet, and this effect was maintained at later time points, i.e. at 10 and 22 months of CR diet. LDL levels tented to be lower at both 2 and 10 months of CR diet, and such decrease became significant at 22 months. Levels of serum lipids (TG, total and LDL-cholesterol) increase with age. However,

in CR-fed animals at 22 months of diet they are closer to those observed in AL animals at earlier time points (2 and 10 months of diet), suggesting that CR reverses the aging-dependent alterations of lipids and lipoproteins metabolism.

In summary, the studies presented in this dissertation add to current understanding of the pathogenetic mechanisms linking aging, neoplastic disease and the modulatory effect CR on both biological processes. They provide clear evidence to indicate that the delaying effect of CR on carcinogenesis is mediated, at least in part, through CR-induced changes in the tissue microenvironment. More specifically, CR appears to result in the persistence of “young” phenotypic features in the liver tissue, both at biological and at biochemical/molecular level. The overall effect translates in a delayed emergence of the age-associated, neoplastic-prone tissue landscape. Our findings support the notion that strategies aimed at delaying biological aging, such as CR, are also effective in decreasing the risk of neoplastic disease.

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