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Review

Senescence and DNA Damage in Adipocytes and Fat Tissues and Its Potential Amelioration through Nutritional Interventions

Abbas Ishaq, Gabriele Saretzki *

Biosciences Institute, Newcastle University, Campus for Ageing and Vitality, Newcastle upon Tyne, NE4 5PL, UK; E-Mails: abbas.ishaq@gmail.com; gabriele.saretzki@ncl.ac.uk

* **Correspondence:** Gabriele Saretzki; E-Mail: gabriele.saretzki@ncl.ac.uk

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Abstract

Accumulating evidence demonstrates that senescence and the associated inflammatory phenotype (SASP) also occur in post-mitotic cells such as mature adipocytes. Visceral adipose tissue in humans is susceptible to inflammation due to nutritional imbalance and ageing. However, while adipose tissue has been well researched in the context of obesity, senescence of differentiated adipocytes has not been investigated thoroughly. Our group recently demonstrated that ageing and normal *ad libitum* (AL) nutrition in mice resulted in increased adipocyte size, DNA damage, $p16^{INK4a}$ expression and inflammation in visceral adipose tissue while some of these senescence markers could be alleviated by dietary restriction (DR). Moreover, another dietary restriction study described a "metabolic memory" as protection against AL-induced senescence after shifting mice from DR back to AL nutrition. Other recent DR studies on mice of different ages analysed the transcriptional profile of adipose tissue and described a metabolic memory for AL at high age. Finally, our group modelled nutritional imbalance *in vitro* through treatment of primary human subcutaneous and omental adipocytes with the saturated fatty acid (FA) palmitic acid (PA). This resulted in a significant increase in DNA damage as well as $p16^{INK4a}$ levels correlating with enhanced intracellular lipid accumulation. In contrast, DNA damage could be prevented with the unsaturated FA oleic acid

(OA). With olive oil being an important part of the Mediterranean diet another study found also other oils such as argan oil to have similar effects of preventing DNA damage *in vivo* and *in vitro.* This review is focused on senescence, DNA damage and inflammation in WAT and adipocytes including nutritional interventions *in vivo* and *in vitro*. It also gives some basic background on these topics. However, it is not a systematic review but aims to highlight recent developments and nutritional interventions in the areas of senescence and DNA damage related to adipocyte tissues and cells.

Keywords
Human adipocyte; adipocyte tissue; senescence; ageing; DNA damage; $p16^{INK4a}$; dietary restriction; inflammation

1. Introduction

The prevalence of obesity and overweight are increasing rapidly in most human populations around the world enhancing the risk for many serious diseases. Ageing and obesity are both conditions associated with serious health problems and an increased risk for diseases and mortality. Nutrition is a recognised as a key player in the aim to improve our health span, prevent obesity and metabolic diseases and to achieve "successful" ageing [1].

This review gives some introduction into senescence- and nutrition-related topics discussing and comparing them in the context of ageing and obesity. It also summarises and evaluates the results from recent ageing and senescence studies on fat tissues and cells and sets them into context of basic molecular processes and mechanisms.

Our group has recently conducted several *in vitro* and *in vivo* studies on model systems, such as mice and differentiated human adipocytes in order to combine the different topics of ageing, nutrition, cellular senescence, DNA damage and inflammation [2-5]. In these studies we analysed known interventions such as dietary restriction *in vivo* and employed different fatty acids *in vitro* and food oils both *in vitro* and *in vivo*. These studies focused on the characterisation of parameters such as adipocyte size, lipid accumulation, markers of senescence, DNA damage and inflammation while other groups employed high-throughput analysis of adipocyte transcriptomics [6].

2. Obesity and Ageing

Obesity occurs when energy intake from nutrition exceeds energy expenditure where surplus calories are being stored predominantly in white adipose tissue (WAT). This results in more and/or larger adipocytes *in vivo* which is accompanied by glucose intolerance and insulin resistance leading to type 2 diabetes, lipodystrophy, hepatic steatosis, hyperlipidaemia hypertension, and a chronic systemic inflammation [7]. Together, these symptoms are part of the metabolic syndrome of obesity which increases the risk of other diseases such as cardiovascular disease, hypertension and stroke [8]. Hyperglycaemia and hyperlipidaemia are the main causes for the metabolic syndrome which is often associated with type 2 diabetes and obesity. Obesity is characterized by increases in the number or size of fat cells (hyperplasia and hypertrophy), or a combination of both.

Ageing is a complex process with a number of molecular and physiological pathways as underlying mechanisms. Ageing is defined as a functional decline of physiological functions in an organism at all levels: metabolism, cognition, changed hormone production, reproduction etc. In contrast, senescence is usually associated with the cellular level. However, both processes are intrically connected with each other. For example, one common factor in senescence and ageing is increased chronic inflammation, also called "inflammaging" [9].

Human ageing is associated with a gradual redistribution of fat tissue from subcutaneous to abdominal visceral sites as well as other organs such as liver and muscle [10]. Such age-related changes in body fat distribution and metabolism might be able to contribute to and accelerate the ageing process as well as age-related diseases and will be described in more detail later. Increased visceral fat content in different organs contributes to lipotoxicity and is a major contributor to insulin resistance and the metabolic syndrome [11, 12].

On the other hand, obesity and its consequences get exacerbated during ageing. However, it is not entirely clear how much similarities or joint mechanisms there are for the two processes. One common processes is certainly increased inflammation while senescence and DNA damage have not been thoroughly analysed in obesity.

Parameters well known to contribute to obesity such as adipocyte size hypertrophy have not been frequently addressed together with a characterisation of the extent of senescence in fat tissue during ageing as well as after nutritional interventions such as dietary restriction (DR). Thus, the major focus of this review are processes such as DNA damage, senescence and inflammation in white adipose mouse tissue and cultured human adipocytes.

3. White Adipose Tissue (WAT) and Adipocytes during Ageing and Obesity

Fat is often the largest organ in humans with around 15-30% of body weight in a healthy state [13]. Due to its metabolic activity it is a key regulator of energy homeostasis and also an important endocrine organ [14]. WAT consists of lipid-bearing adipocytes, blood vessels, pre-adipocytes that sheath blood vessels, T- lymphocytes as well as macrophages, fibroblasts and mesenchymal stem cells (Figure 1). Each WAT depot possesses distinct pre-adipocyte populations that determine their physiological specificity [15, 16].

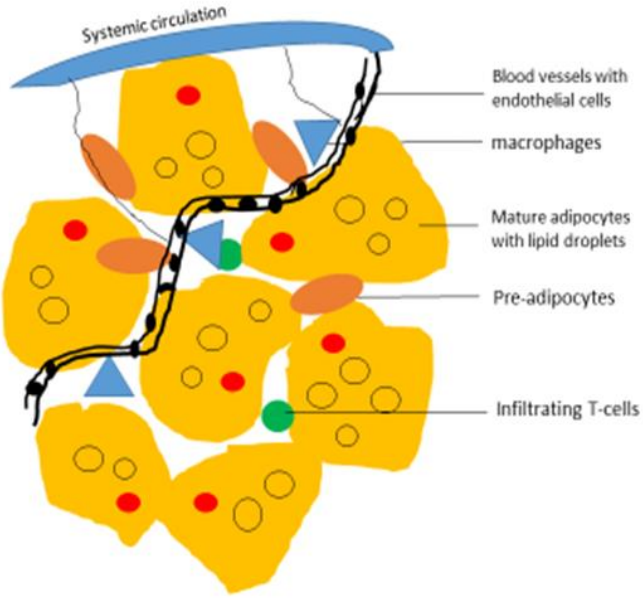

Figure 1 Major cell components of white adipocyte tissue (WAT). Adipocytes co-exist with different other cell types such as pre-adipocytes, blood vessels (mainly endothelial cells), resident macrophages, infiltrating T-cells and are connected to the systemic circulation.

Adipocyte size in both subcutaneous and visceral WAT changes with nutrition state (*ad libitum* (AL), high fat diet (HFD) or caloric restriction (CR)) as well as during ageing. On a standard diet, adipocyte size in mice peaks around 22 months and declines with ageing [17]. Changes in the size of the triglyceride droplets are facilitated by dynamic pathways of lipogenesis, lipolysis, and β-oxidation [18].

Subcutaneous adipocytes function as the primary storage of excess calories as a single, large intracellular triacylglyceride droplet formed through esterification of fatty acids and glycerol. During excessive caloric intake, fat is stored initially in subcutaneous adipocytes followed by visceral adipocytes during continued high caloric intake. Visceral adipose tissue in humans is prone to inflammation during nutritional imbalance and ageing, although with different mechanisms [19, 20]. In particular, hypertrophic adipocytes initiate pro-inflammatory responses in local adipose tissue, which, if sustained, could lead to the development of the metabolic syndrome [21].

Fat mass in humans and mice peaks in middle to early old age while there is a substantial decline at advanced age [16]. During ageing, there develops a dysfunction of fat tissue together with a fat redistribution between subcutaneous and visceral depots [16]. Visceral fat and non-adipose tissue lipid accumulation are correlated with dyslipidemia, insulin resistance, pathology and the metabolic syndrome [12, 16]. There are several potential mechanisms underlying this redistribution. Mitochondrial dysfunction such as decreased energy generation and biogenesis have been discussed recently [22]. For example, a decrease of important biogenesis factors such as PPARγ which regulates adipogenesis and insulin-sensitivity by fat depot redistribution has been described during ageing [23]. During aging, fat distribution was also altered by stimulating lipid uptake, lipogenesis, lipolysis, lipid oxidation as well as fatty acid recycling predominantly in visceral fat compared to subcutaneous fat [23]. Finally, also the reduced replicative capablity of pre-adipocytes to regenerate different adipocyte depots might play a role in age-related fat redistribution [15].

Adipose tissues are populated by pre-adipocytes as adult tissue stem cells accounting for 15-50% of the total cell population in WAT [16] and differentiated adipocytes as well as some other cell types (endothelial cells, macrophages etc., see Figure 1).

Adipogenesis is the formation of adipocytes through differentiation of pre-adipocytes and lipid accumulation in them. Fat tissue increases in volume through both an increase in adipocyte size (hypertrophy) and increase in adipocyte count (hyperplasia). Hypertrophy is predominantly associated with diet-induced changes in adipose tissue mass, while hyperplasia is to a large extend associated with genetic factors [24]. The number of adipocytes in an adult human organism is determined during early childhood and adolescence, with a turnover rate of 50% of total adipocyte population every 8.3 years regardless of whole-body adiposity [25].

Both, adiposity and body fat distribution have strong genetic components and are influenced by depot-specific adipose tissue stem cells. Remodelling of subcutaneous adipose tissue (SAT) via the storage of excess lipids through adipocyte expansion in size (hypertrophy) as well as recruitment of new precursor cells (hyperplasia) impacts the risk of developing cardiometabolic and other diseases.

In obesity an expandable and metabolically flexible SAT is essential for a healthy obesity status (without metabolic dysfunctions) while the inability of SAT to expand results in unhealthy obesity with an increase in VAT volume, dyslipidemia and insulin resistance. The specific negative metabolic effect of visceral fat is most likely caused by a higher mobilisation of FFAs into the circulation. While both fat depots, SAT and VAT, are able to undergo hypertrophy and hyperplasia, the specific contribution of both processes can vary depending on disease type and metabolic status. Visceral adipose tissue is particularly susceptible to chronic low-grade inflammation and its dysfunction causes various metabolic diseases.

Metabolic responsiveness of adipocytes decreases with ageing [26]. There is also a decrease in the level of adipogenic transcription factors such as PPARγ and C/EBPα with age [27].

4. Lipid Metabolism: Lipogenesis, Lipolysis, β-oxidation and Its Relation to ROS and Mitochondria

Lipid metabolism includes the synthesis and degradation of cellular lipids. This process involves the breakdown and storage of fat from food or fat stores for energy. Most fats in mammals are obtained from food in the form of triglycerides and cholesterol. Intracellular lipid droplets are composed of triacylglyceride (TAG) which are the stable state of lipids. TAG consists of a triacylglycerol head with two or three units of fatty acids (FA). More than 90% of dietary fats are composed of TAG consisting of different types of FA. Fatty acids are carboxylic acids with long hydrophobic hydrocarbon chains with a predominantly even number of carbons. While saturated FA contain a single bond at the hydrocarbon tails, unsaturated FA contain covalent double bonds. Unsaturated FA are classified as mono-unsaturated or poly-unsaturated FA (MUFA and PUFA) depending on the number of double-bonds.

The TAG components are taken up by gut enterocytes and are re-synthesised into TAG. During lipolysis, adipocyte TAGs are hydrolysed into glycerol and free fatty acids (FFA) for downstream energy use. Hormones s such as catecholamines and insulin regulate lipolysis with opposing effects [28].

In healthy adipocytes, lipid content is regulated by TAG synthesis to form large lipid droplets (LD), TAG lipolysis and controlled release of free fatty acids (FFA) into the bloodstream [19, 29, 30] or

locally when neighbouring adipocytes incorporate FFA into their TAG droplets without changing overall adiposity of the WAT.

Uptake of FFAs by cells is mediated by multiple cell membrane proteins as an initial step of fatty acid utilisation where protein FA transporters play a major role [31]. This regulated process ensures a rapid clearance of lipids from the circulation postprandially and to facilitate substrate provision to organs and tissues with high metabolic demand. Fatty acid transporters are also implicated in disease pathogenesis of obesity, insulin resistance and type 2 diabetes [31]. FABP4 (fatty acid binding protein 4) is a major target of the mitochondrial biogenesis factor PPARγ (Peroxisome proliferator activated receptor γ) predominantly in adipocytes and macrophages. FABP4 also functions as a fatty acids chaperone, which couples intracellular lipids to signalling pathways and biological target tissues [32].

FAs are utilised as an energy source through fatty acid oxidation (β-oxidation) in organs such as cardiac and skeletal muscles, liver and kidney [33]. Characteristically, adipocytes of white adipose tissue display low β-oxidation activity, unlike those in brown adipose tissue (BAT) [34, 35]. During β-oxidation fatty acids are converted into acetyl-CoA within the mitochondria. β-oxidation begins by addition of a coenzyme A (CoA) group to the acyl group of the FA by fatty acyl-CoA synthase. Carnitine palmitoyltransferase 1 (CPT1) then adds a carnitine group to the long chain acyl-CoA to form acylcarnitine. Acylcarnitine is then shuttled into the mitochondria by the carnitine/acylcarnitine antiporter carnitine-acylcarnitine translocase and reverted to the long chain acyl-CoA by CPT2 [36]. CPT1 is the rate-limiting enzyme in β-oxidation and is inhibited by malonyl-CoA, preventing simultaneous fatty acid synthesis and catalysis.

β-oxidation has been linked to increased reactive oxidative species (ROS) generation in rat cardiac muscle where electron transport was partially inhibited by uncoupling of hydrogen ions [37]. In contrast, another study found that ROS was not produced in the ETC (electron transport chain) but identified mitochondrial fatty acid oxidation as the source of increased ROS generation in kidney tubules of diabetic mice [38]. Moreover, ROS scavenging in a 3T3-L1 mouse adipocyte model promoted insulin-dependent glucose uptake in the presence of increased pro-inflammatory cytokines [39]. Thus, changes in ROS levels due to β-oxidation can differ by cell type, and increased β-oxidation may be rather variable with regards to ROS generation and inflammation. Pharmacological and nutritional promotion of β-oxidation has been suggested as an interventional strategy against obesity.

Lipogenesis consumes a large amount of ATP generated through mitochondrial oxidative phosphorylation. Obesity is associated with increased lipid storage, reduced mitochondrial biogenesis with up to 50% less mitochondria in hypertrophic white adipocytes and increased glycolysis in white adipocytes [40]. This results in decreased oxidative phosphorylation and further decrease of already low β-oxidation levels [41]. Oxidative stress with increased ROS levels is able to inhibit oxygen consumption in adipocytes [42], while uncoupling the ETC instead limits lipid accumulation in adipocytes [43]. It was suggested that exercise and DR, antioxidants and uncouplers can be employed to alleviate oxidative stress in hypertrophic adipocytes [44]. Moreover, transplantation of human brown adipocytes into mice improved systemic glucose tolerance by providing an outlet for glucose consumption instead of conversion into fat by facilitating this process through an uncoupled mitochondrial ETC [45].

5. Inflammation

Inflammation is a process that restores homeostasis following pathogen invasion, lipid stress, DNA damage and other stressors [46]. When inducers of inflammation such as signals from stressed and damaged tissues are not successfully removed, the inflammatory state continues and becomes chronic [47] like in the metabolic syndrome and during ageing.

The best studied inflammatory mediators are chemokines for the recruitment of leukocytes and cytokines for recruitment of macrophages. Cytokine upregulation has been found in many chronic inflammatory, systemic and autoimmune diseases, including metabolic syndrome. Prominent families of cytokines include interleukin IL-1, TNFα, and IL-6. Pro-inflammatory cytokines transduce signals through the master regulator of inflammation, NF-κB in a rapid response fashion. Activated NF-κB translocates into the nucleus to induce gene expression of pro-inflammatory mediators (chemokines, cytokines), immune-receptors, stress response factors, ion channels, growth factors, transcription factors and regulators as well as apoptosis inhibitors [48]. The NLRP3 (NLR family, pyrin domain containing 3) inflammasome is a large, multimeric inflammatory factor that is highly reactive to pathogen-associated and danger-associated molecular patterns (DAMPS), including most of the stress stimuli upregulated by changes in metabolic pathways, metabolic syndrome and inflammaging through NF-κB [49-51].

6. Adipose Tissue Inflammation and Macrophage Infiltration during Ageing and Obesity

Obesity is tightly associated with inflammation in visceral WAT [13, 52]. Adipose tissue-derived pro-inflammatory cytokines such as TNFα can cause insulin resistance in obesity in *ob/ob* obese and *db/db* diabetic mouse models [53]. Other studies described the NLRP3 inflammasome as the central inflammatory driver in WAT during obesity and ageing [54, 55]. In addition, increased FFA levels act as danger-associated molecular pattern (DAMP) that can activate the NLRP3 inflammasome in whole adipose tissue [49].

In WAT from non-obese subjects, saturated fatty acids result in the recruitment and a pro-inflammatory phenotype of macrophages by $CD8^+$ T-lymphocytes and through Toll-like receptor activation [56]. Moreover, macrophages treated with pro-inflammatory signals such as LPS (lipopolysaccharide) and IFNγ (interferon gamma) are predisposed to fatty acid synthesis [57, 58], while anti-inflammatory macrophages activate β-oxidation [59]. The upregulation of PPARγ has an anti-inflammatory effect in macrophages and is required for phagocytosis of apoptotic cells [60, 61].

In obesity and during ageing, visceral WAT is characterised by increased inflammation due to sustained macrophage activation and cytokine paracrine signalling between adipocytes and macrophages [62]. Histologically, these macrophages can aggregate and are able to consume individual dead adipocytes by forming characteristic crown-like structures (CLS) [63]. Macrophage aggregation increases during ageing and in HFD-fed mice as a model for obesity and is more prevalent in the visceral than subcutaneous WAT [62, 64]. Macrophage accumulation has also been reported as a result of weight loss, facilitating adipose tissue lipolysis [65]. While chronic adipose tissue macrophage (ATM) accumulation during ageing and obesity is associated with detrimental and extensively elevated inflammation, data from Kosteli and colleagues suggest that transient macrophage accumulation protects local adipocyte function and is beneficial for the maintenance of adipose tissue health [65].

CPT1, an important transporter during beta-oxidation, is highly upregulated in WAT-infiltrating macrophages which corresponds to their lipolytic function when forming crown-like aggregates around single or fused adipocytes in WAT of obese subjects to degrade them [65]. In addition to this function, the secretory phenotype of macrophages plays an important role in the inflammatory state of WAT. The inflammatory state of adipose tissue is reliant on both downregulation of adipocyte-derived adiponectin and upregulation of infiltrating macrophage-derived TNFα. Activation of macrophages towards a pro-inflammatory phenotype is also dependent on the NLRP3 inflammasome which attenuates the activation of lipolysis in ATM of old mice [66, 67]. Co-culture of 3T3-L1-derived adipocytes with the macrophage line RAW264 without direct contact resulted in a significant upregulation of TNFα in adipocytes in a paracrine manner [68]. In contrast, blocking of TNFα prevented upregulation of *MCP1* (monocyte chemoattractant protein-1) expression in 3T3-L1 adipocytes, while release of free fatty acids induced further TNFα signalling in adjacent macrophages [68]. Many other cytokines as well as factors such as PPARγ and FABP4 are also involved in the inflammation of adipocytes as well as macrophages.

Obesity is associated with adipocyte death and macrophage infiltration [13, 65]. Increase in visceral adipocyte death has been correlated to increased visceral fat macrophage infiltration in elevated adiposity and obesity, while the underlying mechanisms have not been conclusively determined [63, 69, 70].

The intercellular interactions between macrophages and adipocytes contribute to the overall inflammation profile in adipose tissue. The presence of pro-inflammatory ATM in subcutaneous WAT inhibits the differentiation of pre-adipocytes at a higher rate than in visceral WAT from the same obese human subjects [71]. Infiltrating macrophages in adipose tissue produce comparatively less pro-inflammatory cytokines in WAT during ageing than in obesity [72]. Induction of inflammation with LPS elicits a higher upregulation of IL-6 in visceral fat in older compared to younger mice [73].

In contrast, inflammation is rather similar in fat tissue during ageing and in obesity due to the accumulation of senescent pre-adipocytes and upregulation of the senescence-associated secretory phenotype (SASP) [74]. Cytokines associated with the SASP increase in multiple tissues with chronological ageing [75]. However, senescence in postmitotic, differentiated mature adipocytes has not been characterised well until recently [5] and will be described in more detail below.

Cytokines like TNFα decrease insulin sensitivity by increasing serine phosphorylation of insulin receptor substrate IRS-1, reducing insulin/IGF-1 signalling [53]. TNFα also downregulated PGC-1α (Peroxisome proliferator-activated receptor-gamma coactivator-1α) during obesity [76]. The master regulator of mitochondrial biogenesis PGC-1α is highly relevant to adipose tissue since it is modulated by insulin signalling, ROS, calcium/calmodulin interaction and cytokines. Thus, PGC-1α is not only instrumental for mitochondrial biogenesis, but is also involved in the regulation of metabolic pathways as well as an oxidative stress response [77]. Through these mechanisms, TNFα inhibits adipogenesis in visceral pre-adipocytes to a higher degree than in subcutaneous pre-adipocytes [53]. Additional downregulation of adipogenic transcription factors occurs during ageing reducing the differentiation potential of pre-adipocytes [78]. This age-related decrease contributes to an age-associated reduction in insulin sensitivity through phosphorylation of IRS-1 (Insulin receptor substrate 1), as well as a decrease in *GLUT4* expression [79]. Finally, HFD exacerbated the inflammatory effects of transplanted senescent pre-adipocytes in young mouse adipose tissue,

while removing senescent cells with senolytics in human adipose tissue was able to decrease pro-inflammatory cytokine secretion [17, 80].

Importantly, senescence-associated pro-inflammatory cytokines and chemokines are secreted by these cells which can lead to the development of metabolic diseases. This was shown in adipocytes of transgenic mice with increased *p53* expression and translation, as well as late generation *Tert*$^{-/-}$ mice with critically short telomeres, which resulted in increased insulin resistance and upregulation of pro-inflammatory cytokines in visceral WAT [81]. Visceral WAT macrophage infiltration was also increased in these *Tert*$^{-/-}$ mice, showing that telomere-dependent senescence in adipose tissue can promote inflammation resulting in insulin resistance. Moreover, dying adipocytes can also release toxic cargo leading to paracrine damage [82].

7. Senescence

Senescence was originally defined as an irreversible arrest of cell division and is therefore an important tumour suppression mechanism. Multiple types of senescence have been characterised, depending on their mechanism of induction: replicative senescence, oncogene-induced senescence (OIS) and stress-induced premature senescence. Recently also senescence in postmitotic (PM) tissues was described as a separate entity [83]. Common for all senescence types is that it is a stress response that involves a DNA damage response (DDR) [84, 85]. Here only some of those types will be described briefly because many excellent reviews exist for each of them [83, 84, 86, 87].

7.1 Telomere-dependent Replicative Senescence

Ongoing DNA replication generates cells with progressively shorter telomeres in the absence of telomerase (a reverse transcriptase able to add telomeric repeats *de novo* and thereby counteracting telomere shortening) [88]. Telomeres are tandemly repeated sequences of TTAGGG (in vertebrates) at the ends of linear chromosomes with variable repeat lengths between species. Telomeres form a T-(telomeric) and a D- (displacement) loop and are associated with specific proteins, called shelterin which also ensure a proper capping of telomeres preventing them from being recognised as DNA breaks [89].

Telomeres shorten primarily due to the “end replication problem” (ERP) during normal semi-conservative DNA replication [90, 91] as well as due to oxidative stress that results in the accumulation of DNA damage at telomeres and their accelerated shortening [84, 92]. Shortened beyond a critical length, telomeres can no longer form T-loops and become uncapped, leading to a persistent DNA damage response (DDR), cell cycle arrest, cellular senescence or apoptosis [93]. Damage sites at short, uncapped telomeres are called “telomere-dysfunction-induced foci” (TiF) [93] while telomeric damage that occurs within still long telomeres is called “Telomere-associated foci” (TAF) [94]. While the former mainly occur in mitotic cells, the latter are characteristic for postmitotic cells which do not divide and thus do not shorten their telomeres since telomere shortening requires cell division [95], but still accumulate damage in telomeres.

Although telomere length is often used as an ageing marker in human population studies (for example [96, 97]) it is now recognised that accumulating DNA damage in telomeres (TAF) is sufficient to induce senescence, regardless of telomere length [94, 98]. This also includes senescence of postmitotic tissues, such as neurons, muscle cells and mature adipocytes which all still develop senescence but without the cell cycle arrest [83]. DNA damage is recognised by the

DNA damage response (DDR) through a cascade including kinases such as ATM and ATR, mediators such as Chk1 and Chk2 as well as effectors like p53 and its downstream target $p21^{Cip1}$ which halt the cell cycle for damage to be repaired [93]. However, unrepaired damage can induce an irreversible cell cycle arrest-senescence. Telomeric DNA damage is considered to be rather irrepairable as telomeres are sheltered and not easy accessible by DNA repair factors [89]. In the case of irreparable damage (either in telomeres or elsewhere in the genome), senescence is maintained by direct signalling of p53 through $p21^{Cip1}$, as well as by phosphorylating the retinoblastoma (Rb) protein and activation of the $p16^{INK4a}$ pathway [86]. These pathways are described in more detail below.

7.2 The $P16^{INK4a}$ Pathway and Senescence

$P16^{INK4a}$ activation constitutes a main senescence pathway and thus high $p16^{INK4a}$ levels are an indicator and marker of cellular senescence. $P16^{INK4a}$ is an important tumour suppressor and the pathway is considered to be independent of telomeres.

$P16^{INK4a}$ -mediated senescence is activated through the retinoblastoma (RB) pathway [86]. This pathway inhibits cell cycle progression at the G1 state by binding cyclin dependent kinases 4 and 6 thereby preventing the phosphorylation of RB. Hypophosphorylated RB stays bound to transcription factor E2F1 in the cytoplasm and in that way prevents the transcription of cell-cycle factors. The regulation of $P16^{INK4a}$ is complex and involves various transcription as well as epigenetic factors [99]. For example, there is a feedback loop between $p16^{INK4a}$ and RB where RB phosphorylation increases *$p16^{INK4a}$* expression inhibiting CDK4/6 and resulting in increased levels of hypophosphorylated Rb which in turn decrease *$p16^{INK4a}$* expression [100]. Another feedback-loop exists between ROS and $p16^{INK4a}$ involving protein kinase C delta [101]. $p16^{INK4a}$ plays an important role both in the initiation as well as the maintenance of cellular senescence. Importantly, targeting $p16^{INK4a}$- positive senescent cells has been suggested as a mechanism for the eradication of senescent cells in various mouse tissue types [80, 102-104].

Ageing is associated with replicative senescence and $p16^{INK4a}$ levels increase with ageing in most mammalian tissues [105-107]. Sharpless' group has impressively demonstrated that increased $p16^{INK4a}$ levels in different types of tissue stem cells promote organismal ageing while $p16^{INK4a}$ knock-out improved stem cell function and tissue regeneration thereby delaying ageing in mice [106, 107].

Additionally, $P16^{INK4a}$-mediated senescence results in chromatin reorganization which is associated with repression of genes regulated by transcription factor E2F1 and can be visualised as senescence-associated heterochromatic foci (SAHF) [108].

7.3 $P16^{INK4a}$ and Adipocyte Senescence

Recent studies highlight novel roles for $p16^{INK4a}$ in adipocytes in the control of energy homeostasis. They demonstrate that the pathway is able to regulate physiology and adipocyte functions such as insulin sensitivity, lipid storage, inflammation, oxidative stress and metabolic changes in energy generation [109-112]. $p16^{INK4a}$ promotes lipid accumulation and adipocyte hypertrophy via the insulin-signalling pathway. In particular, the cyclin-dependent kinase 4 (CDK4) which in its canonical function mediates phosphorylation of RB and activation of the cell-cycle progression from G1 to S phase, has been suggested as a key molecule involved in adipocyte insulin sensitivity via an E2F1- and cell cycle-independent manner to regulate lipid storage in mature adipocytes [109, 110]. CDK4 is expressed in terminally differentiated adipocytes in humans and mice

under physiological conditions and it's activity in adipocytes correlates with fat mass, lipogenesis as well as insulin sensitivity [112].

$p16^{INK4a}$ seems to inhibit adipogenesis while knock-down of $p16^{INK4a}$ in 3T3-L1 mouse pre-adipocytes increased adipogenesis [111]. Additionally, it has been speculated that increased $p16^{INK4a}$ levels during ageing and obesity might contribute to the recruitment of macrophages in adipose tissue as well as the prevention of macrophage M2 polarisation with an increased risk of developing type two diabetes [113]. Thus, $p16^{INK4a}$ seems to be a key factor that is implicated in adipose tissue during ageing as well as obesity.

Importantly, the $p16^{INK4a}$ pathway was demonstrated to be involved in energy metabolism and glucose homeostasis in different tissues including beiging adipocytes [113, 114]. The E2F1/pRB repressor complex might function as a molecular switch of cellular glucose utilisation from glycolytic to oxidative metabolism and the adaptation to energy demand. Both, E2F1 and pRB, are able to bind promoters of genes involved in oxidative metabolism. For example, pRB can bind the promoter of the mitochondrial biogenesis master regulator PGC-1α and to repress its transcription [115]. Moreover, pRB-deficient fibroblasts displayed characteristics of brown adipose tissue (BAT) with an activation of BAT-specific genes and an increase in mitochondrial activity [116].

These findings of multiple functional implications of $p16^{INK4a}$ in adipocyte physiology and metabolism are in good accordance with those from Baker and co-authors who demonstrated that eradicating $p16^{INK4a}$ -positive pre-adipocytes in INK-AATAC mice prevented age-related loss of fat mass and attenuated age-related lipodystrophy [17]. In summary. adipose tissue senescence is characterised by tissue dysfunction such as increase of adipocyte size, decrease of adipogenesis including the downregulation of adipogenic transcription factors such as PPARγ as well as changes in energy metabolism [11, 113].

7.4 The p53/$p21^{Cip1}$ Pathway of Senescence in Adipocytes

Senescence is also mediated by the p53-$p21^{Cip1}$ pathway leading to G1 arrest of the cell cycle. This p53-related pathway is independent of $p16^{INK4a}$ -mediated senescence. The p53-$p21^{Cip1}$ pathway is normally activated early in senescence while increase in $p16^{INK4a}$ usually occurs at a later time point. Cells undergoing $p21^{Cip1}$-mediated replicative senescence can continue growth after inactivation of the p53 pathway while cells undergoing $p16^{INK4a}$-mediated senescence have to be removed to recover tissue homeostasis [117]. The p53-$p21^{Cip1}$ pathway also results in the inhibition of the retinoblastoma protein (Rb), suppressing the expression of proliferation-related genes [108]. $P21^{Cip1}$ is an important down-stream target of p53 that inhibits CDK2, which in turn inhibits the cell cycle, as well as PCNA, a scaffold protein involved in DNA replication and DNA damage repair. $p21^{Cip1}$ functions to preserve genome stability by allowing DNA repair, but in the absence of p53, $p21^{Cip1}$ can be detrimental to genomic stability [118].

P53 and $p21^{Cip1}$ have been studied in detail in adipocytes, but not necessarily under the topic of senescence. P53 is upregulated in WAT of obese humans and mice [119]. Increased levels of senescence, p53 induction, *TNFα* and *CCl2* expression were found in diabetic compared to non-diabetic humans [81].

Increased levels of ROS and DNA damage, coupled with upregulation of p53 and $p21^{Cip1}$, were found in visceral WAT of mice after diet-induced obesity, primary adipocytes and in 3T3-L1 cells [120]. In p53 knockout mice under high fat and high sugar diet, there was a reduction of visceral and

subcutaneous adiposity, body weight and inflammation as well as an improved insulin sensitivity compared to wild type (WT) mice [121]. $P21^{Cip1}$ knockout mice on a high fat, high sugar diet also gained less weight and had improved insulin sensitivity compared to controls [122]. Thus, both knockout mouse models were protected from diet-induced hypertrophy. Moreover, a decrease in the levels of NAD^+ and SirT1 can promote WAT senescence by increasing the levels of p53 and p21 [123].

7.5 SASP and Inflammation as Part of Senescence

It is well known that an increase in inflammation as well as oxidative damage are major characteristics of senescence and ageing. Like other inducers, inflammation caused by ageing and senescence results in the activation of NF-κB as the major transcription factor that responds to inflammatory signals and increases transcription of inflammatory factors [124]. Moreover, ageing results in a down-regulation of PPARα which is able to inhibit NF-κB [125]. Independently of whether senescence occurs in dividing or postmitotic (PM) cells, they have a number of common characteristics. Senescent cells secrete different pro-inflammatory cytokines such as TNFα, IL-6, IL-8, IL-1 and others, various CXCL chemokines, metalloproteinases, serine proteases as well as insulin-like growth factor into their local environment [126]. This senescence-associated secretory phenotype (SASP) is able to stress neighbouring cells through paracrine inflammatory mediator secretion as well as ROS signalling, causing local inflammation and senescence in those cells called "bystander effect" [127]. Through the bystander effect, senescent cells affect the entire local tissue environment. Transplanting senescent pre-adipocytes into the visceral adipose tissue of young mice is sufficient to impair their physical performance [80].

While $p16^{INK4a}$ and $p21^{Cip1}$ are well-known markers for senescence [126] and necessary to induce replicative senescence in dividing cells, it is unclear why they are upregulated in post-mitotic senescence. Other studies have shown that leakage of nuclear or mitochondrial DNA into the cytoplasm and their detection by the cGMP-AMP synthase (cGAS) – Stimulator of IFN gene (STING) system is crucial for the development of a senescence-related inflammatory phenotype [128-130]. DNA binding to cGAS activates NF-κB as well as respective downstream inflammatory factors.

7.6 Senescence in Postmitotic (PM) Tissues

Senescence in post-mitotic cells had previously been shown in mouse cortical and Purkinje neurons, hepatocytes and cardiomyocytes [102, 131, 132]. As these cells do not divide, the irreversible cell cycle arrest is not a required feature of post-mitotic senescence while most other characteristics such as mitochondrial dysfunction and development of SASP occur as in senescent cells derived from dividing cells. Moreover, while there is no telomere shortening in PM cells, an accumulation of telomeric damage has been described generating a DNA damage response indistinguishable from that caused by telomere shortening [131, 132].

It was established recently that senescent cells contribute to detrimental effects in liver and heart [102, 132]. In cardiomyocytes, increase in *p16, p21* and *p15* expression, SADS (Senescence-associated distension of satellites), and TAF were detected during ageing of wild type mice between 3 and 30 months [132]. In neurons, senescence was characterised by activation of p38 MAPK, increase in TAF, chromatin modification (SADS), lipid peroxidation, and IL-6 levels during ageing in wild type mice [131].

Two recent studies found an accumulation of lipid droplets in correlation with senescence in different tissues [102, 103]. While senescence in liver promoted hepatic steatosis, clearance of senescent hepatocytes decreased liver lipid accumulation [102]. Hepatocytes are quiescent under healthy conditions. Treating cultured hepatocytes with irradiation induced senescence markers such as sen-β-gal (senescence-associated beta-galactosidase) staining and DNA damage foci, a decrease in mitochondrial β-oxidation capacity and increased lipid accumulation (steatosis) [102]. However, hepatocytes have a higher β-oxidation capacity compared to WAT adipocytes [133]. Importantly, there was a correlation between steatosis and increased hepatocyte senescence in mice *in vivo*. Liver steatosis occurs in the metabolic syndrome as well as during ageing. The same group demonstrated that preventing lipid accumulation in adult mouse fibroblasts ameliorated the presence of cytoplasmic chromatin despite increased nuclear DNA damage [103]. Intriguingly, it was recently demonstrated that obesity also induces senescence in glial cells in the lateral ventricles of mouse brain, inhibiting neurogenesis and promoting lipid accumulation in glial cells of the brain resulting in anxiety-driven neurological dysfunction [103]. This phenotype was termed "accumulation of lipids in senescence" and presents a newly discovered association between lipid accumulation and senescence that begins in the liver, affecting lipid accumulation and adipocyte senescence in adipose tissue and subsequently leads to lipid accumulation in senescent glial cells in the brain. In contrast, the removal of senescent cells by using senolytic drugs or preventing senescence by exercise is able to counteract many effects of senescent cells in different tissues including the brain and WAT [102-104, 134]. In general, it seems that the relation between lipids and senescence might differ between tissues and cell types.

White adipocytes are highly susceptible to senescence both during ageing as well as obesity [135]. Senescence in WAT is characterised by an expansion of fat tissues, hypertrophy of adipocytes, dyslipidemia as well as insulin resistance [135]. It is also assumed that VAT (visceral adipose tissue) is more prone to senescence than SAT (subcutaneous adipose tissue) [22]. Importantly, obese humans can have up to 30 times more senescent subcutaneous WAT pre-adipocytes, compared to lean individuals [11, 136].

8. The Role of Mitochondria and ROS in Senescence

Mitochondria play a central role in ATP synthesis through oxidative phosphorylation, the Krebs cycle and fatty acid β-oxidation, cell signalling through products of various metabolic pathways, differentiation, apoptosis and senescence. Mitochondria are also the main source of endogenous cellular ROS. In contrast, mitochondrial dysfunction including increased mitochondrial biogenesis, ROS levels and less ATP generation and coupling, is a major hallmark of senescence [137]. Mammalian mitochondria are composed of more than 1500 proteins with the majority not being encoded by the mitochondrial DNA [138]. Human mitochondrial DNA (mtDNA) encodes two ribosomal RNAs, 22 tRNAs, and 13 protein components of complex I, III, V, and cytochrome oxidase while complex II is entirely nuclearly encoded. Consequently, nuclearly encoded proteins play an important role in mitochondrial biogenesis as well as in intra-organellar cross-talk [139]. This cross-talk involves transcription factors like NRF-1, NRF-2 and the regulators of mitochondrial biogenesis PGC-1α and PGC-1β. The transcription factors regulate genes for the mitochondrial transcription, translation and replication processes as well as proteins that compose the respiratory chain [140].

The PGC-1 coactivator family is regulated by various environmental signals such as cold stress, energy deprivation as well as the availability of nutrients and growth factors [140].

At the same time, PGC-1α and PGC-1β regulate transcription factors NRF-1, NRF-2 and TFAM (mitochondrial transcription factor A) [141, 142]. TFAM is upregulated by NRF-1 and stimulates transcription of mitochondrial genes [140]. Importantly, in addition to regulating mitochondrial biogenesis PGC-1α is as well involved in in adipocyte beiging and senescence [116]. A study in human fibroblasts demonstrated that overexpression of *PGC-1α* induced senescence [143].

Increased mitochondrial ROS generation due to mitochondrial dysfunction has been suggested as a contributing factor to the onset as well as maintenance of senescence [137]. Moreover, there is a positive feedback loop between ROS, mitochondrial signals and nuclear DNA damage [137]. Nuclear DNA damage by ROS generated through the electron transport chain, in particular at complexes I and III, has been recognised as an important cause of senescence induction [137]. Intriguingly Passos' group even demonstrated recently that senescence induction strongly depends on the presence of mitochondria [144]. Induction of mitochondrial depletion through a system consisting of PARKIN and the oxidative phosphorylation uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) prevented many features of the senescence phenotype [144]. Upon mitochondrial depletion, expression of 33% of the genes reduced in senescence were upregulated. Conversely, around 60% of SASP genes were downregulated, thereby strongly decreasing inflammation. This result demonstrates that a large number of senescence-associated changes is dependent on mitochondria, in particular the pro-inflammatory phenotype. On the other hand, other parameters, such as the expression of the cell cycle inhibitors *p21* and *p16* could not be prevented by depletion of mitochondria [144]. The paper also demonstrated that induction of DDR with different stimuli in normal cells with mitochondria resulted in increased mitochondrial mass due to increased levels of PGC-1α and PGC-1β, while PGC-1β-/- MEFs (mouse embryonic fibroblasts) had a delayed senescence onset. Increase in mitochondrial mass in mouse liver was also correlated to increased amounts of DNA damage foci, as well as an age-associated increase in PGC-1β [144]. These results demonstrated that mitochondrial biogenesis is central to the development and maintenance of senescence *in vitro* and *in vivo.* Mitochondria in senescent cells also lose their ability to efficiently utilise fatty acids [102].

Mitochondria play a key role in adipogenesis where mitochondrial biogenesis is upregulated during differentiation [44, 145]. Adipocyte differentiation also promotes increased mitochondrial metabolism and ROS generation, while decreased ROS levels prevent adipocyte differentiation [146]. Consequently, ROS play a signalling role here as well known for other processes [147]. Mitochondrial dysfunction is increasingly recognised as causal for various age-related processes in adipose tissues [22].

9. Dietary Restriction (DR) and Its Effects on Health and Metabolic Changes in Adipose Tissues

Caloric and dietary restriction (CR, DR) are a reduction of calories/food intake without malnutrition. It is the only known non-genetic intervention that significantly increases longevity in a large number of organisms and models from yeast to primates. This suggests a role for regulators of energy metabolism in the mechanism of DR which can be described as a metabolic reprogramming. In addition to extending lifespan, DR also improves health span by improving

metabolic health thereby delaying the onset of age-related disorders in many different species including mammals.

DR results in a reduction of body weight and adipose tissue mass in humans, monkeys and rodents. 25% to 65% DR is suggested to be the beneficial range, based on studies in mice [148]. Reduction in visceral fat through caloric restriction, limiting fat tissue development with rapamycin, surgical removal, and induction of apoptosis in visceral fat in mice generally results in an increased lifespan [11]. In addition to changes in fat mass, hyperglycemia and hyperinsulinemia seem to be associated to modifications of lipid and energy metabolism. Importantly, induced changes in energy homeostasis, mitochondrial function and lipid metabolism are the most likely underlying mechanisms for the beneficial health effects of dietary restriction. During DR, the levels of plasma leptin and insulin are low. Importantly, DR shifts the source of energy from glucose to lipids [149]. While AL-fed animals are able to use various energy substrates such as glucose and fats, DR animals mainly utilised glucose after food intake while using endogenous fatty acids from white adipose tissue and mitochondrial β-oxidation in the absence of food associated with the activation of PPAR-α, which induces the expression of genes related to mitochondrial β-oxidation. Thus, DR improves the efficiency of lipid metabolism and also inhibits lipid deposition in non-adipose tissues [6, 150].

A recent study by Hahn and co-authors characterised lipid metabolism in WAT and metabolic reprogramming under DR condition using various high-throughput techniques, in particular transcriptomics [6]. The study confirmed a differential regulation of lipogenic genes as a key signature of dietary restriction in mammals. The authors identified that WAT responds to DR by upregulating genes involved in mitochondrial biogenesis, lipogenesis and fatty acid metabolism. Interestingly, newly synthesised FA were predominantly built into membrane lipids including mitochondrial cardiolipin. The authors suggest that long-term DR shifts WAT metabolism from fat storage to phospholipid synthesis. In addition, the authors established that AL feeding promoted a pro-inflammatory gene expression in pre-adipocytes [6]. In contrast, weight loss in obese humans results in reduced plasma cytokine levels of TNFα and IL-6 [151]. Importantly, the study found a significant correlation between the amount of visceral fat loss and the percentage reduction in TNF-α and IL-6 plasma levels.

Moreover, dietary restriction and dietary restriction mimetics such as rapamycin are able to reduce mitochondrial ROS levels [152]. However, rapamycin compared to DR does not change any gene expression in adipose tissue suggesting different mechanisms of actions of both interventions [153].

DR has been shown to extend health span and lifespan and to delay oncogenesis in various animals including rodents [154-156]. The effect of DR was also translated into longer-lived animals such as rhesus monkeys with some contradictory results possibly due to methodological problems (overfeeding of AL controls) at the University of Wisconsin and the NIH [157, 158]. Both studies performed a long-term DR of 30% food decrease but only In the Wisconsin-located study there was a general decrease of age-related and all-cause mortality. These discrepancies in rather similar DR studies emphasises that the effects of CR in long-lived mammals are complex and might depend on a multitude of environmental, nutritional, and genetic factors. Additionally, there was a sex dimorphism effect of CR in rhesus monkeys, where male monkeys were more likely to have lower plasma lipoproteins and adiposity than female monkeys [159, 160] which might also apply to humans.

However, there were improvements to health span in both DR studies with CR monkeys showing an improved metabolic profile and slightly less oxidative stress measured as plasma isoprostane levels. Importantly, despite lack of consistent effects in longevity, in these studies CR reduced various age-related pathologies such as the incidence of diabetes, cancer and cardiovascular disease. Due to its beneficial effects on health and longevity, various human studies have been performed or are ongoing demonstrating beneficial health outcomes such as weight loss and decrease of cardiometabolic risk factors as well as inflammatory parameters [161, 162].

Ogrodnik and co-workers demonstrated that genes involved in lipid modification and inflammatory pathways changed during DR in mouse hepatocytes while it also protected against lipid accumulation and telomeric damage (TAF) in those cells while high fat diet increased the level of senescence measured as $p16^{INK4a}$ using RNA *in situ* hybridisation [102]. Importantly, treatment of two year old wild type mice with senolytics prevented lipid accumulation and steatosis in liver [102]. However, Hahn et al. emphasised the tissue-specificity of effects between liver, BAT and WAT regarding their metabolic reprogramming and transcriptional changes during DR in mice [6].

Four main signalling pathways have been suggested as mediators and mechanisms underlying the benefits of DR: the insulin/IGF-1 (insulin-like growth factor-1), the mTOR (mammalian target of rapamycin), the AMPK (AMP-activated protein kinase) and SirT1 (Sirtuin-1) pathways [163]. SIRT1 and AMPK are involved in major metabolic activities such as fatty acid oxidation, inhibition of glycolysis as well as protein, nucleotide and fatty acid synthesis through PGC-1α [164]. Decrease in plasma glucose, insulin or IGF-1 and circulating adipokines are physiological hallmarks of DR and are associated with the activation of the nutrient-sensing AMPK, SIRT1, PPARα, and PGC-1α molecular pathways [164, 165]. The activation of SirT1 during DR downregulates the pro-adipogenic mitochondrial biogenesis factor PPARγ and results in higher lipolysis and reduction of fat mass and thereby contributes to a longer life span. Importantly, all these mechanisms of beneficial effects during DR are not mutually exclusive [166].

While the beneficial role of caloric restriction on reduction of fat mass and metabolic changes is well known [6, 150, 151], senescence has not been analysed in detail in adipose tissue under this condition. The Saretzki group has performed two studies using dietary restriction on mice and characterised senescence and DNA damage in visceral adipose tissue [2, 3]. These results are summarised and set into context of current research of nutritional interventions below.

10. Effects of Ageing and Dietary Restriction on Senescence in Visceral Adipose Tissue

Senescence is implicated in many homeostatic processes such as tumour suppression, embryonic development, and wound repair [167]. Both, senescence and low-grade chronic inflammation are common to the ageing process in many tissues. However, the significance of senescence for lipid accumulation and hypertrophy in adipocytes has not been determined in detail yet. Instead, most studies on senescence in adipose tissues, pioneered by Kirklands' group at Mayo Clinics in Rochester (USA), have rather focussed on senescence of pre-adipocytes, the progenitors of mature adipocytes [11, 78, 168].

Thus, the recent studies from the Saretzki group describe some novel direction in nutrition research combining research on senescence and ageing in adipose tissue with different nutritional interventions. The group performed an initial study on wild type mice of different ages as well as a late onset, short term DR and examined changes in adipocyte size and presence of senescence

markers during ageing and DR [2]. Until recently, the presence of non-replicative senescence in post-mitotic cells had only been described in tissues such as mouse brain (Purkinje neurons) as well as heart muscle [131, 132]. Consequently, our study aimed to analyse whether senescence also occurs in post-mitotic fat tissue during mouse ageing and whether it can be ameliorated by dietary restriction [2, 3].

10.1 Changes in Adipocyte Size and AMPK Activity during Ageing and DR

Both ageing and obesity are characterised by an increased adipocyte tissue mass. However, adipocytes are larger in obesity and they get smaller at very high age [16]. The study by Ishaq and co-workers characterised retroperitoneal visceral adipocyte size as area and perimeter during ageing in male mice from 5 months on until an age of 30 months with *ad libitum* (AL) nutrition [2]. Adipocyte area and perimeter increased from 5 months till 17.5 months. At higher ages (25 and 30 month), according to what is known already [16], adipocyte size declined again. Others have described a peak of adiposity for mice at the age of 22 months which could be due to different genetic strains [17].

Interestingly, as body weight decreased to the levels of young mice under short term DR at 17.5 months, adipocyte size changed similarly after 2.5 month of DR to the level of young mice [2]. This result suggests that adipocyte size seems to correlate to body weight during DR, while there was no such correlation during ageing. This disparity between body weight and adipocyte size could be caused by additional age-related changes such as less adipogenesis due to pre-adipocyte senescence, increased lipolysis or dyslipidemia at higher ages [13].

AMPK is a kinase that monitors energy levels and gets activated by phosphorylation when energy levels decline, for example during exercise and fasting [169]. AMPK activity increased at 25 month of age and was highest at 30 months suggesting a possible caloric or energy deficiency at that high age. Together with a steeply declined adipocyte size this might suggest that visceral adipocytes in 30-month-old mice might either promote lipolysis or undergo a decrease in the uptake of fatty acids. Surprisingly, AMPK activity was not increased after the 2.5 months of short-term DR.

10.2 Changes in DNA Damage, Inflammatory Markers and Macrophage Numbers during Ageing and DR

Phosphorylation of histone H2A, subtype X (γH2A.X) is a biomarker for DNA damage and often used to characterise senescence in the form of damage foci [93]. Ishaq and co-authors found that the percentage of cells containing DNA damage in visceral adipose tissue steeply increased from 5 months to 25 months and was still high at 30 months [2]. The number of DNA damage foci per nucleus also increased from 5 till 25 months but was decreased at 30 months of age. The steady increase in DNA damage was paralleled by a similar increase in *p16*^{INK4a} gene expression during ageing while this parameter could not be analysed at the protein level since p16^{INK4a} antibodies do not work on mouse tissues. As expected, most senescence parameters were decreased after the 2.5 months of late-onset, short-term DR [2].

An increase of inflammatory markers (in particular *TNFα* expression) in WAT at high age [2] corresponds well to the study of Hahn and co-authors who described a pro-inflammatory gene expression pattern during long-term AL feeding conditions [6]. However, both studies did not discriminate between adipocytes and macrophages as gene expression was analysed on total WAT.

While studies on obesity and ageing have described an activation of the NLRP3 inflammasome including upregulation of IL-1β expression and secretion in WAT [53, 66], the study by Ishaq *et al.* did only find an increased IL-1β expression in retroperitoneal WAT during ageing in mice [2]. During short-term DR there was a trend for decrease in all three inflammatory markers with a significant more than 3-fold lower *IL-6* expression. This result seems to suggest that indeed a short-term, late onset DR was able to reduce adipose tissue inflammation which is known to be increased during the ageing process.

Due to the important role of macrophages for tissue adipose inflammation [51], the study by Ishaq and co-workers determined adipose tissue macrophage infiltration. While there was a general trend for increase in macrophage numbers with increasing age, these were more than 10 fold elevated at 25 months [2]. Additionally, most of the macrophages in the 30 months old visceral adipose tissue formed aggregates of macrophages. Interestingly, there was an association between TNFα expression and macrophage aggregation in 30-month-old mice in the study in accordance to suggestions that most of pro-inflammatory cytokines such as TNFα are initially expressed and secreted by macrophages and that there exists a paracrine loop between them and adipocytes [68]. TNFα it is involved in various aspects of adipocyte metabolism. For example, it affects glucose homeostasis in adipocytes, promotes lipolysis in cultured adipocytes and is able to inhibit adipocyte differentiation and lipogenesis [170]. Macrophage aggregates are indicative of removal of dead or lipodystrophic adipocytes by macrophages [171, 172]. Macrophage recruitment and CLS formation is caused by signalling from stressed adipocytes, either through hypertrophy, FA secretion or lipolysis [65, 171, 173]. Thus, together with the AMPK phosphorylation and inflammation results, our findings might suggest that lipolysis in very old adipocytes could cause various stages of macrophage aggregation. In contrast, there was no change in macrophage numbers or aggregation with DR [2].

In summary, at a very high age of 30 months mouse WAT had a low adipocyte size, high DNA damage, macrophage aggregation, increase of inflammatory markers like TNF-α as well as activated AMPK signalling. Moreover, a 2.5 months long dietary restriction at middle age from 15 months till 17.5 months decreased adipocyte size as well as DNA damage and some inflammatory parameters but did not change macrophage numbers or AMPK activity.

The findings from this study also suggest that hypertrophy in white adipocytes caused by *ad libitum* nutrition is damaging to adipocytes resulting in senescence, which can be ameliorated by short-term dietary restriction until mid-life, but seems to be refractory to DR changes at high age (24 months in mice) [6]. Importantly, Baker and co-authors have demonstrated that an inducible removal of $p16^{INK4a}$-positive senescent adipocytes is able to decrease systemic inflammation [17, 174]. In contrast, feeding mice with a high fat diet (HFD) increased levels of senescence-associated β-galactosidase and the SASP in visceral WAT, while exercise reduced this increase in senescence markers [134].

11. An Age-dependent Metabolic Memory for Nutrition

In addition to well-known effects of dietary/caloric restriction-induced changes in lipid composition of fat tissue as well as a global metabolic reprogramming [6, 175], various studies reported a metabolic memory for dietary/caloric restriction with differential effects in specific tissues such as liver and fat [6, 102]. However, also the age of onset of DR seems to be important

for DR effects. While many mouse studies used a long-term DR, some also attempted a later onset at 11-24 months or as part of switch-experiments [2, 3, 6, 155, 156]. Importantly, the study by Hahn et al. demonstrated that an introduction of DR at an age of 24 months (very late onset), when already 20% of AL-fed mice have died, did not exert the expected beneficial effects [6]. Under these conditions, it seems that the life-long metabolic memory of AL nutrition prevented any transcriptional reprogramming due to DR after two years of age.

11.1 Effects of DR on Adipocyte Size, DNA Damage and Inflammatory Markers as well as Macrophages

After having established effects of ageing and late-onset short-term DR in visceral fat from wild-type mice [2], Ishaq and co-workers analysed visceral adipose tissue from another DR study on male C57BL/6 mice that underwent a longer dietary restriction of 40% less food consumption starting at 3 months until 12 or 15 months [3, 155]. In addition, after 12 months either on AL or DR diet, mice were switched back for 3 months to the opposite nutrition regimen. On the same mouse cohort a previous study had described a metabolic memory of increased glucose tolerance and lowered plasma insulin levels [155]. Others have demonstrated that a metabolic memory of improved glucose tolerance after DR could be retained for up to 10 months in mice while other parameters such as body mass, fasting insulin levels and insulin sensitivity were similar to AL-fed mice at this time point [156].

Like in the previous DR study [2] the authors characterised adipocyte size in retroperitoneal adipose tissue to determine the effects of AL and an early-onset, longer-time (9-12 months) DR of 40% on visceral adipose tissue. Long-term AL-feeding resulted in adipocytes of similar sizes to those of the 17.5 months old AL-fed mice from the previous study [2] described above, suggesting a plateau in adipocyte size at this middle age (see Figure 2 for representative H&E images) [3]. As expected from the results of the first short-term, late onset DR study [2], early-onset DR for 9 and 12 months also generated significantly smaller sizes of around 30% of adipocytes under AL [3].

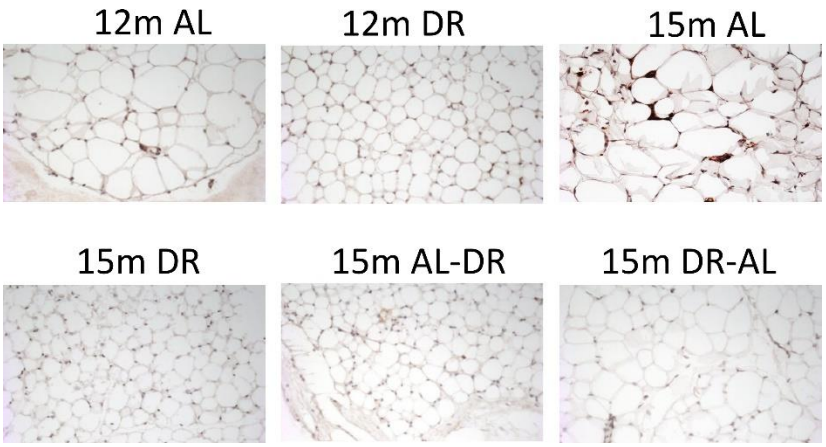

Figure 2 Representative Haematoxilin & Eosin (H&E) phase contrast images of adipocyte sizes of all conditions as indicated.

Interestingly, a switch to DR from AL for 3 months after 12 months of AL was able to reduce adipocyte size to similar levels as long-term DR. This is remarkable as it suggests that a short-term, late-onset DR of 3 months has similar effects on adipocyte size as a longer-term DR of 9 and 12

months. Importantly, a switch from DR to AL for 3 months in 12 months old mice which were previously under DR for 9 months did not show an increase in adipocyte size [3]. This result suggests that the DR condition preserved adipocyte size even after switching back to AL in a memory effect as described for other physiological parameters in this mouse cohort previously [155].

The authors also determined whether DR is protective against DNA damage and the onset of senescence in VAT. There was a strong trend of a 50% decrease in average numbers of γH2A.X DNA damage foci in all DR-related conditions compared to 12 and 15 months of AL [3]. Moreover, there was a significantly higher percentage of DNA-damaged cells after 15 months of AL compared to 15 months old DR mice as well as both switches with a similar trend for 12 months AL and DR [3]. This result suggests that switching to DR for 3 months after 12 month of AL feeding ameliorated the occurrence of DNA-damaged cells to the level of 12 months DR (at an age of 15 months) while there was no increase in DNA damage after switching back to AL for 3 months after 9 months of DR. Thus, there was a similar effect of short-term DR as for adipocyte size demonstrating firstly, that short-term, late onset DR had a very similar effect on decrease of DNA damage as early-onset, longer term DR and secondly, that there was a memory effect to maintain low DNA damage levels in VAT after switching back to AL after DR feeding. Strikingly, the average number of DNA damage foci per nucleus strongly correlated to adipocyte size (Figure 3). Thus, one could speculate, that both parameters reflect senescence-related processes and have a potential to be used as indicators of senescence as well as for anti-senescence interventions, for example when using exercise or senolytic treatments that remove those cells in tissues from aged organisms.

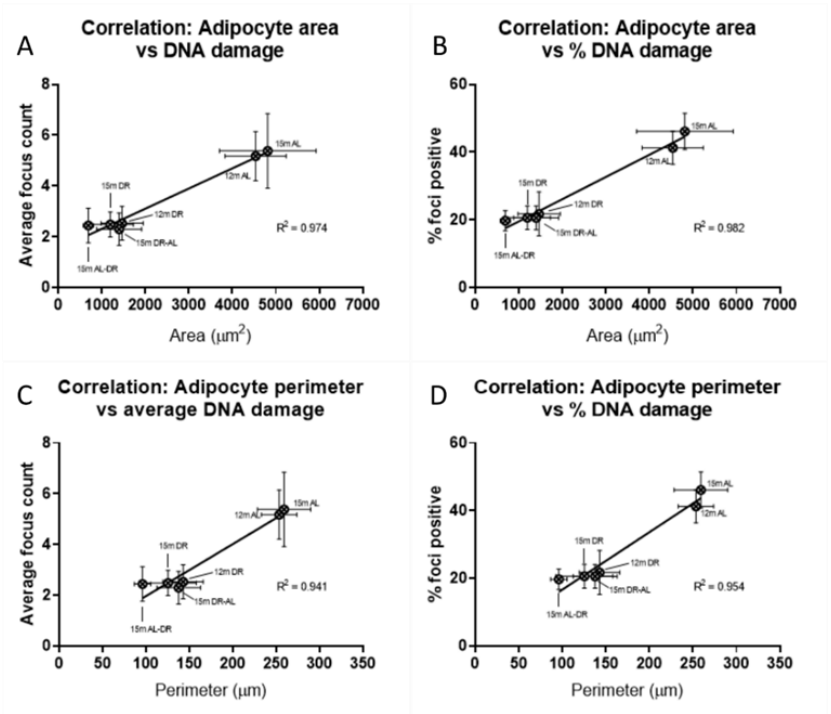

Figure 3 Correlation of adipocyte size to DNA damage in a long-term DR study [3] A) Adipocyte area versus DNA damage focus number. B) Adipocyte area versus percentage of cells positive for DNA damage. C) Adipocyte perimeter versus DNA damage focus number. D) Adipocyte perimeter versus percentage of cells positive for DNA damage. N=4-6 mice per groups. R^2 are indicated in the graphs and reflect a highly significant correlation between parameters.

Activation of AMPK promotes β-oxidation and glucose uptake in skeletal muscles [176, 177] and induces a metabolic activation state in mouse muscle, heart and brain. AMPK activation has thus been proposed as a therapeutic target for treatment of obesity and Type 2 diabetes [178]. It is also a hallmark of caloric restriction, since CR generally generates a low intracellular ATP environment. Treatment of human subcutaneous and visceral primary adipocytes with metformin (an AMPK activator) for 24 hours increased insulin-stimulated glucose uptake [179]. Thus, AMPK phosphorylation can act as a marker for adipocyte ATP levels and insulin-sensitivity.

Interestingly, while there was a trend for increase in AMPK activity after 9 months of DR, 12 months DR showed a significant increase while it was lost after switch-back to AL and also stayed low after short-term DR (see Figure 4) which corresponds to the results of the previous short-term DR study [2]. This result suggests that apparently only a continuous longer-term DR induces a consistent increase in AMPK activity which however is lost as soon as the DR feeding is switched to AL. Thus, there seems to be no memory effect for AMPK activity as shown for other physiological parameters in other studies [156].

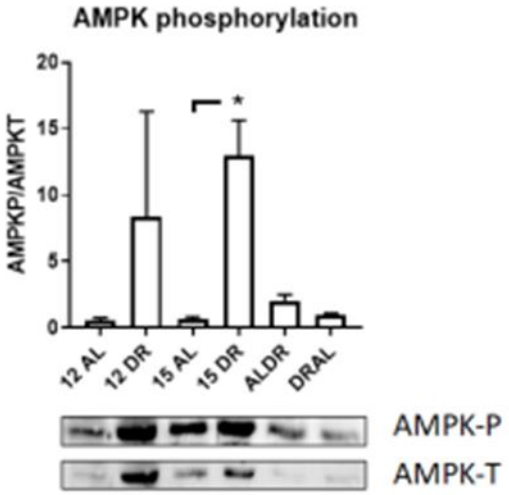

Figure 4 Cellular nutrient sensing state depending on feeding regimen in a DR study [3] shown as AMPK phosphorylation using Western blot technique. Age of mice and treatments are indicated. Comparison between treatments was analysed by one-way ANOVA with Holm-Sidak post-hoc test. * = $p < 0.05$ vs indicated. 4-6 mice were used per group.

In addition to a similar trend for changes in $p16^{INK4a}$ expression as for adipocyte size and DNA damage in this DR study, the late-onset switch to DR for 3 months also reduced expression of $p21^{Cip1}$ compared to the 15 months AL-fed mice while all other DR-related conditions had a similar trend as the parameters described above demonstrating a good consistency and robustness of all those chosen senescence markers [3].

Interestingly, there was no significant change or trend for *IL-6* expression under DR. However, *TNF-α* and *IL-1β* expression was consistently low at 15 months of DR and in the two late-onset dietary switches in feeding conditions [3]. This result seems to suggest that firstly, long-term as well as short-term DR are able to induce a reduction in some inflammatory markers, similarly to that seen in the previous DR study [2] and secondly, even after switch-back to AL for 3 months these

markers stayed at a low level confirming again a memory effect for some inflammatory markers after terminating the DR treatment. This result is also in accordance to the data from the study of Hahn et al. who described a pro-inflammatory state in pre-adipocytes caused by long-term AL nutrition [6].

Interestingly, there was no change in numbers of adipose tissue macrophages with any feeding condition. The study described a rough ratio of 1:2 for macrophages and adipocytes across all treatments and a trend to low levels of aggregation under all DR conditions at 12 and 15 months as well as after switch-back to AL for 3 months [3]. However, differently from most other analysed parameters, there was no decrease in aggregates after short-term DR from 12 till 15 months which is consistent with the results of the previous short-term DR study [2]. This result seems to suggest that similar to AMPK activation, only long-term DR seems to decrease the amounts of macrophage aggregates while it stays low after a 3- month switch-back to AL feeding. Strikingly, only a relatively small subset of non-macrophages in the adipose tissue were damaged while almost all macrophages were positive for DNA damage while there were around twice as many adipocytes in the tissue than macrophages. It would be interesting to discriminate also other senescence parameters between different cell types from the WAT in order to better characterise the contribution of each cell type to the general WAT phenotype.

In summary, the second DR study from Ishaq and colleagues [3] found consistent and anticipated changes in various but not all analysed parameters between AL and long-term DR at 12 and 15 months of age. The findings from both DR studies [2, 3] also suggest that even a late-onset, short-term DR is able to alleviate some deleterious senescence-associated effects caused by AL and that long-term DR even protects against the effects of a following short-term AL switch-back by a metabolic memory. Consistently, in both studies late-onset, short-term DR was sufficient to reduce adipocyte size and DNA damage [2, 3].

Net accumulation of lipid in adipocytes is a function of rate of lipogenesis versus lipolysis. In the two DR studies of Ishaq and co-workers [2, 3], this nutritional intervention promoted a decreased lipid accumulation in visceral retroperitoneal adipocytes suggesting either increased lipolysis or lower lipogenesis. Importantly, the decrease in adipocyte size during DR coincided with a decrease in DNA damage foci per cell nucleus as well as the amount of damaged cells in the tissue (see Figure 3). However, the authors did not analyse any transcriptional or metabolic parameters in the adipose tissue.

The effect of a metabolic memory suggests that a period of caloric restriction can have a longer lasting health benefit at mid-age even after transition away from a DR diet to prevent DNA damage and senescence in visceral adipose tissue and adds to previous evidence that DR protects against senescence and ageing [102, 155]. In contrast, at a rather high age of two years in mice, it was demonstrated that mice rather had a memory for the previous AL nutrition making them refractory for a DR setting in at that age [6].

An earlier study has analysed a switch from DR to AL feeding at 11 month for up to 10 months, with a glycaemic memory of improved glucose tolerance persisting for up to 10 months after switching from DR to AL despite other parameters of AL-feeding such as body mass, insulin levels and insulin sensitivity returning to AL levels [156]. However, no direct senescence markers or fat tissues were analysed in that study. In contrast, Hahn and co-authors found that a DR onset at a high age (24 months) rendered WAT refractory to beneficial changes while a shift from long-term

to back to AL even increased age-related mortality of mice [6]. These results are important for a possible implementation of DR to a human setting.

For a better mechanistic characterisation of nutritional stress other than general *ad libitum* feeding in mice, the authors progressed to *in vitro* cultured and differentiated primary human adipocytes in order to model the process of induction and alleviation of senescence employing free fatty acids which is described in the next headings.

12. Suitability of Adipocyte *in Vitro* Models for the Characterisation of Senescence

In vitro adipocyte models can be generated from primary pre-adipocytes, embryonic stem cells, induced pluripotency cells (iPSCs) or mesenchymal stem cells [179, 180]. 3T3-L1 is a sub-cultured cell line of 3T3 mouse fibroblasts with the capacity to accumulate lipid droplets upon treatment with insulin to increase glucose uptake, 3-isobutyl-1-methyxanthine (IBMX) to increase cAMP through protein kinase A (PKA) activation [181] and dexamethasone to increase expression of adipogenic factors such as PPARγ and C/EBPα [179, 181-183].

Mouse 3T3-L1 cells are often used as an easy-to-culture cellular system [179] due to their immortal nature in obesity-related and biochemical studies and to characterise the effects of nutrients on adipogenesis [184, 185], but very rarely for the analysis of senescence-associated parameters such as DNA damage. Vergoni and co-workers [120] reported an increased chemokine production and p53 activation after doxorubicin-induced DNA damage in differentiated 3T3-L1, and that DNA damage promoted lipolysis analysed through release of glycerol into the growth medium [120]. However, these cells are immortalised and thus unsuited for any senescence-related studies since they do not undergo real senescence and do not show any typical markers of it such as sen-β-gal staining or DNA damage after inducers of senescence such as irradiation and H_2O_2 treatment (own unpublished results). A similar result was also found by Zeng and co-authors who described an effect of PA (palmitic acid) onto DDR via prevention of $p21^{Cip1}$ induction in embryonic mouse fibroblasts and osteoblasts, but not in immortalised NIH-3T3 cells and various cancer cell lines [186].

Although the expression of some senescence markers *($p16^{INK4a}$* and *$p21^{Cip1}$* expression) as well as selected inflammatory factors (*TNF-α, IL-6*) were increased in these cells when treated with palmitic acid (PA) and linoleic acid (LA) most important senescence markers were not present using common stressors such as 20Gy x-irradiation and 150µM H_2O_2 treatment (own unpublished results). Consequently, this cell model seemed unsuitable for analysing FA-induced senescence.

Cultured human adipocytes are differentiated from pre-adipocytes by inducing a lipid-storing phenotype due to upregulation of PPARγ, C/EBP and cAMP levels, similarly to that in 3T3-L1 cells [179]. Human visceral and subcutaneous adipocytes are differentiated from primary pre-adipocytes isolated from the stromal-vascular fraction of fractionated adipose tissue. Thus, human primary pre-adipocytes are a more physiologically relevant model for the analysis of senescence compared to immortalised 3T3-L1 cells.

While in human differentiated adipocytes $p16^{INK4a}$ staining and the induction of DNA damage foci worked well under irradiation and H_2O_2 treatment used as positive controls for senescence markers (see Figure 5), sen-β-gal staining as a common senescence marker was still not suitable since already differentiated adipocytes showed a high degree of positive staining (unpublished results). A possible reason for this could be that these cells are already postmitotic. Interestingly, H_2O_2 treatment induced even higher DNA damage than irradiation with 20Gy did (Figure 5A-C). The occurrence of a

proliferation arrest was determined using an antibody against the proliferation-associated Ki67 antigen (see Figure 5D left image for representative staining).

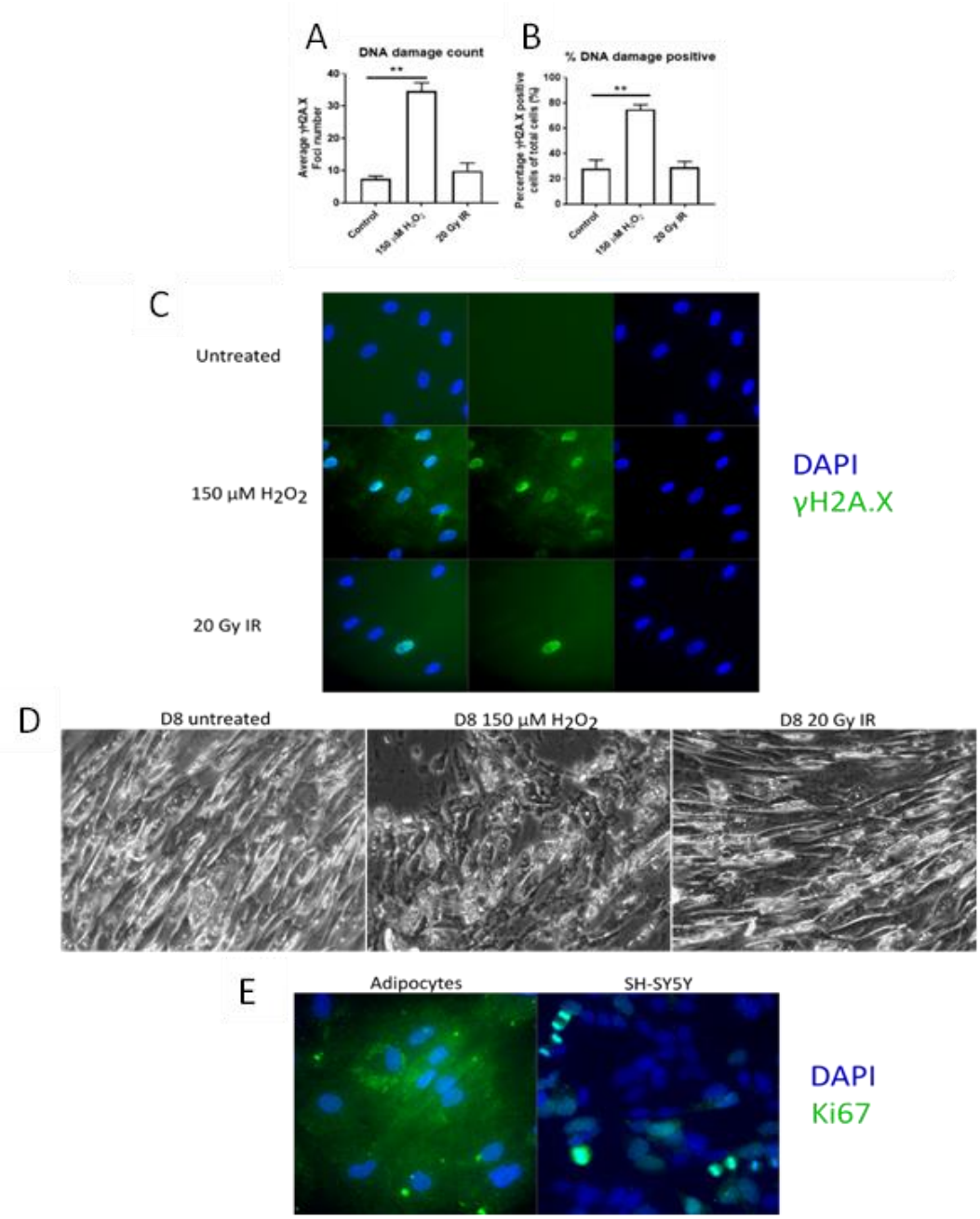

Figure 5 Establishment of senescence markers in human adipocytes at day 8 using 150 µM H_2O_2 and 20 Gy irradiation. A) Average γH2A.X DNA damage focus count per nucleus. Comparisons between treatments were analysed by one-way ANOVA with Holm-Sidak post-hoc test. ** = $p < 0.001$ vs indicated, n = 3. B) Average percentage of DNA damage positive nuclei per z-stack image per sample for 7 images. C) Representative images showing γH2A.X DNA damage foci after H_2O_2 treatment and irradiation. D) Representative phase contrast images for DNA damage after induction, imaged at 400x magnification. E) Immuno-fluorescence staining of adipocytes with a Ki67 proliferation marker using SH-SY5Y neuroblastoma cells as a positive control for dividing cells, 400x magnification.

13. Palmitic Acid as an Inducer of Senescence in Human Adipocytes *in Vitro*

Following the discovery of lipid-based hypertrophy and senescence in mouse visceral WAT, Ishaq and co-workers modelled senescence in human primary differentiated adipocytes treated with palmitic acid as nutritional intervention *in vitro*. The study employed subcutaneous adipocytes from a cell repository as well as subcutaneous and visceral adipocytes from the same human donors as isogenic pairs [5]. The authors aimed to determine if lipid droplet hypertrophy due to free fatty acid storage is associated with DNA damage and senescence in adipocytes *in vitro*. Based on the known biological effects of saturated and unsaturated fatty acids, the expectation was that the detrimental effects of saturated FA can induce DNA damage and promote a senescence phenotype. Consequently, the *in vitro* study compared the effects of palmitic acid (PA) and oleic acid (OA) in human adipocytes. Palmitic acid is the most common saturated fatty acid and a major component of palm oil, meat, butter and cheese with a content of 50-60% of total fats. In contrast, oleic acid as a monounsaturated omega-9 fatty acid is a natural component of various animal and vegetable fats and oils. Triglycerides of oleic acid are the major component of olive oil and thus an important ingredient of the Mediterranean diet.

In order to create an adipocyte senescence model, differentiated human subcutaneous (see Figure 6 for representative images) as well as omental adipocytes were treated with 100 nM PA or OA for 2 to 4 days. As expected, PA treatment increased lipid droplet (LD) size around 2-to 3-fold after 2 and 4 treatment days, respectively, while there was no increase in untreated or OA-treated adipocytes [5] confirming earlier findings of hypertrophy induction by PA [187]. However, PA did not affect nutrient sensing in adipocytes while there was a trend towards an increase in phosphorylated AMPK due to OA treatment (Figure 7B). The latter finding might suggest signalling of a low energy state after OA treatment.

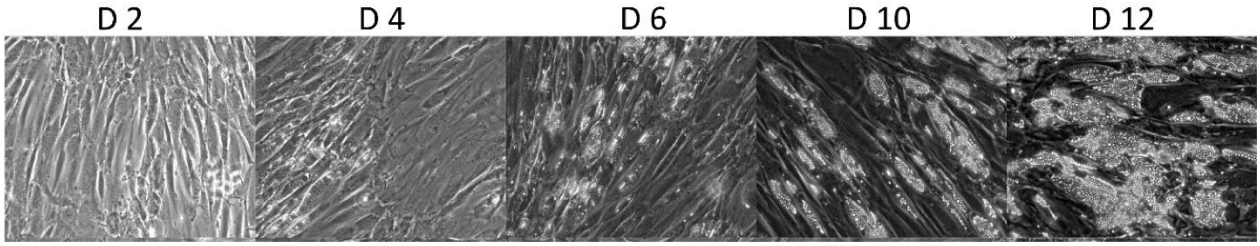

Figure 6 Representative phase contrast images of progressive of lipid accumulation during differentiation of human subcutaneous adipocytes *in vitro.* (100x magnification). Cells organised in a more parallel fashion, indicating high local confluence, are more likely to accumulate lipid droplets.

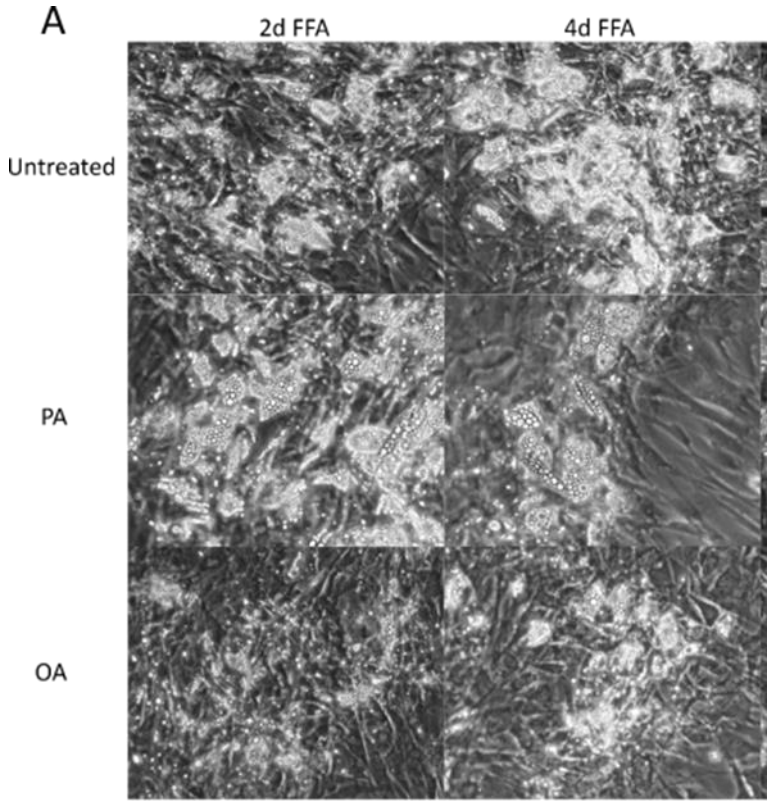

Figure 7 Lipid droplet accumulation and AMPK activation in subcutaneous adipocytes after FFA treatment. A) Representative phase contrast images (100x magnification). B) Phosphorylation of AMPK measured by Western blot.

PA treatment induced a 3-5-fold increase in general DNA damage and even double that amount in TAF (co-localisation of DNA damage foci with telomeres) while OA treatment did not induce any telomere-associated foci [5]. TAF are the gold-standard for senescence biomarkers since telomeric damage cannot be repaired [93, 94] and accumulates in non-dividing cells such as PM cells [95]. Others have also shown the presence of DNA damage after PA treatment [186, 188].

However, while most studies have detected increased oxidative stress after PA treatment in other cell types [189, 190] as the most likely cause of DNA damage the study from Ishaq and co-workers [5] did not find an increase in mitochondrial superoxide after PA treatment suggesting alternative mechanisms for the induction of DNA damage. There are some alternative pathways that could explain the occurrence of DNA damage without measurable mitochondrial superoxide levels. For example, it was recently demonstrated that senescence induction by PA in endothelial cells works through stimulation of autophagy [191]. Wen and co-workers found that AMPK was able to inhibit ROS generation as an upstream pathway [54]. The authors suggested that PA can decrease

AMPK activity causing a defective autophagy function. In addition, Zeng and co-authors suggested that palmitic acid, stearic and myristic acid prevent the induction of $p21^{Cip1}$ and BAX, thus, negatively regulating the DNA damage response [186]. Moreover, it was also shown that PA induces mitochondrial damage and release of mitochondrial DNA into the cytosol activating the cGas-STING pathway in endothelial cells [192]. Interestingly, STING is required to be palmitoylated for its activity [193] and resides within the endoplasmatic reticulum (ER) as well as on ER- mitochondria-associated membranes [194], while PA is known to induce ER stress [195]. STING seems to be upregulated after

PA treatment [196] and can induce autophagy that is however compromised due to PA [191]. Thus, there exist multiple alternative signalling pathways that could be responsible for the damaging phenotype of PA that requires further investigation.

Unlike in mouse tissues, the senescence marker $p16^{INK4a}$ can be analysed by immuno-staining in human cells. The percentage of $p16^{INK4a}$-positive nuclei increased 3-4-fold with PA treatment in subcutaneous adipocytes while OA did not show any effect. In contrast, omental adipocytes showed a large heterogeneity and thus no significances [5]. However, the increase in $p16^{INK4a}$ protein was not matched by an increase in *p16*^{NK4a} gene expression, suggesting that $p16^{INK4a}$ protein-upregulation could be rather due to increased translation or protein stability. Surprisingly, there was no change in the expression of *p21*^{Cip1} or any inflammatory marker (*IL-6, TNF-α* and *IL-1β*). In contrast, PA treatment has been shown to create a pro-inflammatory environment in various cell lines, such as HeLa, COS-7 and 3T3-L1 [197] as well as primary cells such as bone marrow-derived macrophages [198].

In summary, the study by Ishaq and co-authors [5] established that treatment of human primary adipocytes with extracellular PA increased both lipid accumulation and various senescence parameters but not inflammatory markers.

13.1 Nitrate Treatment was not Able to Alleviate Senescence in Primary Human Adipocytes

As a possible intervention to alleviate the damaging effects of PA, a reduction of lipid accumulation by increasing lipid turnover using nitrate treatment was attempted by the study of Ishaq and co-workers [5]. Nitrate consumption is thought to increase β-oxidation capabilities of treated cells after reduction to nitric oxide [199, 200]. Prior studies had shown that β-oxidation can be increased during rodent white adipocyte differentiation, inducing a beiging effect [200]. The hypothesis was that increased β-oxidation would also be able to alleviate senescence by reducing lipid droplet size and hypertrophy in adipocytes. However, in the study from Ishaq et al. [5] nitrate treatment following differentiation did not alleviate PA-induced senescence neither in subcutaneous nor omental human primary adipocytes. In contrast, it had been demonstrated previously that inorganic nitrate was able to counteract the increase in oxidative stress after hyperglycaemia in older obese subjects *in vivo* [201]. Another *in vivo* study on HFD fed mice also demonstrated a beneficial effect of nitrate counteracting fat accumulation and improving glucose metabolism as well as inducing lower inflammation parameters in VAT [202]. The same study also treated primary mouse subcutaneous adipocytes with PA and found higher mitochondrial respiration and a higher protein levels of mitochondrial respiration complexes. Different treatment protocols, as well as different metabolic conditions of nitrate metabolism *in vivo* and *in vitro* could be responsible for the differing effects of nitrate on adipocyte parameters in the above described studies.

13.2 Co-treatment of OA and PA in Donor-derived Isogenic Subcutaneous and Omental Human Adipocytes

The study from Ishaq et al. [5] compared the effects of PA and OA on cultured human isogenic subcutaneous versus omental adipocytes. Lipid accumulation during subcutaneous adipocyte differentiation started at day 6 of differentiation and was variable between donors and was as well highly dependent on cell density prior to differentiation. Omental adipocytes accumulated

substantially less lipids (only around 50% more) compared to subcutaneous adipocytes after PA treatment where there was a 2-4 fold increase compared to untreated adipocytes [5]. Thus, these *in vitro* results confirmed the different capabilities of subcutaneous and omental adipocytes to accumulate lipids *in vivo* [16, 203].

In order to analyse whether OA was able to counteract the negative effects of PA, subcutaneous and omental adipocytes were either supplemented with OA first and then with PA or the other way around or co-treated with each OA and PA. While there was a significant increase in lipid droplets in subcutaneous adipocytes after PA only treatment, omental ones did not show any significant change under this condition [5]. None of the 3 combination treatments decreased LD significantly in subcutaneous adipocytes while visceral ones had in general very muted effects of for all treatments.

PA increased the average amount of γH2A.X DNA damage foci per nucleus around 3-fold in subcutaneous adipocytes while OA did not induce any DNA damage at all [5]. In contrast, co-treatment of PA and OA generated significantly lower DNA damage compared to PA treatment only [5]. Thus, OA seems to be able to counteract the effects of PA on DNA damage when subcutaneous adipocytes were exposed to both FFAs simultaneously. In contrast, while there was a similar trend for DNA damage of PA and OA treated OM adipocytes compared to untreated cells, the majority of treatments were inconclusive due to a large variation and heterogeneity between donors. While $P16^{INK4a}$ immunofluorescence analysis showed similar differences as DNA damage after PA and OA treatment, no significances were found for any combination in subcutaneous adipocytes due large variations between donors. Again, there were no differences in omental adipocytes in any treatment.

In conclusion, for the three analysed parameters of either consecutive or combined treatment of PA and OA only co-treatment of OA and PA on DNA damage demonstrated a significant decrease in subcutaneous adipocytes. Still, LD number and $p16^{INK4a}$ staining showed similar trends without reaching significance. Possibly, a larger number of donors might have also rendered those parameters significant in subcutaneous adipocytes. The reason for the large heterogeneity of the omental samples could be an *in vitro* effect since the initial pre-adipocytes proliferated and differentiated less well as was described previously [16].

Remarkably, $p16^{INK4a}$ positive nuclei showed a three times higher amount of DNA damage foci than $p16^{INK4a}$ negative nuclei suggesting a good coincidence of both senescence markers (Figure 8). The actual function of $p16^{INK4a}$ in post-mitotic cell senescence is not clear, but it is known that removal of naturally occurring $p16^{INK4a}$-positive cells from tissues can rejuvenate mice [17, 103, 174]. However, it is unknown whether the association between increased DNA damage in $p16^{INK4a}$-positive nuclei holds true in other cell types and *in vivo* or whether it is specific for adipocytes. Interestingly, while normally $p16^{INK4a}$ accumulation develops during a time frame of around 10 days after stress treatments *in vitro* in fibroblasts, in human subcutaneous and visceral adipocytes analysed by Ishaq and co-workers, $p16^{INK4a}$ accumulation occurred within 4 days of PA treatment [5]. Thus, it is possible that there is a difference between mitotic and postmitotic cells such as differentiated adipocytes in the kinetics of $p16^{INK4a}$ accumulation after stress treatment.

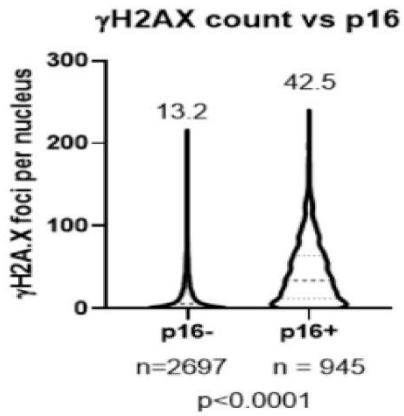

Figure 8 Co-staining of $p16^{INK4a}$ and γH2A.X on FFA-treated adipocytes. Violin plot for the distribution of DNA damage count, grouped by $p16^{INK4a}$ -positive or $p16^{INK4a}$ -negative nuclei in all treatments (OA, PA and all combinations).

It is known that high circulating levels of saturated free fatty acids such as palmitate induce inflammatory responses and cause insulin resistance in peripheral tissues *in vivo* [204]. However, Ishaq and co-authors only found trends for increase of inflammatory markers after PA treatment in subcutaneous adipocytes *in vitro* [5]. Previous studies had demonstrated that increased contact of adipocytes with PA promoted direct detrimental intracellular effects [189-191]. There are two main pathways for PA-induced cellular stress: endoplasmic reticulum (ER) stress and mitochondrial ROS generation. A study using co-treatment of OA and PA in bone marrow-derived cells demonstrated that the ratio of PA-derived phosphatidylcholine to unsaturated FA-derived phosphatidylcholine in the ER membrane determined whether the inflammasome was upregulated in tissue-infiltrating macrophages [198]. Kwon and co-workers [204] demonstrated that PA induced mitochondrial dysfunction and ROS generation as well as nuclear translocation of NF-κB-related p65. In contrast, preconditioning of mouse neuroblastoma cells with OA and removing it again was sufficient to fully block palmitate-induced intracellular signalling and metabolic dysfunctions. Interestingly, the decrease in mitochondrial ROS generation by OA was associated with increased levels of PGC-1α [204]. However, Ishaq and co-workers [5] have not analysed the beneficial effect of OA mechanistically in their adipocyte model.

Mono- or poly-unsaturated FFAs such as oleic acid seem to be protective against saturated FFAs. The results from Ishaq and co-authors who did not find any senescence markers after OA treatment in cultivated adipocytes add to this beneficial effect of OA [5]. Oleic acid is a major component of the Mediterranean diet and able to reverse inflammation-associated insulin resistance [205]. Replacement of dietary PA with OA increased insulin sensitivity in humans [206] and can alleviate detrimental cellular effects of PA such as apoptosis in human β-cells [207].

14. Argan Oil is Protective Against DNA Damage *in Vitro* and *in Vivo*

In addition to oleic acid which is a major component of olive oil [208], there are other oils with a similarly beneficial effect to olive oil. Bouchab and co-authors performed a study on liver tissue in mice which were challenged with iron as a liver-toxic agent that induced DNA damage [4]. Co-treatment of iron with olive oil and argan oil from the tree *Argania spinosa* ameliorated DNA damage in liver cells by 50% while there were no significant changes in the expression of the senescence

markers *p16*^{INK4a} and *p21*^{Cip1} and four inflammatory markers (TNFα, Cox1, IL-6, IL-1β) [4]. The authors also used human fibroblasts as an *in vitro* model which was challenged with hyperoxia during culture and found that both, olive oil and argan oil decreased DNA damage in these cells. In addition, argan oil, but not olive oil was able to decrease intracellular peroxide levels. The latter effect is remarkable as in a cell-free assay olive oil showed a larger radical scavenging potential than argan oil [4]. However, in biological systems argan oil seems to be more efficient in decreasing oxidative stress. Argan oil contains 45% oleic acid and 35% linoleic acid as well as minor antioxidant compounds. In summary, these results from Bouchab and colleagues [4] confirm the protective effect of olive and argan oils *in vitro* and *in vivo* while it has not been assessed on fat tissue or cultured adipocytes yet.

15. Conclusions

Obesity and ageing are thought to share various common pathways and mechanisms. While obesity together with the metabolic syndrome have been investigated intensively, mechanisms of ageing and senescence in adipose tissue are less well researched. Differentiated adipocytes are postmitotic cells. Senescence in postmitotic cells differs from that in mitotic cells as it lacks any cell cycle arrest. However, it is still characterised by increased DNA damage, p16^{INK4} staining and inflammation associated with the "senescence-associated secretory phenotype" (SASP).

Ageing in wild type mice on *ad libitum* (AL) nutrition generated hypertrophy in visceral adipose tissue which is associated with increased amounts of DNA damage, but a rather less pronounced inflammatory component at the gene expression level. In contrast, dietary restriction (DR) which is known as the only non-genetic nutritional intervention to extend longevity and health span in various model organisms, was able to ameliorate adipocyte size and decrease levels of DNA damage [2, 3].

Remarkably, as was shown previously for other systemic physiological parameters such as glucose and insulin levels in a mouse study of dietary restriction [155], visceral adipose tissue also showed a metabolic memory for decreased adipocyte size and low amounts of DNA damage after switching from a long-term DR back to normal *ad libitum* feeding. In addition, for several parameters, a late-onset short-term DR was as efficient in increasing health parameters and decreasing senescence markers as was an early-onset, long-term DR [3]. In contrast, a DR at very high age has been identified as refractory to many health-improving effects on the transcriptional level [6]. This study has also complemented the analysis of metabolic memory by demonstrating important transcriptome changes in adipocyte tissue which was largely connected to mitochondrial biogenesis as well as membrane lipids such as cardiolipin [6].

It is well known that some food components such as the saturated palmitic acid (PA) which is mainly contained in meat, cheese and processed food, are detrimental for human health. Employing cultured human primary subcutaneous and visceral adipocytes as a model a recent study demonstrated that PA induced various senescence markers in cultured adipocytes such as hypertrophy, elevated DNA damage and the senescence-related factor p16^{INK4a} [5]. In contrast, components of the Mediterranean diets such as oleic acid (OA) and argan oil where able to ameliorate the occurrence of senescence-associated parameters such as DNA damage in different models while nitrate treatment in the adipocyte culture model was not able to counteract PA-induced senescence. These studies advance our knowledge of senescence and the ageing process

in adipocyte cells and tissue and identify some potential interventions to counteract these processes (summarised in Figure 9).

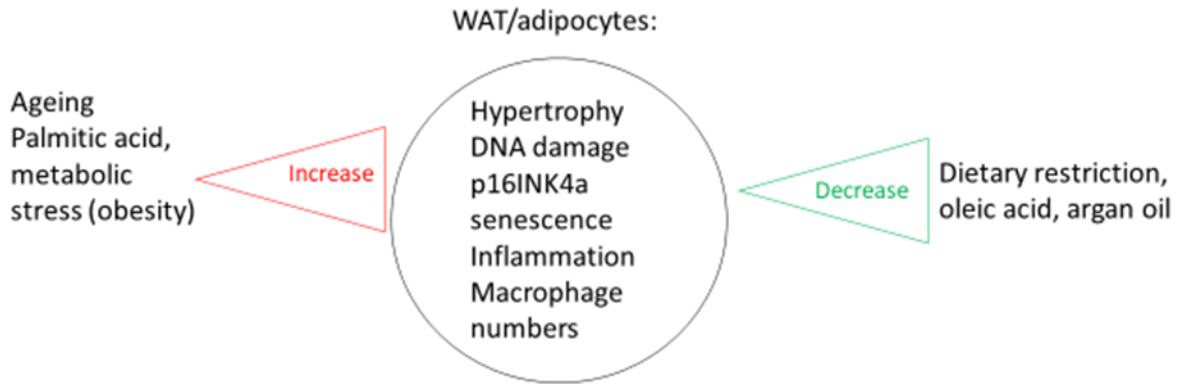

Figure 9 Summary scheme of the opposing effects of ageing, stress and palmitc acid in contrast to dietary restriction, oleic acid and argan oil on stress and senescence markers in WAT and adipocytes [2-5].

Consequently, nutritional interventions such as caloric and dietary restriction (CR, DR) or the use of olive and argan oils as major parts of the Mediterranean diet confer health benefits by ameliorating DNA damage, cellular senescence, adipocyte hypertrophy or reprogramming the adipocyte transcriptome and lipogenic genes in particular [2-6]. While most nutritional studies employ model organisms, it will be important in the future to perform direct human interventional studies in order to demonstrate whether various nutritional interventions are able to improve human health parameters and to ameliorate and to delay the ageing process.

Author Contributions

Both authors jointly designed the review and wrote the manuscript. AI composed most of the figures.

Competing Interests

The authors have declared that no competing interests exist.

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