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Animal models of NAFLD from the pathologist's point of view☆

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ABSTRACT

Fatty liver disease is a multifactorial world-wide health problem resulting from a complex interplay between liver, adipose tissue and intestine and initiated by alcohol abuse, overeating, various types of intoxication, adverse drug reactions and genetic or acquired metabolic defects. Depending on etiology fatty liver disease is commonly categorized as alcoholic or non-alcoholic. Both types may progress from simple steatosis to the necro-inflammatory lesion of alcoholic (ASH) and non-alcoholic steatohepatitis (NASH), respectively, and finally to cirrhosis and hepatocellular carcinoma. Animal models are helpful to clarify aspects of pathogenesis and progression. Generally, they are classified as nutritional (dietary), toxin-induced and genetic, respectively, or represent a combination of these factors. Numerous reviews are dealing with NASH animal models designed to imitate as closely as possible the metabolic situation associated with human disease. This review focuses on currently used mouse models of NASH with particular emphasis on liver morphology. Despite metabolic similarities most models (except those with chemically or genetically induced porphyria or keratin 18-deficiency) fail to develop the morphologic key features of NASH, namely hepatocyte ballooning and formation of histologically and immunohistochemically well-defined Mallory-Denk-Bodies (MDBs). Although MDBs are not universally detectable in ballooned hepatocytes in NASH their experimental reproduction and analysis may, however, significantly contribute to our understanding of important pathogenic aspects of NASH despite the obvious differences in etiology.

1. Introduction

Fatty liver disease is a multifactorial and still expanding world-wide health problem [1,2]. It is the result of a complex interplay between liver, adipose tissue and intestine and may be caused by alcohol abuse, overeating, intoxication, adverse drug reactions, genetic or acquired metabolic defects or combinations of several factors. According to etiology, this disorder is commonly categorized as alcoholic or non-alcoholic. The latter (non-alcoholic fatty liver disease; NAFLD) exists in the absence of significant alcohol consumption and is the hepatic manifestation of the metabolic syndrome, which is characterized by visceral obesity, dyslipidemia, type II diabetes mellitus, insulin resistance (IR), elevated arterial blood pressure and related cardio-vascular disorders [2–6].Both types of fatty liver disease may progress from simple steatosis to the necro-inflammatory lesion of alcoholic (ASH) and non-alcoholic (NASH) steatohepatitis (SH), respectively, cirrhosis and hepatocellular carcinoma [1,2].

In the analysis of NAFLD/NASH animal models the pathologist is inclined to concentrate on morphologic aspects, since the morphologic picture usually reflects the endpoint of common pathogenic pathways notwithstanding differences of the primary etiology. In contrast, less morphologically oriented scientists will prefer models based on special diets, administration of toxins or genetic alterations resembling the metabolic background of human disease with less emphasis on morphology. This review focuses on attempts at experimental reproduction of morphologic key features of human NAFLD/NASH in mice and their contribution to our understanding of pathogenesis.

2. Morphologic features of steatohepatitis of alcoholic and non-alcoholic etiology

Histopathological evaluation of liver biopsy material represents the gold standard for the diagnosis of SH [2,3]. In principle, SH is characterized by variable degrees of predominantly macrovesicular steatosis and enlarged and rounded (ballooned) hepatocytes with increased nuclear and nucleolar size and flocculent, cleared cytoplasm preferentially in centrilobular (perivenular; acinar zone 3) position; most of them contain cytoplasmic inclusions, termed Mallory-Denk bodies (MDBs)

Abbreviations: NAFLD, non-alcoholic fatty liver disease; SH, steatohepatitis; ASH, alcoholic steatohepatitis; NASH, nonalcoholic steatohepatitis; MDB, Mallory-Denk body; H&E, hematoxylin & eosin; IF, intermediate filament; p62, sequestosome1/p62; SAMe, S-adenosylmethionine; FA, fatty acid; TG, triglyceride; IR, insulin resistance

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[2,3,7]. Ballooning is a key lesion of human SH [2,3,8–11]. Its pathogenesis is multifactorial. Derangement or even loss of the hepatocytic keratin intermediate filament (IF) cytoskeleton is characteristic and may adversely affect cell stability and intracellular organization [11]. However, additional factors not directly related to the IF cytoskeleton, like microtubular failure and impaired secretory capacity with retention of proteins and fluids cannot be excluded [2]. Moreover, electron microscopy revealed accumulation of small lipid droplets in ballooned hepatocytes which could also contribute to their morphologic appearance [9,10,12]. A significant correlation exists between ballooning and pericellular/perisinusoidal fibrosis. This is in line with the observation that ballooned hepatocytes produce Hedgehog (Hh) ligands and are in close proximity to Hh-responsive myofibroblasts [13].

In hematoxylin and eosin (H&E)-stained sections MDBs appear as irregular eosinophilic aggregates of variable size. They have to be distinguished from condensed cytoplasmic areas and can be specifically identified by immunostaining since they contain keratins, ubiquitin, sequestosome 1/p62 (p62) and other stress proteins [14–16]. For detection and identification of MDBs immunohistochemistry is superior to H&E staining due to higher sensitivity and specificity [17], and their presence may be underestimated in conventionally stained sections [2]. Longitudinal studies revealed MDBs as indicators of poor prognosis and predictors of disease progression [18–21]. Hepatocyte necrosis, apoptosis, bilirubinostasis, and ductular reaction may be present [2,3]. The predominantly lobular inflammation is usually of mixed type including mononuclear cells, polymorphonuclear leukocytes, clustered Kupffer cells and occasional microgranulomas. Hepatocytes in zone 3 are often surrounded by a rim of collagen (pericellular fibrosis; Fig. 1B) or are replaced by fibrous tissue (central sclerosis), eventually with obliteration of the central vein. Pericellular fibrosis appears to be more pronounced in the presence of MDBs [18,19]. In the absence of active disease this fibrosis pattern suggests prior episodes of SH. With disease progression incomplete fibrous septa arise and bridging septal fibrosis and cirrhosis may follow [2].

In their morphologic appearance ASH and NASH closely resemble each other but in most instances the lesions in NASH are less severe than in ASH. For example, in NASH central sclerosis and veno-occlusive lesions are very rare, MDBs are less distinct and clusters of neutrophils are much less common than in ASH [2,3,10]. Hence, intense neutrophil granulocyte infiltration points to alcoholic etiology [2]. In this situation, about 25% of MDB-containing hepatocytes are associated with, or penetrated by, neutrophils (termed “satellitosis”; Figs. 1, 2).

Of note, immunohistochemistry revealed a correlation of MDBs and satellitosis with TNF-α and IL-1 expression [22]. Pediatric NASH may differ from the adult form by showing either panacinar or periportal steatosis, increased portal mononuclear inflammation and fibrosis, few polymorphonuclear leukocytes, minimal or no hepatocyte ballooning or MDBs, particularly in younger children [2,10,23].

3. Animal models

NAFLD/NASH animal models are classified according to etiology as nutritional (dietary), toxin-induced and genetic, respectively, or constitute a combination of these factors. This review focuses on commonly used mouse models [24–47].

The more frequently used models are summarized in Table 1, whereas less common models are listed in Table 2.

3.1. Nutritional (dietary) models

Dietary models intend to reproduce a metabolic syndrome-like situation but most of them show differences to human disease regarding

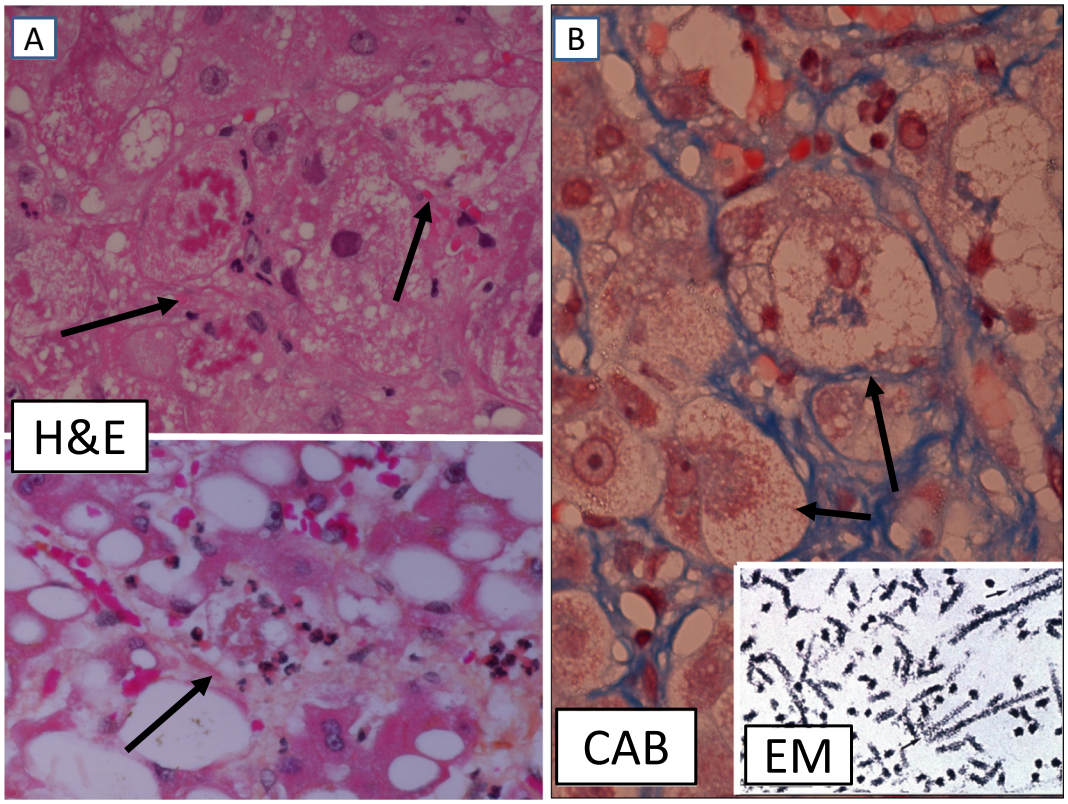

Fig. 1. Morphologic key features of steatohepatitis. A, Ballooned hepatocytes with large nuclei and prominent nucleoli containing MDBs in the upper part of the figure (arrows); in the lower part a ballooned hepatocyte is surrounded and penetrated by inflammatory cells (mostly neutrophils) resembling satellitosis (arrow); hepatocytes show macrovesicular steatosis (H&E staining). B, Ballooned hepatocytes some of them containing MDBs with pericellular fibrosis (arrows; CAB staining). Inset in B depicts the filamentous ultrastructure of the MDB (EM, [electron micrograph]).

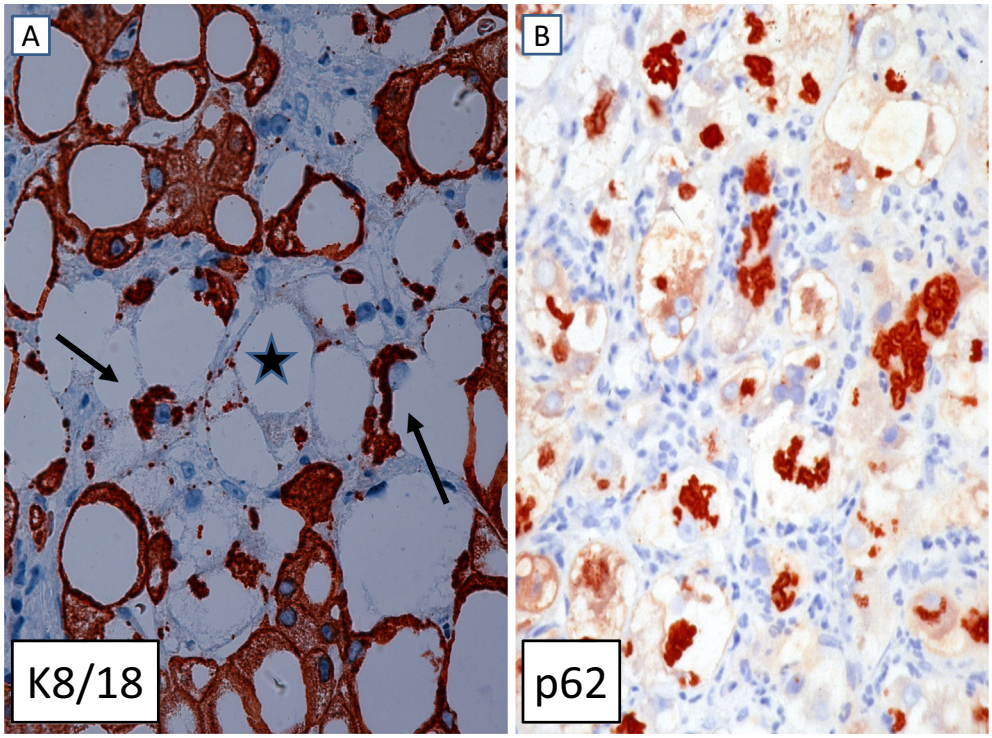

Fig. 2. A, Ballooned and MDB-containing (arrows) hepatocytes lack keratin immunostaining (asterisk). MDBs are immunostained for keratin (arrows). Steatotic hepatocytes at the periphery are keratin-positive (immunohistochemistry with antibodies to K8/K18). B, Immunohistochemical staining with p62 antibodies proves p62 content of MDBs.

clinical, metabolic or morphologic aspects. The convergence of dietary manipulations with a specific genetic background often exaggerates liver damage.

3.1.1. Methionine-and choline-deficient (MCD) diet [8,27,28,31,35,37,38,48–58]

This diet contains high amounts of sucrose (40%) and fat (10%) but lacks methionine and choline. The latter substances are required for hepatic secretion of triglycerides (TGs) as very low density lipoproteins (VLDL) and are also involved in mitochondrial fatty acid (FA) β-oxidation [28,37].

In contrast to human NASH, MCD-diet-fed mice lack increased FA plasma levels and lose weight. However, by modification of the diet the relevance for human disease can be improved. For example, in the semisynthetic choline-deficient L-amino acid-defined (CDAA) diet proteins are substituted by L-amino acids; after prolonged CDAA feeding the mice develop obesity, IR and elevated plasma TG and cholesterol levels [31,35].

As shown by Ishioka et al. [49] the composition of the gut microbiome is markedly altered in mice fed MCD or CDAA diet. Lipopolysaccharides (LPS) administered to MCD-diet-fed mice induced TNF-α production by Kupffer cells and increased fibrogenesis by hepatic stellate cells [50]. Dietary fructo-oligosaccharides (FOS) restored gastrointestinal microbiome and intestinal barrier leading to a decrease of CD 14-positive Kupffer cells and improved liver injury [48]. Moreover, activation of the hedgehog (Hh) pathway promoted natural killer T (NKT) cell enrichment in the liver followed by hepatic stellate cell activation and fibrogenesis [8,57].

The severity of liver damage induced by MCD or related diets depends on gender, mouse strain and duration of feeding and is exaggerated by additional high fat (HF) diet administration [31,35,38,48]. Morphologic features are macrovesicular steatosis, perisinusoidal fibrosis, mitochondrial abnormalities, hepatocyte ballooning, apoptosis and necroinflammation; proinflammatory and

Table 1
Morphologic characteristics of common NASH models.

Model	Etiol.	IR	Stea.	Ball.	MDBs	Infl.	Fibr.	References
MCD	D	+[a]	+	+	−	+	+	[8,27,28,37,38,48–58]
CDAA	D	+[a]	+	+	−	+	+	[31,35,49]
HF	D	+	+	+/−	−	+/−	+/−	[28,31,35–37,39,41,45,47,49,59–85,89]
HF, fructose	D	+	+	+	−	+	+	[28,31,41,76,77,79,80]
HF, fructose, cholesterol	D/G	+	+	+	+	+	+	[31,59,64,78]
Atherogenic	D	+[a]	+	+	−	+	+	[27,28,81–83,86,87,90]
Atherogenic/HF	D	+	+	+	+	+	+	[81,86]
DDC	T	n.d.	+	+	+	+	+	[15,16,92–105]
Fech/fech	G	?	+	+	+	+	?	[96,99]
Ob/ob	G	+	+	+	−	+	−	[34,108,116,118]
Db/db	G	+	+	+	−	+	−	[24,28,36,119]
Ob/ob, HF, fructose, cholesterol	G/D	+	+	+	−	+	+	[33,64,109]
Db/db, HF, iron or MCD	G/D	+	+	+	−	+	+	[24,28,36,119]
$K18^{-/-}$	G	n.d.	+	+	+	+	+	[120]

Etiol., etiology (D: dietary; G: genetic; T: toxin-induced); IR, insulin resistance; Stea., steatosis; Ball., ballooning; Infl., inflammation; Fibr., fibrosis; for abbreviations of the models, see text.

[a] Only hepatic.

Table 2
Morphologic characteristics of less common NASH/NAFLD models.

Model	Etiol.	IR	Stea.	Ball.	MDBs	Infl.	Fibr.	References
NSTZ/Diabetes +HFD	T/D		+	+	−	+	+	[31,90]
MSG	T	+	+	?	+	+	+	[90,106]
JVS	G	−	+	−	−	−	−	[32,36]
AOX deficiency	G	−	+	+[27]/−[28]	−	+	−	[27,28]
MTPα mutation	G	+	+					[36]
PPARα deficiency. HF/Starved	G/D	−	+	+	−	+	+[42]/−[28]	[28,37,54,67,70,112]
Aromatase deficiency	G	−	+	−	−	−	−	[36]
SREBP-1c transgenic	G	+	+	+	−	+	+	[27,28,36,37,121,122]
KK-Ay/a (+/−MCD)	G/D	+	+	+[a]	+[a]	+[a]	+[a]	[36]
ALR deficiency	G		+			+	+	[123]
NEMO deficiency	G	n.d.	+	+	−	+	?	[124]
CD36 deficiency	G	+	+			+		[36]
STAT5B deficiency	G	−	+	−	−	−	−	[36,37]
MAT1A deficiency	G	−	+	+	−	+	+	[27,42]
PTEN deficiency	G	−	+	+	−/+[27]	+	+	[27,28,125,126]
TSOD	G	?	+	+	+	+	+	[90]
PEMT deficiency	G		+					[127]
Foz/foz	G	+	+	−	−	−	−	[24,36]
Foz/foz +HFD	G/D	+	+	+	−	+	+	[24]

Etiol., etiology (D: dietary; G: genetic; T: toxin-induced); IR, insulin resistance; Stea., steatosis; Ball., ballooning; Infl., inflammation; Fibr., fibrosis; ?: not described. For abbreviations of the models, see text.
[a] With MCD diet.

profibrogenic cytokines, COX-2 and macrophage chemotactic protein-1 (MCP-1) are responsible for accumulation and activation of neutrophils and mononuclear cells. However, MDBs as defined by light microscopy, electron microscopy and immunohistochemistry have not been reported. In mice fed choline (CD)- and choline/cysteine (CCD)-deficient-diet ballooned hepatocytes with increased nuclear size and MDBs were observed in H&E-stained sections but not analysed regarding ultrastructure and protein composition [38,51,58]. LPS administration in addition to MCD diet significantly upregulated TNF-α production and induced NASH-like lesions but without MDB formation [50]. MCD diet fed to p16-deficient and PPAR-α-deficient mice, respectively, enhanced oxidative stress as well as steatosis and inflammation but without MDB induction [54,56].

3.1.2. Fat-enriched diets: high fat and high fat/fructose/sucrose diet (+/−cholesterol) [28,31,35–37,39,41,45,47,49,59–85]

These diets vary in their content of fat and additional ingredients; with the combination of fat, sugar and cholesterol they resemble Western style/fast food nutrition prone to development of NAFLD [36,37]. The American Lifestyle Induced Obesity Syndrome (ALIOS) diet is enriched in trans-fats (30% of fat content) and fructose (applied by corn syrup-containing drinking water) [76]; the Amylin Liver NASH (AMLN) diet contains cholesterol (2%) in addition [31,59,64].

Content and type of fat, composition of the diet, duration of the dietary regimen as well as gender, species, genetic background/strain and physical activity (particularly sedentary behavior) determine the metabolic and morphologic consequences. Unsaturated FAs are more harmful than saturated ones [77]. Lard has more pronounced adverse effects on hepatic insulin sensitivity than palm oil [35,36]. Sources of oxidative stress as important player include CYP2E1, NADPH oxidase, mitochondrial dysfunction and cytokines. Hepatic mitochondrial damage causes ATP depletion in addition to ROS production [66,73]. Endotoxin levels in the portal blood are increased due to bacterial overgrowth and increased gut permeability [75,78]. Fructose significantly contributes to obesity, IR and oxidative stress [31] and promotes expression of proinflammatory cytokines via increased intestinal translocation of bacterial endotoxin and Kupffer cell activation [41].

Diets rich in fat without other ingredients induced only mild SH and minimal fibrosis, lard enhanced steatosis and fibrosis [35,36]. Addition of fructose exacerbated liver damage with progressive fibrosis and cirrhosis together with increased TNF-α levels, whereas sole application of fructose resulted in mild steatosis only [28,41,77,79,80]. MDBs were absent in most studies under these dietary conditions [39,41,49,59–67,69,73,76,77,79,81–83,85–88] and only rarely their presence was described on the basis of H&E staining but without further immunohistochemical characterization [68,72].

Interestingly, Asgharpour et al. [78] reported a unique isogenic mouse strain (B6/129) derived from C57JBl/6J and 129S1/SvImJ strains fed high fat/high fructose/glucose/cholesterol diet for 24–52 weeks that reproduced, in addition to characteristic metabolic, transcriptomic and cell signaling alterations, the key pathological features of human NASH including hepatocyte ballooning with decreased K18 immunostaining and MDB formation in contrast to the parent strains.

HF-diet-induced steatosis primed the liver to additional insults: (i) Deletion of PPARα increased the susceptibility to liver disease by HF feeding [73]; (ii) gold thioglucose, which causes damage in the ventromedial hypothalamus resulting in hyperphagy and obesity, enhanced severity of NASH-like lesions including hepatocellular ballooning and MDBs (as shown in H&E stained liver sections without further characterization) in lard-fed mice [74]. (iii) A single intraperitoneal injection of diethylnitrosamine (DEN) resulted in hepatocyte ballooning and MDB formation in HF-diet-fed mice, whereas MDBs were absent in DEN-treated mice on standard diet [72]. (iv) Intraperitoneal injection of tetracycline increased steatosis and expression levels of proinflammatory cytokines in HF-diet-fed mice [71]. (v) LPS in low doses led to severe fibrosis and liver injury in HF-diet- but not in chow-fed mice [36]. (vi) Application of a low dose of CCl_4 together with a nuclear liver X receptor (LXR) agonist in combination with HF diet induced NASH-like morphology including hepatocyte ballooning with MDBs as reported on the basis of H&E-stained sections without additional immunohistochemical characterization [89].

3.1.3. Atherogenic (high cholesterol and high cholate) diet

This diet contains cholesterol (1–1.25%) and cholate (0.5%) and thereby emulates some etiologic aspects of human NASH. However, the resulting weight loss, attenuation of IR and lowered serum TG levels are not found in human NASH. Conversely, combination with HF diet (e.g. addition of 60% fat derived from cocoa butter) induced hepatic IR and NASH-like lesions, including MDBs [81] in association with ballooned hepatocytes, after prolonged feeding [86]. Down-regulation of genes of antioxidant enzymes accompanied disease progression indicating the

synergism of dietary fat overload and cholesterol. In line with these observations, oxidative stress and mRNA levels of inflammatory cytokine-, innate-immunity- and fibrogenesis-related genes were significantly elevated in livers of diet-treated Nrf2-deficient mice [28,82,83,86,87,90].

3.2. Toxin-/drug-induced models

3.2.1. Drug-induced steatosis/SH

Adverse drug reactions may lead to steatosis and steatohepatitis [91]. Such animal models are discussed in detail by H. Jaeschke in this issue.

3.2.2. Porphyrinogenic agents (DDC and GF) as inducers of NASH-like liver lesions

Intoxication of mice with the porphyrinogenic agents griseofulvin (GF) or 3,5-diethoxycarbonly-1,4-dihydrocollidine (DDC) causes protoporphyria with porphyrin pigment deposition in hepatocytes, macrophages and bile duct lumina and, after prolonged (> 2 months) treatment, leads to SH with mild macrovesicular steatosis, predominantly centrilobular hepatocyte ballooning, MDB formation, spotty hepatocyte necroses and variable (mild or missing) neutrophil-granulocytic infiltration resembling satellitosis [14–16,92]. Murine MDBs contain keratin, p62, and ubiquitin and show filamentous ultrastructure ([15,16,92]; Fig. 3).

As in human ASH and NASH, immunohistochemistry using keratin antibodies revealed derangement or even disappearance of the hepatocytic keratin IF cytoskeleton concomitant with MDB formation. Liver tumors often observed in long-term GF- or DDC-treated mice expressed MDBs in greater numbers than non-neoplastic hepatocytes [15,16]. MDB formation is associated with increased keratin synthesis, disturbance of the 1:1 relationship of keratins type I (K18) and II (K8) with excess of K8 [15,16]. Keratin (particularly K8) aggregation seems to be the initial step in MDB formation, at least in the DDC mouse model, followed by co-aggregation of additional components, like ubiquitin, the stress and adapter protein p62 and heat shock proteins. P62 seems to be responsible for enlargement and stabilization of MDBs [93]. Since both porphyrins and retained bile acids induce oxidative stress two mechanisms may cooperate in the development of the SH-like phenotype in the DDC/GF model, namely porphyrin- and cholestasis (bile acid)-induced oxidative stress [94–96]. Transglutaminase 2 (TG2)-mediated cross-linking also plays a role in the aggregation process and TG2-deficient mice were unable to develop MDBs [15,16]. Malondialdehyde might also contribute to MDB formation since it cross-linked K8 and disrupted the keratin IF network in isolated hepatocytes [97].

The combination of DDC intoxication and fat-enriched diet significantly boosted steatosis and MDB induction [98]. Moreover, older age predisposed to MDB formation [99], possibly due to elevated oxidative stress combined with decreased protein degradation [99,100].

DDC-induced cytoskeletal alterations and MDBs disappeared within about 1 month of recovery on normal diet but rapidly reappeared (within 3–4 days) when re-challenged with DDC and a variety of other agents, including alcohol. This is reminiscent of an anamnestic reaction ("toxic memory") in which oxidative stress and impairment of cellular rescue mechanisms (e.g. impaired chaperone action and proteolysis) may be involved [15,16,101,102].

According to recent studies, downregulation of PPAR-α is intimately associated with DDC-induced murine SH leading to decreased FA metabolism and delayed stress response; by administration of the PPAR-α agonist fenofibrate oxidative stress and inflammation were reduced, lipid metabolism was restored, and disruption of the IF cytoskeleton and MDB formation were prevented (Nikam, A. et al., in preparation).

In patients, the genetic background may determine the severity of NASH. In line with the clinical observation, strain differences regarding SH and MDB formation were also observed in chronically DDC-intoxicated mice disclosing high-, low- and non-responders [103,104]. Differences in gene expression and metabolic data provided hints concerning the pathogenic principle involved. In high responders particularly S-adenosylmethionine (SAMe) metabolism was dysregulated by DDC intoxication. As key methyl group donor for phosphatidylcholine synthesis, SAMe plays an important role in the export of VLDL from the liver and in glutathione synthesis. Moreover, prostaglandin E2 and TNF-α were upregulated in the sensitive strains in contrast to the resistant strain and this may explain the differences in inflammatory response [104,105].

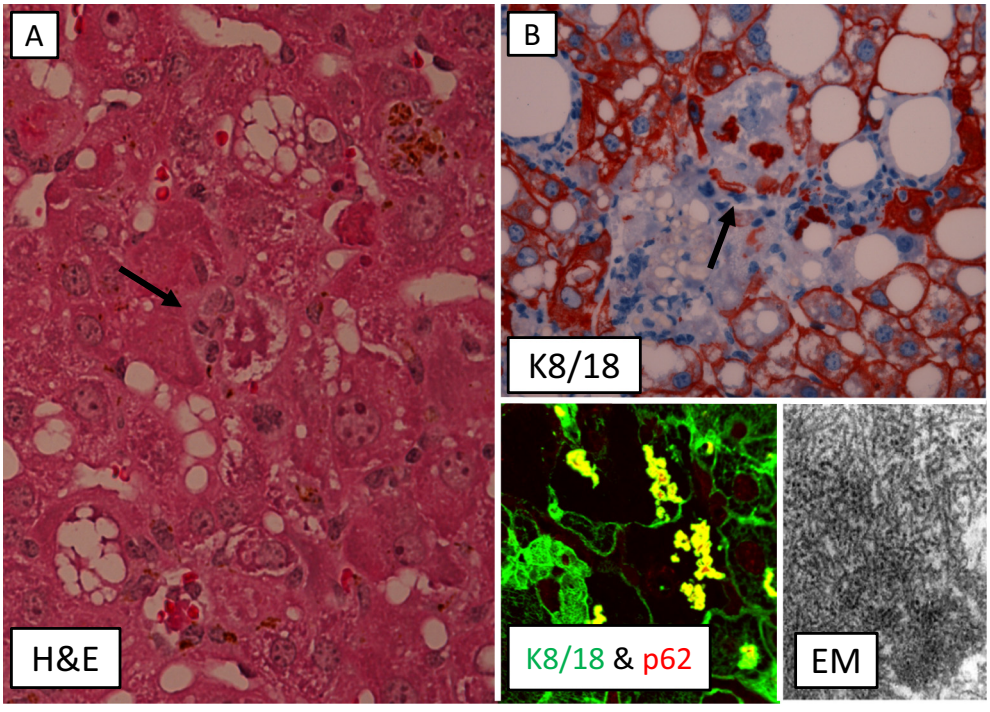

Fig. 3. Morphology of the DDC mouse model. A, DDC treatment of mice for 10 weeks leads to steatohepatitis, ballooning and MDB formation (arrow) [H&E: hematoxylin & eosin staining]. B, Like in human disease ballooned MDB-containing hepatocytes lack keratin immunostaining in contrast to MDBs (K8/K18; arrow). MDBs are immunostained with both keratin (green) and p62 (red) antibodies; p62- and keratin- positive MDBs (yellow) are present in keratin-negative ballooned hepatocytes (K8/K18 & p62). Filamentous MDB ultrastructure is identical to that in human disease (EM [electron micrograph]).

3.2.3. Less commonly used models

3.2.3.1. Neonatal streptozotocin (NSTZ)/Diabetes model. Intraperitoneal or subcutaneous injection of a low dose of STZ shortly after birth caused type 1 diabetes due to damage of the pancreatic islets. Feeding HF diet to STZ mice for 6 to 8 weeks led to hepatic steatosis, inflammation, ballooning (without MDB formation) and progressive pericellular fibrosis. Upon continuation of HF diet steatosis decreased but lobular inflammation with foamy macrophages and chicken-wire fibrosis increased. Eventually, liver tumors developed [31,90].

3.2.3.2. Monosodium glutamate (MSG). MSG applied as single dose (4 mg/g b.w.) within 5 days after birth was followed by obesity, oxidative stress, IR, type 2 diabetes, hyperinsulinemia and hyperlipidemia in 3–4-months-old mice. In the liver, marked microvesicular steatosis, centrilobular hepatocellular ballooning with MDBs, megamitochondria, scattered neutrophil granulocyte foci and mild perivenular fibrosis could be detected. However, MDBs were only diagnosed on the basis of H&E-stained sections without further characterization. In mice older than 10 months tumors ('hepatocellular adenomas' and 'carcinomas') were observed in the non-cirrhotic liver [90,106].

3.3. Genetic models

Based on their genetic background, transgenic or gene knock-out mice mimic the human metabolic syndrome with its consequences. In addition, the animals may be more sensitive to harmful external influences, such as diets, microbial components or drugs and toxins [32–34,37,107–109].

3.3.1. Ferrochelatase deficiency

These mice (fech/fech) carry an inactivating mutation of the ferrochelatase gene causing protoporphyria due to inhibition of the last step in heme biosynthesis similar to the DDC model [96]. At the age of around 20 weeks they spontaneously developed NASH-like liver disease with MDB formation concomitant with oxidative stress, mitochondrial dysfunction, reduced ATP production, increased protein oxidation, decreased proteasomal activity and upregulation of Nrf2 and several oxidative stress response genes. Biochemical prerequisites for MDB formation, including K8 overexpression and increased TG2 activity, were already present in younger animals before appearance of MDBs. In addition, fech/fech mice were more sensitive to DDC and intoxication for 3 weeks already sufficed to induce MDBs (instead of 6–8 weeks required in wild type animals). Since keratins and lamins aggregate upon exposure to protoporphyrin IX and other porphyrins, this mechanism may contribute to MDB formation in this model as well as in DDC/GF-induced protopophyria [96,103].

3.3.2. Impairment of FA oxidation

FA oxidation occurs in mitochondria (β-oxidation), peroxisomes (β-oxidation) and microsomes (ω-oxidation). Mutation-related impairment contributes to steatosis [32].

3.3.2.1. Juvenile visceral steatosis (JVS). This model is characterized by mutation of the carnitine transporter gene *Octn2* (organic cation transporter 2) resulting in systemic carnitine deficiency. Since carnitine is essential for the transport of FAs into the mitochondria for β-oxidation its deficiency causes impaired degradation of long-chain FAs (LCFAs). Shortly after birth the animals developed hypoglycemia, hepatomegaly, steatosis and increased hepatocyte proliferation [32,36].

3.3.2.2. Fatty acyl-CoA oxidase (AOX) deficiency. AOX is the rate limiting enzyme of peroxisomal β-oxidation of LCFAs. Two to 4 months old AOX-deficient mice showed transient diffuse steatosis, spotty hepatocyte drop-out and mainly neutrophil granulocytic inflammation. Steatotic hepatocytes were, however, replaced by fat-free cells in 6 to 8 months old animals. While peroxisomes were rare in steatotic hepatocytes, their number increased in regenerating cells indicating activation of PPAR-α. Later non-metastasizing liver tumors developed [27,28].

3.3.2.3. Heterozygous mutation of Mitochondrial Trifunctional Protein (MTPα). Mutation of this key enzyme of mitochondrial β-oxidation is associated with elevation of serum ALT, IR, hyperinsulinemia, impaired glucose tolerance and progressive hepatic steatosis [36].

3.3.2.4. Deficiencies of peroxisome proliferator-activated receptors (PPARs). PPARs are nuclear receptors that regulate genes responsible for FA uptake, transport, intracellular binding, storage and catabolism. They are activated by a variety of exogenous ligands, including drugs (fibrates); meanwhile endogenous ligands have been described (reviewed in [110,111]) that link these receptors to lipid metabolism and antioxidant stress response. In mouse models, both simple steatosis and SH are associated with reduced PPAR-α [54,67,112,113] (Nikam, A. et al., in preparation), in contrast to the human situation, where only SH, but not simple steatosis is associated with reduced PPAR-α expression and activity [114,115].

The liver-enriched transcription factor PPAR-α counteracts obesity-induced chronic inflammation by reducing hepatic fat content and down-regulation of inflammatory genes [67]. In addition, it inhibits NF-κB signaling and its consequences [34,37,116,117]. PPAR-α-deficient mice, when stressed by starvation or chronic ingestion of HF diet, reacted with upregulation of many inflammatory genes, pronounced centrilobular macro/microvesicular steatosis, SH, hepatocyte apoptosis and ballooning without MDB formation [28,37,54,67,70,112].

PPAR-γ is predominantly expressed in adipose tissue, colonic epithelium and macrophages whereas its level is very low in normal liver. In several murine obesity and diabetes models overexpressed PPAR-γ is linked to lipogenesis; the expression of its target gene *Srebp-1c* is increased in HF-fed animals. PPAR-γ deficiency in adipose tissue results in fatty liver and increased gluconeogenesis. Mice fed fat (predominantly saturated FAs from lard)-enriched diets developed obesity, IR and steatosis concomitant with elevated proinflammatory cytokine levels and activation of hepatic stellate cells [37].

3.3.2.5. Aromatase-deficiency. Aromatase is a key enzyme in estrogen synthesis. Female aromatase-deficient C57BL/6J mice gradually accumulated abdominal fat and developed hyperinsulinemia and hepatic microvesicular steatosis in zones 2 and 3 due to impaired hepatic mitochondrial and peroxisomal β-oxidation of FAs, but without necroinflammation and MDB formation. Impairment of β-oxidation could be corrected by 17β-estradiol administration. Moreover, treatment with the peroxisomal proliferator benzafibrate restored PPAR-α function and improved steatosis [36].

3.3.3. Impairment of leptin function

The peptide hormone leptin is produced by adipocytes and participates in the hypothalamic regulation of feeding behavior by reducing food intake. Leptin function can be thwarted by mutation of the gene resulting in a truncated inactive product (in ob/ob mice) or by resistance to leptin action due to mutation of the receptor gene (in db/db mice, Zucker rats). Leptin interacts with several nuclear receptors, including glucocorticoid receptor, hepatocyte nuclear factor (HNF) 4α and the PPARs. It is involved in fibrogenesis and modulation of innate and acquired immunity by its effect on maturation and activation of lymphocytes and macrophages. The antisteatotic effect of leptin depends on PPAR-α [37,118].

3.3.3.1. Ob/ob mice. This mouse model simulates many aspects of the metabolic syndrome in humans. However, in contrast to ob/ob mice, leptin plasma concentrations in NASH patients are higher than in control subjects. Ob/ob mice with free access to standard chow are

inactive, hyperphagic and extremely obese; they exhibit hyperglycemia, IR and hyperinsulinemia, develop intestinal bacterial overgrowth and overexpress TNF-α. TNF-α causes lipolysis of adipose tissue with increased delivery of LCFAs to the liver. In addition, SREBP-1c is activated and accumulates in hepatocyte nuclei promoting de novo synthesis of FAs. The increase of mitochondrial uncoupling protein 2 (UCP-2) impairs ATP synthesis and makes hepatocytes more vulnerable [34,108].

Ob/ob mice fed normal chow develop fatty liver without fibrosis. To trigger progression to SH additional insult(s) is/are necessary, such as feeding MCD or fat-enriched diet, ethanol exposure, ischemia-reperfusion injury or LPS toxicity. The fact that these mice were still protected against fibrosis proves leptin's function in hepatic fibrogenesis [116]. However, feeding a HF diet containing fructose and cholesterol to ob/ob mice caused fibrosis in addition to severe steatosis, inflammation and ballooning [64]. Treatment of ob/ob mice with TNF-α antibodies alleviated NASH [33,109]. Moreover, norepinephrine increased the number of stellate cells, up-regulated TGF-β mRNA levels, elevated hepatic collagen mRNA expression and induced diffuse perisinusoidal fibrosis [33]. PPARα-deficient ob/ob mice developed excessive obesity and steatosis without MDBs due to decreased FA oxidation [116,118].

3.3.3.2. Db/db mice. This model simulates human metabolic syndrome in many but not all its aspects. Db/db mice exhibit normal or elevated leptin levels but are resistant to the leptin effect due to mutated leptin receptor. The animals are hyperphagic, develop early onset obesity, hyperglycemia, IR, hyperinsulinemia and macrovesicular steatosis without spontaneous progression to SH and fibrosis. Additional insults, such as trans-fat or MCD diet feeding or iron supplementation, are required to trigger oxidative stress and NASH-like morphology, characterized by hepatocellular ballooning (without MDBs), fibrosis due to activation of stellate cells, increased ALT and TNF-α levels, impaired hepatic mitochondrial fatty acid β-oxidation and reduced hepatic antioxidant levels, together with overexpression of genes responsible for inflammation, fibrogenesis and lipid synthesis and storage. Transition from fatty liver to NASH was associated with SAMe depletion [24,28,36,119].

3.3.4. Keratin 18 deficiency and keratin 8 overexpression

Aged (18–20 months old) K18- deficient mice develop SH-like liver morphology with steatosis, spotty hepatocyte necrosis, hepatocyte ballooning, MDB formation and fibrosis which is preceded by simple macrovesicular steatosis (Fig. 4). Immunohistochemically, MDBs resemble those in human disease (Fig. 5). The appearance of liver tumors preferentially in males underlines the relevance of this model for human NASH [120].

3.3.5. SREBP-1c-transgenic mice

Lipid homeostasis in mammalian cells is controlled in a feedback regulatory way by sterol regulatory element-binding proteins (SREBPs) which are membrane-bound transcription factors. The isoform SREBP-1c is insulin-dependent and regulates genes required for synthesis and uptake of cholesterol, FAs, TGs and phospholipids as well as glucose metabolism. In transgenic mice selective overexpression of SREBP-1c in adipose tissue reduces its differentiation. The animals develop severe IR and hepatic steatosis, lobular inflammation, ballooned hepatocytes and perivenular/pericellular fibrosis similar to human NASH. Ballooned hepatocytes with MDBs have also been described by some authors in older mice, but further characterization regarding morphology, ultrastructure and composition was not provided [121]. Liver damage was aggravated by feeding HF diet [27,28,36,37,121,122].

3.3.6. Less common genetic models

3.3.6.1. KK-Ay/a mice. These mice carry a heterozygous mutation in the *agouti* gene and are hyperphagic due to diminished hypothalamic suppression of food intake. They develop obesity, IR, hyperglycemia, and steatosis. SH can be triggered by additional stimuli such as feeding MCD diet [36].

3.3.6.2. Augmenter of liver regeneration (ALR) deficiency. ALR is a widely distributed pleiotropic protein originally identified as hepatic growth factor. It is essential for hepatic mitochondrial function and lipid homeostasis. Two weeks after birth, deficient mice develop mitochondrial defects resulting in low ATP levels, increased oxidative stress, excessive steatosis, hepatocyte apoptosis and stellate cell activation. After temporary improvement hepatic inflammation, hepatocellular necrosis, ductular proliferation, fibrosis and neoplastic nodules resembling 'hepatocellular carcinoma' were observed [123].

3.3.6.3. NEMO deficiency. The IκB kinase subunit NEMO/IKKγ is essential for activation of the transcription factor NF-κB that regulates cellular responses to inflammation. Hepatic NEMO-deficient mice developed NASH-like liver disease with inflammation, mitochondrial abnormalities, hepatocyte ballooning (without MDB formation) and increased hepatocyte apoptosis followed by oval cell proliferation and hepatocellular carcinoma. Moreover, the liver was sensitized to LPS toxicity reflected by increased expression of TNF-α and other cytokines. Liver damage could be prevented by antioxidants or genetic ablation of FADD indicating that oxidative stress- and death receptor-mediated death of NEMO-deficient hepatocytes is involved [124].

3.3.6.4. Fatty acid translocase (CD36) deficiency. The transmembrane protein CD36 is an important FA transporter expressed in peripheral tissues, including muscle and adipose tissue. Elevated circulating LCFAs and TG levels, hepatic IR, steatosis and inflammation were observed in CD 36-deficient mice [36].

3.3.6.5. STAT 5B deficiency. The ubiquitous transcription factor Stat 5B is a substrate of the insulin and the growth hormone receptor and activated by a variety of cytokines and growth factors. Promoter elements of certain lipogenic genes contain Stat 5B binding sites. STAT 5B-deficient mice developed obesity, IR, hyperglycemia, hyperlipidemia and steatosis without inflammation or fibrosis [37]. Studies have shown that some of the suppressors of cytokine signaling proteins are impaired in the livers of STAT5B-deficient mice [36].

3.3.6.6. Methionine adenosyltransferase-1A (MAT1A) deficiency. MAT1A is a liver-specific rate limiting enzyme of methionine metabolism and catalyzes the synthesis of SAMe [28]. Deficient mice have decreased levels of antioxidants, including glutathione, and decreased expression of genes involved in lipid peroxidation [42]. The animals were hyperglycemic but with normal insulin levels and did not show other features of metabolic syndrome. At 4–8 months of age SH and also liver tumors arose spontaneously [27].

3.3.6.7. Phosphatase and Tensin Homolog (PTEN) deficiency. PTEN acts as tumor suppressor and negative regulator of several cellular signaling pathways involved in apoptosis, cell proliferation and tumor formation [28]. Overexpression of adipogenic, lipogenic and β-oxidation-related genes was demonstrated [125]. In contrast to the human situation liver-specific PTEN-deficient mice show decreased body fat mass and hypersensitivity to insulin [27]. The morphologic liver phenotype starts with simple steatosis and by 40 weeks the lesions closely resemble human NASH with macrovesicular steatosis, hepatocyte ballooning, lobular inflammation, perisinusoidal fibrosis and finally development of liver tumors ('hepatocellular carcinomas'). MDBs have been described on the basis of H&E-stained tissue sections only [125,126].

3.3.6.8. Tsumura-Suzuki Obese Diabetes mice (TSOD). TSOD mice are obese with hyperglycemia, glucosuria, hyperinsulinemia and hyperlipidemia. Increased levels of TNF-α and IL-6 are released from

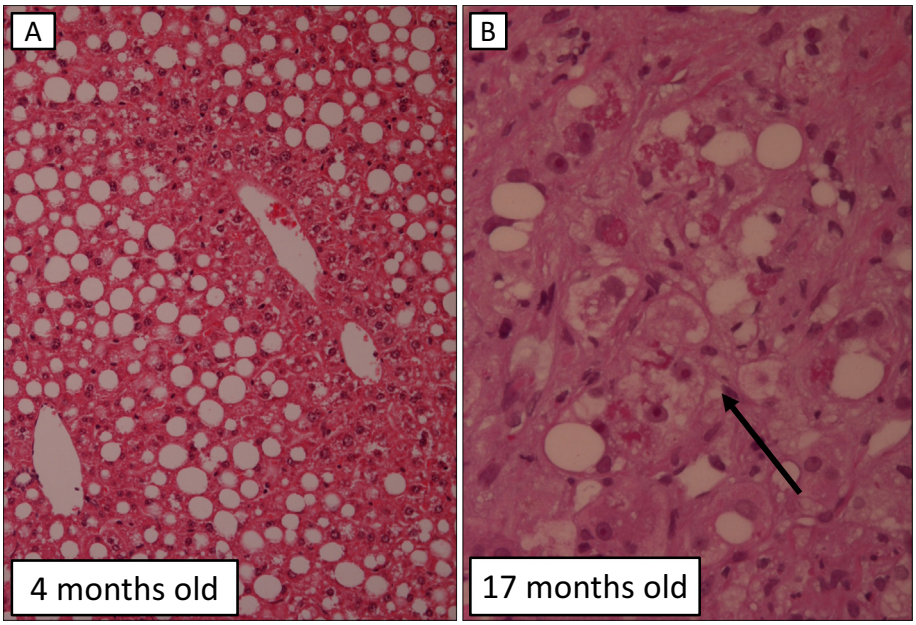

Fig. 4. Liver histology of K18-deficient mice. A, Young (4 months old) keratin 18-deficient mice show macrovesicular steatosis, B, old (17 months) mice develop steatohepatitis with steatosis, hepatocyte ballooning and MDB formation (arrow).

visceral adipose tissue by injured lipocytes and macrophages. In 6 months old mice steatosis, hepatocyte ballooning, MDBs, lobular neutrophil inflammation and mild perivenular and pericellular fibrosis, and in 10 months old and older animals liver tumors, resembling in their morphology and immunostaining human hepatocellular adenomas and carcinomas have been described [90].

3.3.6.9. Phosphatidylethanolamine-N-methyltransferase (PEMT) deficiency. The deficient mice were protected from obesity and IR when fed HF diet but developed severe steatosis mainly due to inadequate secretion of VLDL particles [127].

3.3.6.10. Foz/foz mice. They carry a mutated *alstrom syndrome 1* (*Alms 1*) gene which encodes a protein present in the basal body of the primary cilium and also plays a role in intracellular transport and appetite regulation. Foz/foz mice are obese and hyperphagic with IR, reduced adiponectin levels, hypercholesterolemia and steatosis. HF diet promoted progression to NASH with severe fibrosis in a strain-dependent manner caused by inhibition of PPAR-α-mediated peroxisomal FA oxidation [24,36].

4. Relevance of animal models in human NAFLD/NASH research

A variety of interacting genetic and environmental factors determine human NAFLD/NASH. To obtain firm insights into the pathogenesis of the disease it is important to reproduce pathologic situations

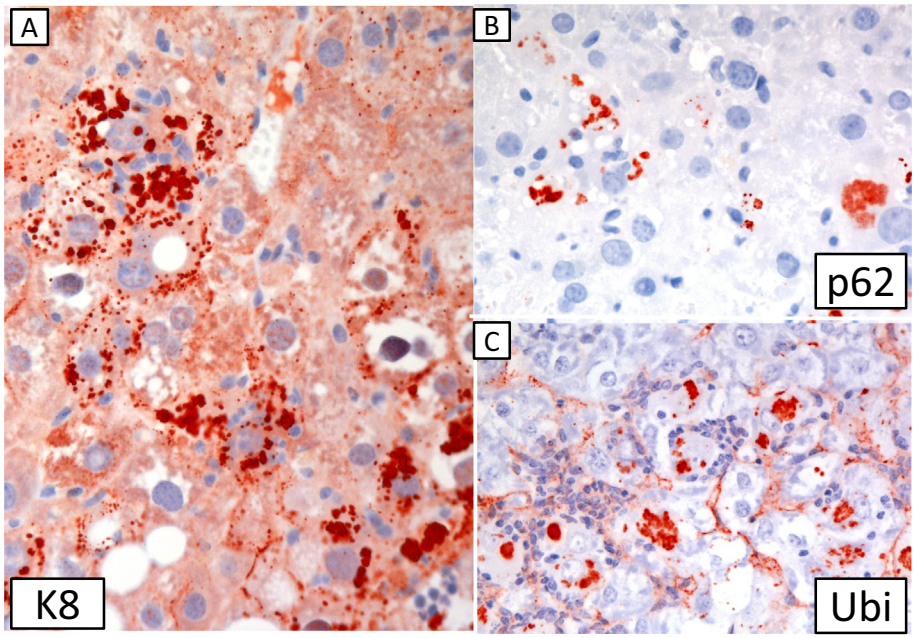

Fig. 5. Immunohistochemical staining of livers of old K18-deficient mice. MDBs (partly granular) in old keratin 18-deficient mice are immunostained with antibodies to keratin 8 (A; K8), p62 (B; p62) and ubiquitin (C; Ubi [Ubiquitin]) indicating the presence of these antigens in MDBs.

under standardized conditions in vitro, in isolated perfused organs and/or in whole animals [36]. Considerable efforts have been made to generate mouse models, since mice share many physiological, anatomical and metabolic features with humans and are particularly suitable for genetic manipulations.

As evident from this and other reviews, the ideal animal model, that resembles human NAFLD/NASH in clinical manifestations, etiology, pathophysiology and pathomorphology, does not exist. Since experimental systems reproduce only certain aspects of human disease it is necessary to focus on decisive features and interpret them appropriately [31,36].

Many discoveries in NAFLD/NASH research are based on dietary models or leptin-deficient (ob/ob) or leptin-resistant (db/db) rodents [32,34]. Feeding fat-enriched diets with or without additives produces features closely resembling human metabolic syndrome and its pathophysiologic consequences. The MCD model differs in its clinical consequences from human disease since the mice lose weight and lack IR. Although the leptin-related models imitate human metabolic syndrome, leptin or leptin receptor mutations do not prevail in NASH patients and leptin levels only poorly correlate with progression of simple steatosis to NASH [28]. Striking is the lack of clear-cut MDB formation in most models, which together with hepatocyte ballooning is regarded as diagnostically and prognostically important morphologic feature in human disease. Although ballooning and MDBs are mentioned in some reports they were usually not adequately characterized. To unequivocally identify MDBs, light microscopy should be complemented by immunohistochemistry using antibodies to keratins and/or p62 and/or ubiquitin. Consequently, only the chronic DDC and GF intoxication and the K18 deficiency models, although etiologically different from human NASH, resemble the entire morphologic spectrum of human NAFLD/NASH [15,16,120]. Since the keratin IF cytoskeleton is evolutionary highly conserved and keratins as major components of MDBs are in addition to their mechanical functions involved in a variety of vital cellular pathways [95], it can be expected that mechanisms leading to MDB formation, irrespective of their cause, are particularly relevant to the understanding of essential pathogenic principles in NASH.

5. Pathogenesis of key morphologic features of NASH as revealed by human and animal studies

5.1. Hepatocyte injury

Hepatocytes are the primary targets in NAFLD and their damage triggers the inflammatory response.

5.1.1. Steatosis

Hepatic steatosis results from imbalance between import or synthesis versus catabolism or export of FAs in the liver [13,34,36,128,129]. Excess dietary lipid supply or release from peripheral adipose tissue, when storage capacity is overwhelmed, increase FA inflow into the liver where they are either esterified to TGs, oxidized or secreted as VLDLs [6,130]. When mitochondrial oxidative capacity (i.e. PPAR-α-regulated mitochondrial β-oxidation) is exceeded, alternative pathways in peroxisomes (β-oxidation) and endoplasmic reticulum (microsomal ω-oxidation) take over. Thus, mitochondria, microsomes and peroxisomes cooperate in hepatic FA metabolism and excessive steatosis develops when these systems fail. FA oxidation generates ROS, and an adaptive response to the detrimental effects of oxidative stress is activated [32].

Increased ROS production is an early event in steatotic liver and precedes NASH. Fat-laden hepatocytes resemble replicatively senescent cells. Despite oxidative DNA damage most hepatocytes are viable due to their ability of compensating oxidative stress by mechanisms that limit ROS production and enhance tolerance to ROS [5,33]. However, insufficient adaptation results in lipotoxicity and cell damage [6,13,130].

5.1.2. Hepatocyte ballooning, MDB formation and disturbance of the keratin IF cytoskeleton

MDBs present in ballooned hepatocytes are morphologic hallmarks of, but not restricted to, ASH and NASH. They also occur in Wilson's disease, chronic cholestasis (e.g., primary biliary cirrhosis), Indian Childhood Cirrhosis and other types of copper toxicosis, chemical and drug toxicity and in hepatocellular carcinomas [15,16,131]. Of note, MDBs and morphologic steatohepatitic features are also found in about 15% of patients with chronic hepatitis C [20]. The association of MDBs with etiologically different chronic liver diseases suggests similarities in pathogenic pathways. Oxidative stress might be a common denominator. MDBs are indicators of disease severity and progression [18–21]. However, it is as yet unclear whether they are only markers of a specific type of cell injury or harmful per se.

The central role of overexpressed K8 without its type I partner K18 in MDB formation is supported by several observations (reviewed by Strnad et al., [15,16]): (i) MDB formation in the $K18^{-/-}$ model starts with small K8 aggregates which then coalesce to larger inclusions with MDB-typical features, i.e., immunoreactivity with the MDB-specific antibody M_M 120-1 and antibodies to p62, ubiquitin and heat shock proteins; (ii) K8-deficient mice fail to develop MDBs [132]; (iii) mice overexpressing K8 spontaneously develop MDBs [15,16]; (iv) HF diet triggers MDB formation and ballooning in transgenic mice overexpressing human K8 (K8tg) [98], whereas in non-transgenic mice HF diet feeding only results in macrovesicular steatosis and mild inflammation. Hepatic heat shock protein (HSP) 72 levels are lower under these experimental conditions which might render hepatocyte keratins more prone to misfolding [15,16,133]; (v) K8 in contrast to K18 is a preferred substrate for TG2-induced cross-linking; consequently, TG2-deficient mice fail to produce MDBs. Phosphorylation of K8 at S74 by p38 kinase facilitates TG2-mediated crosslinking. Moreover, mice that overexpress the phosphomutant K8S74A (or overexpress the human variant G62C) with a conformational change that blocks kinase access have decreased capacity for MDB formation [15,16,95]. Of note, drugs inducing SH in humans, like amiodarone, perhexiline maleate or 4,4`-diethylaminoethoxyhexestrol, trigger keratin cross-links in hepatocytes [97]. Fig. 6 depicts a schematic representation of the process of MDB formation.

According to their chemical composition MDBs represent an aggregation of keratins and stress proteins (i.e., p62, HSPs) resulting from overexpression of keratins with excess of K8, hyperphosphorylation and misfolding of K8 (i.e., acquisition of β-sheet conformation [133], decreased degradation and TG-mediated cross-linking [15,16]). Therefore, stressed MDB-producing hepatocytes have to be viable and competent to protein synthesis despite concomitant loss or at least disturbance of the IF cytoskeleton characteristic of ballooned hepatocytes; this is in line with the observation that MDB-containing ballooned hepatocytes contain large nuclei with prominent nucleoli typical of actively protein-synthesizing cells [134]. On that basis we propose that MDB formation reflects an adaptive response, by which protein synthesis is shifted to the production of stress-associated proteins, possibly at the expense of other proteins.

As “guardians of the cell” [135] keratins not only provide cell stability in their polymerized filamentous form but also take part in metabolic processes. Abnormal keratin phosphorylation due to activation of stress-induced kinases (“phosphate sink”) can be the consequence of cell injury and may also abrogate phosphorylation-dependent inactivation of other vital cellular functions [95,136]. Keratins protect hepatocytes from apoptosis in a pathway-dependent manner; for example, K18 mutations predispose to Fas- but not TNF-α-mediated apoptosis [95,136]. After initiation of apoptosis the type I K18 is subject to caspase-mediated proteolysis whereas K8 as type II keratin is resistant due to lack of a consensus caspase cleavage sequence. This is a possible explanation of the imbalance of K8:K18 ratio (inhibiting IF formation) to the advantage of K8 required for MDB formation [15,16]. Whether keratin deficiency in intestinal epithelium adversely affects

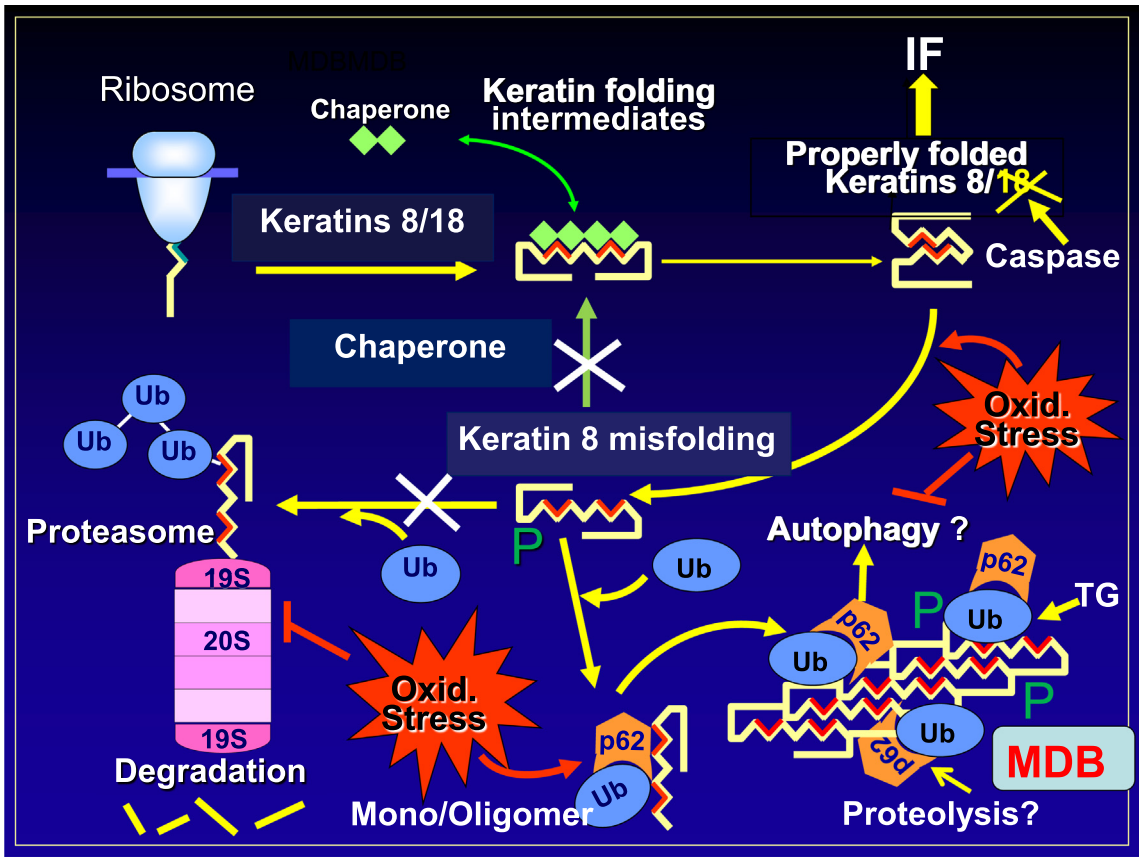

Fig. 6. Schematic representation of MDB development indicating the complex interplay between keratin synthesis, misfolding, decreased degradation and aggregation with oxidative stress as major player. Ub: ubiquitin; P: phosphorylated site; IF: intermediate filament; TG: transglutaminase.

the intestinal barrier function in K18-deficient mice as contributing factor to NASH deserves further studies [120].

5.1.3. Mitochondrial damage

In human NASH and related animal models mitochondrial dysfunction contributes to altered liver morphology. This includes (i) accumulation of lipids as a consequence of impaired mitochondrial metabolism – such as reduced β-oxidation, tricarboxylic (TCA) cycle, oxidative phosphorylation, or lipid export –with metabolic consequences, e.g. IR, and impact on cell survival, and (ii) morphologic changes of mitochondria indicating insufficient mitochondrial quality control resulting in redox imbalance, oxidative damage, and inflammation [137]. A typical example of these alterations are megamitochondria, observed in adult [138] and pediatric NASH [139], supposedly as adaptive phenomenon to oxidative stress [138–140].

Dysfunction of the mitochondrial respiratory system limits metabolite flux through the TCA cycle resulting in ATP deficit with ultimate shift from apoptosis to necrosis. Extensive oxidative damage and defective respiration with reduction in hepatic ATP content were found in DDC [141] and in HF diet mouse models [142]. Moreover, downregulation of PPAR-α also affects mitochondrial redox balance by reducing the expression of antioxidant genes [143] in PPAR-α-null mice [112,144], HF-diet-fed and DDC-intoxicated mice (Nikam, A. et al., in preparation).

While damaged mitochondria can be eliminated by mitophagy, de novo production that relies on the correct balance between fusion and fission processes [145] as part of mitochondrial quality control is more often involved. Its importance has been demonstrated in mouse models of fatty liver disease [146]: HF-diet feeding of fission-competent mice (B6SJL/129) led to impaired glucose tolerance, elevated blood glucose, excessive weight gain and liver histology consistent with steatosis and hepatocellular ballooning. These mice also showed smaller mitochondria, indicative of mitochondrial fragmentation due to excessive fission.

Mitophagy is an important protective mechanism against liver injury, steatosis and SH essentially by preventing the accumulation of damaged mitochondria. The loss of inner membrane potential triggers their removal by macroautophagy as shown in ASH [147,148] and NASH models [149].

Microtubules and microfilaments are involved in maintaining shape and position, as well as migration of mitochondria (reviewed in [150]). Mitochondrial function and morphology have also been linked to keratins and the IF cytoskeleton in the liver of K8-deficient mice [151].

5.1.4. Cell death

5.1.4.1. Apoptosis. Apoptotic cell death in NASH and related animal models can be initiated by an intrinsic and an extrinsic pathway. The extrinsic pathway starts with the activation of surface death receptors, i.e., Fas receptor (CD 95), TNF-α receptor 1 and TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2. In NASH upregulation of death receptors is responsible for increased sensitivity to apoptotic stimuli. Moreover, excess free FAs promote ROS production, which can induce apoptosis via mitochondrial dysfunction or Fas ligand/death receptor induction. In addition, endoplasmic reticulum (ER) stress caused by saturated free FAs is increased in obese patients and animal models and can lead to cell death ([152] - consult this reference for further information on this topic). Moreover, keratin alterations mentioned above also play a role in apoptotic cell death [136].

5.2. Inflammation and fibrosis

The immune system is in various ways involved in the pathogenesis

of NAFLD/NASH and related animal models. Damaged hepatocytes release molecular signals that trigger inflammation in the sense of a wound healing response [13,44]. The central role of TNF-α and other inflammatory cytokines in the progression of steatosis to NASH is obvious from the correlation between circulating cytokine levels and severity of steatosis, inflammation and fibrosis [38,58]. TNF-α may be produced by Kupffer cells, adipose tissue, hepatocytes, stellate cells, monocytes, neutrophils, dendritic cells and natural killer cells [38,153]. Moreover, dietary factors modulate the intestinal microbiome, and the hepatic immune system is exposed to gut-derived inflammatory mediators resulting from increased gut permeability in patients with NAFLD as well as in animal models [13,33,36,153].

In several rodent NASH models the Hh signaling pathway is activated, as indicated by increased numbers of Hh-producing cells, and promotes liver inflammation through osteopontin (OPN)-mediated macrophage activation [8,154,155]. Moreover, Hh activation correlates with fibrosis by stimulating transformation of hepatic stellate cells into matrix-producing myofibroblasts [154].

5.3. NASH and liver tumors

Hepatocellular carcinoma (HCC) is a well-known complication of cirrhosis in NASH patients, but may also appear in non-cirrhotic livers [2]. The occurrence of liver tumors has been reported in a variety of NASH animal models of dietary, genetic or toxic etiology (e.g., MCD diet, HF diet + DEN; DDC/GF, NSTZ-Diabetes model, MSG, AOX deficiency, K18-deficiency, ALR deficiency, NEMO deficiency, MAT1A deficiency, PTEN deficiency, TSOD mice). Although these models, as discussed in this review, do not entirely reflect the human situation and the resulting tumors are difficult to categorize as benign or malignant they can still contribute to our understanding of pathogenic principles of NASH-associated hepatocellular neoplasia. Moreover, some of these models seem to be particularly sensitive to exposure to carcinogens (e.g. injection of diethylnitrosamine; [72,76,156]). The development of liver tumors of uncertain biologic behavior in chronically DDC- or GF-fed mice and in old K18-deficient mice with NASH-like liver pathology suggest pathogenic similarities (e.g. oxidative stress). Old $K18^{-/-}$ mice are particularly interesting in this context. Hepatocellular tumors with steatohepatitic features, considerable cellular and nuclear pleomorphism, atypical mitoses and nodule-in-nodule formation, thus suspicious of malignancy but without detectable metastases, prevailed in male animals whereas in females tumors were less often observed and predominantly monomorphic [120].

6. Conclusions

NAFLD is a complex and multifactorial disorder that in its various manifestations is determined by environmental and genetic factors and interplay of liver, adipose tissue and gut. It results from hepatocellular lipotoxic damage, altered intestinal microbiome and related immune response. Analyses of human disease and animal models disclosed that oxidative stress due to imbalance between pro- and antioxidant production plays a critical role in the progression of simple steatosis to SH.

The sequence of events in the pathogenesis of SH can be summarized as follows: (i) Steatotic hepatocytes undergo lipotoxic injury; (ii) damage-associated factors are released and initiate an immune response by activation of resident stroma cells; (iii) locally produced cytokines enhance hepatocellular steatosis and lipotoxic injury; (iii) intestinal dysbiosis and impaired barrier function boost the process by exposure of hepatocytes and stroma cells to gut-derived bacterial products; (iv) activation of hepatic stellate cells promotes fibrosis.

Animal models are indispensable tools to elucidate pathogenic principles. However, no single animal model can reproduce a complete picture of the mechanisms responsible for appearance, variations, progression and outcome of human disease. In addition, few models take into account environmental conditions despite the fact that this may have a significant impact on the meaningfulness of experiments. For a given question it is crucial to determine which aspects of human disease are essential and to assess their representation in the model. In the pathologist's view morphology denotes an end stage of a disease process and its analysis can, therefore, significantly contribute to its elucidation. Since hepatocyte ballooning and MDB formation are key features of SH of alcoholic and non-alcoholic etiology we feel that models that reproduce this morphology, irrespective of etiology, are most appropriate to achieve this goal.

Disclosure statement

The authors have nothing to disclose.

Transparency document

The Transparency document associated with this article can be found, in online version.

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