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The role of senescence in the development of non-alcoholic fatty liver disease and progression to non-alcoholic steatohepatitis

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Abbreviations

1. NASH: Non Alcoholic Steatohepatitis
2. NAFLD: Non Alcoholic Fatty Liver Disease
3. ROS: Reactive oxygen species
4. ATM: Ataxia-telangiectasia mutated
5. SA-β-GAL: senescence-associated β-galactosidase
6. SASP: senescence associated secretory phenotype
7. IL: interleukins
8. AT: Adipose tissue
9. HFD: high fat diet
10. HCC: hepatocellular carcinoma
11. HSC: hepatic stellate cells

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ABSTRACT

In recent years cellular senescence has generated a lot of interest among researchers due to its involvement in normal aging process and also in common human diseases. During senescence, cells undergo alterations that include telomere shortening, nuclear area enlargement, genomic and mitochondrial DNA damage, leading to irreversible cell cycle arrest, and secretion of proinflammatory cytokines. Evidence suggests that the complex process of senescence is involved in the development of a plethora of chronic diseases including metabolic and inflammatory disorders and tumorigenesis. Recently, several human and animal studies have emphasized the involvement of senescence in the pathogenesis and development of liver steatosis including the progression to Non-Alcoholic Steatohepatitis

(NASH) as characterized by the additional emergence of inflammation, hepatocyte ballooning and liver fibrosis. The development of Non-Alcoholic Fatty Liver Disease (NAFLD) and its progression to NASH are commonly accompanied by several pathophysiological events including metabolic dysregulation and inflammatory phenomena occurring within the liver which may contribute to or derive from cellular senescence, implying that the latter may be both a stimulus and a consequence of the disease. In this review we summarize the current literature on the impact of cellular senescence in NAFLD/NASH, and discuss the effectiveness and safety of novel senolytic drugs and therapeutic options available to delay or treat the disease. Finally we identify the open questions and issues to be addressed in the near future.

1.Introduction: Molecular characteristics of senescence

The term “senescence” derives from the Latin word “senex” meaning a man of old age. Cellular senescence describes a decline in cell division capacity whereby normal diploid differentiated cells enter a state of cell cycle arrest and lose their ability to proliferate (1,2). It is triggered by DNA damage in chromosomes and telomeres, provoked by internal or external stimuli such as aging, oncogene expression and reactive oxygen species(ROS) accumulation or radiation and chemotherapies respectively (3,4). More specifically, there are two major mechanisms of cellular senescence; one is replicative senescence which depends on telomere shortening or erosion, predominantly upon aging, and the other is stress-induced premature senescence which is mostly telomere-independent and refers to intracellular or environmental stress factors leading to DNA damage (1,2). Both mechanisms induce a complex multigenic pathway known as DNA damage response(DDR) which can either activate a repair mechanism or lead to the inhibition of cell cycle(5). In the latter case, DDR triggers ataxia-

telangiectasia mutated(ATM) and Radd3-related protein kinases leading to p53 phosphorylation and subsequent activation of p21 resulting to cell cycle arrest(6,7). Concurrently p21 and p16 inhibit the phosphorylation of the retinoblastoma factor(Rb) allowing it to bind to E2F transcription factor and stop the progression of the cell cycle(8).

Cells undergoing senescence appear enlarged and flattened with enlarged nuclei under light microscope observation, while biochemical assessment of senescent cells shows the presence of the senescence biomarker senescence-associated β-galactosidase(SA-β-GAL)(9). Senescence-associated heterochromatin foci (SAHF) constitute another senescence biomarker. SAHFs consist of heterochromatin and a group of proteins that contribute to senescence by repressing the expression of proliferation-promoting genes (10). Importantly, the senescence-associated secretory phenotype(SASP), a complex mixture of molecular mediators secreted by senescent cells, serves as an important molecular signature for senescence(3). SASP includes proinflammatory cytokines, such as interleukin-1b(IL-1b), IL-6, IL-8, chemokines such as Monocyte Chemoattractant Protein-1(MCP-1), growth factors such as Human Growth Factor (HGF) and Fibroblast Growth Factor(FGF), proteases such as matrix Metalloproteinases (MMPs), fibronectin, ROS and nitric oxide(3,11). These factors alter the tissue microenvironment as they induce inflammation, attract immune cells to remove senescent cells and induce senescence in neighboring cells in a paracrine manner (12,13)(Figure 1).

This review aims at providing a synopsis on the current literature dealing with cellular and molecular aspects influencing cellular senescence during non-alcoholic fatty liver disease (NAFLD), as well as during its progression to non-alcoholic steatohepatitis(NASH).

2.Senescence in health and disease

2.1:General aspects

Cellular senescence is a complex process that has a dual function, both beneficial and detrimental in human health. Under physiological conditions, senescence eliminates damaged cells and is involved in tissue restoration upon acute stress or injury. The secretion of chemokines and cytokines such as IL-1b, IL8 and MCP-1 through SASP attracts immune cells leading to immunological clearance of the senescent cells (14,15). Consistently, aging and chronic stress induce telomere attrition and excessive SASP, leading to accumulation of senescent cells and insufficient tissue regeneration (14,16). Concurrently, hematopoietic stem cells decrease with age leading in fewer immune cells and a decline in the immune response, provoking a vicious cycle of defective clearance of senescent cells(17). The combination of ineffective regeneration, excessive SASP and inefficient clearance may explain the accumulation of senescent cells in aged organisms, thus increasing the risk for chronic age-related diseases such as dementia, osteoarthritis, metabolic dysregulation and carcinogenesis(16,18).

2.2: Senescence and metabolic dysregulation

Metabolic dysregulation refers to a complex wide range of alterations in glucose and lipids' metabolism, taking place mainly during diabetes and the metabolic syndrome, which can lead to several secondary complications such as NAFLD and cardiometabolic disease(19). Importantly, the expansion of the adipose tissue(AT) and in particular the increase of adipocyte size during obesity leads to upregulation of leptin and downregulation of adiponectin production by the AT(20,21). Besides, the increased adipocyte size leads to hypoxia-induced oversecretion of cytokines and chemokines such as Tumor Necrosis Factor(TNF), IL-6 and MCP-1 by the adipocytes as well as by the inflammatory immune

cells that accumulate in the obese AT(19,22). When reaching the liver, the aforementioned mediators together with the increased levels of free fatty acids and apolipoproteins, observed during metabolic dysregulation, lead to liver injury and development of NAFLD/NASH(20,21).

Metabolic dysregulation is thought to favor cellular senescence in metabolic tissues, such as the AT and the pancreas, further perpetuating a status of metabolic dyshomeostasis of these tissues. For instance, obesity induces excessive ROS production, increased production of cytokines and high expression of SA-β-GAL as well as p53, p16 and p21 in the AT of both mice and humans (23,24). Along this line, p53 ablation in mice ameliorated insulin resistance under obese conditions (24). Consistently, high expression of p14 has been reported in subcutaneous AT from diabetic individuals, having also a positive correlation with p21 in the same tissue(25). Consistently, SA-β-GAL was more abundant in preadipocytes and endothelial cells isolated from obese rats and humans as compared to that of lean ones(26). Senescence-related phenomena are also implicated in pancreatic islet dysfunction during obesity. Deletion of p27 in Irs2-deficient or db/db diabetic mice improved hyperglycaemia by inducing compensatory insulin production due to improved maintenance of their islet mass (27). Besides, senescent endothelial cells express $p16^{INK4a}$ and SA-β-GAL at atherosclerotic plaques and gene polymorphisms in p21 affect the risk of development coronary artery disease and myocardial infraction (28,29).

3.The role of senescence in NAFLD/NASH

NAFLD is one of the most common chronic liver diseases affecting approximately 25% of the population worldwide and its prevalence increases along with aging, obesity and diabetes (30). Its diagnosis depends on clinical and histological criteria which include triglyceride accumulation in hepatocytes, defined as steatosis, in individuals that do not consume

excessive amounts of alcohol. NAFLD frequently progresses to NASH which is characterized by the presence of steatosis, inflammation, necrosis and fibrosis (30,31). Advanced fibrosis observed in some NASH patients may lead to cirrhosis in 10-15% of these patients or even to hepatocellular carcinoma(HCC) (30). Currently there is increasing interest in the association between NAFLD/NASH and senescence.

3.1:Evidence from animal studies

Animal studies have shown a relation between senescence and steatosis. Zhang et.al. investigated whether the p21 and p16 senescence-associated pathways were involved in NAFLD pathogenesis, by studying rats on a high-fat diet(HFD). Upon categorizing the rats into obesity-prone and obesity-resistant ones, they observed that the former, which developed more severe steatosis, displayed increased hepatic mRNA levels of p16 and p21 and decreased p53 and phosphorylated-Rb as compared to the latter(10). The acetylation levels of histones H3 and H4 were increased, the trimethylation of H3-Lys-27 was reduced at the p21-promoter and the dimethylation levels of the H3-Lys-4 of the p21-coding region was higher in livers of obesity-prone rats compared to obesity-resistant rats(10). Likewise, Ogrodnik et al., in order to establish a relationship between hepatic fat deposition and hepatocyte senescence tested three different dietary regimens in C57BL/6 mice whereby one group was fed ad-libetum(AL), the other received restricted-diet(RD) and the third group, which consisted of half AL and half RD animals, was subjected to a dietary crossover at nine months, when the regimen switched for the following three months prior to sacrifice(32). As expected, long-term AL-feeding induced fatty liver development and presented several markers of senescence in the hepatocytes, including increased senescence-associated damage foci, as determined by the presence of γH2AX, increased senescence-associated distention of satellites and larger nuclear areas(32). Aiming to identify the relation between senescence and NAFLD, two different experimental strategies were implemented; p16-expressing

senescent cells were genetically eliminated by utilizing the INK-ATTAC mice, while a senolytic drug cocktail was administered into db/db mice. In both cases a reduction in the number of senescent cells was accompanied by ameliorated hepatic lipid accumulation(32). Finally, analysis of human liver biopsies from NAFLD patients, further confirmed that the senescence markers telomere-associated damage foci and p21 are related to the severity of the disease(32).

Several senescence-related proteins have been shown to be associated with the development of NAFLD and its progression to NASH. For instance, Daugherity et al. showed that ATM increases in HFD-fed mice and obese ATM-deficient mice were characterized by the presence of fewer apoptotic hepatocytes and finally less hepatic fibrosis than the wild type(WT) mice(33). Nevertheless, no difference was observed in liver steatosis between WT and ATM-deficient mice(33). In contrast to the aforementioned improved liver phenotype observed during ATM-deficiency, in another study, HFD-fed ATM-deficient mice were more insulin resistant and were presented with lipodystrophy, predominantly in the subcutaneous AT. This was attributed to impaired adipocyte differentiation under ATM-deficiency due to defective induction of transcriptional factors such as C/EBPa and Peroxisome proliferator-activated receptor gamma(PPAR-γ)(34). Likewise, Schneider et.al. observed glucose dyshomeostasis and acceleration of atherosclerosis in ATM+/- mice compared to ATM+/+ mice, both on a ApoE-/- background, accompanied by deteriorated hepatic insulin signaling(35).

The senescence marker protein 30(SMP30), an antioxidant protein that protects against apoptosis and decreases with aging, is also related to the development of NAFLD(36,37). Both SMP-30-knockout mice on a $Lepr^{db/db}$ background as well as SMP30/Superoxide dismutase-1 double-knockout mice developed hepatic steatosis and presented higher levels of hepatic oxidative stress and superoxide anion radicals compared to WT mice. These findings

were attributed to decreased levels of hepatic Apolipoprotein B, and transcription factors levels involved in lipid metabolism (36,37). Similarly, NASH induction by methionine and choline-deficient diet into p53-deficient male mice resulted in slower disease progression accompanied by lower ROS accumulation and lipid peroxidation as well as fewer apoptotic cells compared to the WT mice(38). Interestingly, experiments performed with primary cultured hepatocytes showed that Transforming Growth Factor-beta(TGF-β) induces ROS production resulting in apoptosis and steatosis, while p53-deficiency decreases its levels(38,39). Likewise, examination of liver biopsies from NAFLD patients showed increased p53 levels compared to controls(38). These results indicate that proteins involved in senescence are overexpressed in NAFLD.

Several lines of evidence suggest that senescence is associated with important changes in DNA methylation affecting also genes related to lipid metabolism(40,41). To investigate this hypothesis, Tryndyak et al. induced NAFLD via a choline- and folate-deficient diet into two different mouse strains, namely the A/J and WSB/EiJ mice, which are known to have different susceptibility to the development of the disease. Indeed, the more severe steatosis, observed in the WSB/EiJ mice, as compared to the A/J mice, was accompanied by different DNA and histone methylation profiles of NAFLD-related genes and genes related to hepatic lipid accumulation and subsequent alteration in their expression (42). These changes suggest that alterations in the epigenetic profile of hepatocytes could determine the severity of NAFLD.

Aging itself, induces cellular senescence leading to development and disease progression of NAFLD through several pathways(1,4,43). Age-related mitochondrial dysfunction and elevated oxidative stress trigger fatty liver disease in aged mice on a HFD(44). Upregulation of the cyclin-dependent kinase-4(cdk4) with aging induces phosphorylation of CCAAT-

enhancer-binding protein(C/EBPα) and formation of C/EBPa-p300 complexes leading to steatosis, while pharmacological inhibition of cdk4 reduces hepatic lipid accumulation (45,46).

Although the abovementioned studies imply that senescence is variously involved in NAFLD pathogenesis and progression, it is of interest whether data from animal studies can be translated into the human system, in order to design therapeutic strategies in the future.

3.2: The involvement of senescence in human NAFLD/NASH

Data from human clinical studies further support the hypothesis that senescence is associated with NAFLD. Telomeres were found to be shorter and nuclear area was lower in liver biopsies from NAFLD patients, as compared to controls, although the latter increased with age in patients with NAFLD (47). DNA damage, indicated by γH2AX expression, was higher in patients with NAFLD accompanied by increased p21 expression, indicating a cell cycle arrest at the G1/S phase(47). Interestingly, both p21 and nuclear area were correlated to the fibrosis stage(47). Another study between NAFLD patients and healthy individuals, demonstrated that the relative nuclear size of hepatocytes in NAFLD patients was significantly larger than the predicted normal value of healthy population, while no difference in telomere length was observed between the two populations(48). Nevertheless, the telomere length negatively correlated to nuclear size both in NAFLD patients and healthy controls, while the increment of nuclear size correlated with age only in healthy individuals, suggesting that in NAFLD the nuclear enlargement proceeds independently of age(48). Consistently, Ping et.al. investigated the validity of telomere length as predictive factor of NAFLD development in T2DM patients, who participated in a 6-year cohort study. Patients who developed NAFLD within this period had shorter telomeres in peripheral blood leukocytes at the end of the follow-up period compared to the patients who did not develop steatosis(49). Similarly, Laish et al. showed that NAFLD patients had shorter telomeres in

their peripheral lymphocytes and higher expression of telomerase reverse transcriptase mRNA as compared to healthy controls(50). These findings support the role of telomere dysfunction and senescence in general in NAFLD.

Accordingly, another study observed that SNP-related variants of the CDKN1A gene, encoding the p21 protein, may be related to the progress of NAFLD. Specifically, genotyping of lymphocyte DNA, collected in parallel to a hepatic biopsy of NAFLD patients, showed that the SNP rs762623 seems to influence the development of fibrosis in NAFLD, but does not affect the progression once fibrosis has started (51). This suggests that the initiation and progression of fibrosis in NAFLD may have different underlying pathophysiology and highlights how genetic variations of senescence-related genes might play a role in the development and progression of the disease. Additionaly, Park etal. demonstrated a significant reduction of hepatic SMP30-protein levels in a stage-dependent manner in NAFLD patients, as evaluated by their NAFLD-activity score, while the levels of SMP30 were inversely correlated to the extent of fibrosis(52).

In addition, genomic instability, featured by the cellular presence of micronuclei(MN), nucleoplasmic bridges(NPBs) and nuclear buds(NBUDs), is considered a form of DNA damage and thus is indicative for cellular senescence. All these three indexes were found to be upregulated in NAFLD patients as compared to control individuals(53).

DNA methylation is another senescence marker which seems to be associated with NAFLD including its progression to NASH(54-57). By performing an epigenome-wide association study of DNA methylation in whole blood for γ-glutamyltransferase, alanine transaminase and aspartate transaminase levels, Nano et.al. showed that methylation of SLC7A11 was associated with reduced risk for hepatic steatosis(56). By determining SLC7A11 gene expression in nine hepatoma cell lines they found that in the HepaRG cell line, SLC7A11 had the highest expression and this was associated to high expression of lipid-related genes (56).

Another gene the methylation of which is considered to be associated with the progression of NAFLD into NASH is that of PPAR-γ as its promoter was found to be hyper-methylated in both liver tissue and circulating DNA of NAFLD patients, while its altered methylation profile in circulating DNA could predict the emergence of fibrosis and thus the severity of the disease (54).

Likewise, Murphy et al. showed that different methylation patterns distinguished patients with mild from advanced NAFLD, with the latter featured by more hypo-methylated genes in their biopsies(55). Further analysis revealed that the tissue repair genes were overexpressed, consistent with methylation and expression being inversely related, while the metabolism associated-genes were hyper-methylated and as a consequence down-regulated in advanced NAFLD(55). Another study identified two different methylated region networks related to NAFLD progression and aging. The first was associated to downregulation of genes related to transcriptional regulation and cell proliferation, while the second to upregulation of genes associated to lipid metabolism(58). A study performing methylation analysis of six tumor suppressor genes and immunohistochemical analysis of oxidative DNA damage in biopsies from NAFLD patients, found that the levels of oxidative DNA damage were closely related to disease progression and to the DNA methylation of tumor suppressor genes (57). The latter likely implies that oxidative DNA damage is an instigator for carcinogenesis-related alterations in DNA methylation during NAFLD/NASH.

Of note, several studies have suggested that DNA methylation signatures can be used as a marker of biological age(40,59). Loomba et.al. recently studied the peripheral blood DNA methylation signatures in biopsy-proven NASH patients and used this marker to assess age acceleration in these patients and compare it to that of healthy controls. They showed that patients with stage 3 fibrosis, scored according to the NASH CRN-classification, had increased age acceleration, which further correlated to the hepatic collagen content(41). A

genome-wide methylation analysis highlighted differently methylated CpG islands which were related to alteration in the expression of a plethora of developmental pathways(41). These data indicate that steatohepatitis may induce altered methylation profiles in a plethora of cells, apart from the hepatocytes, even in peripheral blood cells.

3.3: Senescence and NASH to HCC progression

Hepatocyte senescence is thought to act as a protective mechanism against the development of hepatocellular carcinoma(HCC). Indeed, Xue et.al. showed that restoration of p53 expression in p53-deficient murine liver tumors resulted in cellular senescence and tumor regression into undetectable levels(60). Importantly, the secretion of chemokines and cytokines by senescent cells led to immune cell recruitment and subsequent immunological clearance, wherein several cellular players of the innate immunity, including macrophages and neutrophils, were shown to participate(60). Similarly, transduction of transposable elements expressing the Nras-oncogene into pre-malignant murine hepatocytes induced their senescence and triggered their clearance by monocytes/macrophages in a CD4+ T-cell-dependent manner(61). Of note, the presence of DNA in the cytoplasm, especially under tumorigenic conditions, may act as a senescence-initiating signal and the process depends on a cytoplasmic DNA sensing pathway, composed by the nucleotidyl transferase cGAMP synthase(cGAS) and the adaptor protein 'Stimulator of Interferon Genes'(STING) that is finally essential for the induction of senescence and SASP(62). More precisely, interaction of cGAS with cytoplasmic DNA triggers the formation of cyclic GMP-AMP(cGAMP) from GTP and ATP that in turn activates STING and thereby senescence(63,64). Indeed, under Ras-oncogene-mediated tumorigenic conditions, STING-deficient mice displayed reduced immune cell accumulation and clearance of senescent cells in the liver, leading to increased tumor growth(63).

Intriguingly, although NAFLD/NASH is characterized by increased senescence, the disease occasionally evolves to HCC. Unlike other chronic liver diseases, like viral or alcoholic hepatitis, this may happen even in the absence of cirrhosis(65,66). Indeed, Ertle et.al. demonstrated that nearly half of the NAFLD/NASH-induced HCC patients had no evidence of cirrhosis(65), implying that other mechanisms taking place under NAFLD/NASH may play a role in this process. For instance, obesity was previously linked with hepatic stellate cells'(HSCs) senescence and SASP which promote carcinogenesis(67). Depletion of senescent HSCs or ablation of a SASP inducer in mice, namely IL-1β, prevented HCC development(67). Yoshimoto et.al. related these findings to an increase in gut microbiota population which produces the DNA damaging metabolite, deoxycholic acid(DCA), promoting SASP in HSCs(67). Moreover, in human NASH-related HCC, cancer-associated fibroblasts and non-tumoral HSCs demonstrated increased expression of senescence- and SASP-markers compared to those deriving from conventional HCC(68). Further mechanistic studies are needed to elucidate the role of senescence in NASH-associated HCC.

3.4:NASH resolution and the role of senescence

So far, little knowledge exists about the mechanisms involved in NASH resolution and the requisite tissue restoration, while the majority of studies are focusing on the resolution of NASH-related intratissular inflammation(69). Indeed, during resolution of chronic liver damage, independently of etiology, the removal of the toxic factor causing the injury, provokes a shift of the previously inflammatory hepatic microenvironment into more restorative(69). Dendritic cells, NK cells as well as $CD11b^{hi}F4/80^{int}Ly6C^{lo}$ restorative macrophages are of cardinal importance in this process by secreting fibrolytic MMPs and by provoking senescence or apoptosis of activated HSCs in a paracrine-dependent manner, leading thus to degradation and recession of the excessive extracellular matrix(69,70).

Importantly, senescence of HSCs is thought to be indispensable for the attenuation of hepatic fibrosis and thus NASH resolution. Notably, p53- and p53/INK4a/ARF-deficient mice, as well as mice with HSC-specific p53-ablation treated with CCl4 developed more severe fibrosis than the WT animals, attributed to the presence of activated rather than senescent HSCs(71). Likewise, senescent HSCs are characterized by downregulation of the expression of genes associated with cell cycle progression and extracellular matrix production and upregulation of those encoding SASP components, including fibrolytic MMPs and cytokines, favoring their immune clearance and thus fibrosis resolution(71). Moreover, overexpression or treatment with the SASP component IL-22 was shown to drive HSCs to senescence in vivo and in vitro leading to reduced hepatic fibrosis(72). Similarly, Nishizawa etal. showed that administration of human recombinant Insulin Growth Factor-I(IGF-I) attenuates steatosis, inflammation and fibrosis in mice treated to develop NASH or cirrhosis and drives HSCs to senescence. Importantly, these beneficial effects of IGF-I administration were not observed in p53-deficient mice(73).

Additionally, senescence may influence NASH resolution by affecting the liver regeneration process that is needful for the reformation of the tissue. Importantly, the age- or/and steatosis-associated accumulation of senescent hepatocytes is linked to impaired regenerative capacity of the liver(74,75). Of interest, a spatiotemporal interrelation between SASP and liver regeneration may be of decisive importance during NASH resolution. Indeed, a recent study by Ritschka et al. showed that transient exposure of mouse keratinocytes to SASP led to increased regenerative capacity of those cells, while induction of senescence in single cells in mouse livers resulted to increased stem cell markers in the surrounding tissue. Oppositely, prolonged exposure of keratinocytes to SASP provoked maintenance of the cells to a senescent status, implying overall that SASP acts as a double-edged sword for tissue regeneration in a time-dependent manner(76). In addition, chronic activation of HSCs due to

ageing or NASH and their subsequent chemokine and ROS production leads to decreased activation and proliferation of Liver Progenitor Cells, which are required for liver regeneration following hepatic injury(77). Overall, further studies are essential to reveal the role of senescence in liver regeneration during NASH resolution.

4. Conclusion and future perspectives

Steatotic hepatocytes often display severe DNA damage and express markers of cell cycle arrest, indicating that they have entered a senescent state and implying that senescence is involved in NAFLD/NASH pathogenesis(figure 2)(32,47). Taken that hepatic senescence is causally induced by HFD and aging, both well-established risk factors for NAFLD development, it could be considered as a secondary phenomenon during NAFLD emergence and progression. Nevertheless, depletion of senescent cells in vivo attenuated steatosis, while senescence induction in vitro and in vivo promoted hepatocyte lipid deposition, suggesting that senescence plays indeed an important role in NAFLD pathogenesis(32). Notably, a vicious cycle of cytokine-induced senescence during NAFLD cannot be excluded(78). However, intriguingly, NAFLD-associated senescence may also have beneficial impacts on NAFLD to NASH progression. For instance, senescent HSCs produce less extracellular matrix components and more MMPs, thereby alleviating fibrosis advancement(71). Concurrently, hepatocyte senescence is required for damaged, pre-cancerous cells' detection and clearance, preventing thus liver carcinogenesis(60).

As until now, the golden standard for NAFLD diagnosis and monitoring is the invasive liver biopsy. Thus, current research efforts are focusing on whether senescence markers measured in serum or plasma, defined as 'liquid biopsy', may reflect disease severity and progression. These include altered expression or methylation status of senescence-related genes, metabolomic analyses, markers of oxidative stress or other molecular indicators of

senescence, such as telomere length(41,51,79). Further studies should be conducted in order to prove their potential role as easily accessible biomarkers.

Concurrently, no approved drug exists so far for the treatment of NAFLD/NASH and the current management of the disease predominantly focuses on adequate control of the metabolic profile of the patient putting exercise and diet in the first line for disease prevention and therapy. In NAFLD patients, clinical trials show that long-term diet and exercise reduce hepatic steatosis(80), while in aged obese patients even short-term intervention attenuates hepatic lipid accumulation and improves overall patients' metabolic status(81). A recent retrospective cohort study revealed that in non-obese middle aged individuals, loss of skeletal mass and increase of fat mass with aging is associated with NAFLD development(82). Exercise may be of therapeutic value for these patients as well.

Meanwhile, novel senolytic drugs have already been developed and animal studies currently under way may shed more light on their efficacy. The kinase inhibitors dasatinib and quercetin induce apoptosis in senescent cells in vitro and in vivo(32,83). Administration of these agents in mice with fatty liver eliminated hepatic senescent cells and decreased lipid accumulation (32). SASP may serve as a potential target for senolytic agents. The targeting of signaling pathways such as the Janus kinase–signal transducer and activator of transcription (JAK-STAT) pathway may limit SASP effect (84). Likewise, IL-22 administration in mice limited liver fibrosis and led mouse HSCs to senescence(72). In parallel, restoring telomerase activity by inserting the telomerase gene in senescent murine hepatocytes improved liver function(85). This may constitute a form of gene therapy for humans in the future and a topic for future research.

Whereas, at first glance, senolytic agents may seem as a propitious therapeutic opportunity for several chronic senescence-related diseases, we should bear in mind that cellular senescence is a normal and sometimes beneficial process. Potential side effects such as

rampant cell proliferation and tumorigenesis may prohibit their applicability to human patients. Thus, the therapeutic potential of senescence remains elusive and although senescence has a vast clinical significance, more studies should be conducted in order to clarify its role not only in NAFLD but also in most clinical medical specialties.

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Figure Legends

Figure 1: Molecular aspects of cellular senescence

Aging, oncogene expression, reactive oxygen species accumulation (ROS), radiation and chemotherapy cause DNA damage, telomere shortening and mitochondrial dysfunction, inducing thus the DNA Damage Response (DDR). DDR activates ataxia-telangiectasia mutated (ATM) and Radd3-related (ATR) protein kinases, leading to p53 phosphorylation. p53 activates p21, leading to cell cycle arrest. p21 and p16 inhibit the phosphorylation of the retinoblastoma factor (Rb), allowing it to bind to the E2F transcription factor and stop the progression of cell cycle. Senescent cells appear larger and more flattened; they express the senescence biomarker senescence-associated β galactosidase (SA-β-GAL) and are characterized by the presence of senescence-associated heterochromatin foci (SAHF) and altered DNA methylation signatures. The senescence associated secretory phenotype (SASP) is a complex mixture of pro-inflammatory cytokines, chemokines, growth factors, proteases, fibronectin, ROS and ions such as nitric oxide, secreted by senescent cells. Altogether these factors induce inflammation, attract immune cells to remove senescent cells and at the same time induce senescence in the neighboring cells in a paracrine manner.

Figure 2: Senescence in NAFLD/NASH

Aging and high fat diet (HFD) induce steatosis and senescence in normal hepatocytes via the p53-p21 and the p16-Rb pathway. Hepatocytes of NAFLD patients display senescence markers such as increased expression of γH2AX, short telomeres and altered DNA methylation patterns. These senescent cells secrete interleukin-1b (IL-1b), IL-6, chemokines, and ROS [senescence associated secretory phenotype (SASP) components] leading both to reinforcement of the senescent pathway in the neighboring cells and to disease progression to NASH. NASH hepatocytes also express senescent markers. At the same time, aging and HFD as well as senescent hepatocytes induce inflammation by interleukins' and TNF secretion, macrophage activation and senescence of the lymphocytes. Overall, these factors induce steatosis to normal hepatocytes, while favoring further progression of NAFLD to NASH.