Cytoskeleton alterations in non-alcoholic fatty liver disease

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Declaration of interest: none.

Author Contributions

J.P.: Conceptualization, data curation, writing - original draft, writing - review & editing, visualization

J.T.: Writing - review & editing, visualization

Keywords

Non-alcoholic fatty liver disease, hepatocytes, hepatic stellate cells, cytoskeleton, α-smooth muscle actin, keratin 18

Abbreviations

NAFLD, non-alcoholic fatty liver disease; MAFLD, metabolic-associated fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; HSCs, hepatic stellate cells; α-SMA, α-smooth muscle actin; TGFβ, transforming growth factor β; MCD, methionine-choline-deficient; LAP1, lamina-associated polypeptide; VLDV, very low density lipoprotein; LC3, light chain 3; CYP4A14, cytochrome P450 omega-hydroxylase 4a14

Published article: https://doi.org/10.1016/j.metabol.2021.155115

Abstract

Background: Due to its extremely high prevalence and severity, non-alcoholic fatty liver disease (NALFD) is a serious health and economic concern worldwide. Developing effective methods of diagnosis and therapy demands a deep understanding of its molecular basis. One of the strategies in such an endeavor is the analysis of alterations in the morphology of liver cells. Such alterations, widely reported in NAFLD patients and disease models, are related to the cytoskeleton. Therefore, the fate of the cytoskeleton components is useful to uncover the molecular basis of NAFLD, to further design innovative approaches for its diagnosis and therapy.

Main findings: Several cytoskeleton proteins are up-regulated in liver cells of NAFLD patients. Under pathological conditions, keratin 18 is released from hepatocytes and its detection in the blood emerges as a non-invasive diagnosis tool. α-Smooth muscle actin is up-regulated in hepatic stellate cells and its down-regulation has been widely tested as a potential NALFD therapeutic approach. Other cytoskeleton proteins, such as vimentin, are also up-regulated.

Conclusions: NAFLD progression involves alterations in expression levels of proteins that build the liver cytoskeleton or associate with it. These findings provide a timely opportunity of developing novel approaches for NAFLD diagnosis and therapy.

1. Introduction

Non-alcoholic fatty liver disease (NALFD), also termed metabolic-associated fatty liver disease (MAFLD)[1] is an extremely prevalent disease, affecting about 25 % of the global adult population[2]. It is characterized by fat accumulation in more than 5 % of the hepatocytes, the main liver cells. Its initial stage, non-alcoholic fatty liver (NAFL) is characterized by simple steatosis (fat accumulation within cells), without extensive inflammation. It may evolve into non-alcoholic steatohepatitis (NASH), which is characterized by extensive inflammation and fibrosis (permanent accumulation of scar tissue). NAFLD is an important cause of cirrhosis and hepatocellular carcinoma, a frequent indication for liver transplantation and a large health and economic burden[2].

There is an obvious and urgent need of designing and testing innovative approaches for NAFLD diagnosis and therapy. Such goals demand a detailed understanding of the molecular and cellular basis of the disease. An important source of insight into such basis is the alteration in the morphology of liver cells[3], widely reported in NAFLD patients and disease models. Cellular morphology is mostly defined by the cytoskeleton, an extensive protein network within the cell. Therefore, understanding cytoskeleton alterations in NAFLD is also useful to understand disease mechanism and design novel approaches for NAFLD diagnosis and therapy.

In this narrative review, we discuss documented alterations of cytoskeleton proteins in NAFLD context. Some of those alterations provide potential novel tools of diagnosis and therapy, which have been experimentally tested. Finally, we briefly mention some potential future lines of research.

2. Hepatocyes, Kupffer cells and hepatic stellate cells (HSCs)

The liver has important functions, including storage and processing of nutrients absorbed through the digestive system and also degradation of toxins, including ethanol[4]. About two-thirds of the liver mass is formed by hepatocytes, whose multiple functions include synthesis of bile and also metabolism of carbohydrates, lipids and proteins[5]. Hepatocytes are highly polarized cells. Their plasma membrane contains distinct sets of proteins and lipids, which are vital for hepatocytes to perform many diverse functions. Hepatocyte polarity requires coordinated functions of supra-molecular structures shaping cell structure, including the cytoskeleton[6]. Abnormal hepatocyte polarization may cause liver diseases, including liver cancer[6].

These cells are stacked in lines separated by capillaries, the sinusoids (Fig. 1). Hepatocytes stacks form a radial arrangement converging into a central vein. The sinusoids contain macrophage cells called Kupffer cells (Fig. 1), which form about 15 % of the liver cell population. These phagocytic cells eliminate aged erythrocytes and bacteria that may eventually invade the liver[7]. The ruffling of cell membrane and formation of pseudopodia play an important role in the phagocytosis of Kupffer cells and this is believed to be accomplished by the cytoskeleton. In fact, age-related damage of the cytoskeleton system in Kupffer

cell aging is one of the important mechanisms responsible for a decrease in phagocytosis[7].

Between hepatocyte stacks and the sinusoidal endothelial cells, the perisinusoidal space (or Space of Disse) is found. This space contains hepatic stellate cells (HSCs) (Fig. 1), or Ito cells[8]. These cells represent about 10 % of all liver cells and store vitamin A inside lipid droplets. HSCs have been primarily characterized as the main effector cells in liver fibrosis, due to their capacity to transdifferentiate into collagen-producing myofibroblasts[9]. HSCs play also a fundamental role in liver immunology, representing a versatile source of many soluble immunological active factors including cytokines. They may act as antigen presenting cells and have autophagic activity. The cytoskeleton of HSCs is also critical for their cellular function. In fact, liver fibrosis in NASH is largely due to excessive extracellular matrix deposition, mediated by cytoskeleton alterations in HSCs[10].

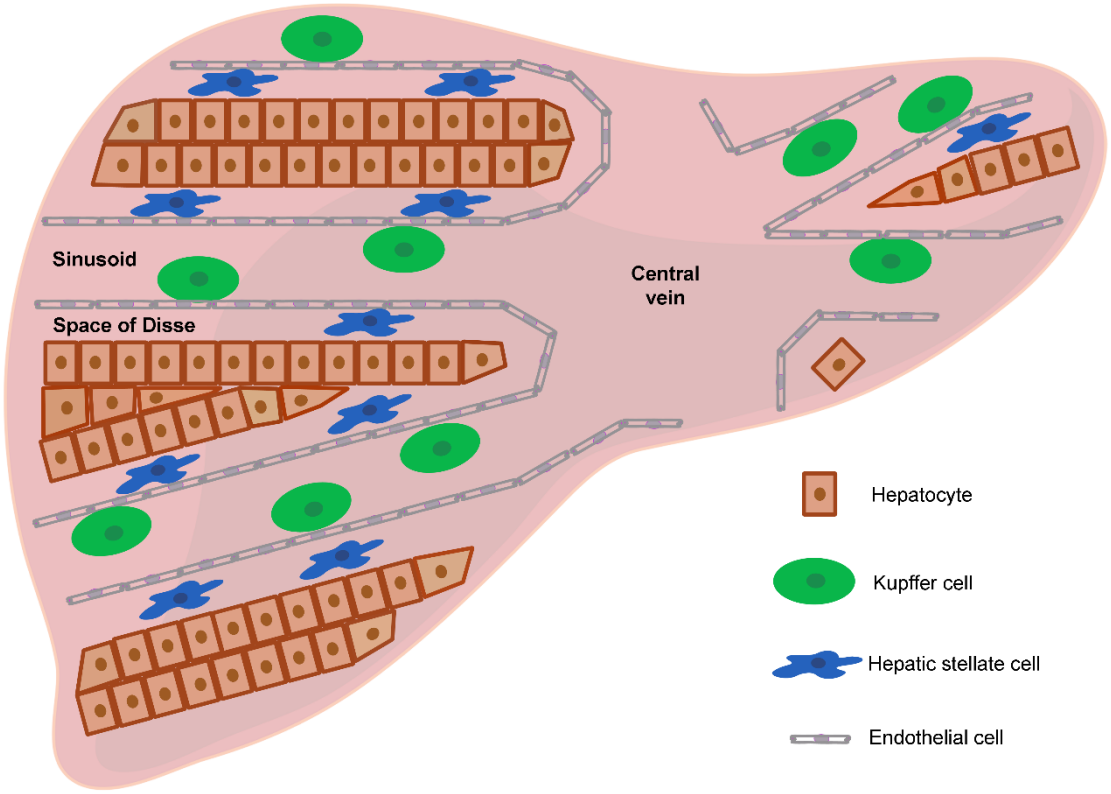

Figure 1: A sketch of liver histology. The sketch represents several sinusoids, defined by epithelial cells (represented in gray), converging into a central vein. Sinusoids contain Kupffer cells (represented in green) attached to epithelial cells. Between sinusoids, hepatocytes (represented in brown) are densely stacked. The space of Disse is comprised between epithelial cells and hepatocyte stacks and contains hepatic stellate cells (represented in blue). The sketch is located within the shape of a human liver.

3. Cytoskeleton

The cytoskeleton is a protein backbone that defines cellular shape and internal structure. It also provides mechanical force and motion for cellular processes, including cell division. The cytoskeleton is divided into microfilaments (or actin filaments), intermediate filaments and microtubules[11].

The actin cytoskeleton is found in monomeric (globular or G actin) or polymeric (filamentous or F actin) forms. Actin filaments are flexible and provide mechanical force to modify movement, shape and contacts. Polymerization, which is mainly regulated by Rho GTPases, is faster in one of the filament ends, generating polarity[12].

Intermediate filaments have two distinct systems, one located in the cytoplasm, whose composition varies among distinct cell types[13] and another located in the nucleus, composed of lamin filaments[14]. Intermediate filaments are diverse in protein composition. While hepatocytes contain two types of keratin, keratin 8 and keratin 18, Kupffer cells have vimentin and HSCs have vimentin, desmin, nestin and glial fibrillary acidic protein[15]. Intermediate filaments provide cellular shape and resistance to physical stress[13].

Microtubules are formed of filaments composed of α- and β-tubulin heterodimers[16]. Microtubules usually originate at the microtubule organization center, which also contains γ-tubulin as a polymerization primer. Microtubules function as transport tracks, being also responsible for organelle sub-cellular localization and in mitotic spindle positioning[16].

Cytoskeleton functioning results of an extensive cross-talk among its components, especially actin filaments and microtubules, which involves cross-linking and mechanical support[17]. Moreover, cytoskeleton proteins can respond to mitochondrial stress, endoplasmic reticulum stress and oxidative stress conditions[18], which are sub-cellular alterations extensively described in NAFLD. Consequently, proteins from the cytoskeleton of liver cells and their alterations in NAFLD might impact the pathophysiology of the disease and emerge as potential diagnostic hallmarks and/or therapeutic targets.

4. Cytoskeleton protein alterations in NAFLD

4.1. α-Smooth muscle actin and vimentin up-regulation in hepatic stellate cells

Upon liver injury, hepatic stellate cells (HSCs)[8] transdifferentiate into myofibroblast-like cells, with a concomitant up-regulation of their α-smooth muscle actin (α-SMA)[10] and vimentin levels[19] (Fig. 2). HSCs transdifferentiation is promoted by activation of the focal adhesion kinase by transforming growth factor β (TGFβ)[20]. These transformed cells produce large amounts of collagen and other extracellular matrix components[21] (Fig. 2). For instance, fibronectin, another extracellular matrix component, was also up-regulated in livers of NAFLD mice[22]. Excessive deposition of extracellular matrix components induces fibrosis, disrupts the normal architecture of the liver and results in hepatic dysfunction[23]. HSCs transdifferentiation into myofibroblast-like cells is the major contributor to liver fibrosis[9] and α-SMA overexpression is a marker of this process.

Studies in human liver biopsies revealed that α–SMA detected in NASH patients was further increased in cirrhosis patients[24]. In pediatric NAFLD patients, liver expression of α-SMA increased with fibrosis severity[25]. α-SMA levels were also up-regulated in NAFLD mice[26], rats[27] and minipigs[28]. These findings show that α-SMA overexpression is a maker of NAFLD progression.

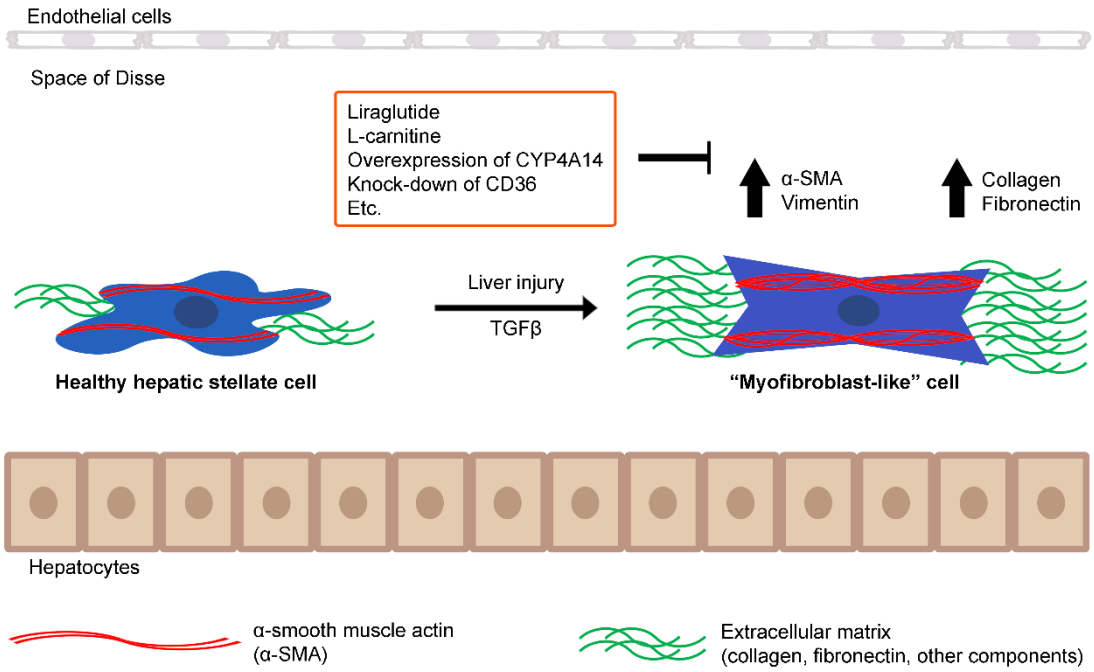

Figure 2: α-Smooth muscle actin (α-SMA) up-regulation in injured hepatic stellate cells (HSCs). HSCs (represented in blue) are located in the space of Disse (between hepatocytes and sinusoid epithelial cells). In a healthy liver, HSCs secrete moderate amounts of α-SMA (represented in red) and produce moderate amounts of extracellular matrix components (represented in green). Upon liver injury, TGFβ induces changes in HSC morphology, transdifferenting into myofibroblast-like cells, which overexpress α-SMA and vimentin. These changes induce production of large amounts of extracellular matrix components, including collagen and fibronectin. Their excessive deposition results in liver fibrosis and dysfunction. Treatment of animal models with a vast array of compounds, including liraglutide and L-carnitine, as well as through overexpression of cytochrome P450 omega-hydroxylase 4a14 (CYP4A14), has been shown to prevent α-SMA up-regulation. Vimentin up-regulation in HSCs and hepatocytes could be prevented through the knock-down of CD36, also reducing liver damage.

4.2. Hepatocyte keratin accumulation into Mallory-Denk bodies

Fat accumulation in hepatocytes causes cell swelling, vulgarly designated as hepatocyte ballooning, which is a steatohepatitis hallmark. This process is mediated by the two types of hepatocyte keratin (also designated as cytokeratin): keratin 8 and keratin 18. Immunofluorescence staining of these keratins revealed extensive decrease or even loss of intermediate filaments in ballooned hepatocytes[29]. It was proposed that, in NAFLD, intermediate filaments are disassembled, with a simultaneous increase in monomeric keratin synthesis[30]. These alterations cause an excess of keratin 8[31], which favors its misfolding, cross-linking and consequent aggregation[32]. These keratin-rich aggregates, termed Mallory-Denk bodies[30], are an established NASH marker and a well-known result of a cytoskeleton alteration in NAFLD.

4.3. Hepatocyte keratin release into the bloodstream

The formation of Mallory-Denk bodies is not the only consequence of hepatocyte intermediate filaments disruption in NAFLD. NAFLD involves hepatocyte apoptosis, which generates keratin 18 fragments in the plasma (Fig. 3). Keratin 18 is a marker of hepatocyte apoptosis and an indicator of NAFLD disease progression. Moreover, serum keratin 18 increased in NAFLD patients[33] and further increased with steatosis, NASH and fibrosis[34]. Several genetic variants of these two proteins were identified in Chinese NAFLD patients, which were also related to insulin resistance[35]. Moreover, keratin 18 was increased in liver and serum of a mouse model of metabolic syndrome, being considered as a reliable indicator of liver damage[36].

Keratin 18 is usually detected using antibodies recognizing its M30 or M65 epitopes. While M30 detects keratin 18 fragments released during apoptosis, M65 fails to discriminate these fragments from intact keratin 18 in the intermediate filaments[37] (Fig. 3). Nevertheless, the keratin 18 M65 epitope has shown to be a better marker to diagnose NASH than M30[38].

Besides intermediate filament erosion and keratin 18 release, NAFLD may also involve disruption of intermediate filaments attachment to the cell membrane. Treatment with HepG2 cells with palmitate down-regulated desmoplakin[39], one of the components of a plaque that anchors intermediate filaments to the cell

membrane. Such down-regulation could lower cell-cell adhesion via desmosomes and disrupt keratin intermediate filaments[40].

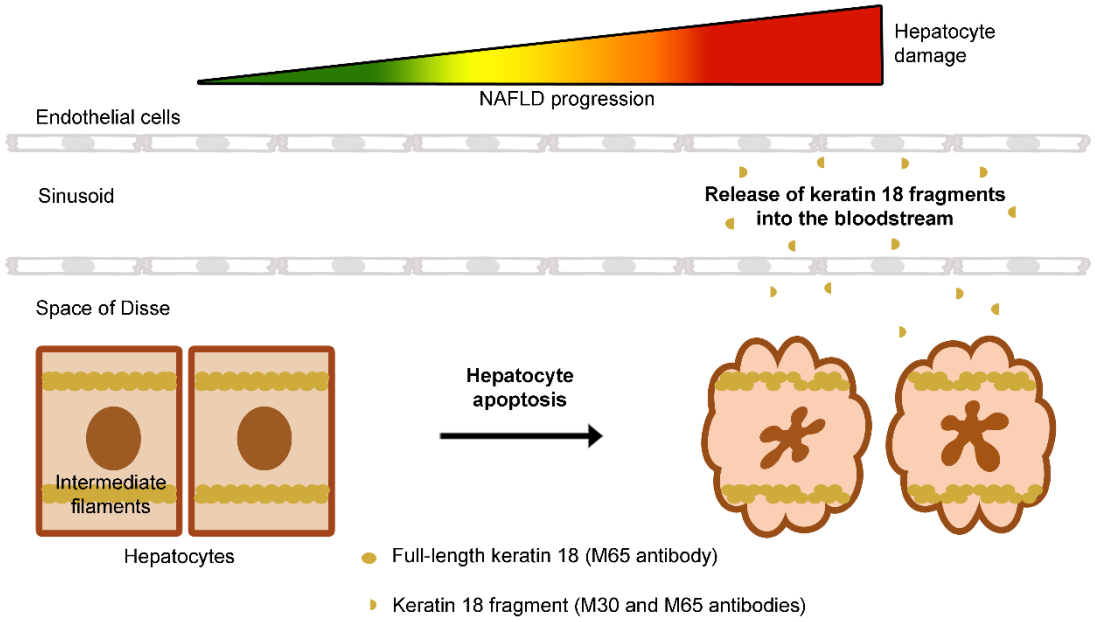

Figure 3: Release of keratin 18 fragments from apoptotic hepatocytes. Upon apoptosis, hepatocyte intermediate filaments (represented in yellow, within hepatocytes represented in brown) release keratin 18 fragments into the sinusoids, which lead to the bloodstream. Released keratin 18 can be detected using specific antibodies. One of these antibodies, named M30, is specific for keratin 18 fragments, while another antibody, M65, detects both full-length and fragmented keratin 18. Serum levels of keratin 18 M30 fragments are correlated with hepatocyte damage and NAFLD progression, which includes severe inflammation, fibrosis, steatosis and ballooning.

4.4. Vimentin and desmin up-regulation in hepatocytes and rodent HSCs, respectively

Vimentin and desmin are additional intermediate filament proteins up-regulated in NAFLD models. In a methionine-choline-deficient (MCD) diet-fed mouse model to induce NASH phenotype, vimentin was up-regulated, cleaved in an apoptosis-dependent manner and secreted. Its up-regulation was associated with inflammation, but not directly with lipid accumulation, as vimentin was not significantly up-regulated in mice fed a high-fat diet[41]. A distinct study also detected vimentin up-regulation in hepatocytes of MCD diet-fed mouse model to induce NASH phenotype[42]. Similarly, vimentin was also up-regulated in a NAFLD mouse model (MCD diet-fed-mice) treated with a pro-inflammatory fibrosis inducer[22]. Vimentin up-regulation and cleavage could also be detected in the HepG2 hepatocyte cell line and also in hepatocytes treated with palmitic acid[43].

Desmin is a marker of rodent HSC activation and was up-regulated in NAFLD rat models[44]. However, it is unclear if this marker is conserved in humans.

4.5. Lamin A/C and lamin-associated proteins dysfunction in hepatocytes

The nuclear cytoskeleton is located beneath the inner nuclear membrane. It consists of a filamentous network, the nuclear lamina, composed of intertwined A-type and B-type lamin proteins[45]. The nuclear membrane is also topologically connected to the endoplasmic reticulum, where lipid synthesis occurs[46]. Emerging evidence indicates that lamins and their nuclear membrane-associated proteins have a role in lipid metabolism and could be involved in the development of NAFLD[47]. Some patients with mutations in the *LMNA* gene, which encodes lamin A/C, an A-type lamin[48], developed NAFLD[49,50]. Two missense heterozygous *LMNA* mutations associated with nuclear misshaping were found in metabolic syndrome patients[51], further confirming lamin involvement in NAFLD. Lamin A/C keeps hepatocyte homeostasis and nuclear shape, being found in NAFLD nuclear hepatic inclusions[52]. Hepatocyte-specific deletion of lamin A/C caused steatohepatitis in male mice only. Lamin deficiency also caused nuclear deformity and male-specific increases in sensitivity to diet-induced

steatohepatitis, fibrosis and hepatocyte growth defects. In these mice, fatty acid metabolism and pro-inflammatory genes were up-regulated[53]. Moreover, sexual dimorphic genes, such as *Cyp1a2* and *Esr1* (coding for a cytochrome P450 family member and an estrogen receptor, respectively) were de-regulated in mouse hepatocytes[54]. The liver is a sexually dimorphic organ[55]. Although it is unknown why only male mice were prone to steatohepatitis induced by lamin A/C deficiency, these evidences could be a link to the enhanced NAFLD propensity in males[56].

Lamin-associated proteins have also been implied in NAFLD. The nuclear inner membrane-inserted lamina-associated polypeptide (LAP1) activates torsinA, an ATPase located in the nuclear intermembrane space, to regulate very low density lipoprotein (VLDL) secretion from hepatocytes. Deletion of torsinA or LAP1 in mouse hepatocytes decreased VLDL secretion and caused profound steatosis. Mice carrying each of these deletions developed hepatic steatosis and subsequent steatohepatitis on a regular diet[57]. These findings indicate that the nuclear cytoskeleton and associated proteins torsinA and LAP1 are required for proper lipid secretion. Their dysfunction could also be involved in NAFLD, through inhibition of lipid secretion.

4.6. α-tubulin up-regulation in mouse hepatocytes

Microtubules and formed by α- and β-tubulin heterodimers. Two α-tubulin isoforms, tubulin alpha 8[58] and α-tubulin1b were up-regulated in tumor tissue of hepatocellular carcinoma patients[58,59]. Although tubulin alpha 8 was also up-regulated in a NASH mouse model[60], it remains unclear if these two α-tubulin isoforms are also up-regulated in human NAFLD patients.

Fat liver accumulation and oxidative stress in NAFLD can induce lipid peroxidation[61]. Interestingly, two important lipid peroxidation products, the aldehydes 4- hydroxynonenal and 4-oxononenal, were able to inhibit polymerization of purified tubulin[62] and to rapidly disrupt microtubules in HepG2 cells[63]. Up-regulation of α-tubulin isoforms is related to hepatocellular carcinoma and possibly to human NAFLD; however, oxidative stress may have a counterbalancing effect.

4.7. Light chain 3 (LC3) isoform distribution alterations

Light chain 3 (LC3), a common subunit of two microtubule-binding proteins, is found in two isoforms. LC3-I is cytosolic, whereas LC3-II locates in autophagosome membranes, being its cellular level correlated with the extent of autophagosome formation and accumulation[64]. LC3 participates in autophagy, eliminating old or damaged mitochondria and endoplasmic reticulum[65]. The LC3-II isoform is likely to attach autophagosomes to microtubules[64].

A NAFLD mouse model has shown impaired autophagy and enhanced conversion of LC3-I to LC3-II[66], which was also observed in the livers of rats fed with a high fat and high cholesterol diet[67]. The excess of LC3-II suggests autophagosome accumulation in hepatic cells, which could in part explain the autophagy deficiency observed in NAFLD[68]. Therefore, the cytoskeleton could also be involved in such a deficiency, through LC3-II attachment to microtubules. In ovariectomized rats, there was also increased hepatic fat accumulation and concomitant decrease in LC3-I[69], which suggests impairment of lipid droplets autophagy (lipophagy). This finding also suggests a possible explanation for increased female propensity to NAFLD after menopause[70], when the ovaries decrease estrogen production.

5. Cytoskeleton protein in NAFLD diagnosis

Release of keratin from hepatocyte intermediate filaments is one of the clinically most useful cytoskeleton alterations in NAFLD. Quantification of keratin 18 in patient serum has been extensively tested, frequently in combination with more established NAFLD markers (Table 1). In NAFLD diagnosis, it emerges as a promising alternative to liver biopsy.

In conjunction with other markers, keratin 18 was proposed as a biomarker for fibrotic NASH diagnosis[71-73], whose quantification provides the most used biomarker of steatohepatitis, despite its low accuracy[74]. Their quantification has shown to have a good predictive ability for NASH diagnosis[75], and associated positively with serum alanine aminotransferase[76], another NASH marker. Keratin

18 was proposed as a non-invasive biomarker of early apoptosis, useful to diagnose NASH and differentiate it from simple steatosis[77].

Quantification of keratin 18 fragments was able to predict prognosis of NAFLD patients[78]. Serum keratin 18 fragment levels decreased in obese, liver fibrosis patients, upon diet-induced weight loss[79] and upon physical exercise therapy[80]. Such a decrease was correlated with alleviation in steatosis and fibrosis[79]. Keratin 18 fragment levels also decreased after bariatric surgery[81] and such decrease was positively correlated with improvement in liver histology in adults or children with NAFLD[82] (Table 1). In fact, using a NAFLD rat model, serum levels of keratin 18 fragments were increased in more severe inflammation, fibrosis, steatosis and balooning NAFLD disease stages[83].

Additionally, quantification of secreted vimentin was also proposed as a potential diagnosis method for NASH[41]. Notwithstanding, more consistent data should be experimentally acquired in the future.

In summary, cytoskeleton proteins, especially keratin 18 fragments, are an important biomarker of NAFLD disease progression and their quantification in the blood emerges as a non-invasive diagnosis tool.

Table 1: Studies on NAFLD diagnosis based on quantification of keratin 18 fragments in patient serum

Antibody type	Participants / model systems	Diet	Disease stage	Remarks	Year	Reference
M30	Adult patients	N/A	Active and fibrotic NASH	Used in conjunction with quantification of aspartate aminotransferase and assessment of insulin resistance	2019	[71]
M30	Rat	High-fat	NASH	Used in conjunction with quantification of interferon-λ3 / IL-28B	2020	[73]
(Not specified)	Adult patients	N/A	NAFL and NASH	Used in conjunction with assessments of fibrosis index and insulin resistance	2019	[72]
M30	Adult patients	N/A	(Not specified)	Used in conjunction with quantifications of alanine aminotransferase and aspartate	2020	[76]
M30 and M65	Adult patients	N/A	NAFL and suspected NASH	Used in conjunction with quantification of fibroblast growth factor 21	2017	[78]
M30	Adult patients	N/A	Fibrosis	Used to monitor the effect of diet-induced weight loss	2019	[79]
M65	Adult patients	N/A	(Not specified)	Used in conjunction with quantification of fibroblast growth factor 21 and to monitor the effect of physical exercise	2020	[80]
M30	Adult patients	N/A	(Not specified)	Used to monitor the effect of bariatric surgery; inconsistent results	2021	[81]
M30	Adult and pediatric patients	N/A	NAFL and NASH	Used to monitor changes in liver histology	2014	[82]
M30	Rat	High-sucrose, high-fat	NAFL and NASH	Used to monitor changes in liver histology	2018	[83]

6. Therapeutic perspectives

6.1. Down-regulation of α-SMA and vimentin

The cytoskeleton has been successfully tested as a potential therapeutic target in NAFLD. Many drugs, in several animal and cellular models, which were effective in NAFLD alleviation, were found to down-regulate α–SMA in HSCs (Table 2). Feeding of NASH minipigs with a chinese herbal medicine significantly inhibited α-SMA expression, as well as hepatocyte fibrosis[28]. In NASH mouse models, several compound treatments were effective in down-regulating α-SMA and inhibited HSC transformation, alleviating NASH features, including steatosis and fibrosis. Those compounds include resmetirom, a liver-directed, selective thyroid hormone receptor β agonist[84], the glucagon-like peptide 1 analog liraglutide[85], eugenol[86], diosmin[87], amlexanox, an inhibitor of nuclear factor κB kinase epsilon and TANK-binding kinase 1[88], L-carnitine, a fatty acid β-oxidation cofactor[89], namodenoson, an anti-inflammatory adenosine derivative[90], gypenoside LXXV, a ginseng-extracted compound[91], fermented black radish[92], zoledronic acid[93], a sirtuin inhibitor[94] and many more.

α-SMA expression in HSCs could also be lowered through overexpression of cytochrome P450 omega-hydroxylase 4a14 (CYP4A14)[95] or through knock-out of renalase (an oxidative stress suppressor) in NASH mice[96]. Inhibition of focal adhesion kinase reduced α-SMA and collagen overexpression in an animal model, in the presence of TGFβ[20] (Table 2). Although these studies pertain to HSCs, α-SMA expression was also lowered in hepatocytes through knockdown of CD36, a glycoprotein that promotes lipid transport[43]. Thus, the HSC cytoskeleton protein α-SMA has been widely shown as a promising drug target in NAFLD, including in advanced disease stages.

Although less extensively tested, down-regulation of vimentin, another cytoskeleton protein, has also shown a promising outcome in NAFLD models. Vimentin was up-regulated in mouse HSCs[19] and in human hepatocytes treated with palmitic acid[43]. Knockdown of CD36 reduced both lipid and vimentin levels in hepatocytes[43]. In plasma samples from patients and rat models, vimentin up-regulation was also correlated to increased fibrosis. Interestingly, knockdown of vimentin in rat HSCs inhibited the transdifferentiation into myofibroblasts[97]. These

studies indicate that down-regulation of vimentin could also be useful in NAFLD treatment, in addition to the many approaches shown to down-regulate α-SMA (Fig. 2).

Table 2: Studies on NAFLD therapeutic approaches based on α-smooth muscle actin down-regulation in hepatic stellate cells

Drug / protein level alteration	Model system(s)	Diet / treatment	Disease stage	Remarks	Year	Reference
Fufang Zhenzhu Tiaozhi formula (a preparation of Chinese herbal medicine)	Minipig, HepG2 cell line	High-fat / oleic acid, palmitic acid	NASH	Effect mediated by the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway	2021	[28]
Resmetirom, a thyroid hormone receptor β agonist	Mouse	High-fat, high-fructose, high-cholesterol	NASH and fibrosis	Treatment reduced liver weight without affecting body weight	2021	[84]
Liraglutide, a glucagon-like peptide 1 analog	Mouse	High-fat, high-fructose, high-cholesterol	NASH	Treatments decreased inflammation and fibrosis; elafibranor was also tested, affecting hepatic lipid composition	2021	[85]
Eugenol, a compound extracted from essential oils	Rat	High-fat	Fibrosis	Treatment affected insulin resistance	2021	[86]
Diosmin, an anti-clotting compound	Rat	High-fat	NASH	Treatment has shown effects in insulin resistance, inflammation and fibrosis	2020	[87]
Amlexanox, an anti-inflammatory compound	Mouse	High-fat and/or lipopolysaccharide	(Not specified)	Treatment has shown effects in insulin resistance and inflammation	2019	[88]
L-carnitine, a fatty acid β-oxidation cofactor	Mouse	Methionine-choline-deficient	NAFL	Treatment has shown effects on inflammation, reactive oxygen species production and fibrosis	2020	[89]
Namodenoson, a compound with anti-hepatocellular carcinoma effect	Mouse	High-fat and streptozotocin	NASH	Treatment inhibited liver ischemia / reperfusion injury	2019	[90]
Gypenoside LXXV, a ginseng-extracted compound	Mouse, HepG2 and LX2 cell lines	Methionine-choline-deficient / palmitic acid	NASH	Treatment prevented liver injury and activation of hepatic macrophages	2020	[91]

Table 2: Studies on NAFLD therapeutic approaches based on α-smooth muscle actin down-regulation in hepatic stellate cells (cont.)

Drug / protein level alteration	Model system(s)	Diet / treatment	Disease stage	Remarks	Year	Reference
Fermented black radish	Mouse	Methionine-choline-deficient	Fibrosis	Treatment suppressed activation of Kupffer cells and other liver macrophages	2019	[92]
Zoledronic acid, a compound that lowers excessive calcium levels in the blood	Rat	High-fat	NASH	Treatment resulted in decreased portal vein hypertension	2019	[93]
EX-527, an inhibitor of sirtuin 1 (a metabolic regulator)	Rat	High-fat	Fibrosis	Treatment up-regulated sirtuins 2, 3 and 4	2020	[94]
Hepatic overexpression of cytochrome P450 omega-hydroxylase 4a14 (CYP4A14)	Mouse	Bile duct ligation	Fibrosis	Knock-out of the same protein in mice aggravated transdifferentiation of HSCs into myofibroblasts	2021	[95]
Knock-out of renalase, an enzyme down-regulated in kidney disease	Mouse	choline-deficient, high-fat, supplemented with 0.1% methionine	NASH	Treatment showed a modest effect in NASH suppression	2021	[96]
Inhibition of focal adhesion kinase	Mouse; mouse HSCs	Carbon tetrachloride	Fibrosis	Treatment inhibited TGFβ effect in HSC transdifferentiation	2017	[20]

Additional studies could not be listed here, due to reference number limitations.

6.2. Inhibition of keratin release and modulation of LC3-II

Inhibition of keratin release from hepatocyte intermediate filaments could be another therapeutic possibility. Treatment of a NASH mouse model with fenofibrate inhibited disruption of intermediate filaments and consequent formation of Mallory-Denk bodies, further reducing oxidative stress and inflammation[98] and improving the phenotype.

Restoring cellular levels of LC3-II, the LC3 isoform attaching autophagosomes to microtubules, has shown to alleviate steatosis by reestablishing lipid droplets autophagy. Treatment of high-fat diet mice and hepatocytes exposed to palmitic acid with liraglutide, a glucagon-like peptide 1 analog, restored the autophagic fluxes in these systems, decreased fat accumulation and improving the NAFLD phenotype[99,100]. These studies show that this microtubule-associated protein could be an interesting element for autophagy restoration in NAFLD.

7. Concluding Remarks

In the present review, we have analyzed available information concerning the roles of cytoskeleton proteins in NAFLD. In the literature, alterations in cytoskeleton proteins have been associated to the development of liver steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma. Several of the major scaffold proteins of the cytoskeleton are up-regulated in NAFLD and their down-regulation could be a therapeutic approach. Concerning cytoskeleton interventions as prospective NAFLD therapies, down-regulation of α-SMA is clearly the most extensively tested approach, which inhibits excessive growth of the extracellular matrix, an important cause of fibrosis. α-SMA down-regulation as a promising NAFLD therapeutic approach is impressive. Consequently, down-regulation of vimentin, inhibition of keratin release and modulation of LC3-II are starting to be tested as other potential therapeutic approaches. Notwithstanding, the roles of cytoskeleton motor proteins, including kinesins, dyneins and myosin, seem to be unexplored in NAFLD. Moreover, cytoskeleton proteins are highly regulated, frequently through post-translational modifications[101], a topic that seem also to be unexplored in NAFLD. It becomes now imperative to explore the physiological relevance of cytoskeleton proteins in liver pathophysiology and metabolic diseases, as well as their roles as potential therapeutic targets in NAFLD.

These findings show that the cytoskeleton has a promising therapeutic potential in NAFLD, which is a still poorly explored system, demanding to be further investigated in the NAFLD context.

Acknowledgements

This work was financed by the European Regional Development Fund (ERDF), through the COMPETE 2020 - Operational Programme for Competitiveness and Internationalisation and Portuguese national funds via FCT – Fundação para a Ciência e a Tecnologia, under projects POCI-01-0145-FEDER-028147 (VISCERAL), UIDB/04539/2020 and UIDP/04539/2020. J.P. (POCI-01-0145-FEDER-028147 (VISCERAL)) and J.T. (2020.01560.CEECIND) acknowledge FCT for the research contracts. The authors have no conflicts of interest to disclose.

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