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EXPERIMENTAL STUDY

Molecular mechanisms in the pathogenesis of metabolically associated fatty liver disease

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ABSTRACT
Inflammation is a common feature of all chronic liver diseases and atherosclerosis. The article discusses the participation of cytokines and inflammasomes in the process of development of metabolically associated fatty liver disease (MAFLD) and the ways of their activation under the influence of inductive stimuli (toxins, alcohol, fat, viruses, etc.), most often in the case of disruption of intestinal permeability through toll-like receptors with an imbalance in the composition of intestinal microflora and bile acids. Inflammasomes and cytokines are the sources of sterile inflammation in the liver in obesity and metabolic syndrome with subsequent lipotoxicity which is followed by fibrogenesis. The prospects for therapeutic modulation of diseases with the participation of inflammasomes are therefore sought precisely at the level of influencing the mentioned molecular mechanisms. The article emphasizes the importance of the liver-intestinal axis and modulation of microbiome, as well as calls attention to the influence of the circadian rhythm of the 12-hour pacemaker on gene production in NASH (non-alcoholic steatohepatitis) developing *(Fig. 4, Ref. 56).* Text in PDF *www.elis.sk*
KEY WORDS: NASH, MAFLD, microbiome, lipotoxicity, bile acids, inflammasomes.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is currently a growing health problem with an approximate global prevalence of 17–45 % in Western countries. It is mainly related to the increase in obesity, insulin resistance/diabetes mellitus type 2 (DM2) and metabolic syndrome (MS) (1). In patients with severe obesity, NAFLD occurs in > 80 % of patients and patients with DM2 in up to 75 %. NAFLD includes a spectrum of conditions from simple steatosis (lipid inclusions in more than 5 % of hepatocytes contents) to non-alcoholic steatohepatitis (NASH), which is characterized by hepatocellular injury, inflammation, necrosis, and fibrosis, which is a direct risk factor for developing cirrhosis and hepatocellular carcinoma (HCC). The prevalence of NASH can be exactly determined based on the histological examination of the liver. The predicted prevalence in the European population is estimated at 1.5–6.5 %, with approximately 1/4 of patients having advanced fibrosis at the time of diagnosis and 10–15 % having advanced cirrhosis. Cirrhosis based on NASH is today the third most common indication for liver transplantation (2). Due to the dramatic worldwide increase in the prevalence of NAFLD and its extrahepatic consequences, in 2020, Eslam et al suggested a redefinition of the term NAFLD to MAFLD (metabolically associated fatty liver disease) (3).

The most known consensus on explaining NASH development lies in the hypothesis (from 1998) of "two-hit" insults (4). The first insult is simple steatosis and insulin resistance, which sensitize the liver to subsequent insults. The second insult is multifactorial. Recently, a "multiple-hit hypothesis" has replaced the "two hit hypothesis". This theory suggests that multiple factors such as insulin resistance, hormones secreted by adipose tissue, nutrition, lifestyle, gut microbiota, inflammatory genes and genetic and epigenetic factors, work together to induce NASH in genetically predisposed subjects. In the foreground are the inflammatory processes, oxidative stress, lipid peroxidation and mitochondrial dysfunction. Inflammatory cells from the circulation induce the activation of myofibroblasts and stellate cells, which leads to inflammation, liver fibrosis, and pathological angiogenesis with NASH development, progression to cirrhosis, or up to HCC (5, 6)

Pathophysiological aspects of sterile inflammation of the liver

The NLRP3 inflammasome is a large intracellular multiprotein complex composed of its sensor molecule, NOD-like receptor (NLR), and adapter proteins (apoptosis-associated "speck-like" protein containing CARD-ASC and pro-caspase-1

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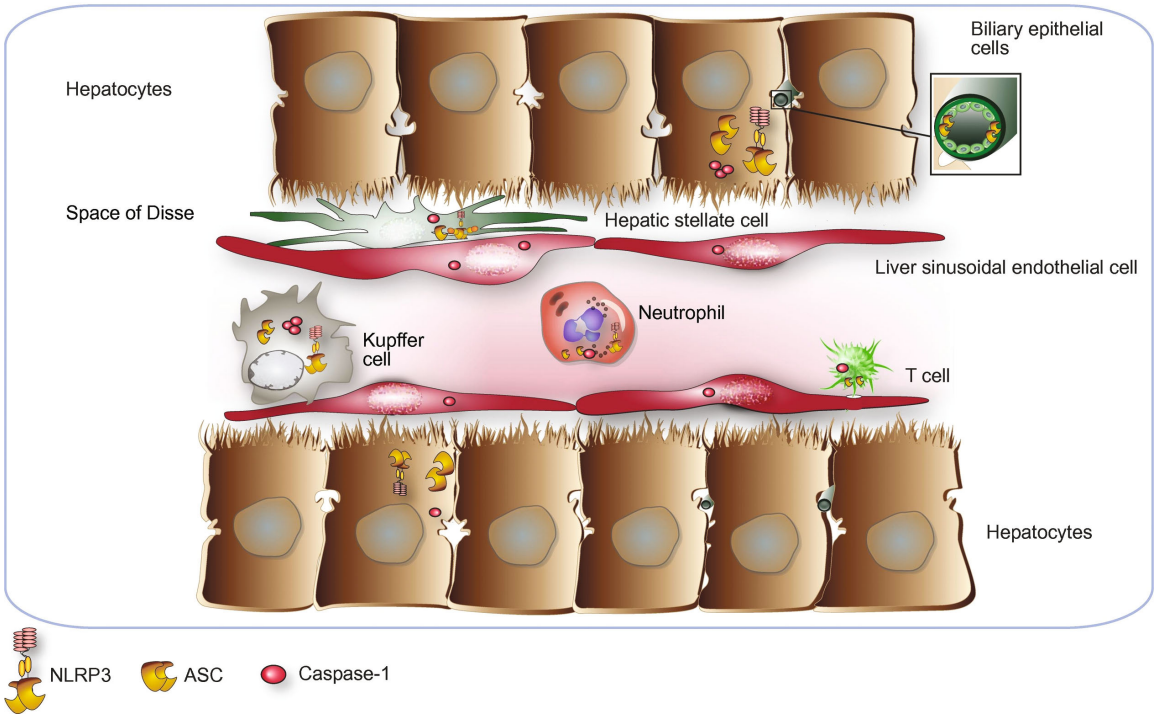

Fig. 1. Cell-specific inflammasome expression in the liver. (Figure was published in (7) under CC BY-NC-ND 4.0 licence).

precursor) that are part of innate immunity. Inflammasomes can be expressed on different liver cells (Fig. 1) (7). They react to pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and environmental pathogens and activate the inflammatory process. They are the main regulators of cell death through pyroptosis and pyronecrosis. Their activation occurs under the influence of various pathogens, most often because of disrupting the intestinal microbial flora or intestinal permeability.

PAMPs are molecular changes induced by the action of various pathogens (viruses, bacteria, ischemia, toxins, alcohol, etc.). DAMPs are molecular sequences formed as a result of cell death, most often in the intestine due to the altered composition of intestinal microbial flora and (or) increased intestinal permeability. They can lead to the activation of sterile inflammation in the liver and other organs and systems (Fig. 2) (8). Uric acid is one of the most common representatives of DAMPs. Mitochondrial components are also a rich source of DAMPs (e.g., ATP, DNA, etc.). Mitochondria thus become a potent stimulator of inflammation.

Activation of inflammasomes is usually a two-step process. The first step is signalling through toll-like receptors (TLR) or interleukin-1-receptor (IL-1R), which leads to the expression of inflammasome components, e.g., NLR, ASC, pro-caspase-1, pro-interleukin-1b, and pro-IL18. Two cytokines, IL-1b and IL-18, require activation by caspase-1 via NLR-receptors (9). The second step is the release of caspase-1 with activation and cytokine production. This signal is mediated by DAMP and PAMP-mediated signalling, which leads to the formation of multiprotein NLR inflammasome and activates pro-caspase-1, cleaving pro-IL-1b and pro-IL18 to their mature forms (10). In the next step, the released active IL-1b activates the IL-1b-receptor, resulting in amplifying inflammasome signalling. Increased production of IL-1b and IL-18 contributes not only to the progression of diabetes and MAFLD but also to alcoholic liver disease (ALD) or to instability of atherosclerotic plaque, which has been confirmed by many experimental and clinical studies (11). In macrophages and animal models, studies have found that oxidized low-density lipoprotein (LDL) and cholesterol crystals activate NLRP3 inflammasomes (12).

Involvement of inflammasomes in the development of NASH

At the beginning of the process, insulin resistance is induced by obesity and DM2 development, together with NAFLD development (13). In patients with untreated DM2, there is significantly increased expression of NLRP3 inflammasomes, proteins associated with apoptosis and pro-inflammatory cytokines (IL1-β, IL-18) with cleavage of caspase-1. The mentioned changes disappeared after initiating antidiabetic treatment with metformin (12). Obesity and insulin resistance are triggers (initiators of the whole process) (14). At the clinical level, the consequences are atherosclerosis development, ischemic heart disease (ICH), DM2, hypertension, dyslipoproteinemia, and complications of atherosclerosis (acute myocardial infarction, stroke) and many cancers (colorectal cancer, pancreatic cancer). In the liver, it is the development of steatosis, from NAFLD to NASH, cirrhosis and HCC, but also the development of alcoholic liver disease or drug-induced damage of the liver (Fig. 3) (15). The process de-

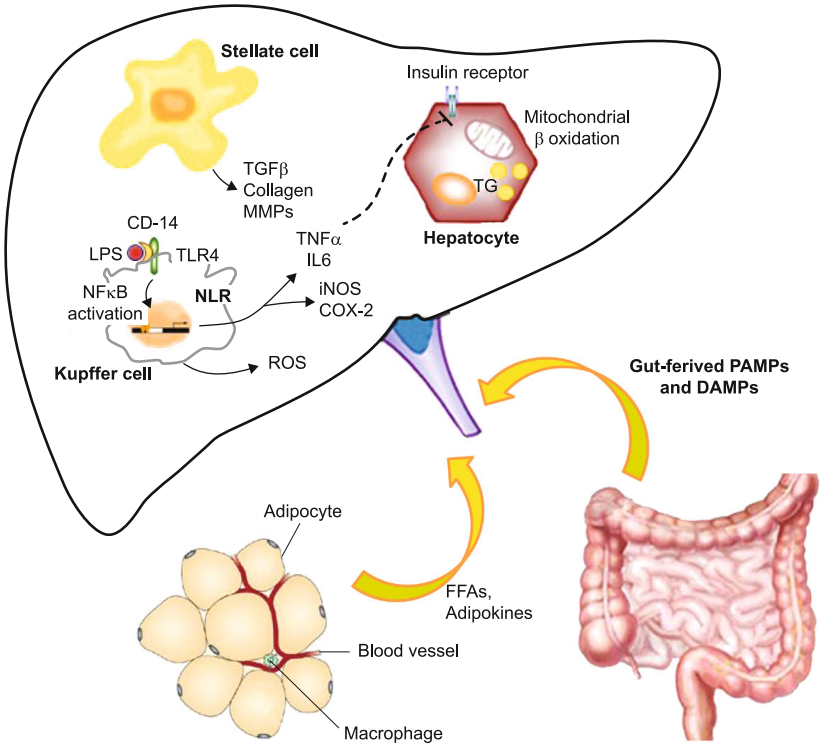

Fig. 2. Triggers of inflammasome activation in liver diseases. Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) bind pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and NOD-like receptors (NLRs). Hepatic innate immune cells initiate and maintain the process of liver inflammation through the production of inflammatory cytokines. Damage to the intestinal mucosal barrier and/or dysbiosis allows bacterial translocation and elevated endotoxin levels during chronic liver disease, worsening inflammation and induction of fibrogenesis through Toll-like receptor (TLR) signaling. Kupffer cells contribute to cytokine production and activation of hepatic stellate cells to maintain the balance between inflammatory and fibrogenic signaling. Adipokines secreted from adipose tissue and inflammatory cytokines impair hepatic insulin signaling, leading to insulin resistance, further inflammation and disease progression (8).

pends on endoplasmic reticulum stress and mitochondrial damage at the subcellular level. There are 4 types of inflammasomes that participate in the process, namely AIM2, NLRC4, NLRP3, and NLRP1, while NLRP3 inflammasome plays the dominant role. The etiopathogenetic trigger of the cascade of changes is dysregulation of the function of white adipose tissue. It is an endocrine organ with the ability to produce a number of endocrine adipokines. It will tip the balance of leptin and adiponectin in favour of leptin along with IL-6, TNFa, resistin, angiotensin II, and other factors. The dominance of pro-inflammatory proatherogenic adipokines leads to platelet aggregation, cholesterol accumulation in macrophages, and increased angiogenesis, thus resulting in an inflammatory reactions in the liver and endothelium of the vascular system. It is believed that obesity itself is an inducer of changes in the intestinal microbial flora. The changed composition of the intestinal microbial flora via toll-like receptors (TLR) activates inflammasomes. Signalling from the intestinal tract through TLRs directly affects receptors in the CNS (Fig. 4). In NAFLD (NASH), the receptors of the central melanocortin system (MC3R, MC4R) play a key role and are responsible for hyperphagia. They also control the synthesis of cholesterol, HDL-cholesterol, as well as secretion of triglycerides and VLDL-cholesterol (Fig. 4).

NASH is pathogenetically also a disease of mitochondria. If we could pharmacologically increase mitophagy, it would slow the progression to the NASH stage. NLRP3 activation contributes to caspase-1-dependent mitochondrial damage and blocks mitophagy. Saturated fatty acids inhibit the regulation of energy stores and fat metabolism by reducing the activity of AMP-kinase, which leads to degradation and recycling of mitochondrial components. The consequence of inhibited mitophagy is the accumulation of dysfunctional mitochondria, formation of reactive oxygen species (ROS), and release of mitochondrial DNA into cytosol with activation of NLRP3 inflammasomes and conversion of pro-IL-1β into the active form of IL-1β (16). This pro-inflammatory state leads to the deterioration of metabolism. IL-1β increases insulin resistance through the expression of TNF-α (17, 18). Inflammasomes are responsible for induced β-cell death and progression of DM2. Long-term exposure of pancreatic β-cells to hyperglycaemia promotes endoplasmic reticulum stress and accumulation of dysfunctional mitochondria with subsequent accumulation of intracellular ROS. ROS induce the activation of NLRP3 inflammasomes. Interleukin-1β induces a local pro-inflammatory environment by activating other chemotactic factors and infiltrating immune cells, which leads to pancreatic β-cell failure (Fig. 3) (16, 15).

Lipotoxicity in the pathogenesis of NASH

Lipotoxicity is dysregulation of the metabolism of lipids and their metabolites in the cell with inducing chronic inflammation and disproportionate intracellular accumulation of lipid inflammatory particles with the destruction of organelles and, ultimately, cell death. Accumulated toxic lipids in cells change the functions of organelles, especially mitochondria and endoplasmic reticulum and link metabolic and pro-inflammatory signals. An increase in triglyceride accumulation in hepatocytes results from the transfer of free fatty acids (FFA) from insulin-resistant adipose tissue and *de novo* lipogenesis in the liver, and increased consumption of dietary fats.

In NAFLD, almost 60 % of hepatic FFAs come from the lipolysis of TG from the adipose tissue. The rest of FFAs is from *de novo* lipogenesis and dietary TG (19). The data show that lipid

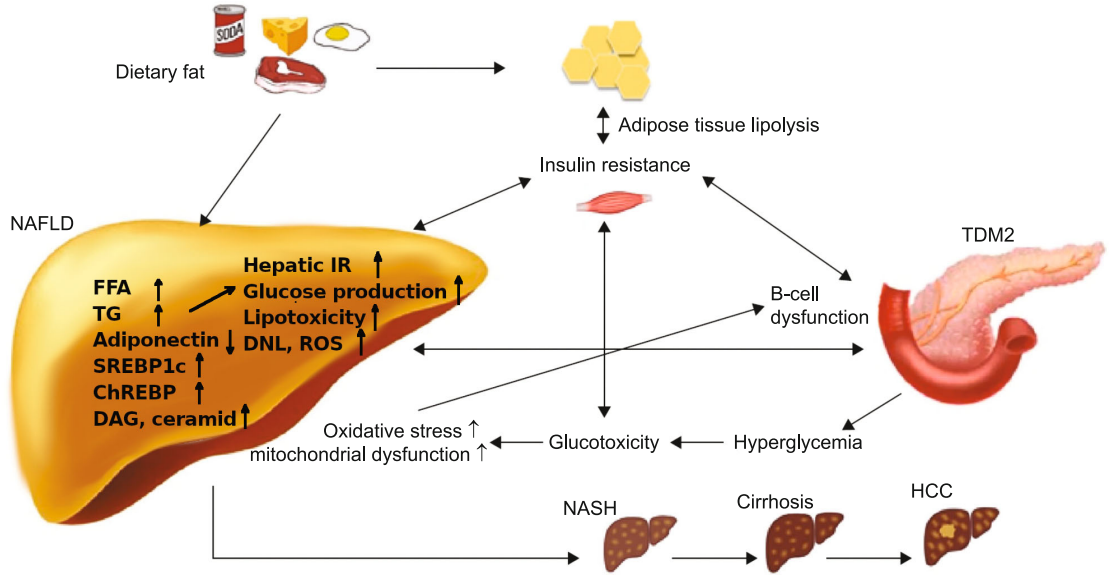

Fig. 3. Consequences of obesity and liver insulin resistance (IR): 1. clinical, 2. etiopathogenetic.
Epidemiological studies noted that T2DM patients have by two-fold increase to develop NAFLD. This complex and intricate association is supported and mediated by insulin resistance (IR).
NAFLD – nonalcoholic fatty liver disease, NASH – nonalcoholic steatohepatitis, HCC – hepatocellular carcinoma, IR – insulin resistance, FFAs – free fatty acids, TG – triglyceride, ChREBP – carbohydrate response element-binding protein, SREBP1c – sterol regulating element-binding protein 1c, DAG – diacylglycerols, ROS – high reactive oxygen species (15).

particles in form of TG in hepatocytes represent a relatively "safe" and "protective" form against the progression of MAFLD to NASH and protect against lipotoxicity. Lipotoxicity is a consequence of the accumulation of toxic intermediates in TG synthesis, including saturated fatty acids (SFA), free cholesterol and complex lipids (glycerophospholipids, sphingolipids) and/or lack of phospholipids, ω-3 polyunsaturated fatty acids (PUFA) and specialized pro-resolving mediators (SPMs) derived from PUFA, responsible for cellular integrity. Yamaguchi et al found that the blockade of TG synthesis through the inhibition of diacylglycerol acyltransferase 2 reduced steatosis but increased the rate of oxidative stress, inflammation, and fibrosis (20).

There are comprehensive data suggesting that the ability to store lipids in a more inert form, such as TGs, at the expense of other species protects individuals from the progression to NASH. An increased level of circulating FFAs is one of the clinical characteristics of patients with obesity and metabolic syndrome. In conditions of insulin resistance, β-oxidation of FFA fails. Elevated levels of FFAs in the liver can damage the hepatocytes by producing toxic metabolites such as ceramides, diacylglycerols, and lysophosphatidylcholine. These metabolites can lead to mitochondrial dysfunction by inhibiting the mitochondrial oxidative respiratory chain, activating NADPH oxidase, or depolarizing the mitochondrial membrane, which results in an increase in ROS production and oxidative stress, and exacerbates liver injury (21). Especially palmitate and stearate (C16:0, C18:0), the main representatives of toxic unsaturated FA, induce apoptosis and inflammation. They are the main dietary components that can be synthesized *de novo* from carbohydrates. Monounsaturated FA, e.g., oleate (C18:1), are less toxic than palmitate despite being factors contributing to steatosis (22).

Some lipids, polyunsaturated fatty acids (PUFA), are protective against cell damage and contribute to fat reduction in hepatocytes. Decreased n-3 PUFA levels are typical findings in patients with NASH. Supplementation with these PUFAs in NASH patients reduced steatosis, although the NASH-activity score and degree of fibrosis remained unchanged. The n-6 PUFA α-linoleic acid has also a hepatoprotective effect by reducing apoptosis and c-Jun N-terminal kinase activation, which, however, leads to the expression of pro-inflammatory compounds (23).

Lysophosphatidylcholine (LPC) is one component of the lipid spectrum and its levels are elevated in patients with NASH. Lysophosphatidylcholines are produced in cells mainly by the enzyme phospholipase A2, which removes one of the fatty acid groups from phosphatidylcholine to form LPC. Lysophosphatidylcholine in the liver upregulates genes for cholesterol biosynthesis and downregulates genes for FA oxidation in the liver. A higher concentration of LPC disrupts mitochondrial integrity in cytochrome C hepatocytes (24). In the vascular system, LPC activates endothelial cells in the initial phase of atherosclerosis (25). Lysophosphatidylcholine also acts as a signalling molecule released by apoptotic cells to recruit phagocytes to phagocytose the apoptotic matrix (26). The optimal level of LPC in the plasma has not been established yet, but we know that LPC levels in LDL positively correlate with the development of the disease. The increase in LPC levels is primarily determined by the activity of the enzyme phospholipase A2 associated with lipoprotein (Lp-PLA2) to produce LPC. Lysophosphatidylcholine acyltransferase 1 (LPCAT) reduces LPC

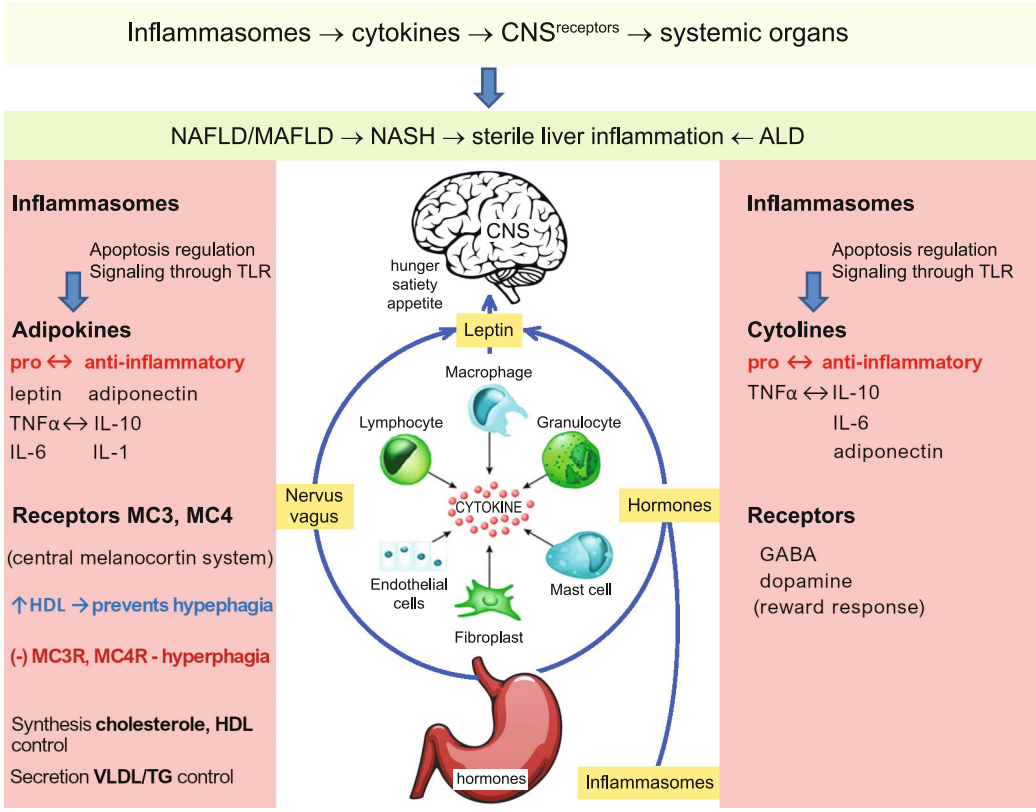

Fig. 4. Etiopathogenetic cascade of the inflammatory reaction in chronic liver diseases.
CNS – central nervous system; NAFLD/MAFLD – nonalcoholic fatty liver disease/metabolic associated fatty liver disease; NASH – nonalcoholic steatohepatitis; ALD – alcoholic liver disease; TLR – toll like receptors; TNFα – tumor necrosis factor α; receptors MC3, MC4 (MC3R, MC4R) – receptors melancortin 3, melanocortin 4; HDL – high-density lipoproteins; VLDL/TG – very-low-density lipoproteins /triglyceride; GABA – gamma-aminobutyric acid.

levels, but its overexpression is associated with oncological diseases. Targeting LPC may be important in treating cardiovascular and neurodegenerative diseases (27).

Other molecules responsible for inflammation, insulin resistance, oxidative stress, and cell death are ceramides, which are formed *de novo* from serine and palmitoyl-CoA by serine cleavage by palmitoyl-transferase in the endoplasmic reticulum or by hydrolysis of sphingomyelin catalysed by sphingomyelinase (28). The involvement of ceramides in the pathogenesis of diabetes and metabolic syndrome, including insulin resistance in the field of obesity, has led to an interest in the role of sphingolipids in MAFLD development. Ceramides are essential second messengers that interact with pathways involved in insulin resistance, oxidative stress, inflammation, and apoptosis, all associated with MAFLD development. Inflammatory cytokines TNF-a and IL-6 and reduced adiponectin together with oxidative stress are risk factors for the progression of NAFLD with ceramide production in hepatocytes (29). Ceramides may contribute to the development of NASH by increasing inflammatory cytokines and oxidative stress. These findings conclude that the role of ceramides in NAFLD and progression to NASH could be partially induced by TNF-a-mediated liver injury (29).

We know from animal and human studies that cholesterol is one of the toxic lipids that accumulates in the liver and contributes to the liver damage in MAFLD and progression to NASH. The accumulation of free cholesterol but not of TG or FA make hepatocytes more sensitive to TNFa and Fas-mediated apoptosis due to mitochondrial glutathione depletion. Other studies suggest that accumulated free cholesterol precipitates in form of crystals in damaged hepatocytes with a subsequent induction of inflammatory response by interaction with NLRP3 inflammasomes in Kupffer cells. These findings are consistent with those of accumulated free cholesterol in Kupffer cell lysosomes with the initiation of an inflammatory response. A disruption of cellular cholesterol homeostasis by nuclear transcription factors, sterol regulatory element-binding protein 2 (SREBP-2), liver X receptor (LXR) and farnesoid X receptor (FXR) has a fundamental role in the accumulation of cholesterol in the liver in NASH. Increased expression of SREBP-2 in NASH causes upregulation of HMGCoA reductase with increased accumulation of free cholesterol in hepatocytes, especially in mitochondria. This process results in apoptosis and induction of a JNK-dependent pro-inflammatory cascade as well as in an increase in free cholesterol in cells and its catabolism with the synthesis of bile acids, which affects the intestinal-hepatic axis (5).

SREBP2 overexpression was not found in obese patients and patients with other chronic liver diseases such as hepatitis C, but its increase was specifically associated with NAFLD/NASH. An accumulation of free cholesterol in hepatocytes should inhibit SREBP2 activation. It is supposed to be a constant activation of SREBP2 as a consequence of the inflammatory process in NASH, while bypassing the negative inhibitory feedback by high cellular cholesterol level, decrease in miRNA-122 (regulator of FA metabolism) and hyperinsulinemia (30). This multifactorial process of free cholesterol deposition in patients with NAFLD/NASH also includes disorders of its elimination with reduced expression of CYP7A1, reduced synthesis of bile acids from cholesterol, and reduced expression of cholesterol transporters ABCG5/G8 responsible for the excretion of cholesterol into bile (31).

Gut microbiome and NAFLD

The intestinal microbiome is a complex of more than 100 trillion microorganisms that live in a harmonious and beneficial symbiosis with the host in the human digestive tract. Its composition is very dynamic during human life and is influenced by several factors, e.g., by method of childbirth, diet composition, age, and antibiotics. It affects immunity, metabolism, and intestinal homeostasis and metabolism of drugs. Qualitative or quantitative changes in the microbiome, so-called dysbiosis, can be a predisposing factor for the development or progression of some chronic diseases, especially those associated with metabolic changes, such as NAFLD and NASH (5). The microbiome includes more than 160 different bacterial species, including anaerobes dominated by Gram-positive strains of *Firmicutes* and Gram-negative strains of *Bacteroidetes* which are involved in producing short-chain fatty acids (SCFAs), i.e., acetate, butyrate, and propionate, and hydrogen. Other strains are represented by *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, and *Verrucomicrobia* (32, 33). The exact function of the gut flora is still largely unexamined. In addition to processing indigestible polysaccharides into SCFA, the microbiome participates in synthesising vitamins K and B, bile acids, and amino acids, as well as in the metabolism of drugs and toxins (34, 35). The first mention of a possible link between the microbiome and NAFLD was from an animal study where intestinal contents from obese rats were administered to non-obese rat models, and the latter animals developed metabolic damage similar to that in their obese donors (35).

The mechanism by which the microbiome affects lipid metabolism in the liver lies in the end products of its metabolism. Intestinal bacteria do not digest fibre and indigestible starch but ferment them with the formation of SCFAs. Increased levels of these metabolites increase satiety and reduce lipogenesis and cholesterol synthesis. They improve the barrier function of the intestine and prevent the transfer of bacterial toxins from the intestine into systemic circulation. SCFAs activate AMPK (AMP-activated protein kinase) and bind to GPCRs (G-protein-coupled receptors). The most studied receptors for SCFAs are GPR43 and GPR41, later renamed as free fatty acid receptor (FFAR2), FFAR3, GPR109a/HCAR2 (hydroxycarboxylic acid receptor), and GPR164, which are expressed in a huge varietycells ranging from those of the gastrointestinal mucosa to cells of the immune system and nerve cells. The effect of activating these receptors varies depending on the cell on which they are located. By binding to enteroendocrine cells, they increase fat oxidation, reduce glycemia, and increase the secretion of peptides such as GLP-1 (glucagon-like peptide 1) and PYY (peptide YY or also known as peptide tyrosine – the anorectigenic hormone of the ileum and large intestine), which are proteins responsible for the sensation of satiety and gastric inhibitory polypeptide (GIP), important for insulin secretion (36).

The damage of the intestinal microbiome is also responsible for inhibiting the synthesis of angiopoietin-associated protein 4 (ANGPTL4), a bactericidal protein modulated by the microbiome in increased concentration of SCFAs, especially C4, which is found in whole milk or produced by fermentation of inulin. It is expressed primarily in the liver, adipose tissue, and intestine. However, influencing its plasma level by dietary measures appears ineffective. ANGPTL4 functions as a specific inhibitor of lipoprotein lipase and a regulator of angiogenesis, and its role is to suppress the release of FFA from VLDL particles. With a damaged microbiome, there is an increased accumulation of lipid particles in the liver (37). While the microbiome and some of its metabolites are of undisputed benefit to the host, other metabolites might well stimulate inflammation or cause hepatocellular damage. The progression of chronic liver disease is directly linked to the transfer of bacteria or bacterial toxins directly into the portal tract.

The most remarkable metabolite of the microbiome in this regard is lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria and an extremely powerful stimulus for inflammatory cytokines. The passage of bacteria and their metabolites activates pro-inflammatory mechanisms after having been bound to specific hepatic receptors. The best-known and most researched are those from toll-like receptors (TLR) family. These multiprotein receptors recognise PAMPs such as bacterial peptidoglycans or LPS, dsDNA and RNA and DAMPs such as cellular stress or death products. The signals inside the cell provided by TLRs also include the activation of inflammasomes. LPS can be detected in the plasma of obese patients, where it correlates with the degree of liver damage. This suggests an association between microbiome-derived LPS and progression to NASH (38).

Brun et al revealed that leptin-deficient ($Lep_{ob/ob}$) and hyperleptinemic ($Lep_{db/db}$) obese mice showed a dysmorphic intestinal mucosal barrier and redistribution of cell junctions and ZO-1 (*zonula occludens* 1 also known as "tight-junction protein 1"). It is a protein on the surface of the cytoplasmic membrane of tight intercellular junctions important for mutual intercellular communication. These changes were associated with a distinct increase in the levels of IL1, IL6, TNF-a, and interferon (IFN) in the portal vein (39). Supplementing the mice fed by a high-fat diet with sodium butyrate repaired their intestinal mucosa, with restoring intestinal levels of ZO-1 and promoting the growth of beneficial bacteria *Christensenellaceae*, *Blautia*, and *Lactobacillus*. Butyrate has also a positive effect on liver damage, reduces the accumulation of fat particles as well as markers of inflammation and fibrosis (40). Patients with NAFLD had a lower representation of

Bacteroidetes strains in their microbiome and a predominance of Gram-negative bacteria from the genus *Prevotella* and *Porphyromonas* spp. compared to healthy controls. NASH patients have higher amounts of ethanol-producing bacteria in the microbiome and higher blood ethanol levels than NAFLD patients or healthy people, suggesting an association with disease progression. Also, a relatively higher abundance of *Bacteroidetes* was found in patients with NASH compared to patients without NASH.

We know from older studies that the degree of liver fibrosis F ≥2 is associated with changes in glucose metabolism, lipids, and amino acids. Therefore, it is clear that liver diseases are associated with dysbiosis of the intestinal microbiome and changes in their metabolic functions (5). In dysbiosis due to NASH, there is overproduction of endogenous alcohol with excessive release of ethanol into the bloodstream, subsequent progression of inflammation in the liver, ROS formation via cytochrome P450 2E1 (CYP2E1), and increased intestinal permeability (41). Several studies are currently underway on the effect of probiotics on liver changes in patients with NAFLD or NASH. Outcome data are not yet available, and the effectiveness of probiotics in metabolic diseases remains a subject of debate.

Bile acids and gut-liver axis in the development of NASH

The liver synthesises primary bile acids (BA), cholic acid (CA) and chenodeoxycholic acid (CDCA), which first accumulate in the gallbladder and then are released into the lumen of the duodenum postprandial with the participation of the hormone cholecystokinin. Primary bile acids can be conjugated with other substances, especially glycine and taurine, which results in conjugated bile acids. In the intestinal lumen, primary bile acids are converted into secondary bile acids, deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) with the participation of intestinal microflora. Bile acids serve as important mediators of several metabolic functions, including absorption of fats and fat-soluble vitamins, elimination of cholesterol, and regulation of the intestinal microbiome and are essential signalling molecules. They have the role of endogenous ligands for receptors, namely for those of farnesoid X receptor (FXR), Takeda G-protein-associated receptor 5 (TGR5) and sphingosine-1-phosphate receptor 2 (S1PR2), which regulate basal metabolism and enterohepatic circulation (42).

A high concentration of bile acids in the ileum activates FXR, which induces the expression and release of the enteroendocrine hormone fibroblast growth factor 19 (FGF19) and is released into the portal circulation (43). FGF19 and its receptor system represent one of the most important gut-derived signals influencing the adipose tissue and liver response during diet-induced NAFLD. FGF19 is transported to the liver and interacts with fibroblast growth factor receptor-4 (FGFR4) with the assistance of β-Klotho co-receptor (βKLB), extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). βKLB is a transmembrane protein expressed mainly in the liver. It is crucial for full activation of the FGF/FGFR complex with the induction of intracellular responses such as decrease in the level of the microsomal enzyme cholesterol-7a-hydroxylase (CYP7A1) and subsequent inhibition of bile acid synthesis (44, 45).

This process is strictly regulated because the accumulation of BA in the liver can lead to hepatotoxicity. Intestinal FGF19 and the hepatic KLB/FGFR4 receptor system indeed represent an endocrine network essential for maintaining BA homeostasis. In general, FXR activation lowers the circulating lipid levels because it suppresses *de novo* fatty acid synthesis, reduces hepatic VLDL secretion and increases TG hydrolysis and clearance as well as fatty acid oxidation. FXR activation can reduce glucose intolerance by decreasing hepatic gluconeogenesis and glycolysis and increasing glycogen synthesis. FXR activation can reduce gluconeogenesis through SHP-1 (small heterodimer partner – also known as NR0B2, a member of the family of nuclear receptors and intracellular transcription factors important for the metabolic circadian clock) by suppressing critical transcription factors involved in gluconeogenesis (46, 47). Over the last decade, two bile acid receptors, namely FXR and TGR5, have come to the fore as targets for treating MAFLD (48, 49), while FXR agonists or analogues could be a successful strategy in NASH treatment (50).

Currently, we have several FXR agonists with hepatoprotective properties (e.g., obeticholic acid). Lipophilic bile acids have emerged as potent modulators of metabolism and insulin sensitivity. By binding to FXR, lipophilic bile acids promote insulin sensitivity and reduce hepatic gluconeogenesis and circulating TG. These effects are mediated by decreased hepatic lipid synthesis and increased peripheral VLDL clearance. Activation of the nuclear receptor FXR also increases the expression of hepatic scavenger receptors (SRB1), which accelerates reverse cholesterol transport by increasing HDL clearance (51). The well-known FLINT study intended to demonstrate the beneficial effect of treatment with obeticholic acid as an FXR antagonist and showed improvement in the histological picture. No patient had worsening of fibrosis. However, a long-term use of obeticholic acid increases the lipid profile and causes pruritus (52). Gradual escalation of dosing the obeticholic acid is associated with a better tolerance in terms of pruritus. Alternative approaches are also being tried to adjust bile acid levels to affect MAFLD and NASH treatment. One alternative lies in the administration of long-chain ω-3 polyunsaturated FA along with gut microbiota modulation, which is still an unexplored area (53).

The 12-hour pacemaker and MAFLD

Another pathomechanism affecting MAFLD development needed to be investigated more deeply, is the influence of biorhythms. All rhythms and biological clocks of organisms provide the advantage that an organism, from unicellular to multicellular, including mammals, is able to anticipate changes in its environment and adapt to them. This advantage has led to the development of various oscillators regulating all aspects of biology, including the cell cycle oscillator controlling cell division, infradian hormonal cycle controlling organismal development (periodicity greater than 24 hours), circadian cycle (period close to 24 hours) and ultradian cycle (period less than 24 hours) which regulate the cyclic activi-

ties of the organism, its metabolism and cellular activity. Studies of the 12-hour pacemaker confirm its ancient origins as a "tidal" adaptation. The 12-hour rhythms of genes involved in ER stress, UPR (unfolded protein response, a cell-autonomous mechanism of endoplasmic reticulum homeostasis control) and mitochondrial genes important for the preservation of species are shown. It was found that the purpose of these rhythms is the regulation of the CEDIF (central dogma information flow) gene capacity. This is to manage the increased capacity of RNA and protein production and processing during the rush hour in time of dawn and dusk (54). These spikes appear to allow increased gene expression at times of transitions between rest and activity. The latest vehicle-cargo hypothesis explains how the 12-hour pacemaker and circadian clock complement each other: the 12-hour pacemaker increases the overall capacity for gene expression at dawn and dusk, and the circadian clock determines which genes are expressed at each rush hour. New data suggest that mammalian 12-hour cycles are regulated by X-box binding protein 1 (XBP1). XBP1 directly transcriptionally regulates more than 500 genes; alternatively, XBP1 transcriptionally regulates 12-hour oscillations of GABP (GA-binding protein) expression. GABP is a transcription factor of the Ets family that controls gene expression in several important biological settings. GABP is currently considered to be a key transcriptional regulator of dynamically regulated, lineage-restricted genes, particularly in myeloid cells and neuromuscular junction. In addition, it regulates genes that are closely involved in the control of the cell cycle, protein synthesis, and cellular metabolism. XBP1 represents a molecular link between ER stress detected in the ER and the transcriptional protein response (UPR) in the cell nucleus. During ER stress, IRE1α oligomerises in the ER membrane, activates its ribonuclease domain through autophosphorylation and cleaves an intron from the unspliced form of Xbp1 (Xbp1us) mRNA. XBP1 then translocates to the nucleus and activates the transcription of UPR genes, including many that have been found to have 12-hour rhythms.

The IRE1α/XBP1s signalling cascade is dominant for maintaining metabolic health at the cellular and whole-organism levels. Several studies have shown that the activation of control pathways of ER stress perception and quality control of Xbp1s and IRE1α is a protective factor against developing hepatic steatosis (54). One of the mechanismslies in the regulation of membrane fluidity by controlling lipid composition. This is made possible by the gene expression for lysophosphatidylcholine acyltransferase 3 (Lpcat3), which promotes the incorporation of polyunsaturated fatty acids into phosphatidylcholines (PCs) in the ER membrane during high-fat diet-mediated ER stress. An increased level of polyunsaturated PCs increases ER membrane fluidity and reduces the initial ER stress (55, 56). An important finding is that 12-hour rhythms of Lpcat3 gene expression and levels of different types of 2-Lyso-PC were found in the livers of mouse models. Another important finding is that liver-specific ablation of XBP1 attenuated the hepatic 12-hour oscillation of Lpcat3 gene expression with a reduction in the level of polyunsaturated PCs in the liver and significant decrease in membrane permeability with impaired lipid metabolism and subsequently accelerated development of NAFLD, impaired glucose tolerance and hyperinsulinemia in mice (55, 56). Interestingly, a downregulation of an average 12-hour gene expression is strongly associated with progression to hepatic steatosis and NASH in humans as well. However, it is not precisely known whether the 12-hour rhythms of MAFLD patients were also disrupted. These investigations open the door to a possible causal role of the mammalian 12-hour pacemaker in maintaining metabolic homeostasis and metabolic health (55).

Conclusion

The last decade has brought a lot of new information in the field of knowledge concerning the effects of inflammasomes in the etiopathogenesis of chronic degenerative diseases in experimental models and first *in vivo* models and clinical studies. A new dimension is being opened into the knowledge and understanding of the progression, induction, and intensification processes of the inflammatory response. In the article, we summarise new molecular pathomechanisms of cellular liver damage in MAFLD and NASH due to the induction of a sterile inflammatory cascade, as well as an overview of microbiome changes and link with bile acid balance in NASH pathogenesis. New knowledge is emerging about the possibility of prospective therapeutic interventions and ways to block the inflammatory cascade in diseases such as NAFLD, NASH, DM2, and other accompanying diseases. There is absolutely new knowledge on clarifying the influence of circadian rhythms not only on the homeostasis of the entire organism but also on individual organ systems. The near future could bring fundamentally new therapeutic approaches in treating diseases impacted by inflammasomes and non-sterile inflammatory cascade.

The gradual completion of the etiopathogenetic mosaic of degenerative diseases of various systems points to common springboards and mediators in the development of most non-communicable diseases which are the most common cause of mortality today.

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