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Strategies, models and biomarkers in experimental non-alcoholic fatty liver disease research

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ABSTRACT

Non-alcoholic fatty liver disease encompasses a spectrum of liver diseases, including simple steatosis, steatohepatitis, liver fibrosis and cirrhosis and hepatocellular carcinoma. Non-alcoholic fatty liver disease is currently the most dominant chronic liver disease in Western countries due to the fact that hepatic steatosis is associated with insulin resistance, type 2 diabetes mellitus, obesity, metabolic syndrome and drug-induced injury. A variety of chemicals, mainly drugs, and diets is known to cause hepatic steatosis in humans and rodents. Experimental non-alcoholic fatty liver disease models rely on the application of a diet or the administration of drugs to laboratory animals or the exposure of hepatic cell lines to these drugs. More recently, genetically modified rodents or zebrafish have been introduced as non-alcoholic fatty liver disease models. Considerable interest now lies in the discovery and development of novel non-invasive biomarkers of non-alcoholic fatty liver disease, with specific focus on hepatic steatosis. Experimental diagnostic biomarkers of non-alcoholic fatty liver disease, such as (epi)genetic parameters and '-omics'-based read-outs are still in their infancy, but show great promise. In this paper, the array of tools and models for the study of liver steatosis is discussed. Furthermore, the current state-of-art regarding experimental biomarkers such as epigenetic, genetic, transcriptomic, proteomic and metabonomic biomarkers will be reviewed.

Abbreviations: ACS, acyl-coenzyme A synthase; adipoR, adiponectin receptor; ADP, adenosine diphosphate; AP2diph, attenuated P2 diphtheria toxin; AOX, acyl-coenzyme A oxidase; ApoE, apolipoprotein E; ATP, adenosine triphosphate; CBS, cystathionine-β-synthase; CD36, cluster of differentiation 36; CoA, coenzyme A; CPT1, carnitine palmitoyl transferase 1; CYP, cytochrome P450; DNA, deoxyribonucleic acid; FAD, oxidized flavin adenine dinucleotide; FADH2, reduced flavin adenine dinucleotide; FFAs, free fatty acids; FXR, farnesoid x receptor; Gal, galectin; HCC, hepatocellular carcinoma; HFD, high-fat diet; IL-6, interleukine-6; IMS, intermembrane space; IR, insulin resistance; LDL, low density lipoprotein; LDs, lipid droplets; MA, macrovesicular steatosis; MAT, methionine adenosyl transferase; MC4, melanocortin 4; MCD, methionine and choline-deficient diet; MI, microvesicular steatosis; (micro)RNA, (micro)ribonucleic acid; MPT, mitochondrial permeability transition; MRC, mitochondrial respiratory chain; MTP, mitochondrial trifunctional protein; MTTP, mitochondrial triglyceride transfer protein; NAD, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PC, phosphatidylcholine; PECAM, platelet endothelial cell adhesion molecule; PNPLA, patatin-like phospholipid domain containing protein; PPAR, peroxisome proliferator-activated receptor; PTEN, hepatocyte-specific phosphatase and tensin homolog; SREBP, sterol regulatory-element binding protein; TGs, triglycerides; VLDL, very low density lipoproteins; VPA, valproic acid.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) covers a spectrum of chronic liver diseases, ranging from hepatic steatosis or fatty liver to non-alcoholic steatohepatitis (NASH), liver fibrosis, liver cirrhosis and eventually hepatocellular carcinoma (HCC). It is estimated that 1 billion people currently suffer from any form of NAFLD [1]. Liver steatosis, marked as the first stage of NAFLD, is closely associated with obesity, type 2 diabetes mellitus, insulin resistance (IR) and drug-induced liver injury [2]. This is substantiated by the fact that NAFLD is the most dominant chronic liver disease with a prevalence of 20–30% in Western countries, 30–50% in patients with type 2 diabetes mellitus and 80–90% in obese adults [3–6]. Steatosis is considered as a benign reversible event, while NASH has the potential to lead to cirrhosis and HCC. As much as 10–25% of liver steatosis patients progress to NASH and 2–3% of NASH patients develop liver cancer [3,7]. Hence, liver steatosis is of major concern for worldwide health, especially since sedentary lifestyle, modern Western nutrition, genetic predispositions, a variety of pharmacological agents and hepatitis B and C infection have been identified as critical causes of NAFLD [2,3,7–12]. At present, 70,000 Europeans are fatally affected by chronic liver disease every year [13]. Furthermore, HCC incidence increased tremendously over the past 25 years [14] and was responsible for the death of 21,670 patients in the United States in 2013 [15]. The economic burden imposed by chronic liver disease and its causes is considerable. Indeed, in the United States, 160–300 billion Dollars are spent every year on obesity, which constitutes 10% of overall health care costs [16]. So far, the only curative therapy for acute and chronic liver failure is liver transplantation, with a cost in Europe of about 100,000 Euro the first year and 10,000 Euro yearly thereafter [17].

Liver steatosis is featured by the accumulation of fat in at least 5% of hepatocytes. These fatty or lipid droplets, typically present in the cytosol, are composed of aggregated triglycerides (TGs) engulfed by a phospholipid monolayer [18]. In physiological conditions, these lipids generate energy and serve as a substrate for membrane synthesis, though in excess, this can lead to hepatocellular damage. Four pathogenic mechanisms are thought to be responsible for the accumulation of TG-based lipid droplets (Fig. 1), namely (*i*) the increased uptake of free fatty acids (FFAs) from high-fat food or adipocytes in body fat, (*ii*) the increased synthesis of FFAs in the liver from glucose or acetate by IR, (*iii*) the decreased mitochondrial β-oxidation of FFAs caused by a multitude of drugs and (*iv*) the decreased secretion of TGs in very low density lipoproteins from the liver [11,19]. The progression from steatosis to NASH is presented in the so-called "two-hit" hypothesis [20]. TG accumulation by IR, increased fatty acid influx, decreased fatty acid oxidation and/or decreased TG transport can be considered as the "first hit", making the liver more sensitive to a "second hit", such as oxidative stress, lipoapoptosis and increased production of inflammatory cytokines. This can ultimately result in the development of NASH [7,20,21]. Being associated with obesity and type 2 diabetes mellitus, IR plays a key role in the origin and maintenance of hepatic steatosis. Increased peripheral lipolysis and downregulation of tissue glucose uptake results in an increase of FFAs in hepatocytes and thus creates the basis for TG production. Together with decreased apolipoprotein synthesis, TG secretion, in the form of very low density lipoproteins, is inhibited. The resulting excess of FFAs induces a rise in mitochondrial β-oxidation, production of reactive oxygen species and oxidative stress as well as elevated lipid peroxidation, all which are hallmarks of the injury observed in the liver of obese patients suffering from steatosis [22–24]. Additionally, hyperinsulinemia and hyperglycemia, both caused by increased insulin secretion by pancreatic β-cells, trigger the transformation of FFAs to glucose and inhibit β-oxidation, resulting in the accumulation of TGs in the cytosol of hepatocytes. Collectively, these triggers induce the production of proinflammatory cytokines *via* release of tumor necrosis factor α, which leads to the development of NASH [11,25–28]. The development of liver steatosis is also closely related to the metabolic syndrome. In this respect, liver steatosis patients show one or more features of the metabolic syndrome, such as IR, glucose intolerance, obesity, hypertriglyceridemia and elevated blood pressure [29]. Additionally, pharmacological agents targeting mitochondria are known to cause oxidative stress or liver steatosis (Fig. 2).

Histologically, no distinction can be made between alcoholic fatty liver disease and NASH, since both conditions have an identical histological pattern. This emphasizes the importance of excluding significant alcohol intake (*i.e.* more than 20 g/day) in patients when diagnosing NASH. The latter is characterized by the presence of steatosis, mixed inflammatory cell infiltration, hepatocyte ballooning, necrosis, glycogen nuclei, Mallory's bodies and fibrosis [30]. Two types of steatosis can be recognized, namely macrovesicular steatosis and microvesicular steatosis. Both present the accumulation of TGs in the cytosol of hepatocytes. However, in case of macrovesicular steatosis, large lipid droplets which displace the cytoplasmic content and nucleus are observed, while in microvesicular steatosis small well-circumscribed lipid droplets that do not displace the nucleus are seen (Fig. 3) [31].

Steatosis is often accompanied by phospholipidosis, implying the intracellular accumulation of phospholipids. As such, phospholipidosis is a lysosomal storage disorder and is considered as a reversible side effect [32]. Phospholipidosis will not be further discussed in this paper. Rather, focus will be put on strategies to experimentally mimic the pathological features of NAFLD induced by drugs, diets or dietary ingredients combined with rodents, zebrafish and genetic modified animals as well as with liver-based *in vitro* models. The clinical diagnosis of liver steatosis and NAFLD is based on a number of read-outs, including clinical chemistry parameters (*e.g.* cholesterol and transaminase levels), histopathological examination of liver biopsies and specific physical tests (*e.g.* computed tomography and nuclear magnetic resonance) [33]. These clinical read-outs will not be considered in this paper. Instead, particular attention will be paid to more experimental diagnostic biomarkers of NAFLD, such as (epi)genetic parameters and '-omics'-based read-outs.

2. Strategies in NAFLD research

2.1. Drug treatment

2.1.1. Valproic acid

With its anticonvulsive activity being discovered in 1963, valproic acid (VPA), or its sodium valproate derivative, is currently the most commonly prescribed anti-epileptic drug worldwide. In addition to its anticonvulsant properties, VPA is also used for the treatment of bipolar disorders [34–36], clinical depression [37], absence seizures [38], tonic–clonic seizures [39], complex partial seizures [40] and juvenile myoclonic epilepsy [41]. More recently, VPA has been characterized as a histone deacetylase inhibitor [42] with therapeutic potential in the cancer field. Although VPA is considered as a drug with a relatively safe profile, adverse reactions, such as encephalopathy, hypersensitivity syndrome and teratogenicity, have been reported in patients receiving VPA [43]. One of the most alarming side effects is hepatotoxicity, in particular microvesicular steatosis, caused by impairment of mitochondrial β-oxidation. Indeed, upon sequestration with coenzyme A (CoA) to valproyl-CoA in the cytosol of hepatocytes, VPA can enter

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