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Novel targets for delaying aging: The importance of the liver and advances in drug delivery

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ABSTRACT

Age-related changes in liver function have a significant impact on systemic aging and susceptibility to age-related diseases. Nutrient sensing pathways have emerged as important targets for the development of drugs that delay aging and the onset age-related diseases. This supports a central role for the hepatic regulation of metabolism in the association between nutrition and aging. Recently, a role for liver sinusoidal endothelial cells (LSECs) in the relationship between aging and metabolism has also been proposed. Age-related loss of fenestrations within LSECs impairs the transfer of substrates (such as lipoproteins and insulin) between sinusoidal blood and hepatocytes, resulting in post-prandial hyperlipidemia and insulin resistance. Targeted drug delivery methods such as nanoparticles and quantum dots will facilitate the direct delivery of drugs that regulate fenestrations in LSECs, providing an innovative approach to ameliorating age-related diseases and increasing healthspan.

1. Introduction

Advances in the treatment and prevention of infectious diseases, together with increased standards of living have led to a rapid increase in life expectancy over the last century [1]. However, this has driven a significant increase in the number of older people with multimorbidity and disability that impair quality of life and productivity [2]. Therefore, research in aging biology largely focusses on delaying aging in order to increase healthspan, the period of life spent free from, or with limited, disease burden [3,4].

2. Aging and the liver

Aging is usually considered to be a process of gradual physiological deterioration experienced over time by most living things, leading to increased risk of disease and death. Aging is a heterogeneous and heterochronic process that can be difficult to evaluate because there are different rates of aging between species and within individuals of the same species, as well as between tissues in the same organism [5]. This heterogeneity has in part prevented the identification of a single marker and/or cause of aging, however there are several biological processes that have been identified as modulators of the aging process [5]. Described as the "hallmarks of aging", these processes include: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication [6].

One method of evaluating the rate of aging that encompasses many of its manifestations is the "transcriptome age" [7]. Age-related changes in the "transcriptome" of whole blood correlate better than chronological age with risk factors for cardiometabolic disease and death including cholesterol levels, body mass index, blood pressure and fasting glucose [8,9]. Studies such as these highlight the conver-

Abbreviations: AMPK, AMP-activated protein kinase; Cd, cadmium; CR, caloric restriction; FOXO, forkhead box O transcription factor; FSA, formaldehyde treated BSA/HAS; GH, growth hormone; HA, hyaluronan; HOMA-IR, homeostatic model of assessment on insulin resistance; IGF-1, insulin-like growth factor 1; IRS1, insulin receptor subunit 1; ISS, insulin/IGF-1 signaling; LKB1, liver kinase B1; LSEC, liver sinusoidal endothelial cell; mTOR, mechanistic target of rapamycin; MEND, multifunctional envelope-like nanocapsule device; NAD^+, nicotinamide adenine dinucleotide; NAM, nicotinamide; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; PEG, polyethylene glycol; PINP, pro-collagen I N-terminal peptides; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; QDs, quantum dots; S, sulphide; SIRT1, sirtuin 1; TRAIL, TNF-related apoptosis inducing ligand; Te, telluride.

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gence of many aging mechanisms with cardiometabolic dysfunction [10]. This importance of metabolic pathways in aging biology is evidenced by the fact that the most robust intervention that delays aging is the nutritional intervention called "caloric restriction" (CR). First described in 1935 by Clive McKay et al. [11], CR is usually defined as a reduction in total calorie intake by 20-40% with micronutrient supplementation to prevent specific malnutrition disorders. Lifelong CR increases mean and maximal lifespan by 30-50% in rodents, dogs, flies, yeast, worms, monkeys and prokaryotes [2]. There is also limited evidence in humans that CR influences life expectancy and reduces the development of diseases [12] as well as the aging process itself [13]. Mice that are subjected to CR are leaner, more insulin sensitive, glucose tolerant and have reduced incidence of disease [29]. The effects of CR on aging are conserved across a range of species, indicating that there are fundamental cellular pathways linking nutrition with aging and health. Research in this area has led to the identification of several key nutrient sensing pathways that regulate aging (insulin/IGF1/GH signaling, SIRT1, AMPK and mTOR) [2], which in vertebrates are most strategically located in the liver, because it is the master regulator organ of systemic metabolism.

The liver regulates most metabolic pathways including glucose metabolism via glycolysis, glucose uptake and gluconeogenesis [14]; the formation and packaging of cholesterol and other lipids; and insulin sensitivity (Table 1) [15,16]. Ingested substances that are absorbed from the gut travel to the liver via the portal vein [17], which also contains pancreatic hormones including insulin, glucagon and C-peptide [18,19]. The hepatic microvasculature is highly specialized to optimize the bidirectional exchange of substrates between blood and hepatocytes. The endothelial cells that line this sinusoidal microvasculature (the "hepatic sinusoids") are called liver sinusoidal endothelial cells (LSEC) and are perforated with pores called "fenestrations" that facilitate size-selective sieving of blood-borne molecules for uptake by hepatocytes [20,21]. The term "pseudocapillarization" refers to age-related changes in the LSEC including a reduction in the size and number of fenestrations, which leads to impaired uptake of some substrates. Old age is associated with reduction in liver metabolism due to three main factors: reduced metabolic capacity due to diminished liver size and/or enzyme activity; decreased liver blood flow; and reduced transfer of metabolites and molecules from the sinusoids to the hepatocytes [22,23]. Together, these changes impact many facets of hepatic metabolism of substrates such as lipoproteins, drugs and insulin [24–26] thus potentially contributing to insulin resistance, vascular disease and adverse drug reactions [7,27,28].

Here we discuss these two hepatic drug targets – nutrient sensing pathway in hepatocytes; and fenestrations in LSECs – that might impact on systemic and hepatic aging. We also discuss the possibility of using nanomedicines to precisely deliver fenestration-active agents directly to the LSECs.

Table 1
Aging and hepatic metabolism, effects of caloric restriction and drug treatments

Summary of age-related changes in the liver and treatments with caloric restriction and drugs that delays the aging process	Reference
Increased protein synthesis and burden of misfolded proteins producing oxidative stress and damage occur in the liver proteome of aging mice. This burden is reduced following 40% CR or rapamycin	[189]
Increased lipogenesis gene expression, cholesterol synthesis and lipid storage are observed in the aging mice liver. CR promotes the reprogramming of mice liver lipidome	[190–192]
Decreased hepatic autophagy has been demonstrated in multiple species with age, impairment leads to accumulation intracellular oxidative proteins, lipid droplets, lipofuscin and impaired mitochondrial function. Hepatic amino acid, glucose and free fatty acid metabolism are impaired. Acceleration of hepatic related diseases. Resveratrol (200 mg/kg) and 30% CR have been shown to promote SIRT1 mediated autophagy and improve oxidative protein stress.	[83,193,194]
Insulin signalling and sensitivity is reduced in aging rats and mediated via visceral fat content. Treatment with resveratrol, Metformin and CR promote increased insulin sensitivity in aged rats	[65,195–198]
Hepatic glycolysis, glycogenesis and glucose uptake are all decreased with aging. FOXO genetic manipulation demonstrates its role in hepatic gene regulation of gluconeogenesis, glycolysis and lipogenesis. SIRT1 and PGC-1α activation promote increased gluconeogenesis and reduced glycolysis in the liver	[65,96,196,199,200]
SIRT1 and mitochondrial biogenesis are reduced in aging mice; SIRT1-mediated HIF-1α and PGC-1α activity regulate mitochondrial biogenesis. CR and resveratrol treatments promote improved mitochondrial function in aging mice	[44,98,178]
Aging mice livers demonstrate increased inflammation, cellular stress, and fibrosis, with decreased apoptosis, xenobiotic metabolism, cell-cycling and DNA replication genomic profiles. 40% CR for a month was shown to improve these profiles in mice.	[96]

3. Targeting the nutrient sensing pathways

Nutrient sensing pathways regulate adaptive cellular responses to changing nutrient availability such as famine or food excess. During times of nutrient scarcity, these pathways promote the survival and resilience of an organism at the cost of reduced reproductive capacity. Presumably these pathways have evolved to promote reproduction only when there are sufficient nutrients to ensure the survival of offspring. In multicellular organisms nutrient sensing pathways form complex networks that detect, organize, use, reconstitute and metabolize nutrients [29]. The downstream physiological consequences of activating or antagonizing these pathways (for example by CR, or drugs that are thought to mimic CR) are shown in Table 1 [40]. Manipulation of these pathways through CR or genetic interventions has been shown to alter the biological process of aging, median and maximal lifespans and the incidence of many age-related diseases. The four canonical nutrient sensing pathways interact with each other and share many downstream targets that regulate cellular processes that are critical for aging including mitochondrial biogenesis, cellular metabolism, autophagy, DNA repair and expression, and translation (Figure 1).

With increasing age, there are changes in the expression and activity of the four nutrient sensing pathways: insulin/IGF-1, mTOR, AMPK, and the Sirtuin/NAD^+ pathways [16,30,31]. Within the key organs involved in homeostatic metabolic regulation – liver, pancreas, muscle and fat – these changes are associated with other aging processes such as oxidative stress [32] and inflammation [29,33,34]. Together these processes in these organs contribute to impaired systemic metabolism, which in turn is linked mechanistically with diabetes, cardiovascular disease, cancers and neurodegeneration [5,31,33,35]. Although the major interventions used are CR and genetic overexpression or knockout models [36,37], recently pharmacological manipulation of these pathways with agents such as metformin, rapamycin and resveratrol, has been shown to delay the aging process, at least in laboratory animal models [38–40].

Therapeutic agents recapitulate the effects of CR on aging have been termed "CR-mimetics" [41] which primarily target individual components of the nutrient sensing pathways. Although these drugs

have been used to treat individual diseases in the past, it is possible that their beneficial effect is in part secondary to delay the effect of aging on these diseases. Moreover, in some studies in animal models, CR mimetics have been found to increase median and maximal lifespan, probably by delaying the onset of aging [16,42,43].

The main features of these nutrient sensing pathways are as follows:

3.1. Mechanistic target of rapamycin (mTOR)

mTOR is a serine/threonine protein kinase that regulates cell growth, proliferation, motility, protein synthesis and autophagy. mTOR integrates input from various pathways, including insulin and IGF-1, and responds to dietary protein, particularly the branched-chain amino acids. mTOR exists in two different complexes mTORC1 and mTORC2, which are differentiated by their accessory proteins, Raptor and Rictor, and only mTORC1 is sensitive to amino acids [44]. Rapamycin is a potent inhibitor of mTOR that was initially utilized as an immunosuppressive drug for patients following renal transplants. It has now been associated with increased lifespan in mice fed standard diets [45,46] and human studies into the effects of rapamycin on healthy elderly patients are currently being performed [30,47].

3.2. 5' Adenosine monophosphate-activated protein kinase (AMPK)

AMPK regulates cellular uptake of glucose, β-oxidation of fatty acids, the glucose transporter 4 (GLUT4), and mitochondrial biogenesis. AMPK is activated in response to increased cellular AMP:ATP ratio. CR is associated with increased AMPK activity as a consequence of reduced energy intake. Metformin enhances lifespan in mice and this is accompanied by an increase in AMPK activity [39]. In humans, metformin has been shown to reduce the progression of diabetic impairment of glucose tolerance by 31%, reduce atherosclerosis development [48–50] and a systematic review has demonstrated improved cardiovascular mortality by 25% [51,52]. Additionally a recent systematic review found that metformin promotes a significantly lower mortality in diabetic patients compared to non-diabetics and also diabetics that were not on metformin [53]. Human observational studies have concluded that metformin decreases the risk of the most common diseases of aging: cardiovascular disease [54–56], cancer [57], depression [58], frailty [54], mild cognitive impairment [59] and dementia [60,61]. Clinical trials in humans to evaluate its effects on disease susceptibility and aging biology (included transcriptome) are planned.

3.3. Sirtuin pathway (SIRT1)

Sirtuins are class III histone deacetylases that require NAD^+ as a cofactor. CR increases cellular NAD^+ as a consequence of reduced energy intake, thereby activating sirtuins. While the sirtuin family consists of seven members [62], in mammals, the key aging homolog is SIRT1 which deacetylates key histone residues involved in the regulation of transcription and multiple non-histone protein targets relevant to aging (p53, FOXO, PGC-1α, NF-κB) [37,63]. A number of pharmacological agents that allosterically activate SIRT1 delay aging (Sirtuin Activating Compounds, known as STACs) including resveratrol and SRT2014 [40,64–67]. STACs promote allosteric activation of SIRT1 and differ in their potency and optimization in the activation of sirtuin [68]. STACs mimic CR, increase mitochondrial function and protect against metabolic and cardiovascular disease progression by reducing metabolic risk factors [66,68]. Notably resveratrol increased lifespan in mice fed a high fat diet [65] but not in mice on standard chow where only health benefits were observed [46,69]. This suggests that activation of the SIRT1 pathway may have its greatest effect on aging where there is high energy intake and greatest inhibition of SIRT activity. In addition, activation of SIRT1 with resveratrol treatment *in vitro* promoted endothelial protection via KLF-2 and MAPK5 [70].

A comprehensive review of human clinical trials, has demonstrated inconsistent findings in older people, with some showing health benefits, particularly in older humans with multimorbidities such as type II diabetes, non-alcoholic fatty liver disease (NAFLD) and coronary artery disease [71–78]. The dose, duration and the source of resveratrol has varied across many of the trials and might account for some of the inconsistencies seen in aging human studies. More recently nicotinamide mononucleotide (NMN), and nicotinamide riboside (NR), biosynthetic NAD^+ metabolites have been investigated for their ability to augment NAD^+ blood levels [79].

3.4. Insulin/IGF-1signaling pathway (ISS)

The insulin/ IGF-1 signaling pathway is continuously modulated by dietary nutrient status: plasma concentrations of protein (IGF-1) and sugars (insulin) and additionally by the levels of circulating growth hormone (GH) [30]. Insulin release is stimulated by glucose and branched-chain amino acids. GH stimulates hepatic production of IGF-1 and acts on the IIS pathway to modulate insulin sensitivity [80]. Lower levels of insulin, IGF-1 and GH induced by either genetic variability or low energy diets are associated with increased lifespan across taxa and including humans [36].

3.5. Recent additions to the classic pathways

Autophagy is an essential cellular process that promotes cell survival, especially as a cellular starvation response, and regulates homeostasis. Autophagy is upregulated in the setting of low ATP and insulin, reduced growth factor stimulation and JNK1-Beclin-1 dependent cellular stress [81–84]. It facilitates the degradation of unnecessary and dysfunctional cellular products, liberating cellular stores of energy [29,85]. It has previously been highlighted in a review by Gracia-Sancho, et al. [81] that autophagy has an important role in inhibiting the development of liver diseases such as NAFLD, however hallmarks of NAFLD: hyperglycemia and impaired hepatocellular clearance of lipids impairs autophagy, suggesting that targeting autophagy pathways may be beneficial in the treatment of NAFLD and other fatty liver disorders.

Another recent potential addition to these four classical nutrient-sensing pathways is Fibroblast Growth Factor 21 (FGF21). FGF21 is produced by the liver and is increased in response to acute starvation, with a recent study showing that low protein intake is the major stimulant for its expression and blood levels [86,87]. It regulates several metabolic functions (gluconeogenesis, mitochondrial activity, ketogenesis, lipid metabolism) which impact on age-related health. FGF21 interacts with the other nutrient-sensing pathways by activating AMPK and SIRT1 [88], suggesting a key role for FGF21 in linking nutrition and aging. Paradoxically in humans, higher levels of FGF21 are associated with obesity and diabetes, probably reflecting FGF21 resistance in these conditions. The role of FGF21 in aging and nutrition is now a key focus for aging research [89] .

4. Targeting the liver in aging

As described above, the liver is a key regulator of metabolism, with significant age-related changes in function secondary to reduced liver size and enzyme activity, blood flow and transendothelial transfer [20,22,23,90–93]. This impairs detoxification and clearance of various endobiotics (e.g. lipoproteins and insulin) [94] and xenobiotics (e.g. drugs, neurotoxins and carcinogens) [95]. Many processes contribute to reduced metabolic enzyme activity and cellular damage in the aging liver, including the effects of aging on oxidative stress, inflammation, stress responses [96], autophagy and apoptosis [96,97], and mitochondrial function [98]. The reduction in blood flow to the liver reflects a general down regulation of splanchnic blood flow in all abdominal organs rather than any specific hepatic effects on vascular resistance. The reduction in the transfer of substrates between blood and hepatocytes occurs mainly as a result of the loss of fenestrations associated with pseudocapillarization [2,24,25,94,95,99]. All these aging liver changes will impact on the responsiveness and activity of the nutrient sensing pathways.

Age-related changes in liver cells also contribute to the increasing risk of the development of liver diseases with older age. The accumulation of oxidative stress and age-related changes in inflammation favors the progression towards NAFLD, steatohepatitis and hepatocellular carcinoma [100]. The development of NAFLD involves impairment of LSEC functions through impaired regulation of nitric oxide synthase and loss of fenestrations, with these features developing prior to the rise seen in inflammatory markers [101–103]. This suggests that targeting LSECs may, in addition to promoting healthy aging, be relevant to preventing the development of NAFLD and its progression to steatohepatitis (Fig. 1).

4.1. Liver sinusoidal endothelial cells

LSECs have a highly specialised morphology. They are perforated by numerous nano-holes/pores [104–106], called fenestrations, with diameters in the range 50-200 nm. Moreover, there is no underlying basement membrane in the extracellular space of Disse. LSECs possess highly efficient endocytic capacity, transcellular transportation and immune-mediated pathways [107–111] that also contribute to the transfer of substrates between the blood and space of Disse. LSECs retain blood cells within the sinusoidal lumen while allowing the bidirectional passage through the fenestrations of substances such as dissolved or albumin-bound drugs, proteins, lipoproteins, and small viruses (Fig. 2). Little is known about the dynamic structure and regulation of fenestrations. To date the majority of studies on LSEC fenestrations have utilized electron microscopy and fixed cells or tissue, but new super-resolution optical methods are now available [112–116], which can visualize fenestrations in unfixed and/or live LSEC, and their responses to stimuli over time [112,115]. This will facilitate the discovery and testing of drugs that can influence fenestrations.

LSEC are potent scavengers, belonging to the family of "scavenger endothelial cells" [110], which also includes bone marrow sinusoidal endothelial cells [117,118] and choriocapillaris endothelial cells [119]. LSECs clear soluble waste including hyaluronan [120–123], advanced glycation end-products [109,124,125], immune complexes [126], collagen alpha-chains [127,128], oxLDL [129] or nano-particles (<200nm) such as viruses [111] and quantum dots [130] via clathrin mediated endocytosis, while the liver resident macrophages (Küpffer cells) clear particulate matter larger than 200nm [110]. This division of labour is called the "dual-cell principle of waste clearance", a concept first introduced by Sørensen et al. [110]. With the sinusoidal surface area equal to the size of a tennis court, the liver sinusoid is the most potent scavenging system in the body. Consequently, the LSEC has become the bane of nano-particle and antisense oligonucleotide based therapies that aim to target other tissues [131], but it can be an advantage if the liver sinusoid itself is the target.

4.2. Aging LSECs

LSECs undergo morphological and functional transformations in disease and aging. One key change is the loss of fenestrations, termed "defenestration", often associated with the deposition of a basement membrane [132]. "Capillarization" is the term used to describe the combination of defenestration, basement membrane synthesis and altered expression of endothelial proteins which are seen in various chronic liver diseases including cirrhosis [133], primary biliary cirrhosis [134], chronic hepatitis [108], and viral infections [135]. "Pseudocapillarization" is the term used to describe similar, but distinct age-related changes which occur in the LSEC without the signif-

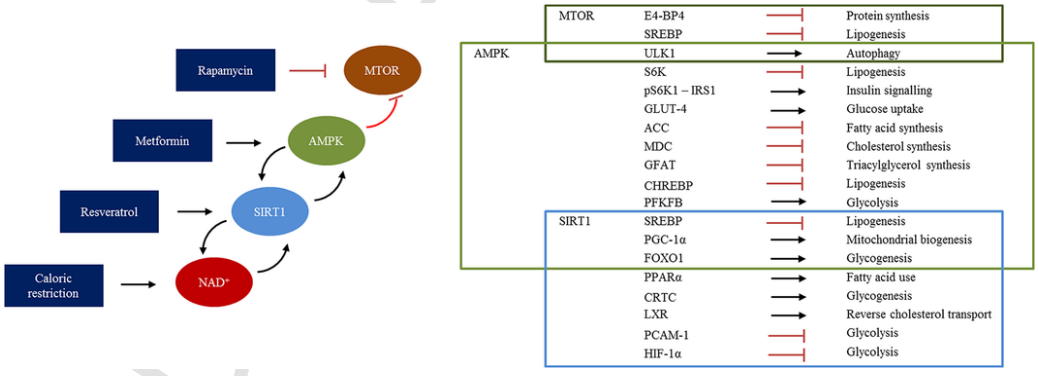

Fig. 1. Drugs that delays the aging process converge on nutrient sensing effectors in the liver. Resveratrol, Metformin, Rapamycin acting on AMPK, mTOR and Sirtuin pathways to regulate ISS and glucose metabolism. All pathways promoted are hepatic. Abbreviations: ACC: acetyl-CoA carboxylase; CHREBP: carbohydrate-responsive element-binding protein; CRTC: creb regulated transcription co-activator 1; E4-BP4: E4 promoter–binding protein 4; FOXO1: forkhead box protein O1; GFAT: glutamine fructose-6-phosphate amidotransferase; GLUT-4: glucose transporter, type 4; HIF-1α: hypoxia induction factor 1α; IRS1: insulin receptor substrate 1; LXR: liver X receptor; MDC: macrophage-derived chemokine; PCAM-1: platelet endothelial cell adhesion molecule 1; PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: PGC-1α: peroxisome proliferator-activated receptor gamma co-activator 1α; PPARα: peroxisome proliferator-activated receptor α; S6K: ribosomal protein S6 kinase; SREBP; sterol regulatory element-binding proteins; ULK1: unc-51 like autophagy activating kinase 1. References: [43,44,68,98,199,201–207]

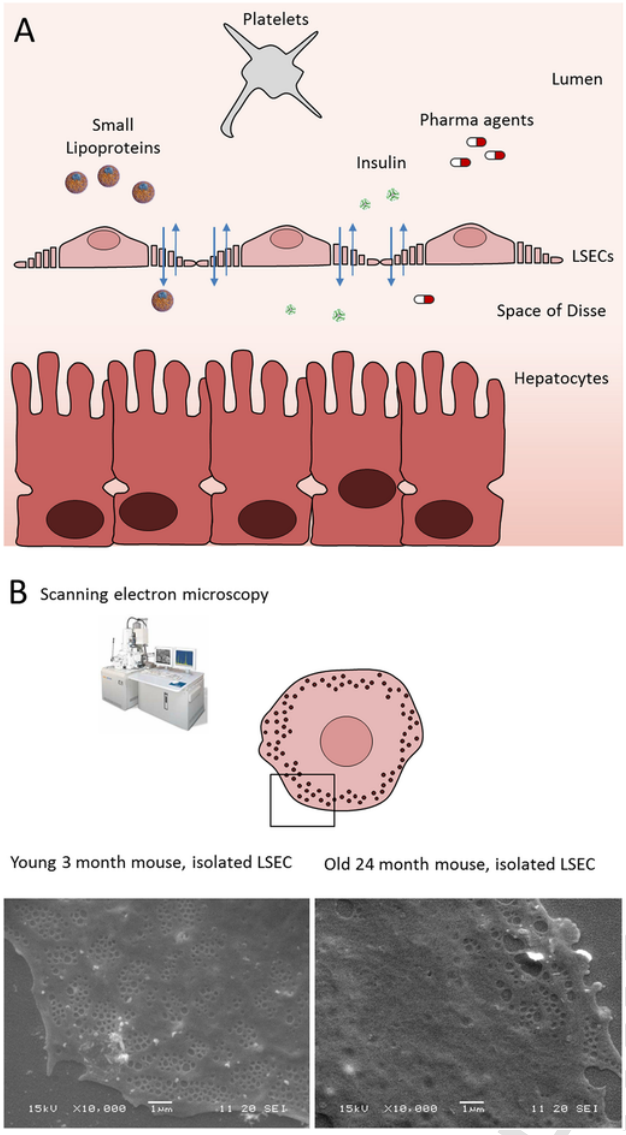

Fig. 2. Loss of filtration and fenestrations in aging mice, schematic of filtration of lipoproteins, insulin and drugs to pass through the blood-liver membrane with scanning electron microscopy images demonstrating defenestration.

icant stellate cell activation or bridging fibrosis seen with chronic liver disease [20,24,25,91,136–140]. Aging is also associated with morphological and/or functional changes with the other cells of the hepatic sinusoid, namely stellate cells and Küpffer cells [21]. LSECs in old age had markedly reduced porosity (% of LSEC surface area perforated by fenestrations) associated with increased cross-sectional thickness of the LSEC in a variety of species from mice to humans. These age-related morphological changes are accompanied by altered expression of many vascular proteins including von Willebrand's factor, ICAM-1, laminin, caveolin-1 and various collagens on LSEC and in the space of Disse [141] but without other features of liver diseases [142]. Thre is reduced scavenging capacity in old mice [143] at the level of endocytosis, as old LSECs were able to degrade endocytosed ligand equally well as young LSECs once it was endocytosed. However, there was no apparent age-related change in the amount of the receptors responsible for endocytosis (stabilin-1 and -2 scavenger receptors), so it was concluded that thickening of LSEC was retarding the transport of internalized ligand to the endo/lysosomal compartment thus slowing the endocytic process – the "traffic jam" hypothesis [143]. Despite the loss of approximately one third of their endocytic capacity, LSEC remain potent scavengers in aging livers.

4.3. Implications of age-related defenestration

Age-related loss of fenestrations has a significant impact on the transfer of substances across the endothelium (Fig. 2). Fenestrations allow passage of smaller lipoproteins including chylomicron remnants, while excluding larger particles such as chylomicrons and platelets. Old age is associated with impaired hepatic clearance of chylomicron remnants and the clinical manifestation of postprandial hypertriglyceridemia. The latter is more closely associated with adverse cardiovascular outcomes in older people than classical dyslipidaemias [136]. Using the multiple indicator dilution method in perfused rat livers, [24] it was shown that the transfer of lipoproteins (average diameter 53nm) across the LSEC was almost totally abolished in livers from old rats. This provides a novel mechanism for age-related dyslipidemia and postprandial hyperlipidaemia [24] and may be a significant contributor to age-related hyperlipidaemia [144] and, hence, vascular disease.

Old age is also associated with insulin resistance and a marked increase in diabetes mellitus. Using multiple indicator dilution methods in perfused livers, [25] it was demonstrated that insulin transfer across LSEC is impaired in old age. Older rats showed a significant reduction in the hepatic volume of insulin distribution, consistent with the restriction of insulin to the vascular space. This was confirmed by whole animal insulin and glucose uptake studies which showed reduced hepatic insulin uptake and concomitant reduced activation of the insulin receptor substrate 1 and insulin pathways in old rats. Measurements of glucose tolerance, HOMA-IR, blood levels of insulin, C-peptide and glucagon showed that the reduced insulin action in the liver was associated with systemic impairment of insulin and glucose metabolism [25]. These findings reveal that fenestrations influence hepatic insulin uptake. Conversely, PDGF-B deficient mice have increased fenestrations which was associated with increased trans-endothelial transport, dramatically lower circulating insulin levels, increased insulin clearance and improved insulin sensitivity [145].

Aging is associated with impaired hepatic metabolism and elimination of many drugs, usually increasing the risk of dose-dependent adverse drug reactions [26]. The reduction in hepatic drug metabolism with aging [146] can in part be explained by changes in the trans-endothelial transport of drugs across the liver sinusoid [147]. For example, hepatic single pass clearance of acetaminophen is reduced in aging rats secondary partly to pseudocapillarization of the LSEC [148,149]. This may explain the age-related decrease in susceptibility to paracetamol-induced hepatoxicity but the increase in nephrotoxicity.

Therefore strategies that maintain fenestration porosity during aging, or increase fenestrations in older people have the potential to improve dyslipidemia and hepatic insulin resistance, thereby providing a novel approach for the treatment and prevention of cardiometabolic risk factors in older people [150].

5. Novel targets to reduce liver aging

In order to discover drug targets that maintain fenestrations into old age, it is necessary to understand the proximate biological processes that regulate fenestrations. The most potent agents for in-

creasing fenestrations are vascular endotheial growth factor (VEGF) and various actin cytoskeleton disruptors [151]. These are mechanistically linked because VEGF acts via its effects on the actin cytoskeleton [152]. A major conceptual advance occurred when 3D-structured illumination super resolution microscopy was used to visualize LSECs [112], revealing the morphological relationship between fenestrations and lipid rafts. It was shown that sieve plates (which are clusters of containing 10-100 fenestrations) were intercalated between thickened areas of membrane identified as lipid rafts [112]. Disruption of lipid rafts and/or actin cytoskeleton increased fenestrations while depletion of non-raft membrane decreased fenestrations. Agents that depleted non-raft membrane prevented actin disruptors from increasing fenestrations, thereby proving that actin disruption increases fenestrations directly by its effects on membrane rafts. VEGF both depleted lipid rafts and increased fenestrations [115] [153]. The results are consistent with a 'sieve-raft' interaction model, where fenestrations form in non-raft regions of endothelial cells once the membrane-stabilizing effects of actin cytoskeleton and membrane rafts are diminished [154]. The sieve-raft model provides a unifying mechanistic pathway for the effects of drugs and other agents that have been reported to increase or decrease fenestrations (Fig. 3).

Prevention of age-related defenestration has been achieved either by using agents that act on nutrient sensing pathways and delay aging more generally; and those that act on specific pathways that appear to regulate fenestrations directly. So far two drugs that delay aging process have been examined and shown to prevent age-related defenestration: resveratrol and metformin.

Resveratrol, which acts on the sirtuin nutrient sensing pathway, has been shown to increase fenestrations in a Werner Syndrome mouse, which is a model for pre-mature aging [155]. Metformin, which acts on the AMPK nutrient sensing pathway, increased fenestration porosity in old mice and improved HOMA-IR and insulin sensitivity [156].

A number of agents that act directly on the pathways associated with the regulation of fenestrations as described above have been tested for their effects on age-related defenestration. The serotonin receptor agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI) promoted increased porosity in young LSECs *in vitro* [157], as well as in old mice *in vivo* [158]. The effects of DOI were shown to be modulated by both VEGF dependent and independent mechanisms. Recent data [159] have demonstrated the utility of multiple pharmaceutical agents in promoting increased fenestration diameter and frequency in isolated LSECs from both young and old mice. These agents all act via the pathways previously discussed in this review: nitric oxide dependent pathways (sildenafil and amlodipine); NAD^+ promotion (nicotinamide mononucleotide); and cell surface death receptor activation (TRAIL) [159].

Lastly, the use of statins has also been shown to promote improvement of autophagy in the setting of liver diseases [53,160–162]. While these drugs have not been specifically tested for this purpose in aging previous findings suggest statin treatment may improve LSEC function through the promotion of KLF-2 similarly to SIRT1 [70] and warrant further investigation.

5.1. Nanoparticles target the LSEC

A fundamental challenge in developing pharmacotherapies is targeting the active agent to the desired cell type or tissue. Fortunately, LSECs have unique properties that can be exploited as a drugable target. The LSEC is the most active and efficient endocytic cell in the body [163] and is densely populated with clathrin coated vesicles and numerous endocytic receptors (e.g. mannose receptors, stabilin receptors, Fc gamma-receptor IIb2 [122,127,164,165]). This endocytic machinery is highly efficient in uptake and degradation of endogenous and exogenous waste material, including all major classes of biological macromolecules. Therefore, it is not surprising that the LSEC has also proven to be target for the uptake of nanoparticles, particularly those with a diameter between 5-20nm. [130,166,167]. Therefore nanoparticles have been proposed as an efficient way of delivering drugs to the LSEC and other liver cells [168,169] (Fig. 3), reducing dosages, off-target effects and adverse drug reactions. Such drug delivery technology would be ideal for therapeutic agents that regulate fenestrations in the LSEC.

5.2. Methods for targeted delivery to LSECs

Several ligands and/or nanoparticles have been investigated for their ability to selectively target the LSEC with a variety of drugs and therapeutic aims.

5.2.1. Specific ligands

Hyaluronic acid (HA) is interesting because the expression of its endocytic receptor, stabilin-2, is restricted to LSECs [170], and the vast majority of HA is cleared by the liver. HA is found within the extracellular matrix and synovial fluids of most human tissues, and HA is known to be biocompatible, degradable and non-toxic [171]. A recent study utilized HA-bound nanoparticles in combination with glycyrrhetinic acid (binds to glycyrrhetinic acid receptors expressed on hepatocytes) to promote targeting within the liver [172]. HA-drug conjugation has been successfully developed for the delivery of anticancer drugs to the liver [173,174]. also It has been pointed out that an extensive range of substrates (e.g. nanoparticles, nanogels, quantum dots, hydrogels and organic molecules) have been bound to HA to target different cancer types. Several carriers with a high affinity

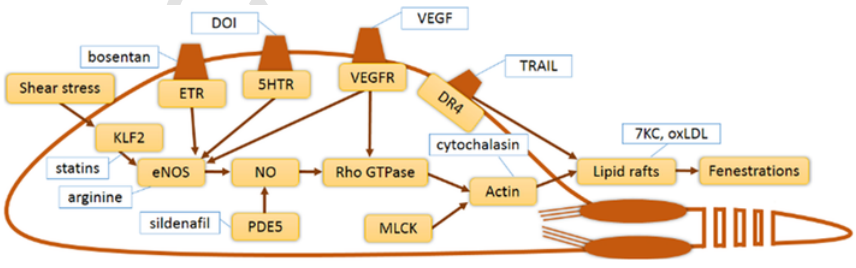

Fig. 3. The sieve-raft model of the regulation of fenestrations, indicating where agents that act on fenestrations have their effects. QDs could be used to deliver these agents specifically to the LSEC *in vivo*. Abbreviations: 5HTR: serotonin receptor; 7KC: 7-ketocholesterol; DR4: death receptor 4; DOI: 2,5-Dimethoxy-4-iodoamphetamine; ETR: endotheial receptor; KLF2: Krüppel-like Factor 2; MLCK: myosin light chain kinase; oxLDL: oxidised low density lipoprotein; PDE5: phosphodiesterase type 5; TRAIL:

TNF-related apoptosis-inducing ligand; VEGF: vascular endotheial growth factor.

for stabilin receptor 2 and mannose receptors (HA, oleyamine and polysaccharide chondroitin sulfate) on LSECs were found to facilitate targeted deliver of miRNA to reduce murine colon cancer metastasis [175].

Chemically modified cytokines with natural ligands can target various liver cell types. IL-10 conjugated with thiophosgene-activated mannose-6-phosphate increased liver uptake compared to intravenous injections of IL-10 alone which accumulated in the kidneys [176]. Receptors for mannose/N-acetyl glucosamine receptors are expressed on hepatocytes, hepatic satellite cells, Küpffer cells and LSECs [169].

Formaldehyde-treated serum albumin (FSA) may have potential for drug delivery to the LSEC because it is avidly taken up by LSECs *in vitro* and *in vivo* [177]. *In vivo*, FSA was almost entirely endocytosed via stabilin-2 receptors in the LSEC with only 2% of the dose taken up by other tissues. [178]. FSA is rapidly endocytosed by LSECs within 5-10 mins of intravenous injection and is degraded without hepatic or LSEC toxicity [110,179].

5.2.2. Nanoparticles and quantum dots

Nanoparticles range in size from 1-500 nm (by definition, one dimension must be within 1-100nm). Nanoparticles demonstrate (i) high motility in a free state, (ii) high surface area, and (iii) may exhibit quantum effects. Quantum dots (QDs) are a type of nanoparticle that exhibits quantum effects and have a restricted size (1-20nm). QDs are semiconductor particles that have different optical and conductive properties than larger sized particles. Nanoparticle and QD uptake within the liver is directed by several properties: size, composition and additional conjugated factors. It has been shown using Cd/Me QDs, that nanoparticles with a diameter of 3-4nm are endocytosed entirely by LSECs [130]. By comparison, polyethylene glycol (PEG)-bound nanoparticles with a diameter of 50 nm are taken up by hepatocytes [169]. PEG promotes greater hydrophilic binding and reduces plasma protein binding within the circulation, which reduces phagocytosis by Küpffer cells. Instead PEG binds to mannose/N-acetyl glucosamine receptors expressed on hepatocytes, LSECs and Küpffer cells leading to endocytosis primarily by hepatocytes [169]. γ-Fe_2O_3 QDs encapsulated within a modified PEG polymer were found to undergo facilitated endocytosis into a ED25 cell line; futhurmore the use of a polymeric micelles was shown to ameliorate toxicity induced by Fe_2O_3 QDs .

Basic nanoparticles with a diameter of >250nm are taken up preferentially by Küpffer cells [180,181]. This is because nanoparticles of this diameter are too large to pass through fenestrations for hepatocyte uptake; instead they remain in the sinusoidal lumen. Küpffer cells generally phagocytose particles ranging in size from 150-300nm in diameter [182]. However, Küpffer cells and LSECs took up 50% of nanocrystals modified with DSPE-PEG_{2000} that had a diameter of just 4-8nm [183]. It appears that both the size and composition of nanoparticles determine which cell type is preferentially targeted [174].

Mercaptosuccinic acid-capped cadmium telluride/cadmium sulfide (CdTe/CdS) quantum dots (QDs) have previously been shown to accumulate selectively in the LSEC with minimal endocytosis by Küpffer cells following intravenous injections *in vivo* in rats [130]. CdTe/CdS QDs are negatively charged nanoparticles that are endocytosed by scavenger receptors on LSECs and Küpffer cells [130]. The limitation of these QDs was the failed clearance via the bile three hours post treatment: this was suggested to contribute to their toxicity in LSECs.

Iron oxide nanocrystals or Cd-Selenide/CdS/Zinc-S (core, shell, shell) QDs encapsulated in a amphiphilic polymer (poly(maleic anhydride-alt-1-octdencene)) have been used to promote selective uptake into the liver, particularly LSECs [184]. These nanoparticles were non-toxic four weeks post-injection with no changes in cell death detected with a TUNEL assay [184]. However, previous studies with iron oxide nanocrystals highlighted cellular changes in actin and tubulin, and low doses of iron may promote cellular stress pathways based on studies performed in HUVECs [185].

Lipophilic, hydrophobic HA-based nanocapsules target LSECs with apparently less toxicity than other nanoparticles [186]. These nanocapsules selectively target LSECs and pulmonary microvascular endothelial cells. The nanocapsules consist of a liquid oil (oleic acid) core stabilized by hydrophobic modified HA. The structure is between 100-150nm in diameter and stable for 15 months *in vivo*. Both intravenous and oral forms of administration were observed to promote accumulation within the liver within 1h for intravenous and within 6ho for oral delivery. Clearance was confirmed in urine of the stable nanocapsule 6h post administration with minimal expression in plasma. These characteristics were considered by the authors to be favorable for drug delivery to the liver.

Another interesting nanoparticle for the delivery of agents to the LSEC is MEND (multifunctional envelope-like nanocapsule device). This nanoparticle is composed of cholesterol and PEG-DMG (1, 2-dimyristoyl-sn-glycero, methoxy ethylene glycol 2000 ether) combined with a cationic lipid (YSK05) to facilitate the endosomal escape of MEND. MEND is targeted to LSECs and hepatocytes via stearylated KLGR, a novel LDL receptor molecule [187] and its 84-118nm diameter. This nanoparticle has been used to deliver siRNA to downregulate Tie2 expression in hepatocytes and LSECs [188] and has been shown to acts via micropinocytosis rather than clathrin-mediated endocytosis.

6. Summary

Pathways that regulate the metabolic responses to nutrition by the liver are a plausible target for delaying aging. Diets and drugs that influence the canonical nutrient sensing pathways in the liver are being extensively studied. However, there are also age-related changes in the liver microcirculation that influence the regulation of metabolism by the liver, particularly the metabolism of lipoproteins and insulin sensitivity. There are several drugs and other agents that can increase fenestrations in LSECs from old animals. Such agents, coupled to nanoparticles that are selectively taken up by LSECs, may prove to be a novel therapeutic target for the prevention and treatment of age-related dyslipidemia and insulin resistance.

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