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Abstract

Ageing is a major risk factor for neoplastic disease. Moreover, what is less appreciated is the fact that some of the most relevant risk factors for humans cancer, such as smoking or UV light, are also associated with accelerated ageing in their target organ. Yet, biological and molecular mechanisms underlying this link are far from being fully elucidated. Studies presented in this thesis stem from the working hypothesis that ageing entails the emergence of a more favorable tissue soil for the clonal expansion of normal or altered cells, including putative pre-neoplastic cells.

Caloric restricted feeding (CRF) represents one of the most effective strategies to delay ageing and age-related pathologies, including cancer. It is almost axiomatically implied that the beneficial effects of CRF are linked to the lower amount of delivered daily calories. However, CRF is often associated with a time-restricted feeding (TRF) schedule and this becomes important to consider in light of the increasing evidence documenting that TRF per se, without reduced caloric intake, can afford several positive effects on the development of age-associated phenotypes.

Given the better translational potential of TRF as opposed to CRF, the aim of the present studies was twofold. Taking advantage of a unique experimental model developed by our research group, we tested the effect of TRF on the emergence of the neoplastic-prone tissue microenvironment associated with ageing. Moreover, we determined the impact of TRF on both local (liver) and systemic age-related alterations.

In order to investigate whether TRF is able to exert any putative effect on either the tissue microenvironment and/or directly on the pre-neoplastic cell population, we set up a simple experimental protocol based on syngeneic hepatocyte transplantation. Hepatocytes isolated

from chemically-induced liver nodules were injected in recipient rats following long-term exposure to TRF regimen. Animals were then continued on *ad libitum* feeding (ALF) and the growth of transplanted cells was evaluated. A significant increase in the frequency of larger size clusters of pre-hepatocytes was seen in TRF-exposed group compared to controls given ALF throughout the experiment, indicating that this dietary regimen was able to delay the emergence of the neoplastic-prone/clonogenic tissue landscape typical of ageing. To our knowledge, this is the first investigation to describe a direct beneficial effect of TRF on carcinogenesis.

While longer follow-up studies are certainly warranted in our experimental setup, it is important to note that the present results parallel very closely those reported by our research group on CRF-treated rats. It was shown that the retarding effect CRF on neoplastic development was attributable, to a significant extent, to a lower clonogenic potential of the tissue microenvironment in caloric-restricted animals.

Parameters related to both liver and systemic ageing were found to be modulated by TRF towards the persistence of a younger phenotype, including a decrease in liver cell senescence, lower incidence of cholangiofibrosis, diminished fat accumulation and up-regulation of Sirt1 in the liver, down-regulation of plasma insulin like growth factor (IGF) 1, up-regulation of hippocampal brain derived neurotrophic factor (BDNF), decreased levels of plasma lipoproteins. Of note, the beneficial effect of TRF on the above parameters was still detectable after 3 months of ALF diet, suggesting that it is based on stable biological changes as opposed to transient metabolic alterations.

As mentioned above, these results have important mechanistic implications. If in fact several effects of CRF can be reproduced by TRF with no decrease in caloric intake, the implication is

that the beneficial effects of the former are mediated, at least in a relevant part, by biochemical/molecular mechanisms set in motion by the latter.

Thus, there is a need for new mechanistic hypotheses to be pursued as to the ageing-retarding effects of TRF and CRF. Placing emphasis on the time component of feeding behaviour, as opposed to the amount of food *per se*, brings attention to the interplay of the pattern of food ingestion with peripheral circadian clocks and to their relation to the rate of ageing. This is in line with the increasing awareness in the literature of the concept of "chrono-nutrition", i.e. the notion that food consumption should be aligned and resonate with body's daily rhythms in order to prolong healthspan. The underlying implication is that a better understanding and fine-tuning of this reciprocal interaction has the potential to widen our opportunities for effective interventions towards delaying ageing.

1. Introduction

1.1. Epidemiology of cancer in ageing.

Cancer is the second leading cause of death globally, and is responsible for an estimated 9.6 million deaths in 2018. Comprehensively, about 1 in 6 deaths is due to cancer. Around one third of deaths from cancer is related to the 5 leading behavioural and dietary risk factors: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, and alcohol use (World Health Organization, *www.who.int*).

Furthermore, cancer is an age-related disease. Individuals over 65 years of age comprise 55-60% and 40-45% of new cases of cancer diagnosis in developed and developing countries, respectively[1]. In the United States of America, ~53% of newly diagnosed tumours and ~69% of related deaths occur in people ≥65 years, and then drop to ~28% and ~44% in the oldest people (≥75 years)[2]. Cancer incidence rate for most cancer types increases until around 75-84 years of age and decreases quite abruptly afterwards[3]. Cancer mortality is 25-46% in

before 80 years of age, 25-20% in people dying at 90-99 years of age and ~5% for oldest-old, 100+ years of age.

Global prevalence and incidence of cancer in aged people reflects the growth of the population and the rise in global life expectancy. Prevalence and incidence of specific cancer types also vary across different ages[1].

1.2. Hallmarks of ageing

Ageing is considered inevitable time-dependent degeneration of physiological functions and integrity, with consequent changes in biochemical and metabolic composition, leading to increased vulnerability to disease and death[4-5].

It is characterized by a progressive loss of homeostatic capacity[6] and an associated decline of fitness and regenerative potential at the tissue, cellular, and molecular levels[7,8].

This impairment is considered to play an important role in the emergence of major human pathologies, such as cancer, diabetes, cardiovascular disease and neurodegenerative disorders, albeit its precise mechanistic role in the genesis of those specific morbidities is yet to be fully elucidated[9].

In an attempt to outline the general phenotypic features of ageing, a number of "hallmarks" have been proposed, including genomic instability, telomere attrition and epigenetic alterations; loss of proteostasis; deregulated nutrient sensing, such as insulin and IGF-1 signalling pathway and nutrient-sensing system; mitochondrial dysfunctions; cellular senescence; stem cell exhaustion; and altered intercellular communication[4,9]. These "hallmarks" will be briefly discussed in the following paragraphs.

1.2.1. Nuclear modifications

Age-related nuclear changes are evident at different levels of nuclear organization, including genomic instability, modification of nuclear structure, telomere attrition and epigenetic alterations. Cellular DNA is constantly exposed to exogenous and endogenous mutagens. Several physical, chemical and biological agents are known to be genotoxic, while endogenous causes of DNA damage are replication errors and reactive chemical species generated during cellular metabolism. Genetic lesions include small point mutations, translocations, chromosomal loss and gain (chromosomal aneuploidies and variations of copy number), and gene disruption through insertion of transposons or viral DNA. Alterations in nuclear structure are particularly evident as defects in nuclear lamins, which provide a scaffold involved in genomic stability, DNA replication and regulation of gene expression via formation of chromatin-protein complexes[9].

Ageing also entails a progressive shortening of telomeres. The latter consist in long repetitive DNA sequences at the end of chromosomes, characterized by the presence of TTAGGG repeats. The tip of telomeres is able to fold into a non-canonical secondary loop-configuration, referred to as G-overhangs, which protect integrity of chromosome ends. Telomere attrition, perceived as DNA damage, occurs at each normal cycle of semiconservative replication, triggering mechanisms of cell protection from DNA-damage, such as senescence or apoptosis[10].

At epigenetic level, increases of histone H4K20 and H3K4 methylation and histone H4K16 acetylation, together with reduction of methylation in H3K9 and H3K27 histones have been indicated has hallmarks of ageing[9].

1.2.2. Loss of proteostasis

The inability to maintain protein homeostasis in cells and tissues is yet another critical feature of biological ageing[11]. Proteostasis is the preservation of a correct balance between proteins synthesis, folding, localization, modification and degradation[12,13]. Protein homeostasis is regulated by two essential mechanisms: one, driven by chaperones, overlooks the correct protein folding and translocation into different organelles; the second is responsible for degradation of misfolded or exhausted proteins and it is carried out by the ubiquitin proteasome pathway and the autophagy system[9,13]. Both the above complementary functions are compromised in ageing, as a consequence of reduced ATP and decreased enzymes activity[6].

Among enzymes implicated in proteostasis whose activity is affected during ageing is Lon protease[14]. This is a key enzyme in protein turnover and regulation, and it plays a critical role in preventing accumulation of mitochondrial protein aggregates, typical feature of ageing and age-associated disease[15].

1.2.3. Deregulated nutrient sensing

Several nutrient-sensing pathways are affected by ageing process, and amongst them insulin and insulin-like growth factor 1 (IGF-1) signalling (IIS), sirtuins and AMPK pathways have been implicated in lifespan regulation and longevity[16,17]. Growth hormone (GH), released by the anterior pituitary, is able to induce IGF-1 production in different cell types, most notably hepatocytes. IGF-1 and insulin exhibit an overlapping regulatory network of anabolic functions, referred to as IIS. Over-activation of IIS pathway is associated with accelerated

aging, while down-regulation of its activity increases longevity and healthy lifespan[18]. Interestingly, plasma levels of IGF-1 appear to decrease with age in humans[19].

Downstream effectors regulated by IIS pathway include mTOR complex and FOXO family of transcription factors, implicated in accelerated ageing (mTOR) and extended lifespan (FOXO), respectively. While the former is activated by IIS, the latter is inhibited by this pathway.

The mTOR protein is a serine/threonine kinase, which plays essential role as a sensor of nutrient and amino acids concentration. Its activity impinges on diverse physiological processes, including metabolism, cell survival and cell growth. On the other hand, such anabolic pathway can also repress autophagy[20]. A decreased of mTOR activity is associated with lifespan-extension[21].

Low extracellular levels of insulin and IGF-1 activate FOXO family of transcription factors, which are involved in a wide range of cellular processes, such as cycle arrest, apoptosis and intermediate metabolism[22].

The other two nutrient sensors, AMPK and sirtuins, have opposite roles to IIS-mTOR anabolic function, in that they can detect low nutrient availability and stimulate catabolism. In fact, AMPK is able to directly inhibit the activity of mTORC1, one of the mTOR multiprotein complexes, while SIRT deacetylases stimulate activation of mitochondrial functions, including fatty acid oxidations and antioxidant defenses.

Collectively, anabolic signalling and utilization of energy stores appear to accelerate or delay ageing, respectively. Various dietary conditions, such as time restricted feeding or caloric restriction, or pharmacological treatments, such as rapamycin, that mimic lower nutrient availability, can promote longevity[23,9,4]. More on these pathways will be discussed in the following paragraphs.

1.2.4. Mitochondrial dysfunction

Mitochondrial dysfunction is due primarily to increased levels of reactive oxygen species (ROS) typical of ageing. Mitochondrial DNA (mtDNA) is particularly sensitive to ROS damaging effects, and alterations of mtDNA increase with age[24]. Rising of ROS levels affect mitochondrial integrity and this, in turn, enhances free radical production, worsening the adverse outcome[4]. Mitochondrial deterioration compromises the efficacy of the respiratory chain, increasing electron leakage and decreasing ATP production, thus resulting in reduced efficiency of mitochondrial bioenergetics.

Ageing is also associated with reduced mitochondrial biogenesis. The family of SIRT deacetylases, whose activity decreases with age, plays a role in mitochondrial biogenesis through mobilization of enzymes involved in energy metabolism and modulation of autophagy. Moreover, sirtuins, especially SIRT1, induce the elimination of damaged mitochondria through autophagy mechanisms[25].

Independently of ROS, other mechanisms can cause deficit in mitochondrial bioenergetics, including accumulation of mutations and deletions in mtDNA, oxidation of proteins, destabilization of macromolecular complexes involved in the respiratory chain and changes in composition of membrane lipids[9].

1.2.5. Cellular senescence

Cellular senescence has traditionally been defined as a cellular condition of persistent cell cycle arrest, which is accompanied by diverse transcriptional, biochemical and morphological alterations[26]. The senescence state is characterized by expression and secretion of soluble factors, collectively known as the senescence-associated secretory

phenotype or SASP, accompanied by peculiar alterations in chromatin structure, denoted as senescence-associated heterochromatin foci o SAHF[27,28]. SASP production is largely mediated by activation of two transcription factors, Nuclear Factor (NF)-kb and CCAAT/enhancer-binding protein beta (C/EBPβ). The secretory phenotype includes cytokines (IL-1α, IL-1β, IL-6 and IL-8), chemokines, growth factors (i.e. hepatocytes growth factor/scatter factor or HGF, and vascular endothelial growth factor or VEGF) and matrix-remodelling enzyme.

Cellular senescence is triggered by oncogenic and mitogenic alterations, such as DNA damage, telomere attrition, oncogene activation and tumour suppressor gene inactivation[29]. The first oncogenic insult identified as promoter of cellular senescence is the overexpression of cell cycle inhibitory proteins $p16^{INK4a}$/Rb and $p19^{ARF}$/p53, both encoded by the same genetic locus, the *INK4a/ARF* locus. The relevance of these pathways in the onset of cell senescence gained support when a correlation between $p16^{INK4a}$/Rb levels and chronological ageing was found in the majority of tissues analysed in humans and in mice. These findings have made the *INK4a/ARF* locus the most studied and the best documented gene associated with ageing and ageing-related pathologies[30].

It has been proposed that senescent cells are constantly removed by immune surveillance and phagocytosis. Accordingly, senescence can be considered as a mechanism to protect tissues, organs and organisms from accumulation of DNA damaged cells or, worse still, proliferation of aberrant cells. On the other hand senescent cells do accumulate with age, probably owing to increased generation or as a result of decreased immune clearance[4]. Considering that the ability to perform tissue repair deteriorates with age, cellular senescence was seen as the possible basis for the ageing process at organ and organismal levels[31,32], as exemplified by the loss of regenerative capacity of tissues in vivo[33,34].

Ultimately, cellular senescence may be viewed as a biological process that can be both beneficial (tumour suppressive) and deleterious (limit tissue renewal).

1.2.6. Stem cell exhaustion

In adult organisms, stem cells are essential for the preservation of tissue homeostasis and regeneration. In old age, quantitative and qualitative decline of stem cells occurs, leading to their progressive exhaustion[35]. Stem cell exhaustion implies a decrease in regenerative potential of tissues and it is considered as one of the most salient phenotypic features of ageing[10].

Examples of stem cells prone to exhaustions in ageing are haematopoietic stem cells, mesenchymal stem cells and intestinal epithelial stem cells. A decrease in the proliferative capacity of these tissue types leads to anaemia, impairment of the immune response, osteoporosis, bone fractures and decline of absorptive and barrier function of the intestine, all of which are common features of ageing[35]. The relevance of this concept to the liver is still uncertain, although the finding of clonality in the majority of human cirrhotic nodules[36] suggests that a similar paradigm might apply.

1.2.7. Altered intercellular communication

Beyond alterations inside cells, the ageing process also includes modifications of the intercellular space, contributing to the emergence of the ageing phenotype in tissues.

The ageing-associated pro-inflammatory phenotype, referred to as “inflammaging”, is a notable example of the above. Inflammaging may result from multiple inciting stimuli, such as the accumulations of damage at cellular and tissue level, changes in the immune system,

the propensity of senescence cells to secrete proinflammatory molecules (SASP), and the ageing-associated increased secretion of cytokines by adipose tissue[9].

Decline of the adaptive immune function, known as “immunosenescence”, consist in a diminished efficiency in the identification and elimination of infectious agents, infected cells and cells expressing an altered antigenic profile, which may aggravate the ageing phenotype. For this very reason, inflammaging and immunosenescence are intertwined in the pathogenesis of ageing-associated diseases[4].

Furthermore, the transmission of the senescence phenotype to adjacent cells through gap-junction-mediated cell-cell contacts (bystander effect) may contribute to accelerate the ageing process at tissue level[4,9]. While such an effect has been characterized in vitro, recent evidence suggests that it may also be operative in vivo in different tissues, including liver[37].

1.3. Ageing and cancer

As already discussed, ageing is a major risk factor for neoplastic disease[38]. However, biological and molecular mechanisms underlying this link are far from being fully understood. Main hypotheses that have been proposed to elucidate the relationship between ageing and cancer include the following: an increased likelihood of time-dependent accumulation of (multiple) mutagenic events in individual cells; a decline of anti-cancer immune-surveillance with age; chronic low-grade systemic inflammation (inflammaging) fuelling carcinogenesis; the emergence of an age-associated, neoplastic-prone tissue landscape.

1.3.1. Time-dependent accumulation of mutagenic events

The most intuitive and widely entertained hypothesis to explain the increased incidence of cancer in elderly postulates that advancing age allows more time for the accumulation of mutagenic events in target cells. This hypothesis stands within the conceptual framework of the Somatic Mutation Theory (SMT), which essentially holds that cancer results from the accrual of multiple (sequential) alterations in critical cellular genes[39]. These alterations translate in the activation of proto-oncogenes and inactivation of tumour suppressor genes, leading to phenotypic changes such as accelerated growth rate; resistance to apoptosis and, consequently, immortality; invasiveness and immune evasion capacity.

However, different lines of evidence point to a lack of correlation between ageing, cancer incidence and mutational load[40]. Firstly, a remarkable portion of epigenetic and genetic alterations occur during ontogeny, when neoplastic disease is virtually absent[40]. In addition, there is no correlation between levels of mutations and incidence of cancer in different tissues. In fact, a large number of healthy individuals exhibit chromosomal translocations, such as *BCR-ABL* fusion, typical of leukaemia, without the later occurrence of this pathological alteration. Similarly, incidence rate of AML1-ETO and TEL-AML1 translocations is much higher than the development of associated leukaemia[41].

These observations indicate that the accumulation of genetic alterations per se are not sufficient to explain the emergence and progression of the neoplastic phenotype[40].

1.3.2. Decline of immune-surveillance

An alternative/complimentary hypothesis to explain the link between ageing and cancer is rooted on the age-related decline in immune-surveillance, a phenomenon which is often referred to as immunosenescence[42,13].

The primary role of immune system is to segregate and eliminate both pathogenic hosts (e.g. viruses, microbes, parasites) and nonconforming self-cells. During ageing, the progressive waning of immunity effector mechanisms leads an increased risk for the development of a wide range of diseases, including infections, chronic inflammatory pathologies and cancer. Immune dysregulation is associated with high levels of pro-inflammatory cytokines, in the absence of activating stimuli, and a concomitant reduced capacity to trigger an efficient inflammatory response against antigenic stimuli[43].

Age-associated decline in immune function involves both innate and adaptive effector mechanisms. Innate immune cells show a constant baseline activation, which translates in increased production of cytokines. On the other hand, the general over-activation is not sufficient to generate effective cellular responses such as phagocytosis, free radical production and chemotaxis, when required. Furthermore, ageing entails a decrease in the number of effector cells of the adaptive response, including a reduced activity of antigen-presenting cells and T cells, due to exhaustion of regenerative capacity of hematopoietic stem cells and thymic involution[44].

Immune-surveillance is thought to play a critical role in controlling the growth of precancerous and cancerous lesions, due to the ability of the immune system to recognise and eliminate cells expressing an altered antigenic profile[13,45].

Such capacity is being exploited in recent therapeutic approaches aiming to enhance the effector function of the immune system via blockade or immune-regulatory pathways[46,47].

1.3.3. Inflammaging.

The term “inflammaging” refers to an age-related systemic state of chronic, subclinical, low-grade inflammation in the absence of triggering stimuli, with associated high levels of plasma pro-inflammatory markers.

Inflammation is generally a beneficial response to tissue damage, aimed at re-establishing structural and functional integrity. However, its chronic, low grade activation is thought to contribute to the progressive derangement of organs and tissues typical of ageing[48].

Several common age-associated diseases, such as cancer, cardiovascular pathologies and diabetes, often display an evident inflammatory background, correlated with increased levels of pro-inflammatory markers. In addition, it is estimated that approximately 20% of cancers are related to chronic inflammation; indeed, all stages of cancer development, from initiation to metastasis, are susceptible to modulation by inflammatory mediators[30].

A main contributing factor to inflammaging appears to be cells senescence, via secretion of SASP components. The latter play a dual role in fuelling inflammation: at tissue level it shapes a pro-inflammatory environment via recruitment of immune cells[29] and by inducing paracrine (bystander) effects on neighbouring cells, thereby amplifying the senescence phenotype; at systemic level, senescent cells can release soluble factors directly into the circulation, promoting a global status of inflammation[43].

Many SASP components are likely to play an active role in tumour progression and metastasis: for example, VEGF is important in angiogenesis while HGF, IL-6 and IL-8 are able to stimulate invasive capacity[30].

1.3.4. The age-associated, neoplastic-prone tissue landscape

A more holistic view to explain the link between ageing and cancer centres on the emergence of what has been referred to as the age-associated, neoplastic-prone tissue landscape[13]. The basic tenet of this hypothesis is that the microenvironment of aged tissues is a favourable soil for the clonal expansion of normal or altered cells, including putative pre-neoplastic cells. Several lines of evidence, obtained with cell transplantation experiments, are in line with the above proposition. Over 20 years ago, McCullough et al. observed that a liver-derived pre-neoplastic cell line injected in animals of different age displayed a tumorigenic potential only in the old recipient, highlighting important biological differences in the hepatic microenvironment of young and old animals[49]. In more recent studies, primary hepatocytes, isolated from chemically-induced liver nodules, were found to clonally expand and form large pre-neoplastic lesions upon transplantation into the liver of aged recipients, while the same nodular hepatocytes remained largely quiescent following injection into young host liver[50]. Most notably, a similar pattern of results was seen when normal hepatocytes were transplanted into either young or old rats: larger clones of donor cells formed in the liver of old recipients compared to those observed in young counterparts[51]. The latter findings were also confirmed using normal hepatocytes isolated from fetal liver: their growth was significantly more pronounced upon injection into old vs. young syngeneic recipients[52].

The combined evidence discussed above has led to the conclusion that the aged liver microenvironment is clonogenic to transplanted normal and altered (including pre-neoplastic) hepatocytes. Such conclusion is also in line, and in fact may help explaining, an ever increasing number of studies showing that clonal expansions are a very common, and possibly universal finding in several tissues in people of old age[53]. Two possible mechanisms

can theoretically account for this outstanding phenomenon. The expanding clonal cell population might be endowed with an absolute growth advantage over surrounding (homotypic) normal counterparts. The presence of cancer-driver mutations in phenotypically normal clones points to such possibility[54]. However, an alternative, albeit not mutually exclusive possibility is that the competitive advantage of rare clonogenic cells might be a consequence of a generalized decrease in proliferative fitness of the bulk of the tissue, favouring the emergence of those cells with a (relatively) preserved growth potential. The finding of a substantial fraction of clonal expansions which does not appear to harbour any candidate driver mutations supports such possible scenario[55]. Moreover, the clonal expansion of normal hepatocytes, isolated from fetal or young donors, upon transplantation into the liver of old (but not young) recipients, again suggests that the growth competitive fitness of aged tissue is compromised[50]. In agreement with the latter conclusion, hepatocytes from aged rat liver displayed a cell-autonomous decrease in clonogenic potential in vivo compared to those isolated from young donors[51].

Studies carried out in mouse hematopoietic system have also provided supportive evidence along these lines. A decreased competitive fitness of lymphoid progenitors associated with physiological ageing was in fact shown to favour the emergence of relatively more proficient clones, including altered cells at risk for neoplastic transformation[56,57].

1.4. Age-delaying mechanisms.

While chronological ageing is obviously not modifiable, the biological phenotype associated with advancing age is amenable to modulation, bearing in mind that prevention of any

chronic alterations before they occur is certainly a more effective approach than any attempt to reverse them once they are already in place[40].

Not surprisingly, research effort throughout the world are devoted to devising strategies towards delaying ageing and age-related morbidities. Among the most investigate are dietary control of caloric intake (under the general heading of caloric restriction, CR) and CR-mimetics, such as resveratrol, rapamycin and metformin, which interfere with pathways thought to be important in mediating the age-delaying effect of CR. In more recent years, the pattern of food intake has gained increasing attention as a possible means to exploit the potential of dietary habits manipulation towards delaying biological ageing. In this regard, time-restricted feeding (TRF), i.e. the temporal restriction of food consumption to a limited daily interval (8-12 hours), is emerging as an alternative strategy to attain a significant positive impact on lifespan and healthspan.

1.4.1. Caloric Restriction (CR)

A reduced caloric intake, i.e. approximately 20 to 40%[58] of ad libitum daily consumption, without malnutrition or inadequate nutritional intake of essential factors[59,60], is the most successful environmental, non-genetic and reproducible intervention that has been found to improve healthy life and enhance lifespan in several animal models, from the unicellular organism to worms, flies, rodents and primates[59–61].

The first report on the effect of CR on ageing dates back to 1935, when Crowell and McCay demonstrated that reduction of daily food intake without malnutrition was able to increase lifespan in rats[62]. After several decades from this preliminary discovery, the effect of caloric restriction on healthspan and lifespan was been confirmed in different animal species, from

unicellular life forms to non-human primates[60]. While the biochemical and molecular mechanisms underlying such beneficial effect remain to be fully clarified, the available evidence points to a number of metabolic pathways as playing a key role. The latter include a down-regulation of insulin and insulin like growth factor (IGF-1) signalling (IIS) cascade[63], the reduced activity of the amino signalling target of rapamycin (TOR)[64], and the inhibition of the glucose signalling Ras-protein kinase A (PKA) pathway[65].

In rodents, the pro-longevity effect of caloric restriction has been tested in animals of different ages, in which dietary treatment started later in life. It was found that initiating CR later in life showed significant effects on lifespan and, moreover, reduced the incidence of spontaneous cancer by more than 50%[60]; however, the beneficial effects were reduced compared to those observed when caloric restriction was started at weaning[58,66]. Caloric restriction seems to share similar mechanisms promoting the extension of lifespan with GH receptor knock-out and GH-deficient mutants mice (more on this issue in following paragraph 1.4.1.1.)[67,68].

The translational potential of caloric restriction was addressed with experiments performed in Rhesus monkeys.

The Rhesus monkeys are the closest model organism to humans in which caloric restriction has been experimentally tested in a controlled environment[69]. Over the past twenty years, substantial data have been generated supporting the concept that caloric restriction 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 restriction of caloric intake. 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 *ad libitum*-fed

controls compared to CR animals, infact the mortality in the control group started at earlier age than the CR group[70]. Animals (male and females) were adults when introduced into the study (7-14 years old), although the classic rodent 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[58].

In contrast to the WNPRC report, 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 calorie-restricted animals. The NIA 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. Monkeys under restricted regimen monkeys received 30% less food than their matched controls. Compared to WNPRC, the NIA study investigated both the effect of early- and late-onset caloric restriction. Late-onset regimen was beneficial on several parameters of metabolic health and overall function. However, no changes in survival were observed. In monkeys exposed to young-onset caloric restriction, no significant effects were noted in survival compared to *ad libitum* group. The incidence of cancer was dramatically reduced in young-onset caloric restriction monkeys, while the incidence was similar between *ad libitum* and late-onset restricted group, indicating that an early intervention may have a more significant impact on cancer development[71].

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[69–71]. This latter approach may provide a slight restriction, preventing obesity in the control monkeys, which may therefore experience survival benefits compared to truly *ad libitum*-fed animals[72,73]. In line with the latter interpretation, final body weights in control groups were higher in WNPRC monkeys compare to NIA counterparts. Importantly, caloric restriction 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[69–71].

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[74].

Finally, in humans, there is some indication that a decrease in caloric intake of around 15% may delay mortality associated with ageing[74,75]. 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[76]. 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 the feasibility, safety and improvements in terms of quality of life and disease risk of caloric restriction strategies. The degree of reduction in food intake 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. Participants under dietary restriction lost weight significantly compared to *ad libitum* 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[77].

Following these results, several studies have attempted to unveil possible metabolic, biochemical, and/or molecular alterations mediating the beneficial effect of dietary rerstriction[78]. Relevant findings are summarized in the next paragraphs.

1.4.1.1. The insulin/insulin like growth factor 1 (IGF-1) signalling (IIS) cascade

A main pathway that has been implicated is the insulin/insulin like growth factor 1 (IGF-1) signalling (IIS) cascade. Exposure to CR results in down-regulation IIS pathway across several species, from *C. elegans* to rhesus monkey, and this appears to be important for the effect of CR on the ageing process[23,79,80]. Indeed, the role of decreased activity of IIS signalling in lifespan extension is well documented. For example, different types of dwarf mice, characterized from low levels of growth hormone, and mutant mice deficient for GH receptor exhibited increase of lifespan compared to wild type animals. Moreover, mice fed a CR diet showed a similar phenotype to dwarf animals, including reduced thyroid hormone, decreased levels of insulin and glucose, lower body temperature and adiposity[81].

In addition to its role in accelerating ageing, IIS signalling pathway is also able to promote neoplastic growth[82]. Consequently, the modulation of ISS activity exerted by CR could be involved in its anti-tumorigenic effect. Interestingly, humans affected by Laron syndrome exhibit mutations in GH receptor, resulting in low level of circulating IGF-1 and a decreased risk of cancer. In addition, a germline mutation affecting the function of the GH-releasing hormone receptor (GHRH-R), leads to decline of GH and IGF-1 levels and is associated with slower progression of spontaneous prostate cancer in a transgenic mouse model of prostate adenocarcinoma[61].

1.4.1.2. The mammalian target of rapamycin (mTOR) pathway

The molecular network comprising the mammalian target of rapamycin (mTOR) is yet another mediator candidate for the effects exerted by CR on ageing and age-related pathologies. This protein is a serine/threonine kinase that is able to assemble with at least two different complexes, mTORC1 and mTORC2. The former is activated by extracellular abundance of amino acids and growth factors, such as insulin and IGF-1. Activation of mTORC1 complex promotes different anabolic processes through activation of different downstream effectors: protein synthesis via ribosomal protein S6 kinase 1 (S6K); fatty acid synthesis via sterol regulatory element-binding protein (SREBP) 1, and adipocyte differentiation via peroxisome proliferator-activated receptor gamma (PPARγ). On the other hand, mTORC1 suppresses autophagy and lysosomal biosynthesis via transcription factor EB (TFEB)[81,83]. Accordingly, mTOR plays a key role in cell growth and proliferation by coordinating the above cellular anabolic pathways.

The role of mTOR in ageing and lifespan has been characterized in simple animals like worms and flies, in which a decrease of mTOR activity was found to be associated with a substantial

increment of lifespan[83]. Notably, *C. elegans* is the first life form in which the beneficial effect of mTOR signalling inhibition in longevity was reported[84]. Furthermore, it has shown that CR-induced down-regulation of mTOR pathway increased lifespan in both in *C. elegans* and in *D. melanogaster*. Similar effects of CR were later described in rodents[85].

In addition, an important role has been attributed to mTOR activity in cancer induction and progression. This is not surprising, given the involvement of this pathway in cellular anabolism and proliferation. Deregulated activation of mTORC1 pathway, due to mutations in its upstream activators, such as mitogen-activated protein kinase or MAPK/Ras and phosphatidylinositol-4,5-bisphosphate 3-kinase or PI3K/Akt signalling pathways, can promote sustained proliferation[86]. Indeed over-expression of Ras, PI3K and Akt oncogenes, owing to different types of genetic alterations, has been found in up to 80% of human cancer[87]. Moreover, mTOR might be implicated in the onset of cancer, given that inhibition of autophagy by mTORC1 complex can interfere with clearance of precancerous cells. There is indeed evidence to suggest that activation of autophagy in pre-malignant lesions might limit the risk of cancer development[88]. Similarly, stimulation of autophagy may also promote longevity by through increased degradation of damaged proteins and organelles, whose accumulation alters cellular homeostasis and accelerates ageing processes[89].

1.4.1.3. The forkhead box transcription factors (FOXO) pathway

Part of the beneficial effects afforded by CR through down-regulation of IIS signalling pathway are ultimately mediated by increased intra-nuclear levels of FOXO protein[90]. When levels of IGF-1 and insulin decrease, FOXO changes its cellular localization and it is shuttled from the cytoplasm to the nucleus, switching to its active form and impinging on numerous pathways; such nucleocytoplasmic compartmentalization is gradually impaired during

ageing[91]. Critical to its anti-ageing effects, FOXO is able to enhance expression of antioxidant enzymes, such as superoxide dismutase and catalase, thereby conferring increased resistance to oxidative stress[92].

This was first demonstrated for the worm orthologue of FOXO family, DAF-16, which activates genes whose products are important in resistance to oxidative stress, pathogens and protein damage. In worms, mutations in insulin receptor or in genes affecting ISS pathway extend longevity up to three fold. On the other hand, this extension is reversed when the gene encoding DAF-16 protein is also mutated[93].

In mammalians, the key transcription factor of FOXO family is FOXO3a and its role has been recently highlighted in the Southern Italian Centenarian study[94]. Moreover, FOXO3a is associated with long healthspan, given that its over-activation is implicated in low incidence of cardiovascular diseases and cancer, linking this increased lifespan phenotype to the integrity of physical and cognitive functions[91].

Recent studies conducted by our research group also found increased levels of FOXO expression in rat liver upon long term exposure to CR diet[95]. Interestingly, such an increase persisted following 3 months of AL feeding after CR diet and, most importantly, it was associated with a decreased growth of transplanted pre-neoplastic hepatocytes[95].

1.4.1.4. The sirtuins family of NAD-dependent protein deacetylases

Sirtuin family of NAD-dependent protein deacetylases plays an essential role in genome stability, since it is able to induce or repress expression of several loci, for example, through chromatin compaction and histone deacetylation. Beyond this function, sirtuins promote activation of diverse target substrates, such as transcription factors, proteins involved in DNA repair mechanism, mediators of autophagy and effectors of glucose and lipid

metabolism. Sirtuins family is implicated in a multitude of cellular functions, including metabolism, stress response, ATP production by mitochondria, handling of reactive oxygen species and ketogenesis[96].

Early studies on *Saccharomyces cerevisiae* identified a conserved gene implicated in transcriptional silencing, indicated as silent information regulator 2 or *SIR2*. Its potential role in longevity was postulated when simple over-expression of Sir2 led to increased lifespan, with a single extra copy of *SIR2* extending lifespan by 30%, while inactivation of same locus decreases lifespan by 50%[83,96]. In addition, *SIR2* was shown to be essential for the induction of pro-longevity effect by a CR diet[97].

In higher organism, homologs of Sir2 (sirtuins) are also involved in the regulation of lifespan. For example over-expression of SIRT6 in transgenic mice led to 15% extension in median lifespan, possibly through regulation of genomic stability, nuclear factor kB (NF-kB) signalling and glucose homeostasis[83,98]. In addition, the absence of SIRT3 is associated with the development of a wide range of age-related pathologies in mice and the ability of CR to prevent unhealthy phenotypes appears to require SIRT3 expression[83].

Furthermore, mice exposed to CR and mutant strains overexpressing SIRT1 display similar phenotypes, including improved glucose tolerance, low serum cholesterol levels, lower plasma insulin and reduced incidence of spontaneous carcinomas.

On the other hand, reduced levels of SIRT3 were reported in several cancers, such as head and neck squamous cell carcinoma (HNSCC), breast and colon carcinomas, while over-activation of SIRT6 was associated with suppression of tumour development, possibly through inhibition of glycolysis[61]. Similarly, SIRT4 knockout mice show development of spontaneous lung, liver and breast cancer and lymphomas; studies on human cancer tissue

samples revealed reduced levels of SIRT4 mRNA in colon, breast, endometrial and oesophageal cancer, confirming previous data in rodents[99].

1.4.2. 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[16].

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

Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenol commonly found in grapes and red wine. This compound became popular as the main responsible for the so-called French paradox, according to which a decreased incidence of cardiovascular diseases in the French population, despite their high-fat diet, is associated with a high consumption of red wine[100]. Resveratrol has been shown to extend lifespan in a wide range of organisms[101–103]. In mammals, resveratrol supplementation has been shown to prolong lifespan of middle-aged mice under high-fat diet[104] and to reduce adipocyte size and inflammatory response while increasing insulin sensitivity in white adipose tissue of obese rhesus monkeys fed high-fat and high-sugar diet for two years[105].

Resveratrol has been also documented 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[106]. Moreover, it causes reduction in proliferation in a number of cancer-cell lines, such as prostate, ovarian and breast[107,108].

The mechanisms by which resveratrol exerts these effects relies on its intrinsic anti-oxidant activity and its ability to induce the expression of several antioxidant enzymes. Furthermore, exposure to resveratrol has been associated to increased SIRT1 activity, possibly by activating AMPK and increasing NAD+ levels[109]. Resveratrol probably mimics CR by SIRT-mediated deacetylation of pro-inflammatory complexes, such as NFkB[107,110,111].

Anothercaloric restriction mimetic is Rapamycin, which, as mentioned before was studied as an immunosuppressant long before its mechanism of action was understood. As it is now well documented, rapamycin inhibits mTOR signaling pathway by binding to FK-binding protein 12 which, by interacting with mTOR, disrupts the mTOR Complex 1[16,61].

Several studies have shown that rapamycin and its analogs, extends lifespan in a wide spectrum of organisms, from yeast to rodents[112,113]. Administration of rapamycin late inlife retards multiple aspects of aging, such as liver dysfunction, alterations in heart and adrenal and endometrial lesions in mice[114]. Importantly, rapamycin has alsobeen 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[115]. In addition to this, rapamycin treatment has been shown to reduce significantly the proportion of mice (16 months 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[116].

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[117].

Despite its beneficial effects, rapamycin treatment has important side effects that limit its usage as an 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.

Metformin is a biguanide commonly used for the treatment of type 2 diabetes and metabolic syndrome since the 1960s. It has anti-hyperglycemic function in diabetic patients by reducing hepatic gluconeogenesis, increasing insulin sensitivity and increasing glucose utilization by muscles and adipocytes[61].

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 in 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[107,118]. 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[107].

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[119]. However, in contrast with CR, metformin does not decrease glycaemia and insulinemia in normal subjects[118].

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[120]. Moreover, retrospective studies on diabetic patients, showed that metformin treated subjects have a lower incidence of cancer and related mortality[121].

1.4.3. Caloric restriction delays carcinogenesis through effects on the tissue microenvironment.

In a recent study, our research group has tested the possibility that caloric restriction may retard carcinogenic process by delaying the onset of the neoplastic-prone microenvironment associated with ageing. When pre-neoplastic hepatocytes were orthotopically injected into rats pre-exposed for 18 months to either *ad libitum* feeding (ALF) or a caloric restricted feeding (CRF) regimen, (70% of food consumed by ALF controls), the size of pre-neoplastic cell cluster at the end of 3 months was significantly lower in animals given the CRF diet. Importantly, similar results were obtained following transplantation of normal hepatocytes, supporting the conclusion that CRF retards the emergence of the clonogenic and neoplastic-prone tissue landscape typical of ageing[95].

1.4.4. Time-Restricted Feeding

A more recent approach that is being considered towards delaying ageing via dietary intervention is time restricted feeding (TRF). In this dietary regimen, daily cycles of feeding and fasting alternate. Feeding time period, restricted to 8-12 hours per day, should match with active phase of animal or human lifestyle[59,122]. The benefits of this feeding schedule appear to be proportional to fasting duration[123].

An increasing number of studies have now indicated that at least some effects of CRF are reproduced by TRF, including stimulation of autophagy, increased mitochondrial respiratory

efficiency, modulation of reactive oxygen species and changes in the profile of inflammatory cytokines[82]. Importantly, under controlled experimental conditions animals exposed to TRF are able to eat ≥90% of the food ratio consumed by ALF fed controls, after a short training period of less than one week[124,125].

Among the best characterized effects of TRF is the prevention of obesity. The prevalence of overweight and obesity is increasing in the many parts in the world, including less developed Countries. Approximately 2 billion people are overweight and one third of these is obese[126]. The increased incidence of obesity translates into a higher burden of major causes of mortality in humans, including cardiovascular pathologies, cancer, chronic liver and kidney disease and diabetes mellitus[126].

The ability of TRF feeding to prevent excess increase in body weight was first shown in Drosophila melanogaster[127] and then confirmed in mammals (mice) fed either regular or high fat diets[125,128–130]. Moreover, TRF could also reverse pre-existing diet-induced obesity, after both long[123]- and short-term[131] exposure.

Together with the beneficial effect on adipose tissue, TRF was able to inhibit diet-induced liver steatosis and the accompanying increase in serum markers of liver disease[124,125],. With reference to the obesity-linked risk of cardiovascular disease, TRF was shown to decrease plasma levels of triglycerides and low density lipoproteins (LDL)[123,124,128,132].

As already mentioned, ageing is associated to low grade of systemic inflammation; TRF was able to reduce inflammatory parameters in white adipose tissue (WAT)[123,124], brown adipose tissue (BAT), plasma[131], liver and jejunum[133]. Thus, TRF decreased levels of pro-inflammatory markers tumour necrosis factor (TNF)-α, IL1β, IL6[133], chemokine ligand 8/monocyte chemoattractant protein 2 (Ccl8/Mcp2)[123], chemokine ligand 2 (CXCL2)[124], were found in WAT and BAT of TRF mice compared to ALF animals. Similarly, monocyte chemoattractant

protein-1 (MCP-1), a potent pro-inflammatory cytokine whose expression correlates with body adiposity, was decreased in animals exposed to TRF regimen for two months[131].

The impact of TRF on insulin-regulated metabolism is also noteworthy. Continuous availability of food, such during ALF, is associated with a "constitutive" high level of insulin the plasma, with limited changes before and after food consumption[134]; this in turn sets the stage for the development of insulin resistance. A TRF schedule is able to prevent such sequence of events, as reported by a number of studies[123]. The beneficial effect of TRF regimen on the emergence of insulin resistance has been confirmed by glucose test tolerance, performed in young and middle-age animals[123,129]. Similarly, metabolic pathways related to IIS axis, such as mTOR, AMP and cAMP response element binding protein (CREB), are sensitive to fasting/feeding cycle[125,135]. In the fasting phase, activation of AMP and CREB leads to catabolic metabolism; on the other hand increased levels of intracellular mTOR are present during feeding period[124,130]. As a result, TRF down-regulates the anabolic state associated to ALF, which correlates with the ageing process.

Very limited evidence is available so far on the possible effects of TRF on the development of neoplastic disease. A retrospective epidemiological study in humans reported a positive correlation between protection from breast cancer risk and duration of overnight fasting period[136].

Given the findings summarized above, studies on TRF regimen deserve further implementation for at least two main reasons. Firstly, a TRF schedule is, at least in principle, more amenable to application to humans compared to a strict CRF diet. If TRF proves to be as effective as CRF in delaying ageing and age-related morbidities, including cancer, it may represent a major step forward in our attempt to exploit diet to prolong healthspan and lifespan. Secondly, if classical effects of CRF on metabolism, microbiota composition, ageing

and age-pathologies can be reproduced by TRF feeding, one has to reconsider the basic postulation that the beneficial effects CRF are inextricably related to caloric restriction *per se*, given the fact that exposure to CRF is generally associated with a TRF pattern.

2. Aim

The overall aim of this thesis is to test whether time-restricted feeding (TRF) exerts any effect on the emergence of the neoplastic-prone/clonogenic liver tissue microenvironment associated with ageing. Furthermore, the effect of TRF on liver and systemic alterations typical of ageing will be determined.

3. Experimental procedures

3.1. Animals and diet

All experiments have been performed with a colony of DPP-IV$^-$ (see paragraph 3.3 below) Fischer 344 male rats bred in-house. Rats were maintained on an alternating 12 hours light/dark cycle, in a temperature and humidity-controlled environment, with water available ad libitum and housed two for each cage. Rats received humane care according to the criteria outlined in the National Institutes of Health Publication 86-23, revised 1985. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Cagliari.

Two sets of experiments have been designed. In the long-term experiment 8-weeks-old male rats were randomly divided into 3 groups: control group (*ad libitum* feeding or ALF), which had *ad libitum* access to food during both light and dark phases; time-restricted feeding group (TRF); and caloric-restricted feeding group (CRF), which received 70% of ALF food

intake. TRF had *ad libitum* access to food for 8 hours during the dark phase, more specifically from T16 to T24 (4 hr after lights off) as depicted in **Figure 1**.

Body weight was measured weekly whereas food consumption was measured daily for TRF/CRF and weekly for ALF respectively. Such feeding regimens were kept for 18 months. Subsequently, TRF and CRF were re-fed *ad libitum* until the end of the experiment. One week after the diet regimens changed, all experimental groups were transplanted with pre-neoplastic hepatocytes derived from syngeneic donor. Three months after transplantation (Tx), at 24 months of age, animals were killed by decapitation (**Figure 2**). Blood samples were collected using heparin and plasma was separated immediately after collection centrifuging at 1800 g for 20' at 4°C. Liver and hippocampus tissues were excised and rapidly frozen for cryostat sections, protein and gene expression analysis. Part of the liver tissue was also fixed in 10% buffered formalin and embedded in paraffin for histological analysis and immunohistochemistry.

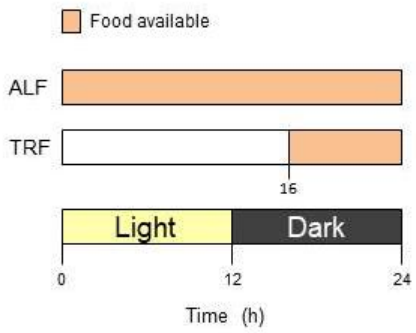

Figure 1. Schedule of food availability.

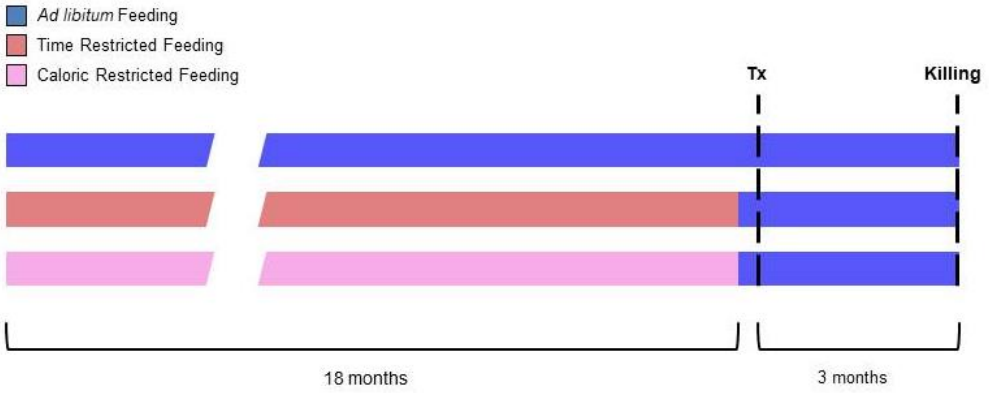

Figure 2. Experimental protocol for the long-term experiment. Animals were fed according to three different nutritional regimens for 18 months, then all groups were fed *ad libitum* until killing.

In short-term experiments animals of different ages were fed for three months only with two different diet regimens, *ad libitum* or time-restricted feeding. The TRF group received their daily food intake in the same time range as described for the long-term experiment. Two different short-term experiments were designed depending on the age, in which TRF started at 18 months of age or at 10 months of age. After three months of diet, all animals were euthanized by decapitation and plasma and tissue samples were collected as previously described (**Figure 3**).

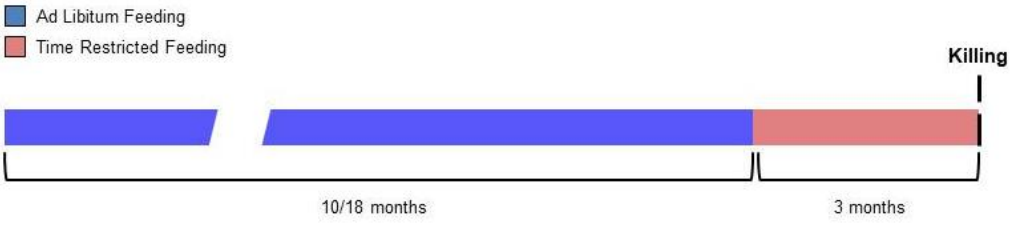

Figure 3. Experimental protocol for the short-term experiment. 10 or 18 months old animals were treated with TRF regimen for 3 months until killing.

Throughout the experiment all animals received a standard rodent laboratory chow (Mucedola Srl, Italy, Cat. #4RF21). Nutrient composition is reported in *Table 1*.

3.2. Induction of pre-neoplastic hepatocytes and their isolation

Hepatocyte nodules were induced according to a modified version of the Solt and Faber protocol[137,138]. Briefly, two-months-old male DPP-IV$^+$ (see paragraph 3.3 below) Fischer 344 rats were injected with a single dose of N-nitrosodiethylamine (DENA, 200 mg/kg of body weight, Sigma-Aldrich, Cat. #N0756) followed, 2 weeks later, by exposure to three consecutive daily doses of N-Acetyl-2-aminofluorene (2-AAF, 20 mg/kg of body weight, Sigma-Aldrich, Cat. #A-7015) given by gavage. The following day, all animals underwent partial hepatectomy.

Nine to ten months later, livers were perfused according to a standard 2-step collagenase perfusion technique[139,140]. After perfusion, large nodules were excised from perfused liver and isolated cells were suspended in 1X PBS. Cell suspension was filtered through a nylon mesh with a pore diameter of 100 μm, in order to eliminate any large cell clusters of pre-nodular cells and normal hepatocytes. Cell viability, determined by trypan blue dye exclusion, was 90-95% in the nodular cell preparation.

3.3. Pre-neoplastic hepatocytes transplantation using the dipeptidyl-peptidase-type IV-deficient rat model

To follow the fate of donor hepatocytes into the recipient liver, the dipeptidyl peptidase type IV (DPP-IV)-deficient rat model was used[141]. Normal F344 rats express a specific exopeptidase, DPP-IV, in a characteristic pattern in the liver, restricted to the apical domain of the plasma membrane[142]. In this transplant system, hepatocytes freshly isolated from the liver of a Fischer 344 rat expressing the enzyme (DPP-IV$^+$) are transplanted into a syngeneic

DPP-IV$^-$ host, such that donor-derived cells can be detected in the recipient liver through simple histochemical and/or immunohistochemical techniques. All animals from the long-term experiment were DPP-IV$^-$ and were transplanted with 6x10^5 pre-neoplastic cells isolated from DPP-IV$^+$ syngeneic donor *via* a mesenteric vein.

3.4. Histological analysis

3.4.1. Standard Histology

Liver tissue histology was observed on formalin-fixed paraffin-embedded tissue sections after standard Hematoxylin and Eosin (H&E) staining.

3.4.2. DPP-IV enzyme detection

To follow the fate of transplanted DPP-IV$^+$ hepatocytes we performed a histochemical staining for the detection of this specific enzyme activity as previously described[143]. Briefly, 5 µm-thick cryostat sections were fixed with 1% v/v acetic acid in absolute ethanol for 5' at -10°C to -20°C, followed by fixation with absolute ethanol for 5' at the same temperature. Sections were then stained with the substrate reagent for 15-20' at RT, then counterstained with Harris Hematoxylin solution (Sigma-Aldrich, Cat. #HHS16).

3.4.3. Detection of senescence-associated β-galactosidase activity (SA-β-gal)

Staining for SA-β-gal was performed according to published procedures[144]. Briefly, 7 µm-thick frozen sections were fixed with 0.5% glutaraldehyde and 4% formaldehyde in PBS.

Staining Reagent was prepared by mixing the following solutions: the first solution was prepared by dissolving 0.6139 g of NaCl (0.15 M) and 0.02849 g of $MgCl_2$ (2 mM) in 14 ml of citric acid/sodium phosphate buffer solution (pH 6.0); adding 3.5 ml of 100 mM potassium ferricyanide, 3.5 ml of 100 mM potassium ferrocyanide and 45.5 ml of H_2O; and warming this resulting solution to 37°C. The second solution was prepared by dissolving 0.07 g of X-Gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside, Thermo Fisher Scientific; Cat. #B1690) in 3.5 ml dimetilformamyde at 4°C. Slides were incubated in Staining Reagent overnight at 37°C.

3.4.4. LipidTox staining

5 µm-thick cryostat sections were fixed in 4% paraformaldehyde in PBS for 10' at RT then stained with Hoechst 1:1000 in PBS (Sigma Aldrich, Cat. #33258) for 10' at RT for nuclei detection. Finally, slides were stained with HCS LipidTOX™ Red phospholipidosis detection reagent 1:200 in PBS for 30' at RT (Thermo Fisher Scientific, Cat. #H34351).

3.5. Protein extraction and Western Blot analysis

Western blot analysis was performed either total or nuclear proteins extracted from liver samples. Total proteins were extracted with RIPA buffer (Composition in *Table 2.*) whereas a commercially available kit (NE-PER™ Nuclear and Cytoplasmic Extraction Reagents, Thermo Fisher Scientific, Cat. #78835) was used for nuclear-protein extraction according to manufacturer's protocol. Protein concentration was measured using the BCA method[145] (dilution factor 1:50 for total proteins and 1:20 for nuclear-protein extraction) (Bicinchoninic

Acid Kit for Protein Determination, Sigma Aldrich, Cat. #BCA1-1KT). Absorbance was read at 560 nm with a microplate reader (Infinite F200 Pro, TECAN).

Samples (75 µg and 45 µg for nuclear and total proteins, respectively) were prepared in 1:4 diluted Laemnli buffer, boiled at 95°C for 5', loaded into SDS-PAGE precast Criterion TGX Stain-Free gels (Bio-Rad Laboratories S.r.l.) and finally run under denaturing conditions. Proteins were transferred into polyvinylidene difluoride (PVDF) membranes (Immuno-Blot™ PVDF Membrane for Protein Blotting, Bio-Rad Laboratories, Cat. #162-0177), blocked with 5% non-fat milk or 3% bovine serum albumin (BSA) in PBS for 45' at room temperature, followed by incubation with primary antibodies overnight at 4°C (antibodies are listed in *Table 3.*). Membranes were then washed and incubated for 2 h at room temperature with the appropriate secondary antibody conjugated with HRP system for successive identification (antibodies are listed in *Table 3.*). Protein bands were detected using a chemiluminescent substrate (Bio-Rad Laboratories, Cat. #1705061) and imaged onto Kodak film.

3.6. RNA isolation, Reverse Transcriptase-PCR and Real-Time qPCR

Total RNA was isolated using TRIzol™ reagent (Thermo Fisher Scientific, Cat. #15596026) 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 (Cat. #A5001). The resulting cDNA was analyzed by quantitative real-time PCR using specific TaqMan assays and TaqMan Gene Expression Master Mix on a StepOne System (Thermo Fisher Scientific). The rat specific assays are listed in *Table 4*. For all assays the

thermal profile was set as follow: 1 cycle at 50˚C for 2 minutes and 95˚C for 10 minutes, and 45 cycles at 95˚C for 15 seconds and 60˚C for 1 minute. Fold change was calculated by the 2-ΔΔCT method[146].

3.7. Blood lipid profile

Lipid profile was assessed by measuring high-density lipoprotein (HDL), low-density lipoproteins (LDL) and Triglycerides (TG), through two different kits.

HDL and LDL concentrations were measured using kit #MAK045 from Sigma-Aldrich, according to manufacturer's instructions. Plasma HDL and LDL were first separated, then concentration of each target was determined by coupled enzyme assay, which resulted in a fluorometric (λ_{ex} = 535 nm / λ_{em} = 587 nm) product, proportional to the cholesterol quantity in the sample.

TG plasma levels were measured using kit #MAK266 from Sigma-Aldrich which detected the concentration of both monoglycerides and diglycerides. In this assay, TG was converted to free fatty acids and glycerol. The glycerol is then oxidized to generate a colorimetric (570 nm) product.

3.8. Enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISA was performed on rat plasm for the quantitative analysis of several target proteins using commercially available kits (*Table 5*), according to manufacturer's instructions.

Briefly, samples were assayed in duplicate at defined dilutions, and incubated to allow the target protein in the sample to bind to the immobilized first antibody on the 96-wells microplate. An enzyme-conjugated second specific antibody was then added and incubated, so to bind to the immobilized target protein. Ultimately, a specific substrate was added to each well, producing a colorimetric reaction proportional to protein concentration. Final absorbance was read on a microplate reader (Infinite F200 pro, Tecan).

3.9. Analysis of plasma amino acids

The amino acids analysis was performed by means of IE-HPLC in an accredited diagnostic laboratory using routine procedures and two identical IE-HPLC setups (HPLC 1 and 2) as previously described[147,148]. Briefly 200 µL of plasma samples were mixed with 20 µL of sulfosalicylic acid solution (40% w/v). The precipitated proteins were removed by centrifugation at 5900g and 10°C for 10'. Then the supernatant was diluted with an equal volume of citrate loading buffer, and 50 µL of the solution was injected on a Biochrom 30 Plus amino acid analyser (Biocherom Ltd). Amino acids were separated by IE-HPLC using a lithium high-performance physiological column (Biochrom Ltd) followed by postcolumn derivatization of the eluting amino acids with ninhydrin and detection of the derivatized amino acids at 570 nm and 440 nm. In total 33 amino acids could be separated over 153' by means of a step-gradient elution using 5 distinct lithium citrate buffers of different ionic strength (from 0.20 mol/L to 1.65 mol/L) and pH (from 2.80 to 3.55). The column temperature was also varied step-wise during the run from initial 33°C to final 78°C, whereas the flow was kept constant at 20 mL/h. The quantification was performed by means of external calibrators for each amino acid using the EZChrom Elite software (Agilent

Technologies Inc). The amino acids were identified according to the retention time and the ratio of the area between the 2 wavelengths (570 nm and 440 nm).

3.10. Image analysis, graphical representation of results and statistical analysis

Acquired microscopic images were processed for quantitative analysis using Image-Pro Premier software (Media Cybernetics, Rockville, USA). Western blots were densitometrically quantified with ImageJ software (NIH, USA). Graphical representation of quantitative results and statistical analysis were performed using Prism 5 software (GraphPad Software, La Jolla, USA). Student “t” test analysis was performed to compare two groups of data and the statistical differences accepted as significant were < 0.05 (*), < 0.01 (**) and < 0.001 (***).

Table 1. **Composition of standard rodent laboratory chow 4RF21 (Mucedola Srl)**

Water	12.00%
Protein	18.50%
Fat	3.00%
Fiber	6.00%
Ash	7.00%
NFE (nitrogen free extract)	53.50%

Table 2. **Composition of RIPA buffer**

Initial concentration	Dilution factor	Final concentration
NaCl 1M	1:6,6	150mM
Triton X-100 (10%)	1:10	1%
Sodium Deoxycholate (5%)	1:10	0.5%
SDS (5%)	1:50	0.1%
TRIS 1M pH 8.0	1:20	50mM

Table 3. **List of primary and secondary antibodies.**

Primary antibody	Diluition factor	Brand	Catalogue Number
p21	1:200	Santa-Cruz	SC-417
p27	1:500	Santa-Cruz	SC-1641
p53	1:200	Santa-Cruz	SC-1311
mTOR	1:2000	Abcam	Ab2732
S6K	1:500	Santa-Cruz	SC-8418
FOXO3a	1:500	Santa-Cruz	SC-11351
SIRT1	1:1000	Santa-Cruz	SC-15404
B-actin	1:10000	Abcam	Ab8227
Secondary antibody	**Diluition factor**	**Brand**	**Catalogue Number**
Anti-Rabbit IgG	1:10000	Abcam	Ab205722

Table 4. **List of rat-specific assays for gene expression**

Gene	Catalogue Code
B-Actin	Rn00667869_m1
B2M	Rn00560865_m1
Il-6	Rn01410330_m1
Il-1α	Rn00566700_m1
Il-1ß	Rn00580432_m1
Il-2	Rn00587673_m1
Il-6	Rn00578225_m1
Bmal1	Rn00577590_m1
Rev-erbα	Rn01460662_m1
Cry1	Rn01503063_m1
Per2	Rn01427704_m1

Table 5. **List of ELISA kits.**

Proteins	Detection Range	Sensitivity	Catalogue #	Company
IL-1α	1.56-100 pg/mL	0.49 pg/mL	HEA071Ra	Cloud-Clone Corp.
IL-1β	6.25-400 pg/mL	2.33 pg/mL	SEA563Ra	Cloud-Clone Corp.
Adiponectin	0.156-5 ng/mL	0.004 ng/mL	RRP300	R&D SYSTEMS
Leptin	100-4000 pg/mL	50 pg/mL	RD291001200R	BioVendor
IGF-1	0.5-18 ng/mL	0.029 ng/mL	E25	Mediagnost
BDNF (plasm)	15-1000 pg/mL	15 pg/mL	CYT306	ChemiKine™
BDNF (hippocampus)	12.29-3000 pg/mL	12 pg/mL	RAB1138	SIGMA-ALDRICH

4. Results

4.1. Time-restricted feeding and liver carcinogenesis

4.1.1. Growth curves

Body weight was monitored weekly for the entire duration of the experiment. Growth curves for ALF, TRF and CRF groups are shown in **Figure 3**. Body weights were significantly lower (~30%) in CR animals compared to other groups. Such difference was already evident after 4 weeks and persisted throughout the study, including the last 3 months, when CRF group was shifted to ALF. Animals given TRF regimen showed a slightly lower weight gain compared to ALF group; the difference became statistically significant after about 5 months and persisted thereafter, until 18 months (89% of ALF, **Figure 4**). However, during the last 3 months, when TRF group was fed *ad libitum* diet, body weight increased and reached values similar to control group (fed *ad libitum* throughout the experiment). Final body weights were 486 g ± 13, 475 g ± 10 and 379 g ± 25 in ALF, TRF and CRF groups, respectively.

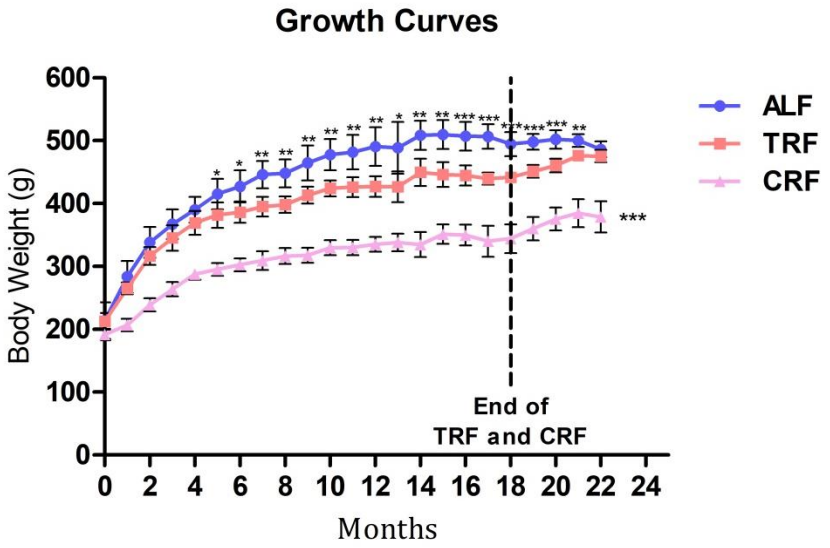

Figure 3. Growth curve of experimental animals treated with ADL, TRF and CRF for 18 months then switched to *ad libitum* diet for further 3 months. Stars at the top represent significance between ALF and TRF groups at each time point. Stars shown laterally identify significant difference between ALF and CRF groups at all time points, except for the first week of experiment (*p <0.05, **p <0.01 and ***p <0.001).

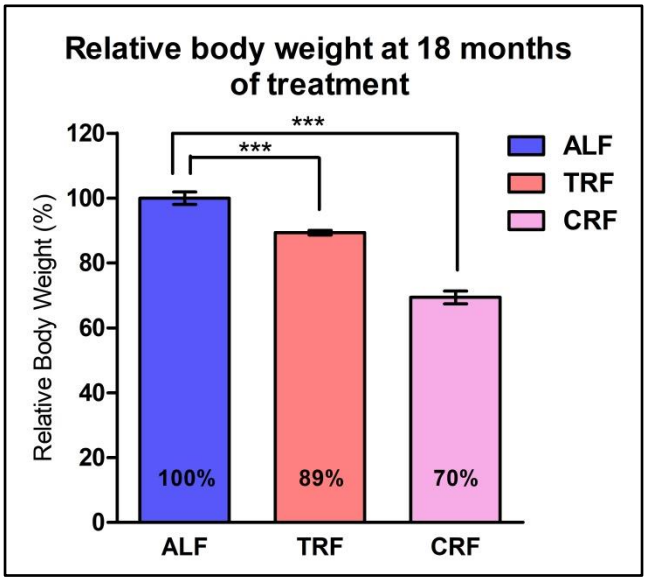

Figure 4. Relative body weight of TRF and CRF experimental groups at the end of the 18 months of treatment, compared to ALF. (P <0.001).

All groups gained body weight mostly in the first 6 months, continuing to gain, albeit more slowly, until 12 months and then remaining steady from 13 to 18 months. During the last 3 months of the study, when TRF and CRF groups were switched to *ad libitum* regimen, animals in both these groups resumed weight gain until the end of the experiment.

However, control ALF group continued to be stable and showed a slight weight loss during the last month (**Figure 5**).

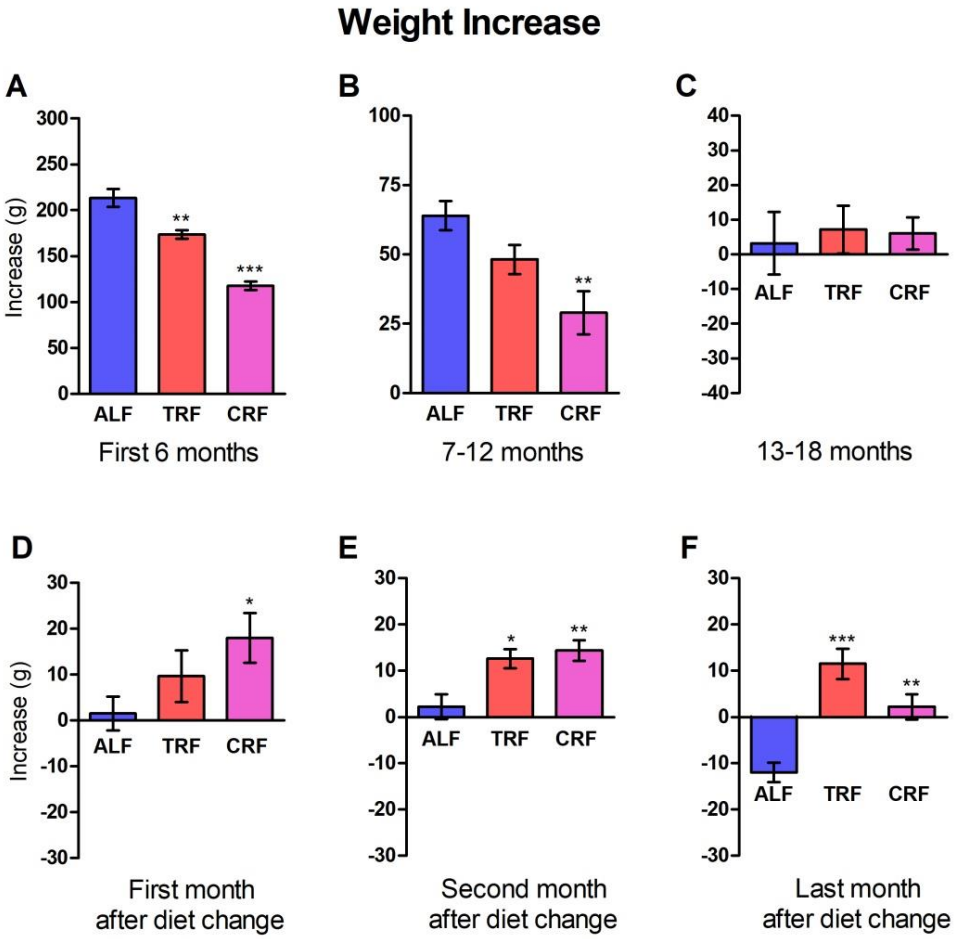

Figure 5. Weight increase during the experiment. The top row shows weight increase during the first 18 months of the experiment, divided into three time periods: first 6 months (A), 7th to 12th month (B) and 13th to 18th month (C). The bottom row shows the last three months of the experiment: first (D), second (E) and third (F) month after diet change. Stars represent significant differences from ALF (*p <0.05, **p <0.01 and ***p <0.001).

4.1.2. Food consumption

Animals were assigned to different experimental groups (ALF, TRF and CRF) at 8 weeks of age and they were maintained in their respective dietary regimens for 18 months. When computed over the entire period, average daily food intake was 19.06 ± 0.67, 17.24 ± 0.51 and 13.41 ± 0.52 g/rat for ALF, TRF and CRF groups, respectively, corresponding to 90.45 ± 2.83 and 70.27 ± 1.13% of ALF animals, for TRF and CRF groups, respectively (**Figure 6**). During the last 3 months of the study, when all groups were continued on *ad libitum* diet, food consumption was 20.36 ± 0.94, 21.20 ± 0.56 and 16.97 ± 0.34 g/rat/day for ALF, TRF and CRF groups, respectively, corresponding to 103.84 ± 1.53 and 83.13 ± 3.03% of ALF controls, for TRF and CRF groups, respectively (**Figure 6**).

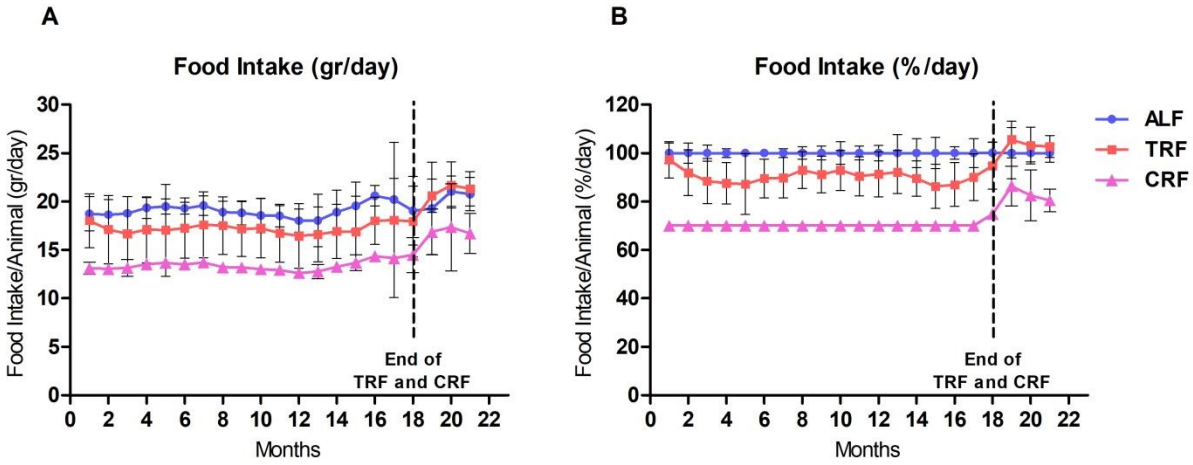

Figure 6. Daily food intake throughout the experiment expressed in grams (A) or as percentage (B).

However, when daily food intake was normalized per body weight, no significant differences were observed among the three experimental groups throughout the 18 months of exposure to either ALF, TRF or CRF dietary regimens (**Figure 7**), indicating that food intake is, at least in part, adjusted with reference to the size of the animals.

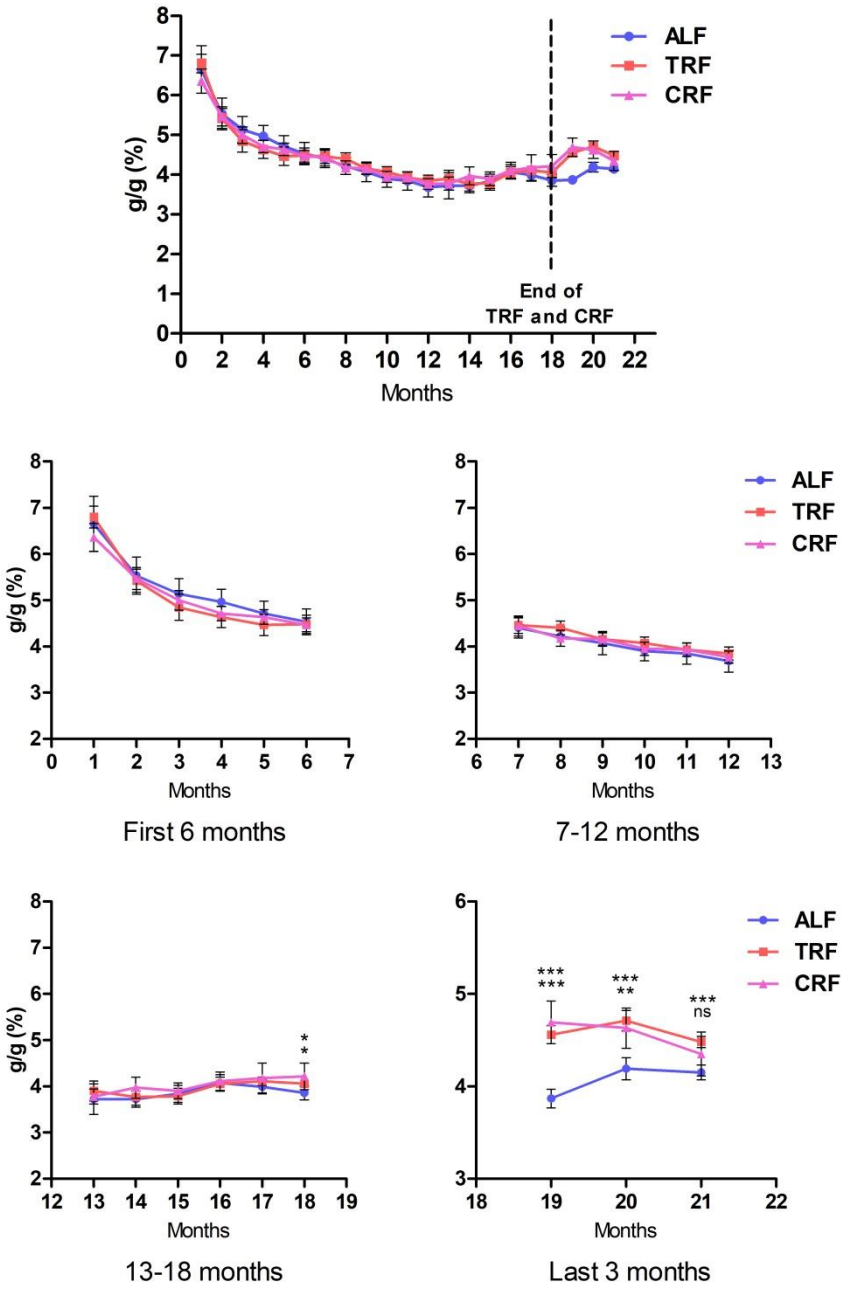

Figure 7. Food intake over body weight. A shows the trend throughout the experiment. The bottom row shows food intake/body weight ratio divided in four different time periods: first 6 months (B), 7th to 12th month (C), 13th to 18th month (D) and last 3 months (E). Stars at the top represent significance between ALF and TRF groups whereas stars at the bottom identify significant differences between ALF and CRF groups (*p <0.05, **p <0.01 and ***p <0.001).

4.1.3. Liver weight

Relative liver weights at the end of the experiment are reported in **Figure 8**. Values were 3.89 ± 0.13, 3.82 ± 0.08 and 3.24 ± 0.09% in the ALF, TRF and CRF groups, respectively and they were significantly lower only in animals receiving CRF diet.

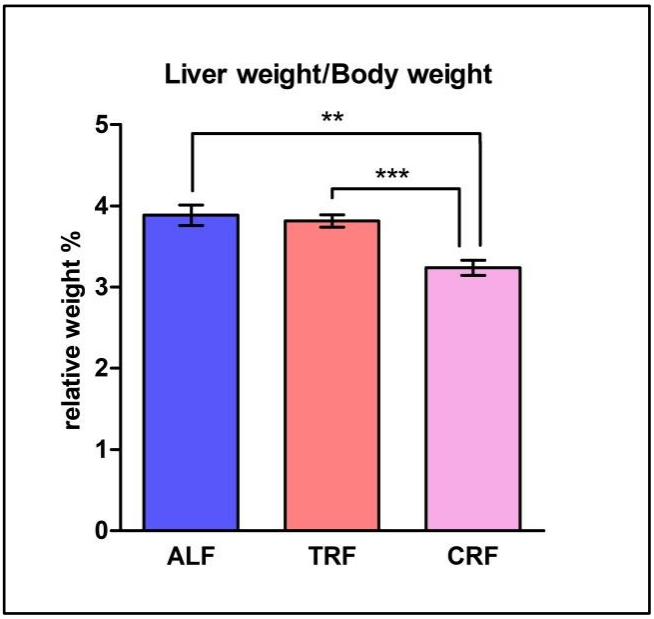

Figure 8. Liver weight over body weight at euthanasia (**p <0.01 and ***p <0.001).

4.1.4. The growth of transplanted pre-neoplastic cells

As already stated in the previous sections, the basic hypothesis of this thesis project is that long-term feeding of a TRF regimen retards the ageing process and this effect translates into a delay in the emergence of the age-associated, neoplastic prone tissue landscape. To test the latter possibility, animals were exposed to ALF, TRF or CRF for 18 months and then they were all continued on ALF during the last 3 months. One week after switching dietary regimen, animals in all groups were delivered a single injection of hepatocytes isolated from chemically-induced preneoplastic nodules, generated as described in Experimental procedures. This specific protocol was selected in order to analyse the effect of TRF, given prior to hepatocyte transplantation, on the growth of the injected pre-neoplastic cell

population, i.e. to study any effect of TRF on the emergence of the neoplastic-prone tissue microenvironment associated with ageing. Animals in all groups were killed 3 months after hepatocyte transplantation. Livers were excised and analysed both macroscopically and histologically.

A few visible hepatocyte nodules were discerned on gross examination; their incidence is reported in *Table 6*. While numbers were unsufficient for statistical analysis, a trend towards a decrease was seen in both TRF and CRF groups.

Table 6. **Hepatic lesions in rats transplanted with pre-neoplastic cells**

Nutritional behaviour	***Total animals with lesion***	***Total lesion per group***	***Size range***
Ad Libitum Feeding	4/4	8	2 mm
Time-Restricted Feeding	3/6	3	1.5-4 mm
Caloric Restricted Feeding	1/5	2	1-0.5 mm

We next performed histochemical analysis in order to detect DPP-IV-expressing, transplanted hepatocyte clusters (see Experimental procedures for details) (**Figure 9**). Ten sections per animal were considered. Results are reported in **Figure 10** as percent cluster size distribution in each group. Singlets, i.e. single DPP-IV-positive hepatocytes, added up to 28.2 ± 12.4, 37.1 ± 9.5 and 41.1 ± 19.3% of all clusters in ALF, TRF and CRF groups, respectively; small clusters, i.e. comprising 2 to 5 cells in cross section, were 21.1 ± 13.0, 26.8 ± 18.2 and 22.2 ± 15.1% of the total in ALF, TRF and CRF groups, respectively; finally, larger clusters, i.e. comprising a minimum of 6 and up to 20 cells, comprised 9.9 ± 7.6, 3.1 ± 3.6 and 4.8 ± 7.8% of total lesions in ALF, TRF and CRF groups, respectively.

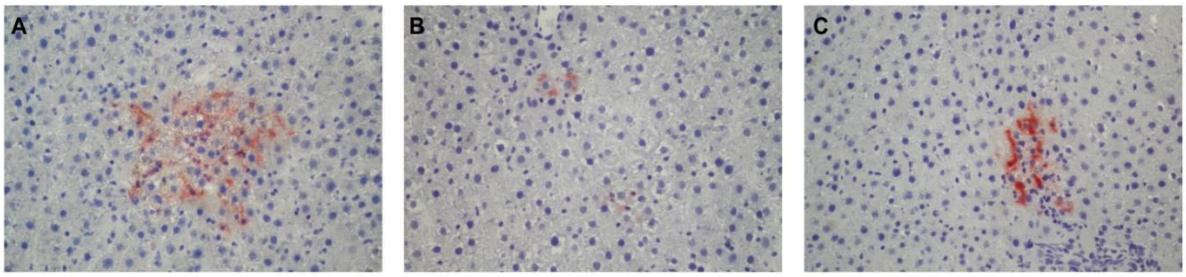

Figure 9. Representative clusters derived from transplanted pre-neoplastic hepatocytes positive for DPP-IV enzyme (rust-orange colour) in the liver of ALF (A), TRF (B) and CRF (C) groups.

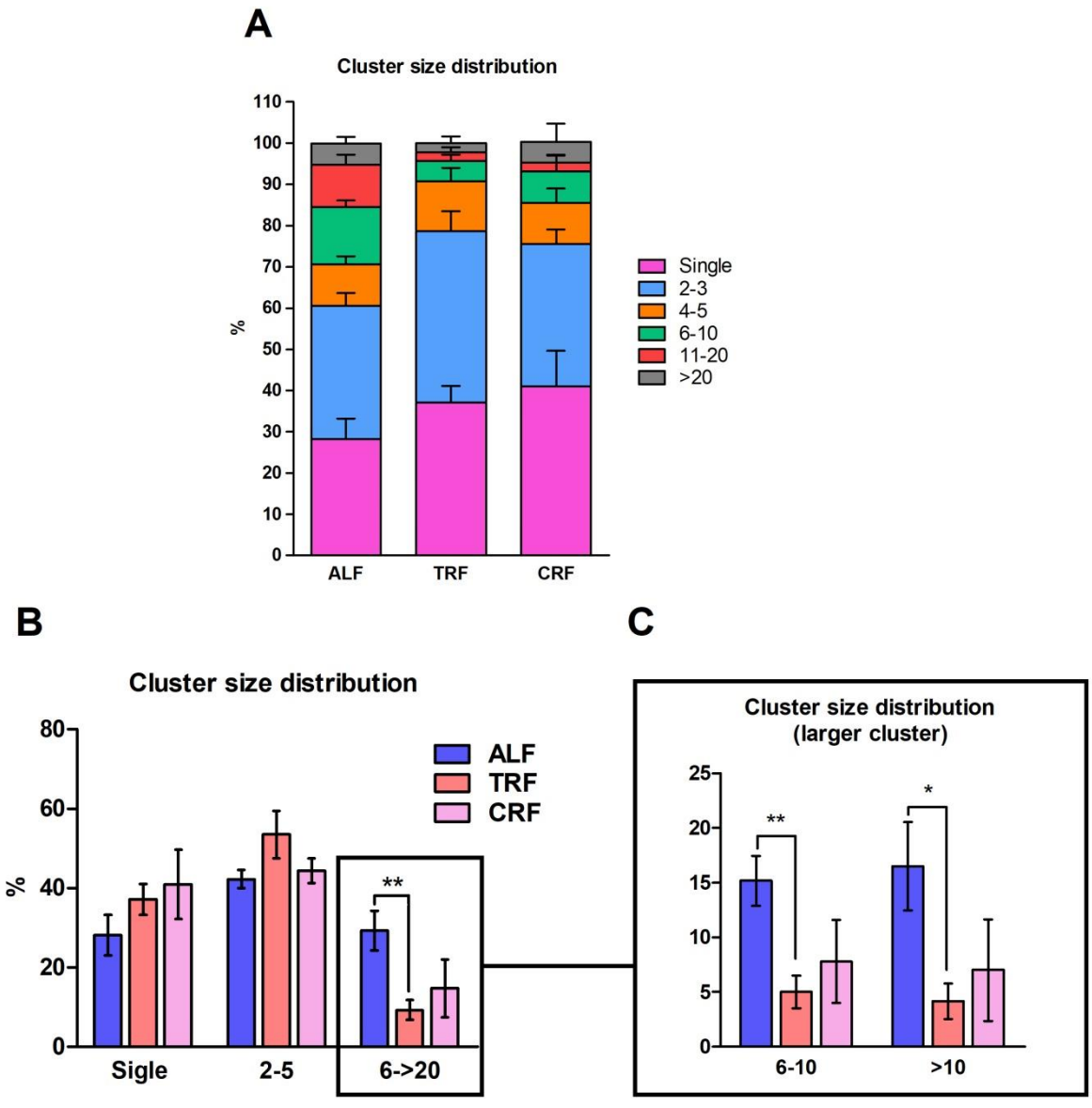

Figure 10. Cluster size of transplanted pre-neoplastic cells. A and B show percentage of clusters according to their size in ALF, TRF and CRF animals. C focuses on cluster size distribution of larger cluster only (*p <0.05 and **p <0.01).

4.1.5. Tissue morphology

Histological analysis of liver samples with standard H&E staining revealed the presence of prominent cholangiofibrosis and bile ductular proliferation, which have long been described in the ageing liver[149]. They were more frequent and larger in size in rats exposed to ALF (**Figure 11**).

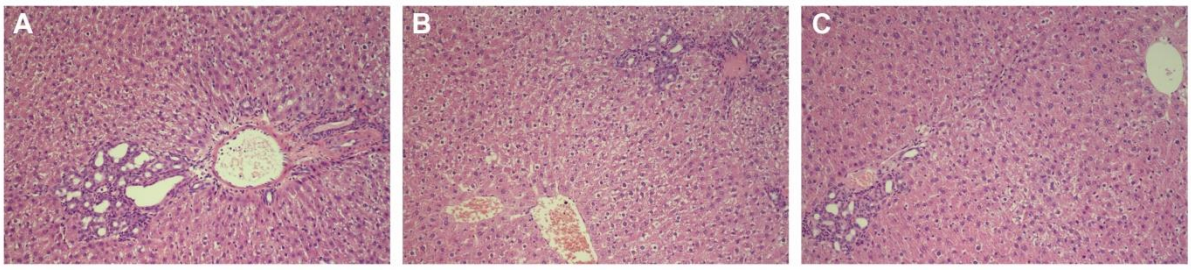

Figure 11. Histological appearance of the liver tissue of ALF (A), TRF (B) and CRF (C) groups after H&E staining.

4.2. Time-restricted feeding and the ageing liver

In the next series of studies, we investigated whether TRF regimen had any effect on the emergence of phenotypic alterations that have been associated with the ageing process in the liver.

4.2.1. Cell Senescence

As already discussed in the Introduction, cell senescence entails a persistent/irreversible arrest of the cell cycle associated with distinct phenotypic changes[150,9]. Senescent cells increase with age in many tissues, including liver, although their precise mechanistic role in the overall ageing process is yet to be defined, if any[151]. While no single property is sufficient to define cell senescence, a series of markers are currently used to identify this phenotype, including increased activity of senescence-associated β-galactosidase (SA-β-Gal), increased expression cell cycle inhibitory proteins (e.g. p53, p21, p27 and p16), increased levels of SASP-related cytokines[30].

4.2.1.1. Senescence-Associated β-galactosidase (SA-β-Gal) activity

The enzyme SA-β-Gal is located in the lysosomal compartment; it is the most widely used biomarker for senescent cells and it is defined as beta-galactosidase activity detectable at pH 6.0[152]. We performed histochemical staining of liver sections obtained from ALF or TRF groups, treated as described in the previous paragraph (18 months on ALF or TRF regimens, followed by 3 months of *ad libitum* diet); three sections from each lobe were examined and expression of SA-β-Gal was estimated using an image analyser (see Experimental procedures for details) (**Figure 12**). Results are presented in **Figure 13**: significant differences were found

in percent SA-β-Gal-stained areas/total between TRF and controls, with values of 1.05±0.27% and 2.47±0.31% in TRF and ALF groups, respectively (P value < 0.01).

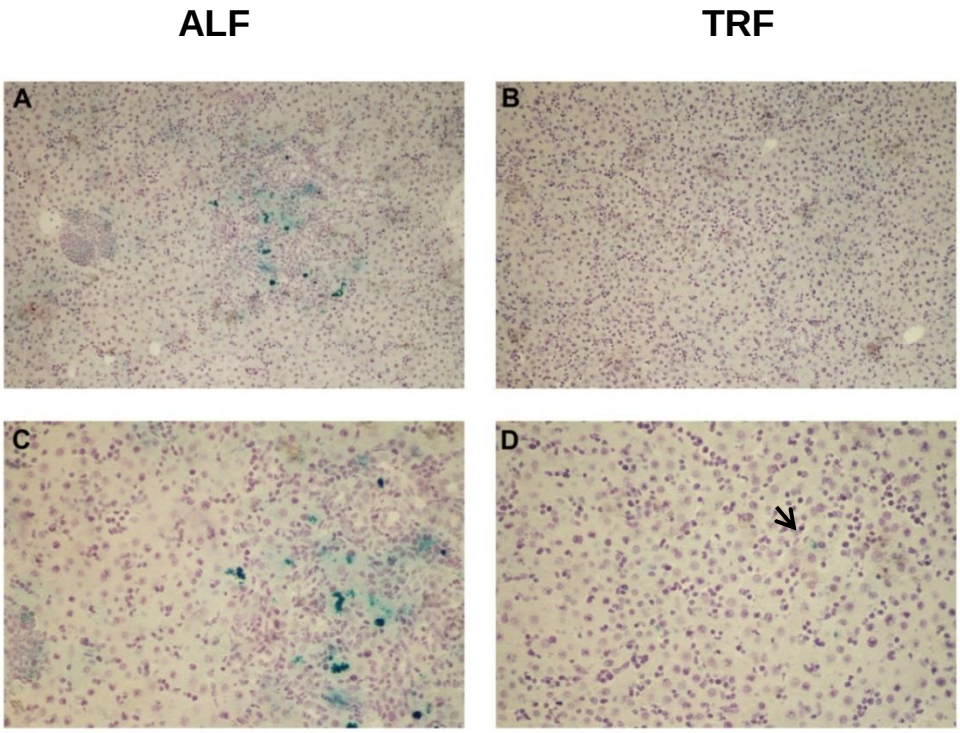

Figure 12. SA-β-gal staining for ALF group (A and C) and TRF group (B and D) (A and B: 100x; C and D: 200x). Only rare positive cells were discerned in TRF group (arrow in bottom-right panel).

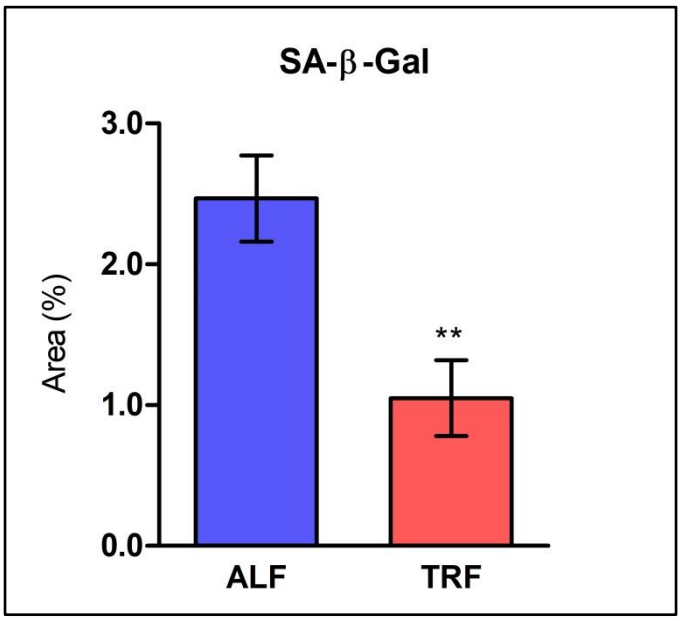

Figure 13. Percent area positive for SA-β-gal staining in long-term treated ALF and TRF groups (P value <0.01).

We also determined the expression of SA-β-Gal in in the liver of rats exposed to 3 months of TRF, starting at 18 months of age. In this setting, animals were killed while on TRF regimen. As reported in **Figure 14** lower levels of the senescence markers were detected in TRF group compared to ALF controls (P value < 0.05), indicating that a 3-month feeding of this dietary protocol, starting at old age, is able to induce at least some of the effects associated with long-term exposure.

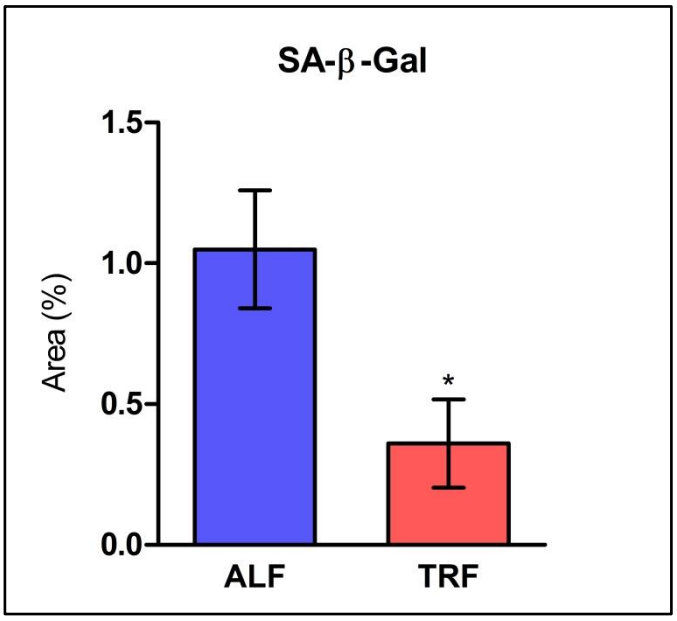

Figure 14. Percent area positive for SA-β-gal staining in ALF and TRF started at 18 months groups (P value <0.05).

4.2.1.2. Cell cycle inhibitory proteins

Up-regulation of cell cycle inhibitory proteins is yet another marker that has been associated with the senescence phenotype. The tumour suppressor p53 is implicated in several mechanisms to contrast tumour development and progression, such as activation of proteins involved in DNA damage repair, induction of apoptosis and cell cycle arrest, in order to avoid proliferation of altered cell.

Cell cycle regulatory proteins also include p21 and p27, which inhibit the function of cyclin dependent kinases (CDKs). Acting downstream of p53, p21 is able to bind different cyclin

complexes, such as CDK2, CDK1 and CDK4/6, leading to cell cycle arrest in G1 or S phase. A similar role is exerted by p27, which prevents activation of CDK2 and CDK4, controlling cell cycle progression at G1 phase.

Expression of p53, p27 and p21 was analysed in the liver of animals exposed to TRF or ALF for 18 months and then continued on ALF for 3 months. As reported in **Figures 15** and **16**, no significant differences were detected in the expression of any of these putative markers of cell senescence between the two groups.

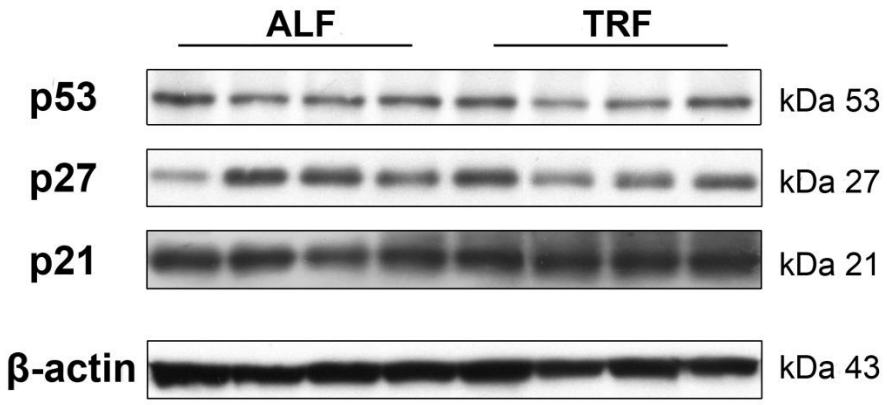

Figure 15. Western Blot analysis of p53, p27 and p21 in ALF and TRF treated animals. WB was performed on nuclear proteins and nuclear β-actin was used as control.

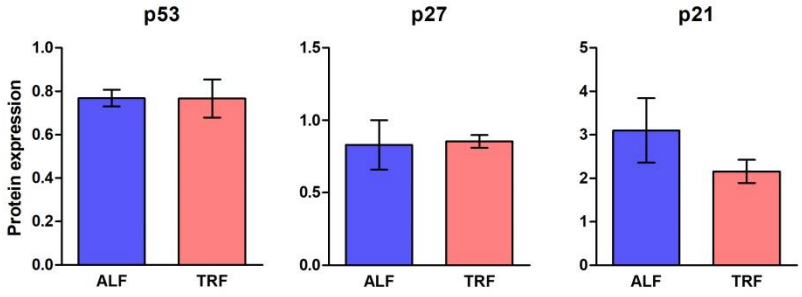

Figure 16. Densitometric analysis of proteins expression for p53, p27 and p21 as assessed by WB.

4.2.1.3. The Senescence-Associate Secretory Phonotype

Inflammatory cytokines play a relevant role in initiation and evolution of various disease processes, including cancer and cardiovascular pathologies, and it is well known that their levels increase during ageing[153]. Such an increase may be due, at least in part, to SASP components[154]. Senescence-associated cytokines can exert their effects both locally, acting in a paracrine fashion on neighbouring cells, but also at systemic level, potentially contributing to inflammaging.

Levels of IL-6, IL-2 and IL-8 expression were measured in both liver and plasma of animals exposed to ALF or TRF for 18 months and then continued on ALF for 3 months. Relative gene expression of IL-6, IL-2 and IL-8 tended to be lower in liver of TRF group, although the difference was not statistically significant; on the other hand, TRF exerted no effect on liver mRNA levels of IL-1α and IL-1β, confirming previous data obtained in mice[133]. In addition, plasma levels of IL-1α and IL-1β cytokines were similar in ALF and TRF animals (**Figure 17**).

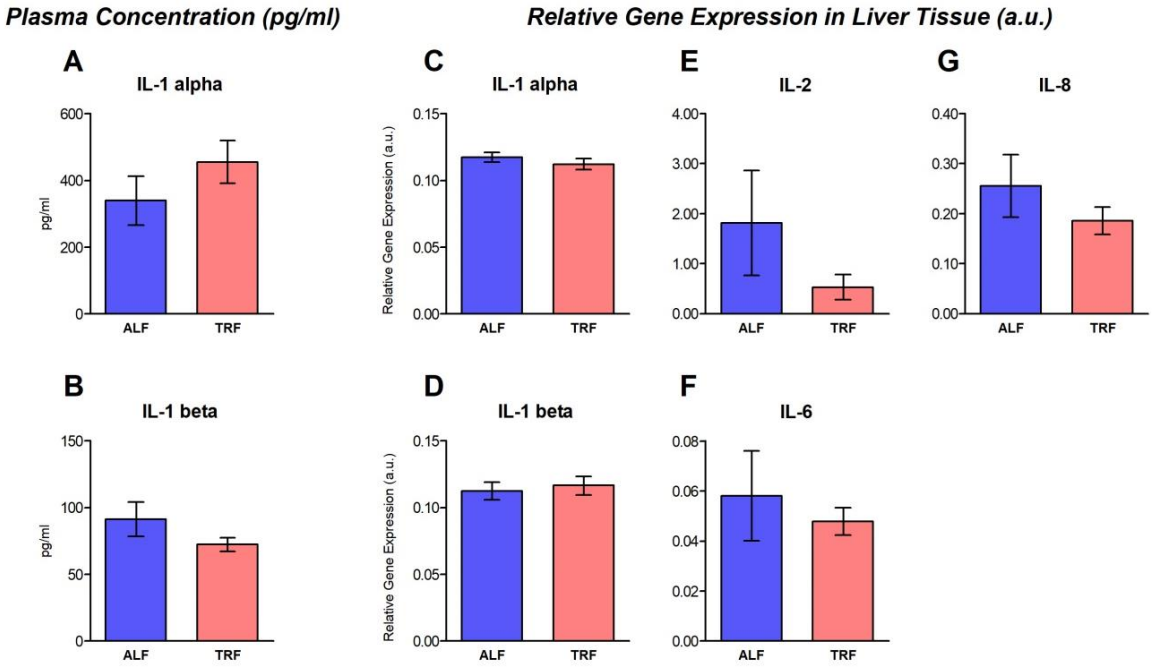

Figure 17. Quantification of ILs in long-term treated ALF and TRF groups. The left column shows plasma concentration of IL-1 alpha and beta. Right side histograms show hepatic mRNA expression of ILs relative to β-actin. No significant differences were observed.

Similar analyses were also performed in animals exposed to TRF for 3 months starting at 10 or 18 months of age. Results are reported in **Figures 18** and **19**. Their overall pattern was very similar to that of the previous study reported above: no significant differences were apparent in the liver expression levels IL-1α, IL-1β, IL-2, IL-6 or IL-8 when animals were given a TRF diet starting at 10 or 18 months. However, plasma levels of IL-1α and IL-1β were generally lower following 3 months of exposure to TRF, albeit statistical significance was not consistently present (**Figures 18** and **19**).

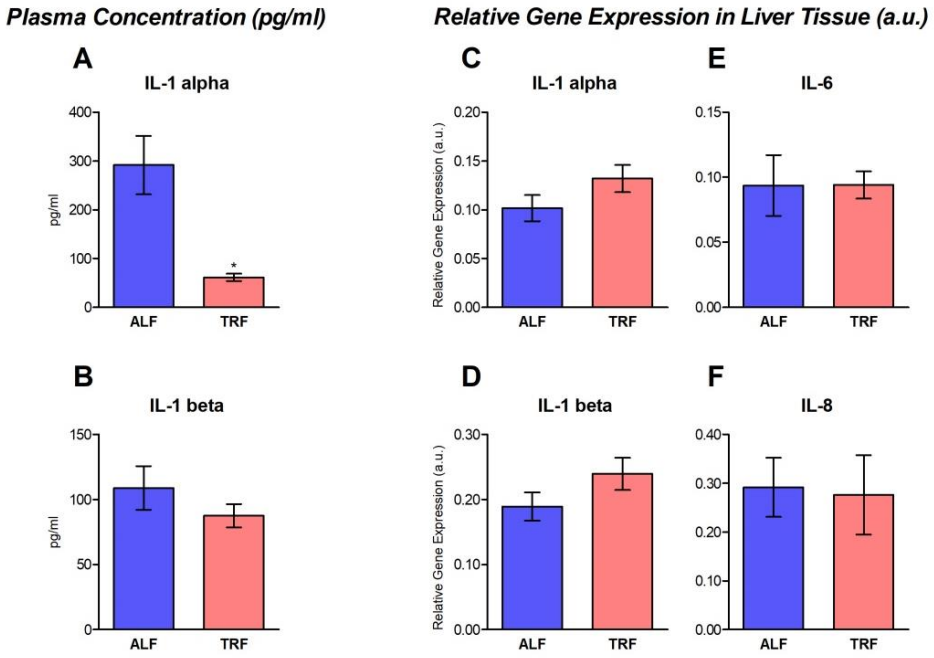

Figure 18. Quantification of ILs in rats started on ALF and TRF at the age of 18 months. The left column shows plasma concentration of IL-1 alpha and beta. Right side histograms show hepatic mRNA expression of ILs relative to β-actin. A significant difference was observed for IL-1 alpha gene expression (P value <0.05).

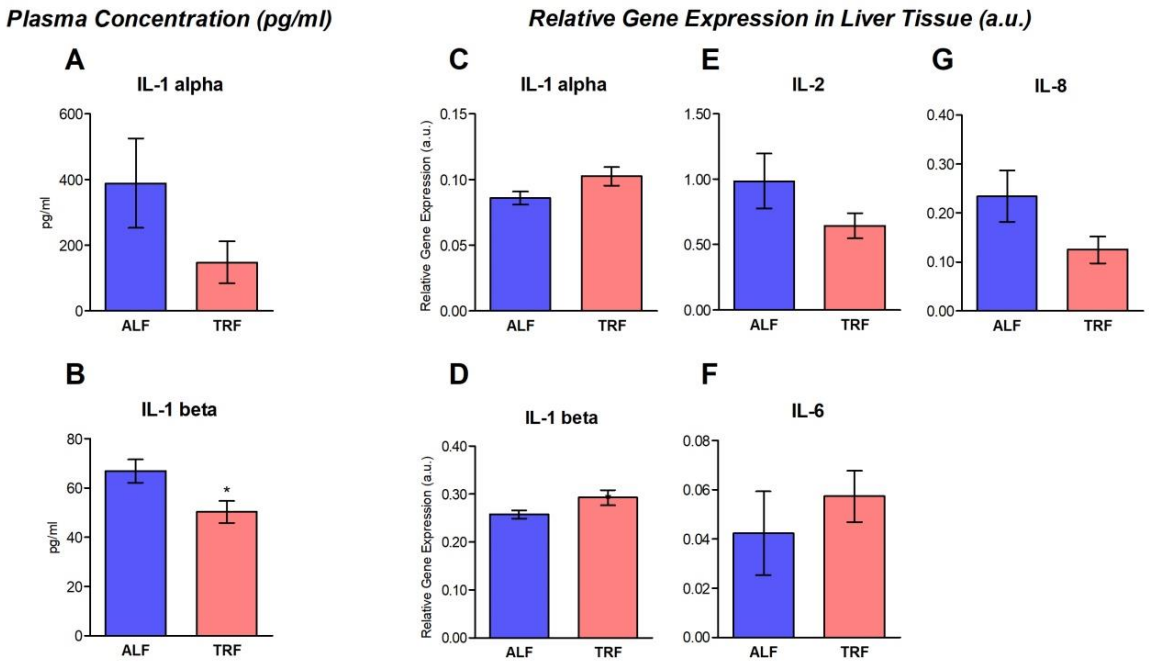

Figure 19. Quantification of ILs in rats given ALF or TRF starting at 10 months of age. The left column shows plasma concentration of IL-1 alpha and beta. Right side histograms show hepatic mRNA expression of ILs relative to β-actin. A significant difference was observed for IL-1 beta gene expression (P value <0.05).

4.2.2. Nutrient sensing pathways

Alterations in nutrient sensing pathways are considered as one of the "hallmarks" of ageing, and several lines of evidence suggest that such altered metabolic regulation does in fact represent an important driver of the ageing process[90,91].

The IIS, mTOR, FOXO and SIRT signalling cascades (discussed in the Introduction) are considered among the most ageing-sensitive and their manipulation has been involved in improving longevity in several experimental settings[90,91]. Thus, it became important to determine whether TRF regimen had any effect on the regulation of any of the above metabolic pathways.

4.2.2.1. Insulin/IGF-1 signalling pathway

The IIS is the first nutrient sensing pathway found to be implicated in the ageing process. It promotes cell growth and proliferation, glucose metabolism and protein synthesis, while it decreases apoptosis.

Evidences of protective effect of decreased IIS activity on longevity are found in several animal species, from *Ceanorhabditis elegans* to mammals, including mutant mice such as Ames dwarf, Snell dwarf and GHRKO mice, which show very low levels of GH and/or IGF-1[98]. Furthermore, epidemiologic studies on centenarians and healthy middle-aged individuals from long-lived families reported reduced levels of insulin and glucose, increased adiponectin levels and improved insulin sensitivity[155,156]. Drugs that reduce the synthesis of growth hormone or prevent IGF-1 release or its binding to the membrane receptor are also being proposed as a means to extend longevity[23].

In light of the above, the effect of long-term exposure to TRF on plasma levels of IGF-1 was determined using ELISA method. Rats were treated with ALF or TRF dietary regimen for 18 months, and were then continued on ALF for 3 months. A significant decrease of IGF-1 was detected in TRF group compared to controls (**Figure 20**, $P < 0.05$).

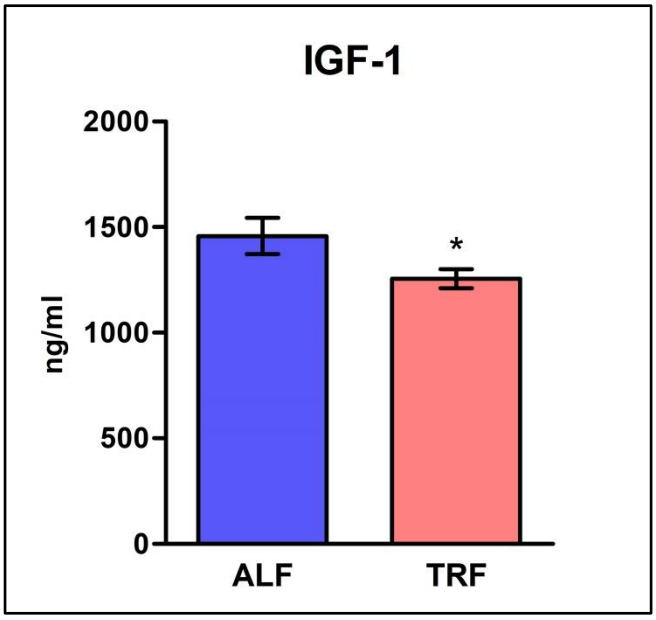

Figure 20. IGF-1 levels in plasma of long-term treated ALF and TRF animals (P value <0.05).

Furthermore, a similar, indeed more prominent result was observed when TRF diet was started at 18 months of age and maintained for 3 months (**Figure 21**, P <0.01), thus confirming that this diet is able to modulate the IIS pathway and that the effect persists after switching to ALF.

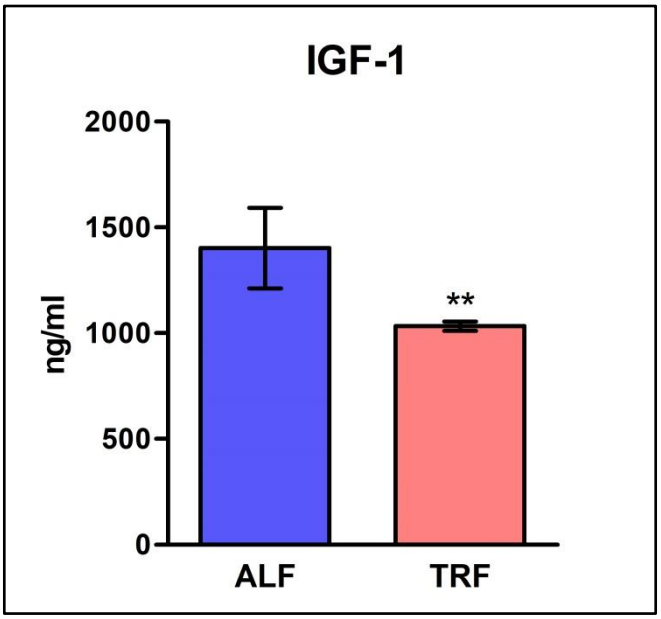

Figure 21. IGF-1 levels in plasma of animals given ALF or TRF for 3 months, starting at the age of 18 months (P value <0.01).

4.2.2.2. Mammalian target of rapamycin (mTOR) and ribosomal S6 kinase (S6K)

Mammalian target of rapamycin, mTOR, is a serine/threonine kinase, belonging to the family of phosphatidylinositol 3-kinase (PI3K). It is a main intracellular downstream effector of IIS pathway and integrates two different types of stimuli, originated from stress agents and from insulin/IGF-1 signalling. At cellular level, mTOR has a key role in proliferation and growth-related processes, such as protein synthesis, regulation of metabolism, particularly anabolic mechanisms; ribosome biogenesis; transcription; and apoptosis. These functions are mediated by two complexes, mTORC1 and mTORC2, which activate downstream

effectors, including the ribosomal S6 kinase or S6K, which has a major role in the regulation of apoptosis[157].

Inhibition of mTOR with rapamycin translates into delayed ageing and increased life span in yeast, worms, flies, and mice[98].

Given these premises, it was important to verify whether TRF could exert any effect on mTOR expression levels under the experimental conditions used in our studies. Western blot analysis of liver proteins revealed no significant differences between animals exposed ALF or TRF for 18 months and then given ALF for an additional 3 months (**Figures 22** and **23**). Moreover, no changes were found when levels of S6K, a main downstream effector of mTOR pathway, were measured (**Figures 22** and **23**). These results are in line with those obtained after long-term exposure to caloric restriction[158].

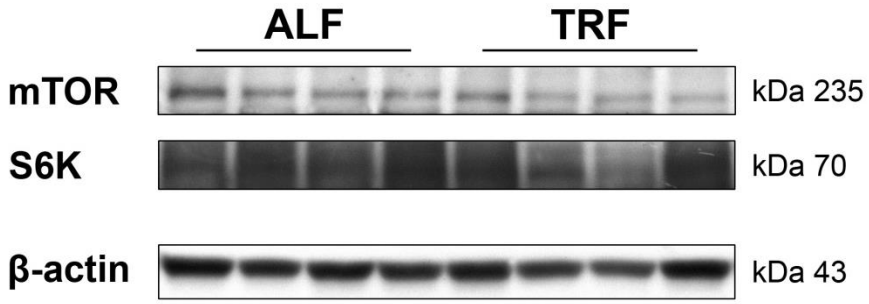

Figure 22. Western Blot analysis of mTOR and S6K in ALF and TRF treated animals. WB was performed on nuclear proteins and nuclear β-actin was used as control.

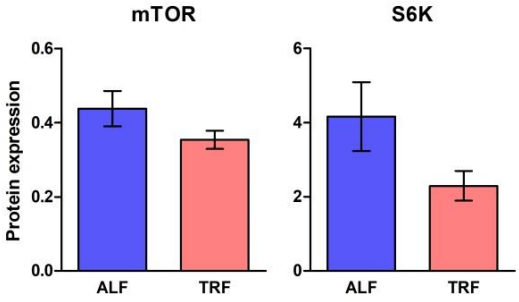

Figure 23. Densitometrical analysis of proteins expression for mTOR and S6K as assessed by WB.

4.2.2.3. Forkhead box protein O (FOXO)

A decreased activity of ISS pathway is associated with upregulation of the FOXO proteins family[93]. Intracellular signalling triggered by IGF-1 and insulin drives inactivation of FOXO transcription factors, through phosphorylation and nuclear exclusion of these proteins. Instead, when both IGF-1 and insulin are down-regulated, the resulting enhanced activity of FOXO potentiates cell protective mechanisms, including stress resistance, autophagy and ubiquitin-proteasome system, cell cycle arrest and apoptosis[5]. Indeed, several studies have demonstrated the positive role of FOXO family in extending longevity, promoting antioxidant capacity through activation of enzymes such as haem oxygenase (HOI), superoxide dismutase (SOD) and catalase[159], and reinforcing the regenerative potential of stem cell[5].

Based on the above considerations, we measured the expression of FOXO3a in rat liver exposed to either ALF or CRF diet for 18 months and then continued on ALF for 3 months. While results indicated a trend towards increased levels in TRF-treated animals, the difference did not reach statistical significance (**Figures 24** and **25**).

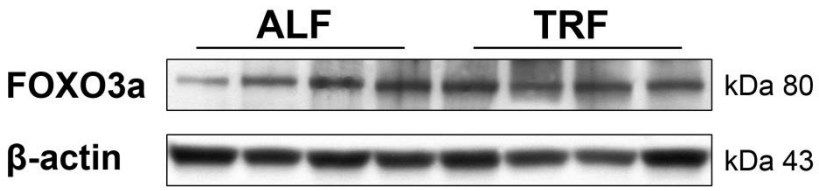

Figure 24. Western Blot analysis of FOXO3a in ALF and TRF treated animals. WB was performed on nuclear proteins and nuclear β-actin was used as control.

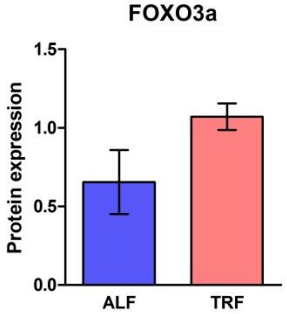

Figure 25. Densitometrical analysis of proteins expression for FOXO3a as assessed by WB..

4.2.2.4. Sirtuin family of NAD-dependent protein deacetylases

Sirtuins are a family of NAD-dependent protein deacetylases and the genes encoding for these proteins are well preserved across species. In mammals, seven members of this family, SIRT1 to SIRT7, have been identified, with cellular distribution: SIRT1 and SIRT2 are localized both in nucleus and cytoplasm, SIRT3, SIRT4 and SIRT5 are mitochondrial, while SIRT6 and SIRT7 are exclusively nuclear[160].

SIRT1, the most studied sirtuin in the context of ageing, is localized predominantly at nuclear level, where it is involved in deacetylation of histone proteins, such as H3, H4 and H1. However it also acts on over 50 non-histone nuclear proteins, including transcription factors and DNA repair proteins[161].

Sirtuins play a central role in diverse mechanisms associated with the emergence of the ageing phenotype, such as regulation of energy metabolism, cell survival, DNA damage repair, tissue regeneration, inflammation, neuronal signalling and circadian rhythms[161].

The role of sirtuins in enhancing lifespan in mammalian systems is not yet clarified; however numerous evidences highlight the supportive effect of sirtuin up-regulation on longevity and/or healthy ageing. For example, transgenic mice overexpressing *Sirt1* do not show increased lifespan, but nevertheless they were less prone to disease[98]; likewise, increased activity of SIRT1 could protect against cardiac dysfunction, apoptosis and oxidative stress[157,160]. Moreover, transgenic mice with moderate overexpression of *Sirt1* were protected against inflammation, liver cancer, steatosis and diabetes[161].

The positive effect of sirtuins on longevity and healthy ageing is possibly linked to its interaction with major conserved pathways of longevity, such as mTOR, FOXO and IIS signalling network. Moreover, the ability of sirtuins to impact on the circadian metabolic clock could explain the correlation between upregulation of sirtuin and healthy ageing,

inasmuch as the preservation of circadian rhythms, altered and dampened in old organisms, is considered as a concrete strategy to delay ageing[162].

In line with above, it was deemed reasonable to consider whether long-term exposure to TRF was able to modify the expression of SIRT1 in the liver. As shown in **Figures 26** and **27**, a 3-fold increase in SIRT1 protein levels was found in TRF group compared to controls. These results parallel those previously reported by our[95] and other research groups[163], who observed an increase in SIRT1 expression in the liver of animals fed a CRF diet.

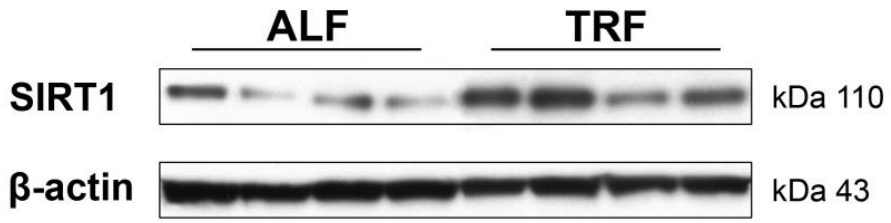

Figure 26. Western Blot analysis of SIRT1 in ALF and TRF treated animals. WB was performed on nuclear proteins and nuclear β-actin was used as control.

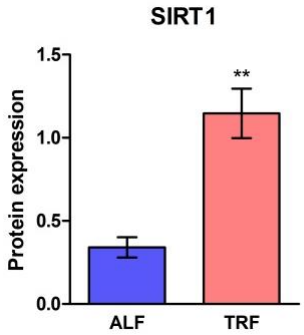

Figure 27. Densitometrical analysis of proteins expression for SIRT1 as assessed by WB (P value <0.01).

4.2.3. Hepatic steatosis

Ageing is an important risk factor for development of metabolic disorders, such as hepatic steatosis, insulin resistance and sarcopenia [164]. Hepatic steatosis is an early phase of non-alcoholic fatty liver disease (NAFLD); as a consequence, ageing also represents a known risk factor for this pathological alteration. Patients with NAFLD are at higher risk for the development of liver cirrhosis and hepatocellular carcinoma [165,166]. A defining feature of NAFLD is the deposition of lipid droplets in the cytoplasm of hepatic cells. Given the potential effect of TRF on the emergence of age-associated phenotypes, we set up a study to determine the extent of lipid droplets accumulation in the liver of animals exposed to this dietary regimen. Cryostat sections obtained from ALF or TRF-treated rats (18 months on their respective diets followed by 3 months on ALF) were stained with HCS LipidTOX™ Red phospholipidosis detection reagent[167] (**Figure 28**). Quantitation of lipid droplets revealed a significantly decreased accumulation in the livers of rats exposed to TRF (**Figure 29**).

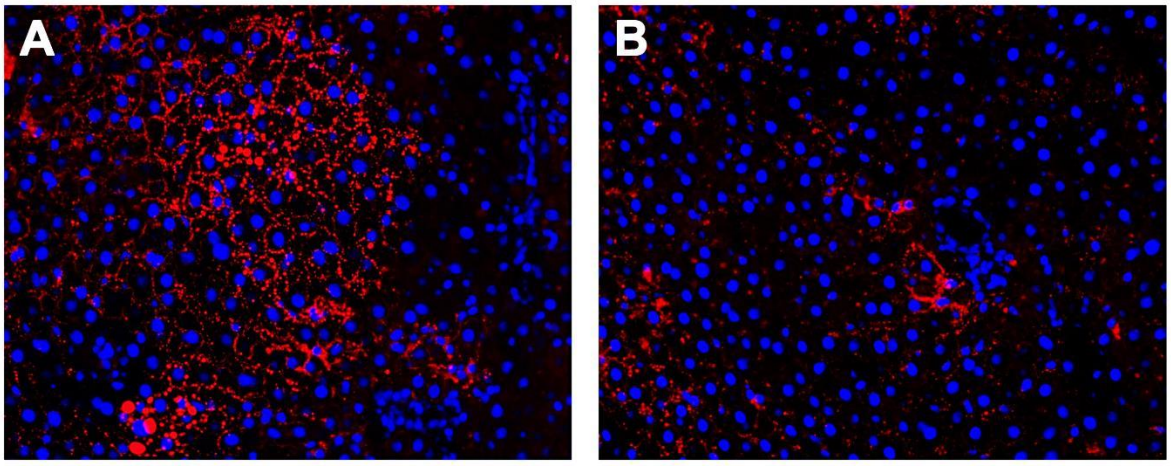

Figure 28. Immunofluorescence analysis of intracellular lipid accumulation (red) in the liver of long-term treated ALF (A) and TRF (B) animals. Nuclei were stained with Hoesct (blue).

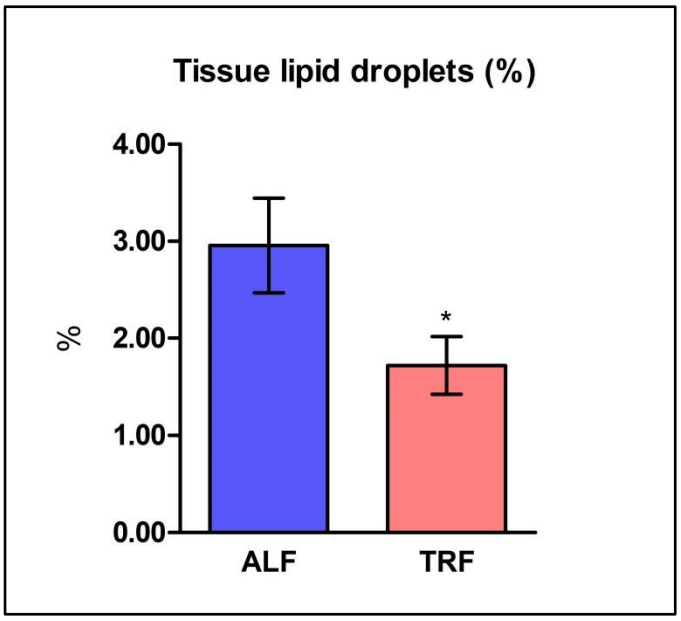

Figure 29. Quantification of immunofluorescence analysis of intracellular lipid accumulation in the liver of long-term treated ALF and TRF animals (P value <0.05).

Similar results were obtained following a 3-mos exposure to TRF diet in animals of different age (10 or 18-mos old) (**Figure 30**), supporting the conclusion that this dietary regimen exerts a protective effect towards the risk of metabolic disease[166,128,124].

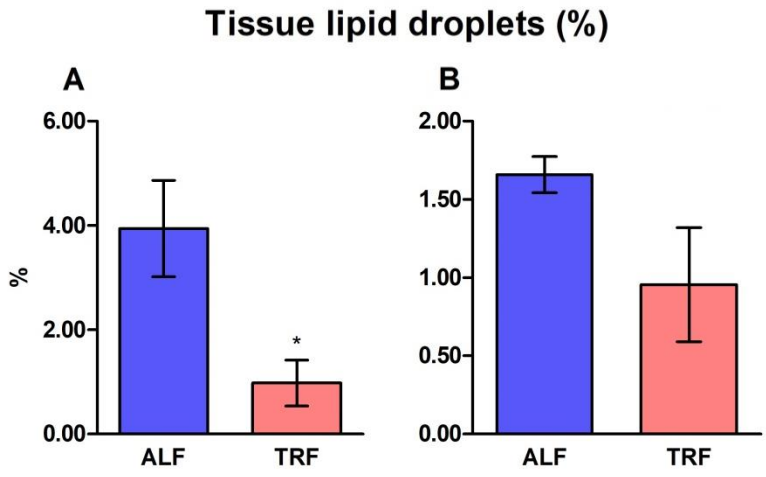

Figure 30. Quantification of immunofluorescence analysis of intracellular lipid accumulation in the liver of ALF and TRF started at 18 months (A) and at 10 months (B) animals (P value <0.05).

4.3. Time-restricted feeding and systemic ageing

4.3.1. Leptin and Adiponectin

Leptin, the "satiety hormone", is an adipokine, i.e. produced primarily by adipocytes, but expressed in various tissues, such as liver, cardiovascular, reproductive, neuronal and musculoskeletal. The major role of leptin is the control of food intake and energy expenditure. Indeed, this hormone links white adipose tissue and central nervous circuits, with the effect of reducing appetite and increasing energy consumption. Its main target for the control of food intake are neuropeptide Y (NPY)-secreting neurons in the nucleus arcuatus of the hypothalamus: leptin inhibits both the synthesis and the release of NPY[134].

Several lines of evidence point to a correlation between increased levels of leptin and rate of ageing and age-related diseases[168]; hyperleptinemia is strongly associated with insulin resistance, hypertension, hyperlipidaemia and metabolic syndrome, typical alterations of old age[169].

If leptin is the "satiety" hormone, adiponectin is the "starvation" hormone. This adipokine has also been involved in the ageing process and it is considered as an enhancer of longevity. In general, leptin and adiponectin show opposite effects on inflammation: leptin induces over-expression of pro-inflammatory markers, such as IL-6 and TNF-α, while adiponectin alleviates the inflammatory process, inhibiting production and release of many immune mediators[170]. The above effects of leptin and adiponectin are also relevant to inflammaging and to the role of inflammation in the pathogenesis of neoplasia[171].

Based on the above, levels of both adipokines were measured in rats exposed to ALF or TRF for 18 months and continued on ALF for 3 months. As shown in **Figure 31**, a trend towards

decreased leptin and increased adiponectin levels was evident in animals exposed to TRF, but differences were not statistically significant (**Figure 31**).

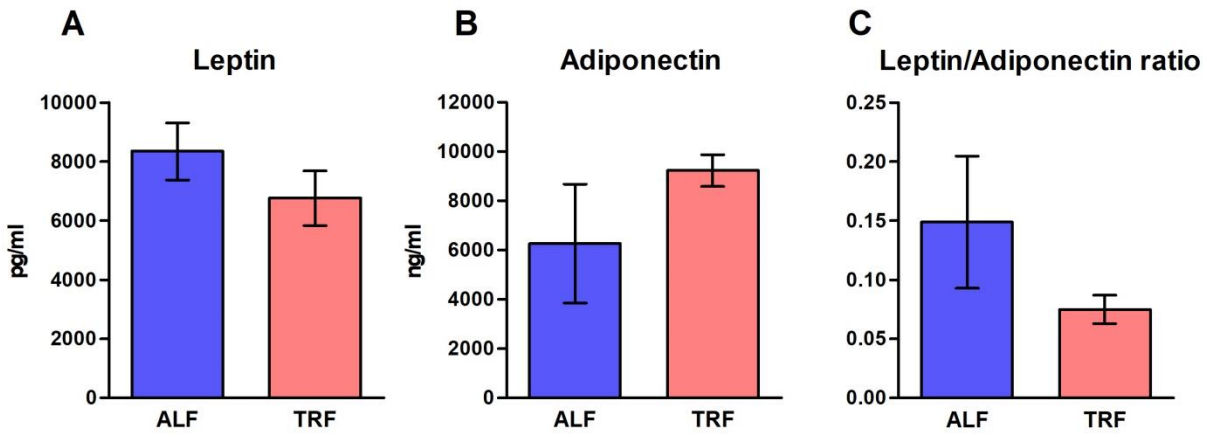

Figure 31. Quantification of plasma leptin and adiponectin, and their ratio in long-term treated ALF and TRF animals. No significant differences were observed.

Similar analysis was also performed on plasma obtained from animals given TRF for 3 months, starting at 18 months of age (**Figure 32**). Importantly, these animals were killed while on TRF regimen. Age matched controls were exposed to ALF throughout the study. Significant differences were observed between the two groups, with lower leptin, higher adiponectin and decreased leptin/adiponectin ratio in TRF-treated rats. Interestingly, these results are in line with those of a study on human subjects undergoing different types of dietary intervention (i.e. caloric restriction, very low caloric diet, intermittent fasting, alternate day fasting)[172].

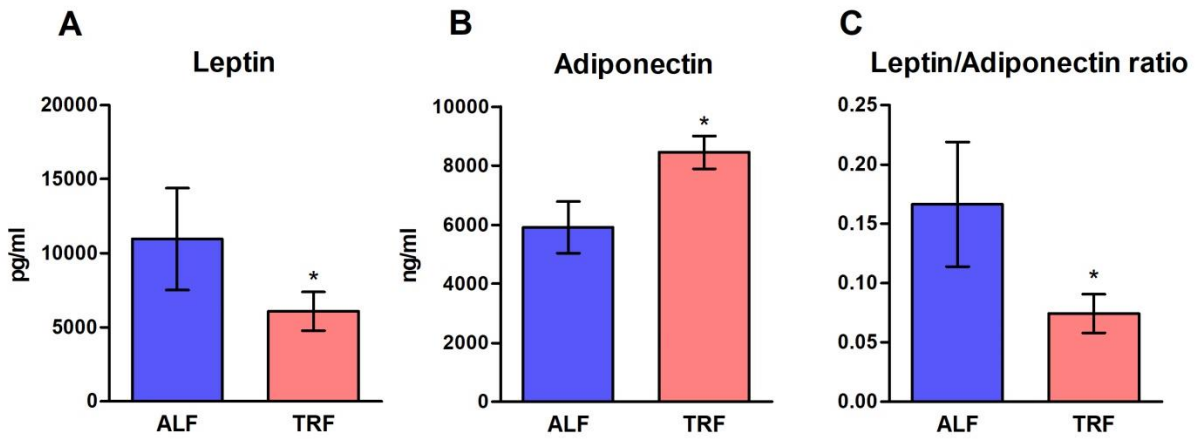

Figure 32. Quantification of plasma leptin and adiponectin, and their ratio following a 3-mos exposure to TRF starting at the age of 18 months (P value <0.05).

4.3.2. Blood lipid profile

Cholesterol plays a key role in the life of any animal species. It is fundamental for all cells as it is a primary constituent of lipid membranes and it is involved in membrane fluidity. Cholesterol is a precursor of steroid hormones, which have essential physiological functions[173]. On the other hand, alterations in plasma lipid profile (hypertriglyceridemia and hypercholesterolemia), are a frequent finding during ageing and are implicated in the pathogenesis of the most common human chronic diseases, such cardiovascular disease[174]. About 25% of men and 42% of women older than 65 years show increased level of total plasma cholesterol[175,176] and higher concentrations of low density lipoprotein (LDL) concentrations are found in old individuals of both sexes[173].

However, lipid profile is susceptible to modulation through interventions on life style, physical activity and nutritional behaviour, among others. Within this context, recent data suggest that TRF might represent a reasonable and feasible strategy to counter the age-associated rise in the incidence of dyslipidemia[124,128].

To test this possibility under our experimental conditions, plasma lipids, including triglycerides (TG), low density lipoproteins (LDL) and high density lipoprotein (HDL), were measured in rats exposed to ALF or TRF for 18 months, followed by ALF for an additional 3 months.

As shown in **Figure 33**, TRF treatment was associated with significantly decreased levels of all lipid parameters examined, i.e. TG, LDL and HDL.

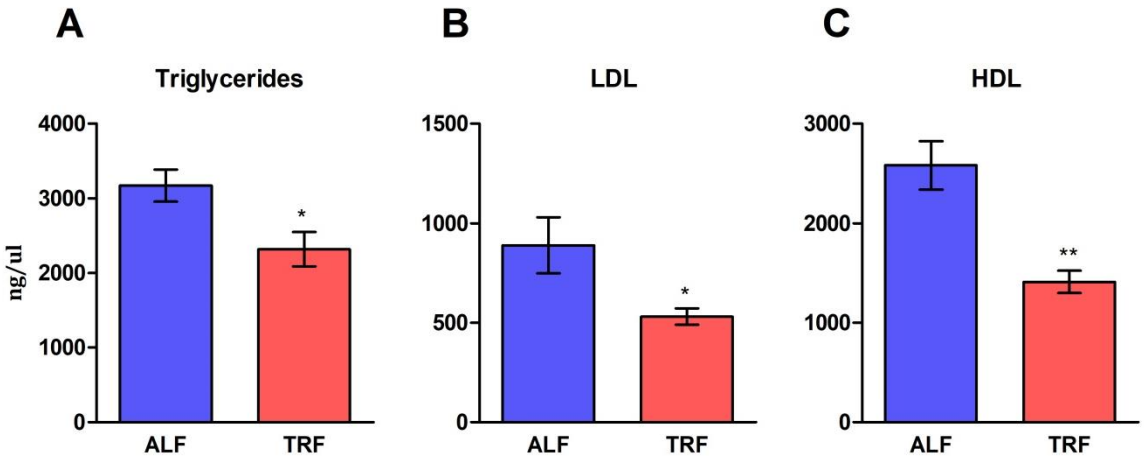

Figure 33. Quantification of plasma triglycerides (A), LDL (B) and HDL (C) in long-term treated ALF and TRF animals (*p <0.05 and **p <0.01).

These results are in agreement with those of previous studies[123]; the ability of TRF to correct dyslipidemia has been proposed as a preventive and therapeutic intervention against various metabolic diseases[123].

As a follow up to the observation reported above, we monitored the effect of TRF on plasma lipids profile in animals exposed to this dietary regimen for 3 months, starting at the age of 10 or 18 months. Results are presented in **Figure 34**. Two sets of findings are noteworthy. Firstly, a 3-mos exposure to TRF was sufficient to lower plasma levels of TG, LDL and HDL, irrespective of whether the dietary regimen began at 10 or 18 months of age.

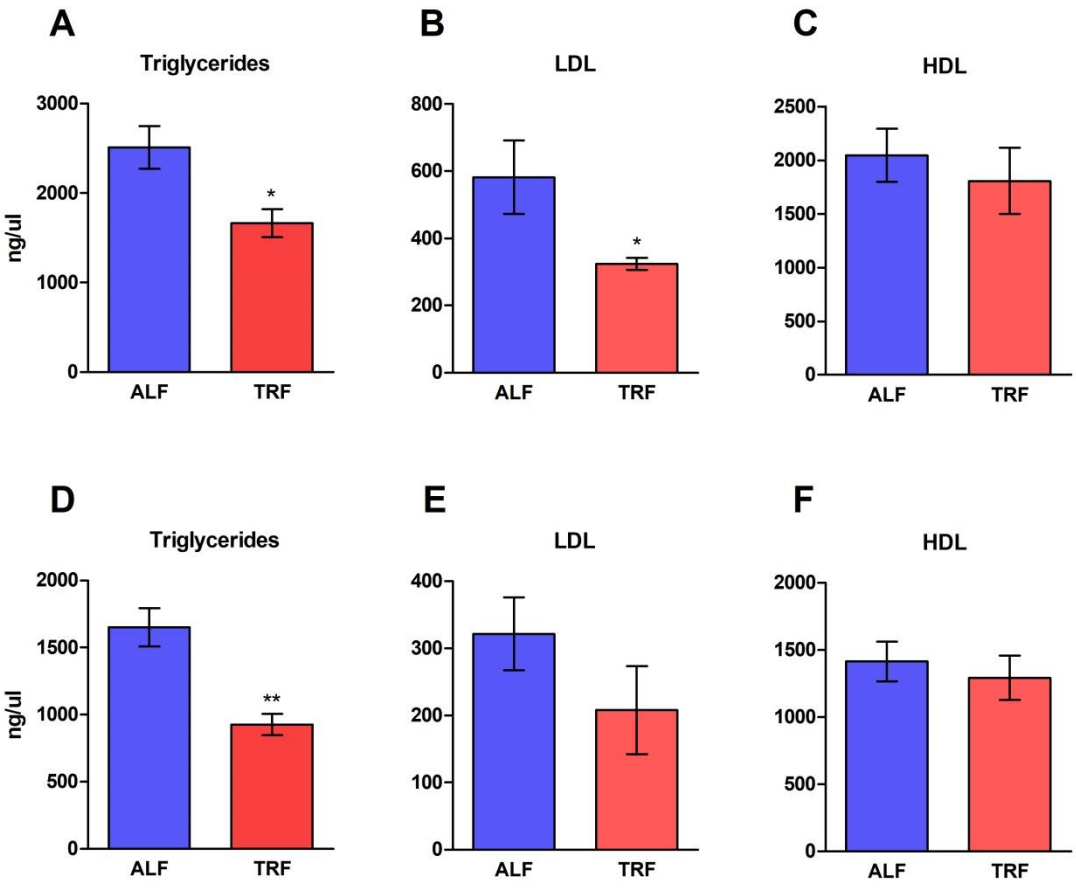

Figure 34. Quantification of plasma triglycerides, LDL and HDL in ALF and TRF started at 18 months (top row) and at 10 months (bottom row) animals (*p <0.05 and **p <0.01).

Secondly, and most importantly, absolute concentrations of plasma lipids increased with age in control ALF group (**Figure 35**), consistent with published results[174,175]. Thus, the lowering effect of TRF on these parameters should more correctly be interpreted as a delay in their age-associated increase, as part of an overall retardation of the emergence of the ageing phenotype.

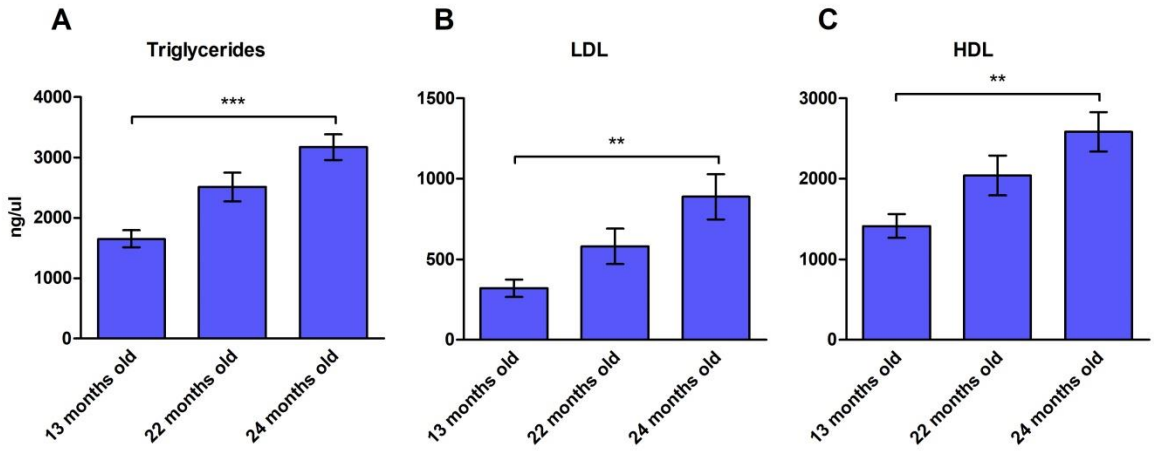

Figure 35. Comparison of triglycerides (A), LDL (B) and HDL (C) plasma concentrations between 13, 22 and 24 mos-old ALF animals (**p <0.01 and ***p <0.001).

4.3.3. Brain derived neurotrophic factor (BDNF)

Ageing is associated with a host of biological changes that contribute to a progressive decline of cognitive and biological functions. Cognitive decline is a common feature in old organisms and affects 10% to 20% of adults aged 65 years and older[157]. Loss of cognitive capacities of the ageing brain is due to a wide range of anatomical, functional and metabolic changes, with or without neuronal cell loss. The hippocampus and cortex are among the most ageing-sensitive regions of the entire brain[177]. Brain atrophy with advancing age is accelerated by oxidative stress, and includes decreased mitochondrial function and loss of essential properties of brain such as neurogenesis, dendritic spine formation and neuronal synaptic plasticity[178]. Furthermore, a decrease of neurotrophic factors, including the brain-derived neurotrophic factor or BDNF, is well documented in the ageing brain[178].

A high concentration of BDNF is found in different areas of brain, such as hippocampus and cortex, where this neurotrophic factor plays an important role in memory formation, neuron plasticity and cell proliferation. Considering the decreased levels of BDNF in the ageing

hippocampus, several studies have suggested a mechanistic role for this neurotrophic factor in age-related hippocampal dysfunction, memory weakening and increased risk to neurodegenerative diseases. Such studies have been conducted in different species, including rodents and primates, in which levels of BDNF mRNA and protein in the hippocampus were reported to decline in old age[177,179].

Thus, to further explore the effect of TRF on the ageing phenotype, levels of BDNF were measured in plasma and in the hippocampus of rats under the specific experimental conditions of our studies. Rats were given either ALF of TRF for 18 months and then fed *ad libitum* for another 3 months. Plasma and hippocampal cell lysate were analysed for BDNF peptide. Higher levels of BDNF were observed in both the hippocampus and in plasma of animals exposed to TRF, albeit values were statistically different only for the hippocampus (**Figure 36**).

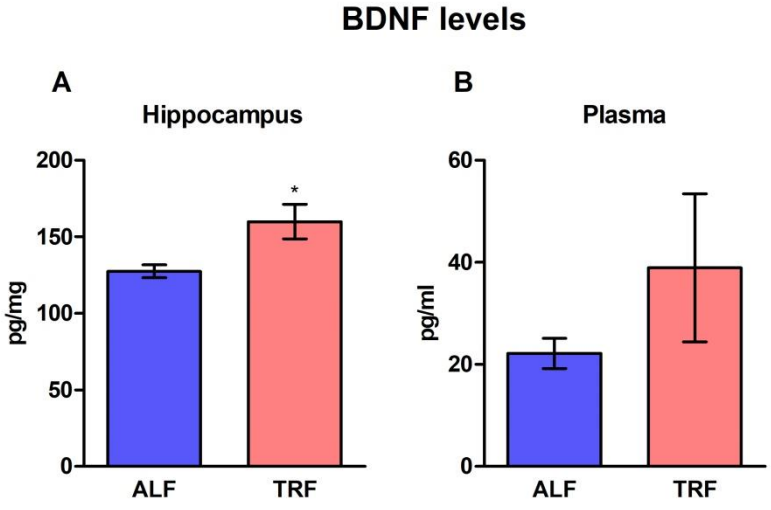

Figure 36. BDNF levels in hippocampus (A) and plasma (B) of long-term treated ALF and TRF animals (P value <0.05).

4.3.4. Amino acid profile

Several studies on dietary effects on ageing and lifespan have focussed their attention on the role of amino acids[180], which are known to represent one of the main substrates to regulate nutrient sensing pathways. For example, amino acids concentration positively correlates with the activity of mTOR, although the precise sensor mechanisms are still not completely defined[181]. As already discussed, low mTOR activity is in turn associated with extended lifespan[182,183]. In light of the above considerations, and as a means to gain further insights into possible systemic effects of TRF, plasma amino acid profiles were analysed in animals given either ALF or TRF for 18 months and continued on ALF for 3 additional months. Overall profiles were similar in both groups. However, plasma levels of histidine, glutamine and glycine were slightly but significantly reduced in animals receiving TRF diet (**Figure 37**). Furthermore, levels of citrulline and urea were also lower following TRF exposure. Interestingly, all of the above metabolites, except for glycine, were found to increase with age (**Figure 38**), as also reported in the literature[184–186]. Thus, it is correct to state that TRF was able to retard such age-associated changes in amino acids profile.

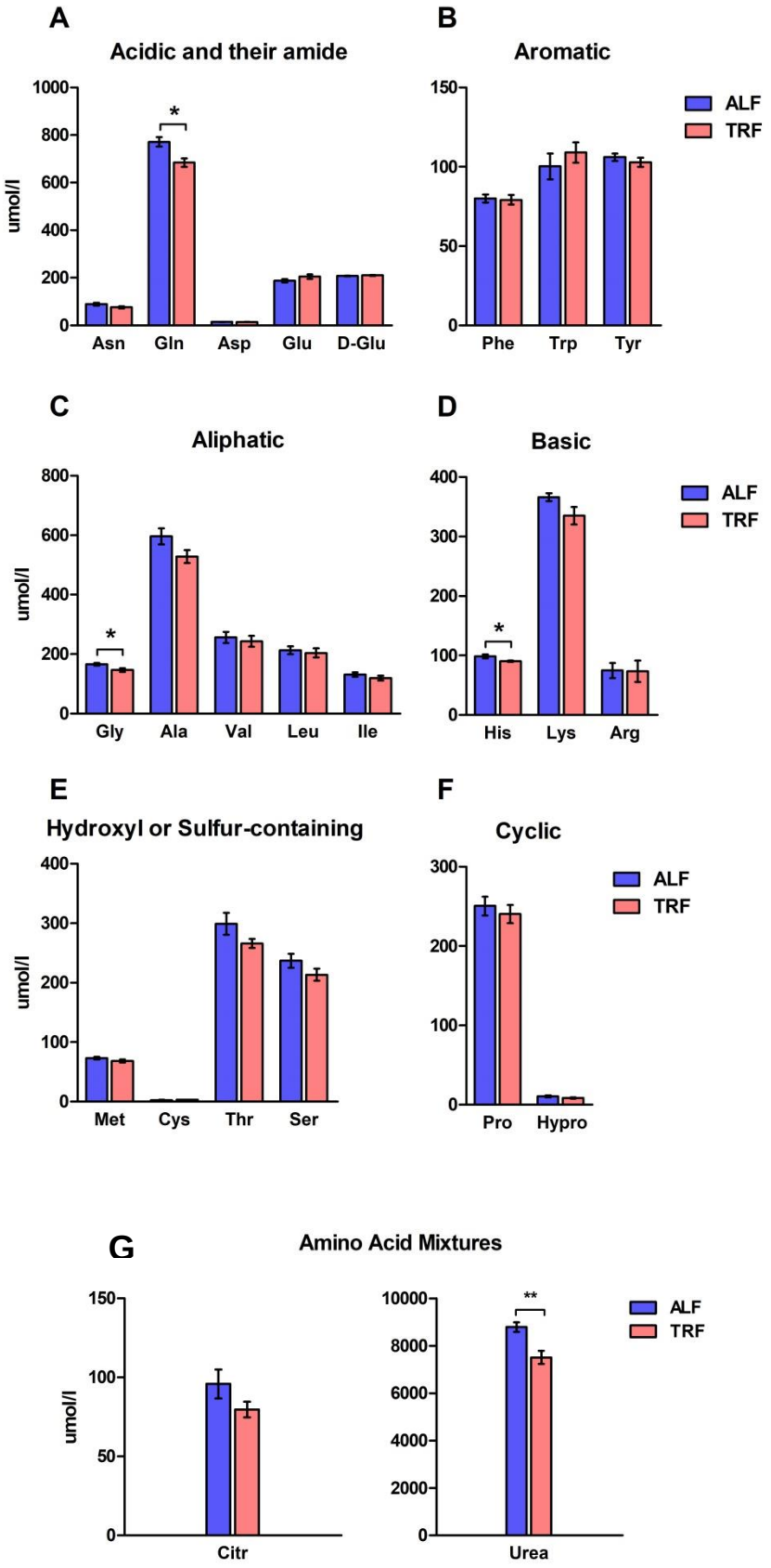

Figure 37. HPLC analysis of plasma amino acids in ALF and TRF experimental groups (*p <0.05 and **p <0.01).

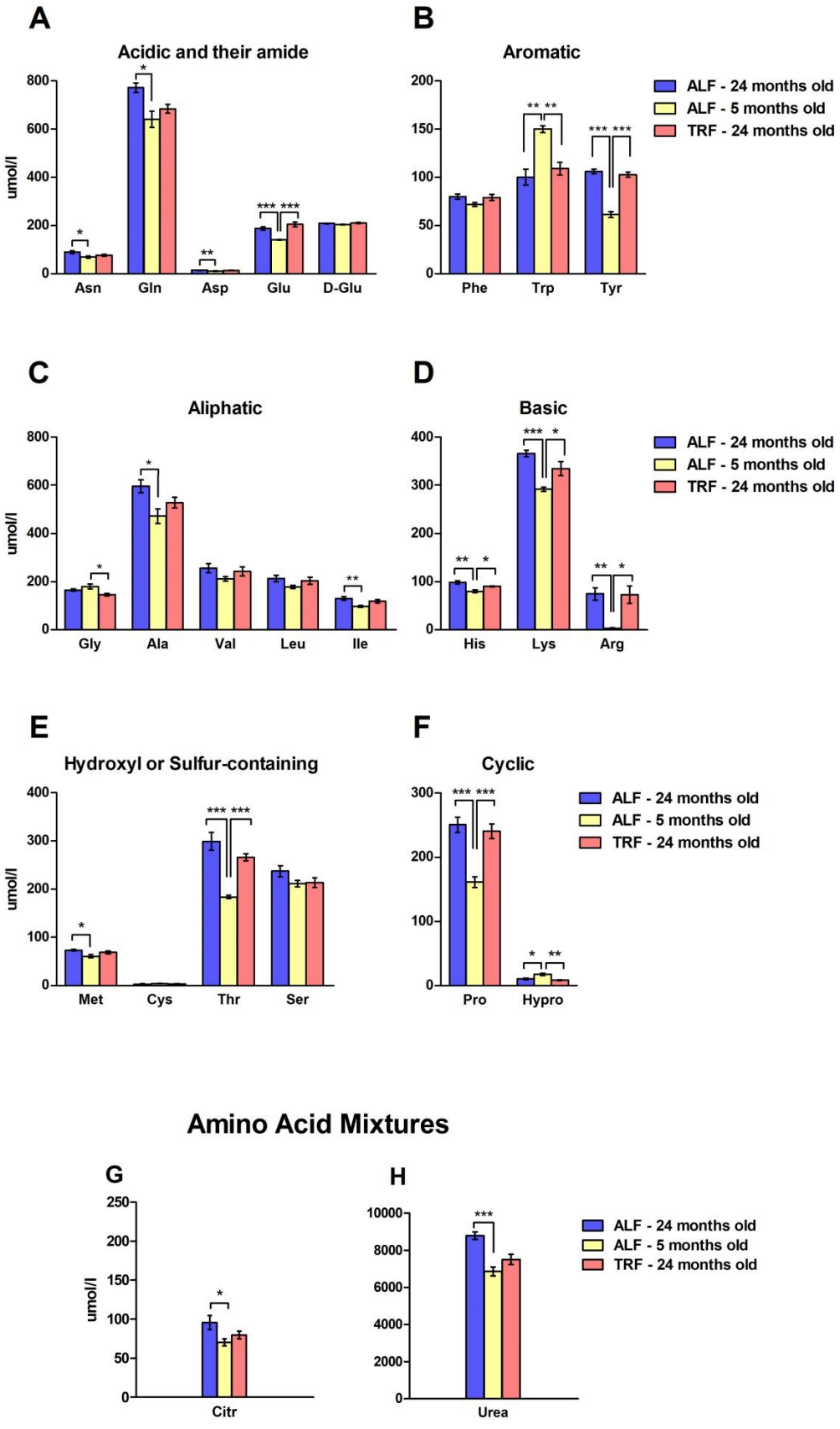

Figure 38. HPLC analysis of plasma amino acids in ALF and TRF animals in comparison with an *ad libitum*-fed 5-month-old experimental group (*p <0.05, **p <0.01 and ***p <0.001).

5. Discussion

While the link between ageing and neoplastic disease is widely acknowledged, what is less appreciated is the fact that some of the most relevant risk factors for cancer in humans are also associated with accelerated ageing in their target organ. Examples include UV light for the skin, smoking for the respiratory mucosa, chronic hepatitis for the liver, among others[39,187–189]. This strengthens the argument that the pathophysiology of ageing appears to be strictly intertwined with that of carcinogenesis, to the point that the emergence of the aged phenotype stands as a major biological driving force towards neoplastic development. And yet, in spite of this overwhelming evidence, the above association is still awaiting a fully satisfactory mechanistic explanation. Studies presented in this thesis stem from the working hypothesis that ageing increases the risk of neoplastic disease through alterations induced in the tissue microenvironment, translating in to the emergence of a neoplastic-prone tissue landscape[13]. More specifically, we contend that the decreased proliferative fitness of aged tissues allows for the selective expansion of variant clones with (relatively) preserved growth potential, including pre-neoplastic clones. Experimental evidence obtained by our and other research groups in support of this hypothesis was discussed in the Introduction and can be summarized as follows. Normal hepatocytes isolated from a young donor and transplanted into either young or old recipients form larger clusters in the latter than in the former, indicating that the aged liver microenvironment is more clonogenic that the younger counterpart[51]. Analogous results were observed when pre-neoplastic hepatocytes isolated from liver nodules were transplanted into either young or aged recipients[50]. Furthermore, albeit the liver is endowed with an enormous regenerative capacity, such property is nevertheless compromised in old age[50], due, at least in part, to a cell-autonomous decrease

in proliferative potential of the aged hepatocyte[50]. An overall similar conceptual framework has been derived from studies conducted in the hematopoietic system: the declining cellular fitness of the aged tissue microenvironment was found to be more conducive to the clonal growth of altered, potentially neoplastic cells[190–193].

Furthermore, the selective clonogenic drive of aged, phenotypically normal tissues has been highlighted in recent years by several studies indicating that clonal expansions are a frequent finding in old healthy individuals (including humans), to the point that they should come to be considered as a normal phenomenon, and in fact a universal marker of advancing age[41]. Since such clonal expansions include (but are not limited to!) cell populations with pre-neoplastic potential (e.g. carrying mutations associated with cancer[40]), it follows that the clonogenicity of the aged tissue microenvironment constitutes a favourable soil for the emergence of neoplastic disease.

Based on the above, strategies aimed at preventing or delaying the establishment of such a clonogenic/neoplastic-prone tissue landscape represents a reasonable strategy towards decreasing the burden of neoplastic disease in old age.

Dietary interventions are among the most effective strategies to delay ageing and age-related morbidities[60,122]. More specifically, a caloric restricted feeding (CRF) regimen has been repeatedly reported to extend lifespan and to decrease the incidence of most common chronic diseases of the old, including cancer, in several species, including monkeys[59,73]. In line with the working hypothesis proposed above, previous studies from our research group have indicated that the retarding effect of CRF on carcinogenesis is exerted, at least in part, through modifications of the tissue microenvironment, i.e. by delaying the emergence of the clonogenic/neoplastic-prone tissue landscape typical of ageing[95].

The remarkable effect of CRF on the rate of ageing has provided a powerful experimental tool to unravel the molecular and biochemical pathways involved in such complex biological process. However, a relatively neglected facet of standard CRF protocols is that animals given such a dietary regimen tend to eat their food in a relatively short period of time. For example, when a 70% CRF diet (i.e. 70% of ALF amount) is given, complete food consumption occurs in less than 8 hrs (Serra and Laconi, unpublished observation). Thus, the CRF regimen also includes a time-restricted feeding (TRF) schedule. The latter consideration is far from trivial, in light of the increasing evidence documenting that a TRF regimen is also effective in modulating several age-related phenotypes. Most importantly, TRF is associated with marginal or no reduction in daily caloric intake, and this bears two relevant implications. Firstly, on mechanistic grounds, it questions the role of reduced calories *per se* in the CRF-induced effects on ageing. Secondly, on practical grounds, it renders TRF a more appealing and amenable dietary strategy to be implemented in humans[59].

Studies presented in this thesis stem from the above considerations. The aim was twofold. Taking advantage of a unique experimental model developed by our research group, we tested the effect of TRF on the emergence of the neoplastic-prone tissue microenvironment associated with ageing. Moreover, we determined the impact of TRF on both local (liver) and systemic age-related alterations.

In order to investigate whether TRF is able to exert any putative effect on either the tissue microenvironment and/or directly on the pre-neoplastic cell population, we set up a simple experimental protocol based on syngeneic hepatocyte transplantation. Hepatocytes isolated from chemically-induced liver nodules were injected in recipient rats following long-term exposure to TRF regimen. Animals were then continued on ALF diet and the growth of transplanted cells was evaluated. A significant increase in the frequency of larger size

clusters of pre-neoplastic hepatocytes was seen in TRF-exposed group compared to controls given ALF throughout the experiment, indicating that this dietary regimen was able to delay the emergence of the neoplastic-prone/clonogenic tissue landscape typical of ageing. To our knowledge, this is the first investigation to describe a direct beneficial effect of TRF on carcinogenesis. It was recently reported that a time-caloric restricted diet could inhibit progression from cirrhosis to hepatocellular carcinoma induced by chronic administration of diethylnitrosamine[166]. However, feeding time in this study was reduced to 2 hours, resulting in a sizeable (~30%) decrease in daily caloric intake. Interestingly, epidemiological studies in women have suggested that prolonged nightly fasting may decrease the risk of breast cancer[194] and breast cancer recurrence[136], possibly by improving biomarkers of glucose control[195]. While longer follow up studies are certainly warranted in our experimental setup, it is important to note that the present results parallel very closely those reported by our research group on CRF-treated rats. It was shown that the retarding effect CRF on neoplastic development was attributable, to a significant extent, to a lower clonogenic potential of the tissue microenvironment in caloric-restricted animals[95].

The conclusion that TRF is able to retard the onset of age-associated phenotypic alterations is supported by an increasing body of evidence published in the literature[60,129,196] and by additional results presented in this dissertation. Parameters related to both liver and systemic ageing were in fact found to be modulated by TRF towards the persistence of a younger phenotype, including a decrease in liver cell senescence, lower incidence of cholangiofibrosis, diminished fat accumulation and up-regulation of SIRT1 in the liver, down-regulation of plasma IGF1, up-regulation of hippocampal BDNF, decreased levels of plasma lipoproteins. Of note, the beneficial effect of TRF on the above parameters was still

detectable after 3 months of ALF diet, suggesting that it is based on stable biological changes as opposed to transient metabolic alterations.

Cellular senescence is considered a hallmark of ageing since senescent cells accumulate in many tissues of old vertebrate organisms[26–28]. While it can be interpreted as a fail-safe mechanism to limit the risk of neoplastic transformation following genotoxic insult, it is also clear that cellular senescence can fuel carcinogenesis through different mechanisms (e.g. SASP-related inflammaging), as discussed in the Introduction. Long-term treatment with TRF resulted in decreased levels of SA-β-Gal positive areas in the liver. Livers samples obtained from TRF-treated animals were also screened for other senescence markers, such inflammatory cytokines and cell-cycle inhibitors; however, no significant differences were found between the ALF and TRF groups. The latter results are in line with previous findings reported in the literature[133]. Indeed TRF seems to influence inflammatory markers in other tissues, such as white and brown adipose tissue, as suggested by studies performed in mice[123,124].

Nutrient sensing pathways were also affected by TRF. Specifically, IGF-1 was down-regulated in animals fed under a time-restricted schedule. The IIS pathway is geared towards anabolism. Thus, it is expected that reducing the feeding period, such as under TRF regimen, likely reduces its signalling during fasting and switches energy usage from glucose to fat, thereby contributing to reduction in adiposity and amelioration of lipoprotein metabolism[123], as also observed in our study. In a similar vein, up-regulation of SIRT1 in the liver of TRF-treated animals can contribute to the transition from anabolic to catabolic processes under prolonged fasting periods[197], thereby promoting longevity.

Plasma levels of two main adipokines, leptin and adiponectin, were also investigated, given their association with ageing and age-related morbidities. Increased levels of leptin in

plasma are in fact associated with obesity, cardiovascular disease, hypertension and hyperlipidaemia[198]. Furthermore, leptin is an angiogenic and pro-inflammatory factor, and it has been implicated in the origin of human breast carcinoma[199]. On the other hand, adiponectin exerts anti-inflammatory effects[200]. Long-term TRF, followed by ALF, did not result in significant alterations in plasma levels of the two adipokines, albeit a trend towards decreased levels of leptin and increased levels of adiponectin was evident. Interestingly, both cytokines were significantly affected in animals exposed to TRF for 3 months, starting at the age of 18 months, in line with results of Hatori *et al*[124].

Finally, the ability of TRF to affect levels of BDNF in the hippocampus points to the potential of this dietary regimen to exert generalized effects at systemic level on biological ageing. This neurotrophic factor is implicated in diverse cognitive functions and its decreased levels observed in old-age have been associated with loss of learning and memory skills. Thus, the retarding effect of TRF on ageing appears to extend to at least several aspects involved in healthy life.

As anticipated in the Introduction, the finding that TRF is effective in delaying ageing phenotypes has two major implications. Firstly, from a practical point of view TRF stands as a dietary manipulation that is more amenable to implementation in humans compared to CRF[123]. The latter is in fact perceived with a negative connotation due to the limit imposed on caloric intake; moreover, it might be associated with important side effects such as infertility, depression, osteoporosis, slower wound healing and others[159]. In fact it is well known that dietary restriction in adults to may decrease reproductive potential in both sexes, possibly due to re-allocation of resources from reproduction to survival[201,202] . In addition, CR in young and growing animals is associated with a reduced skeletal bone

acquisition, resulting from suppression of bone formation and activation of bone resorption[203].

Since TRF does not imply a (major) reduction in total calories, the above contraindications do not apply. Secondly, as a follow up to the latter consideration, if several effects of CRF can be reproduced by TRF with no decrease in caloric intake, the implication is that the beneficial effects of the former are mediated, at least in a relevant part, by biochemical/molecular mechanisms set in motion by the latter.

Thus, there is a need for new mechanistic hypotheses to be pursued as to the ageing-retarding effects of TRF and CRF. Placing emphasis on the time component of feeding behaviour, as opposed to the amount of food per se, brings attention to the interplay of the pattern of food ingestion with peripheral circadian clocks and to their relation to the rate of ageing[204,205]. In fact, several metabolic pathways are controlled by the circadian clock. Among others, the heterodimer CLOCK:BMAL1 regulates the expression of the enzyme nicotinamide phosphorybosil transferase, which catalyzes the rate-limiting step in the NAD+-salvage pathway. This implies that the circadian availability of NAD+ is clock-directed and it can impact on the activity of enzymes utilizing this substrate, such as the NAD-dependent deacetylase SIRT1, whose role in cellular metabolism and aging is well characterized[206]. It is also noteworthy that SIRT1 directly interacts with CLOCK:BMAL1 complex, thereby regulating its function by physically interacting with these circadian regulators[207]. This is in line with the increasing awareness in the literature of the concept of “chrono-nutrition”, i.e. the notion that food consumption should be aligned and resonate with body’s daily rhythms in order to prolong healthspan[204,208]. The underlying implication is that a better understanding and fine-tuning of this reciprocal interaction has the potential to widen our opportunities for effective interventions towards delaying ageing.

As a note of caution, we are aware of the fact that, in our studies, animals exposed to TRF had also a significant, albeit limited component of CRF, since food consumption was about 10% less, on average, in TRF compared to ALF group. While such a small difference in food intake would appear unlikely to be effective in retarding ageing unless it is coupled with a TRF regimen, such an intriguing possibility cannot be ruled out at the moment and deserves further investigations.

In summary, studies presented in this dissertation provide the first direct evidence that a TRF regimen, with about 10% reduction in daily caloric intake, is able to delay the emergence of the neoplastic prone/clonogenic tissue landscape associated with ageing. This specific outcome is part of a more generalized beneficial effect of TRF on several biological, biochemical and metabolic parameters related to the ageing phenotype.

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