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Position Paper

# AISF position paper on nonalcoholic fatty liver disease (NAFLD): Updates and future directions☆

The Italian Association for the Study of the Liver (AISF)

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ABSTRACT

This review summarizes our current understanding of nonalcoholic fatty liver disease (NAFLD), a multifactorial systemic disease resulting from a complex interaction between a specific genetic background and multiple environmental/metabolic "hits".

The role of gut microbiota, lipotoxicity, inflammation and their molecular pathways is reviewed in-depth. We also discuss the epidemiology and natural history of NAFLD by pinpointing the remarkably high prevalence of NAFLD worldwide and its inherent systemic complications: hepatic (steatohepatitis, advanced fibrosis and cirrhosis), cardio-metabolic (cardiovascular disease, cardiomyopathy, arrhythmias and type 2 diabetes) and neoplastic (primary liver cancers and extra-hepatic cancers).

Moreover, we critically report on the diagnostic role of non-invasive biomarkers, imaging techniques and liver biopsy, which remains the reference standard for diagnosing the disease, but cannot be proposed to all patients with suspected NAFLD.

Finally, the management of NAFLD is also reviewed, by highlighting the lifestyle changes and the pharmacological options, with a focus on the innovative drugs.

We conclude that the results of ongoing studies are eagerly expected to lead to introduce into the clinical arena new diagnostic and prognostic biomarkers, prevention and surveillance strategies as well as to new drugs for a tailored approach to the management of NAFLD in the individual patient.



## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) has prompted a growing clinical and research interest over the last 25 years. Paramount information has progressively accumulated on the genetics and behavioral risk factors for disease development and progression [1,2], on hepatic and extra-hepatic complications [3,4] and on putative treatment strategies [5,6]. Nevertheless, the burden of disease is still increasing, mainly due to the rising tide of obesity and type 2 diabetes mellitus (T2DM) epidemics ("*diabesity*") and the lack of effective treatment options. NAFLD which, in a closed loop fuels the "*diabesity*" epidemics [7], may start very early – even in utero [8,9] – and the longer the exposure, the higher the risk to develop an advanced disease and its complications [10].

So, what can be done to halt the development or reduce this burden of disease? European clinical guidelines on the management of NAFLD have been recently published by the three sister societies of Liver Disease, Diabetes and Obesity [11]. At the time of publication of such guidelines, the participating Experts raised considerable concern about the difficulties expected in conducting universal screening and appropriate surveillance strategies and follow-up in such a potentially huge population of individuals. Indeed, the number of individuals at risk seems too large to be affordable by the National healthcare systems, but selection criteria do not guarantee satisfactory sensitivity and specificity to identify disease progression [12]. Surrogate, non-invasive markers of NAFLD can be used for their negative predictive value to spare patients from undergoing liver biopsy; however, their results may assist physicians in dictating the prognosis, not in guiding treatment strategies, given that validated pharmacological interventions are lacking [13].

Disease control may, therefore, only be achieved by appropriate societal interventions aimed at keeping at bay the behavioral risk factors also involved in the pathogenesis of "*diabesity*". Nonalcoholic steatohepatitis-cirrhosis (NASH-cirrhosis)

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☆ Report of 2015 AISF Monothematic Conference on NAFLD.

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and NASH-hepatocellular carcinoma (NASH-HCC) are only the "*tip of the iceberg*" of the unhealthy consequences of non-communicable diseases [14].

Against this background, this updated position paper aims to summarizing the chief topics discussed during the Associazione Italiana per lo Studio del Fegato (AISF) Single Topic Conference on NAFLD held in Modena (October 8–10, 2015) in memory of late Professor Paola Loria, who was the Coordinator of the first AISF clinical practice guidelines for management of NAFLD [15].

## 2. Pathophysiology of NAFLD

### 2.1. Pathogenesis

NAFLD is a multi-factorial disease resulting from a complex interaction of environmental "hits" and a genetic background (Fig. 1). A high-calorie diet, often coupled with a sedentary behavior, contribute to the development of NAFLD, both directly and via weight gain. Dietary excess of saturated fats and refined carbohydrates has been associated with NAFLD and a high fructose intake may increase the risk of NASH [16,17]. Development and progression of NAFLD are strongly associated with insulin resistance (IR) and metabolic syndrome (MetS) components, particularly abdominal obesity and T2DM [18]. The most common cause of NAFLD is an altered whole-body energetic homeostasis, due to caloric intake exceeding caloric expenditure, with consequent spillover of extra-energy in the form of non-esterified fatty acids (NEFA) from visceral adipose tissue into ectopic fat depots, such as the liver, skeletal muscles and pancreas [19]. NAFLD will invariably develop when the rate of hepatic triglyceride flowing to the liver via the bloodstream or synthesized within the liver exceeds the rate of hepatic triglyceride oxidation and VLDL secretion into the bloodstream [19]. Approximately 60% of hepatic lipids derives from increased peripheral lipolysis of triglycerides (due to adipose tissue IR and failure to adequately suppress peripheral triglyceride lipolysis), while dietary fats and sugars contribute approximately 35–40% [20]. The liver itself may also contribute to steatogenesis by synthesizing triglycerides from dietary carbohydrates through de novo lipogenesis. The contribution of de novo lipogenesis to liver fat content is less than 5% in healthy subjects and may increase to approximately 25% in NAFLD patients [20]. Intra-hepatocytic accumulation of diacylglycerol intermediates impairs hepatic insulin signaling and fuels gluconeogenesis, so promoting hyperglycemia and predisposing to the development of T2DM [21]. Increased amounts of circulating and intracellular NEFAs are also associated with an increase in nuclear factor kappa-B (NF-κB), eventually leading to the expanded and dysfunctional adipose tissue overproducing multiple pro-inflammatory cytokines and under-producing anti-inflammatory adipokines, such as adiponectin which, collectively, may further dictate NAFLD progression [22,23].

### 2.2. Lipotoxicity and inflammation

As shown in Fig. 1, NASH progression results from numerous events originating within the liver and in distal organs, including the visceral adipose tissue and the gastrointestinal tract [24]. Fat-induced damage to the hepatocytes (lipotoxicity), is more linked to the abundance of specific toxic compounds, such as NEFAs and ceramides, than to the total amount of stored fat [25]. Evidence from genetic studies, on the other hand, suggests that the amount of fat accumulation is important [26]. Toxic lipids can determine cell injury through a variety of mechanisms, including increased oxidative stress and mitochondrial dysfunction. Saturated fatty acids are increased in NASH [24,25] and induce inflammation and hepatocyte apoptosis by activating Jun N-terminal kinase (JNK) and mitochondrial pathways [27]. Free cholesterol is a prominent mechanism for NASH development and progression. Interestingly, necroptosis has been recently described as a cell death mechanism potentially involved in lipotoxicity, which is morphologically comparable to necrosis, though characterized by definite biochemical pathways [28].

Endoplasmic reticulum (ER) stress also takes part in NASH pathogenesis, as the result of the induction of the unfolded protein response, which is an adaptive mechanism potentially triggering apoptosis. JNK, an activator of inflammation and apoptosis implicated in NAFLD progression, is one of the major mediators of ER stress [29]. Hypoxia perturbs lipid homeostasis and insulin signaling pathways. Moreover, reduced oxygen availability induces secretion of pro-inflammatory cytokines [30]. These adverse effects are mediated by two hypoxia-inducible transcription factors (HIF-1α and HIF-2α), which regulate cellular response to oxygen deficiency and can be activated by additional stimuli involved in NASH, including oxidative stress or inflammatory signals [30].

Chronic inflammation is a key factor in NASH pathogenesis. Kupffer cell activation occurs at an early stage, and precedes the recruitment of other cells. Attention has been paid to the different phenotypes of Kupffer cells, i.e., M1 and M2, considered primarily immuno-regulatory [31]. Hepatocyte death is a strong trigger of inflammation and fibrosis, through signaling pathways that include tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptor, Fas and TNF receptor, and promote the expression of several cytokines and chemokines. Differentiation toward a pro-inflammatory 'M1 phenotype' is driven by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) interacting with toll-like receptors (TLR), and induces expression of pro-inflammatory factors, such as interleukin (IL)-1β, IL-12, TNF-α, and chemokines CCL2 and CCL5 [32]. Remarkably, chemokines such as CCL2 and CCL5 may induce hepatic stellate cell activation, triggering fibrogenesis. Besides resident and infiltrating macrophages, the role of other inflammatory cells, such as neutrophils, lymphocytes, NK cells and dendritic cells is actively being evaluated [24].

TLRs recognize endogenous danger signals, such as DAMPs or PAMPs [32]. TLR-induced pathways play a central role in the activation of hepatic cells, primarily Kupffer cells, but also hepatocytes and stellate cells. TLR2 interacts with multiple PAMPs, which are increased in NAFLD, and its inhibition prevents hepatic/systemic IR in high-fat diet–fed mice [32]. TLR9 is activated by unmethylated DNA, typically expressed in viruses and bacteria but rare in mammalian cells. TLR9 downstream signaling involves IL-1, and is associated with NASH severity and fibrosis. The pivotal role of TLR4 in the pathogenesis of NASH has been shown in TLR4-deficient mice that display lower levels of inflammation and fibrosis. TLR4 is primarily activated by bacterial lipopolysaccharide, triggering expression of cytokines and chemokines (e.g., TNF-α, IL-1β, IL-6 and IL-12) [33]. Reactive oxygen species are also induced in TLR4-activated Kupffer cells. TLR4 is expressed by other hepatic cells, including stellate cells and hepatocytes, where TLR-4 exerts actions relevant for the pathogenesis and progression of NAFLD toward fibrosis [33]. TLR4-mediated inflammatory responses can also be elicited by DAMPs released by necrotic cells, such as high mobility group box-1 [34].

The NOD-like receptors (NLR), which participate in the assembly of inflammasomes (multi-protein complexes required for initiation of inflammatory signals) play a major role in NASH pathogenesis. Activation of the inflammasome is induced by TLRs together with signals linked to cellular damage, e.g., uric acid, reactive oxygen species or adenosine triphosphate, and results in the secretion of mature IL-1 and IL-18. A role for NLRP3 inflammasome in NAFLD development and progression

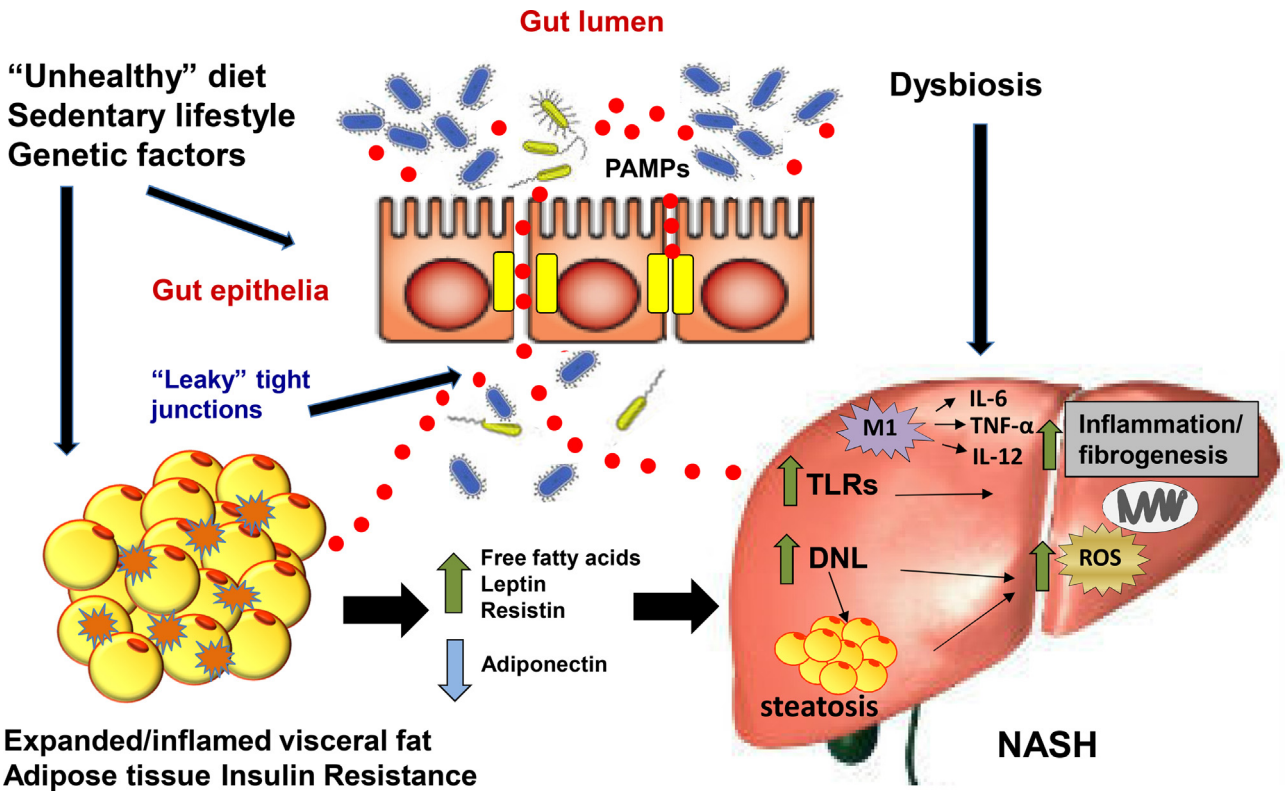


**Fig. 1.** Pathogenesis of NASH. Unhealthy lifestyle and high fat diet not only results in excess fat accumulation (including expanded visceral fat) but also in dysbiosis, where FFAs cause steatosis and exert lipotoxic effects. Moreover, the inflamed adipose tissue releases adipokines, including leptin and resistin associated with increased hepatic fibrosis, while the release of adiponectin is reduced. PAMPs = pathogen-associated molecular patterns; TLRs = toll-like receptors; DNL = de novo lipogenesis.

to NASH has been shown both in humans and in animal models [35]. However, it has also been demonstrated that the lack of NLP3 promotes gut dysbiosis and chronic inflammation [36]. Activation of NLRP3 inflammasome has been associated with hepatocyte pyroptosis, a recently described, inflammasome-mediated, cell death mechanism.

Different nuclear receptors have been implicated in the pathogenesis of NASH and development of fibrosis. Importantly, these proteins may be targeted by drugs already present in the clinical arena. The receptors on which more information has accumulated include the nuclear farnesoid X receptor (FXR), the peroxisome proliferator-activated receptor (PPAR)-gamma and the PPAR-alpha [37]. Agonists of these nuclear receptors improve hepatic steatosis, inflammation and fibrosis, and are being tested in clinical trials (such as discussed at Section 5 in this review).

Adipose tissue is recognized as an endocrine organ that secretes a variety of adipokines, which control systemic metabolism and energy homeostasis. Among these, leptin and adiponectin are involved in the pathogenesis of NAFLD and its progression to NASH, leptin being identified as a pro-fibrogenic adipokine, whereas adiponectin decreases inflammation and fibrogenesis [38].

### 2.3. Microbiota

A huge group of microorganisms, collectively defined as the gut microbiota, colonizes the human intestine. It has been estimated that human gut harbors at least 100 trillion microbial cells, which are differently represented along the intestine, reaching the greatest concentration in the large bowel [39]. Despite a large inter-individual variation, adult gut microbiota is fairly stable and is dominated by the phyla Firmicutes and Bacteroidetes, followed by Actinobacteria and Verrucomicrobia [40].

Gut microbiota confers the host several physiologic benefits, including immune system development, protection from pathogens, and maintenance of intestinal and metabolic homeostasis [41]. Quantitative and qualitative changes of gut microbiota composition, also called *dysbiosis*, have been associated with the development of both intestinal and extra-intestinal diseases, including the MetS. A role of *dysbiosis* in the development of obesity and NAFLD has been demonstrated in both animal and human studies. For example, a seminal study showed that the transfer of gut microbiota from obese to lean individuals induced in the recipients, the same metabolic alterations of the donors [42]. Gut microbiota changes were mainly characterized by an increased Firmicutes/Bacteroidetes ratio. However, other investigators failed to confirm these findings and suggested that metagenomic-based functional aspects of gut microbiota are likely to be more important than phylum specificity [43].

Several mechanisms are involved in the development of obesity and its associated metabolic complications. In particular, gut microbiota can trigger (directly or via the synthesis of end-products of bacterial metabolism, such as short-chain fatty acids), different signaling pathways, which eventually lead to an increased deposition of peripheral fat [42,44]. Furthermore, several intestinal microorganisms can increase the efficiency of caloric extraction from the food, thus contributing to the development of obesity [45]. Lastly, diet-induced *dysbiosis* has been associated with increased gut permeability in both mice [46] and humans [47]. This could lead to increased translocation of bacterial products from the intestinal lumen into the portal circulation thereby triggering chronic inflammation. Moreover, within the liver, these circulating pathogens can also activate the family of pattern recognition receptors (TLRs) and induce pro-inflammatory pathways, which contribute to liver disease development and progression [48–50].

### 2.4. Genetics

Genetic factors account for about of half of the variability in intra-hepatic fat content, and fibrosis tends to be co-inherited with steatosis [26].

Recent genome-wide association studies have begun to unveil the specific common genetic determinants of NAFLD. By far the most important one, due to high frequency and large size effect, is the p.I148 M loss-of-function variant of the Patatin-like phospholipase domain containing-3 (*PNPLA3*) gene. This gene encodes for a lipase involved in the remodeling of lipid droplets in hepatocytes and the release of retinol from hepatic stellate cells [51,52]. In the presence of endogenous/exogenous noxious stimuli (obesity, IR, alcohol and chronic viral hepatitis), carriers of the PNPLA3 I148M variant are at increased risk of developing steatohepatitis, cirrhosis and HCC [52].

The loss-of-function variant p.E167K variant of the transmembrane 6 superfamily member 2 (*TM6SF2*) is more rare, is carried by about 10% of individuals, and also predisposes to NAFLD and advanced fibrosis/cirrhosis by reducing lipid secretion from hepatocytes, while protecting at the same time from dyslipidemia and cardiovascular disease [53,54]. Other genetic determinants of NAFLD include variants of the glucokinase regulatory protein, regulating lipogenesis and the membrane-bound *O*-acyltransferase domain containing-7, influencing phospholipid metabolism [55,56].

Multiple mutations in genes regulating lipid secretion, such as apolipoprotein B, are also associated with increased risk of severe NAFLD, cirrhosis and HCC, and are responsible for familial cases of the disease [57].

All these new discoveries are expected to carry out an improvement in our ability to gauge the risk in the individual patient, identifying new disease mechanisms and tailored therapeutic targets.

## 3. Epidemiology and natural history of NAFLD

### 3.1. Epidemiology

A recent systematic review and meta-analysis estimated that 25% of the world adult population has NAFLD as diagnosed by imaging. Although NAFLD was highly prevalent in all continents, the highest prevalence rates were reported from South America and the Middle East, whereas the lowest prevalence was reported from Africa [58]. In Italy, the prevalence of NAFLD ranges approximately from 20% [59] to 25% [60–62].

Demographic (age, sex and ethnicity) and metabolic modifiers (MetS components) modulate the risk of developing NAFLD. The prototypic NAFLD patient is a 60-to-85 year-old Hispanic man; in contrast, individuals of European and, in particular, African descent are more spared from NAFLD [63]. Patients with either severe obesity (~98%) or T2DM (~70%) are maximally prone to NAFLD development; NAFLD is equally prevalent in either sex among patients with T2DM which, therefore, abrogates the higher prevalence of the male sex typically observed in non-diabetic NAFLD individuals [63]. The risk of NAFLD is also maximal (~85%) among patients with combined dyslipidemia and elevated serum aminotransferases [64].

The prevalence of NASH ranges from as many as 59.1% among biopsied NAFLD patients to 29.9% for North America and 6.7% for Asia, respectively, among patients who are not candidates for liver biopsy [58].

The pooled regional NAFLD incidence estimates for Asia and Israel were reported to be approximately 52 per 1000 and 28 per 1000 person-years, respectively [58]. In Italy, a pioneering study performed on a cohort of hysterectomized women reported an incidence rate of NAFLD of approximately 2 per 1000 women/year [65].

A recent meta-analysis estimated that the pooled mean fibrosis progression rate per year for patients with biopsy-proven NASH was 0.09, and approximately 40% of these patients progressed to the more advanced stages over time [58]. The annual incidence of HCC in NAFLD patients was 0.44 per 1000 person-years, whereas the corresponding figure for patients with NASH was 5.3 per 1000 person-years [58]. Compared to non-NAFLD control subjects, patients with NAFLD are exposed to an almost two-fold increased risk of liver-related mortality [58] and to a substantially increased risk of developing fatal and non-fatal CVD events [66].

Recent studies have described the specific features of NAFLD epidemiology in lean individuals [67], elderly people [68] and children [69].

### 3.2. Natural history of NAFLD: hepatic risk

NAFLD course has four clinical-pathological entities: steatosis, NASH, advanced fibrosis/cirrhosis, and HCC. Research has focused on risk factors for each of these steps and factors regulating the migration of patients from each entity to the others [70].

#### 3.2.1. Steatosis

Normal liver is almost totally devoid of any fat content [71]. Conversely, most patients with NAFLD will have simple steatosis, (fatty changes affecting >5% of hepatocytes), usually associated with mild chronic inflammation [72,73].

Steatosis results from the interaction of multiple factors including age, sex and lifestyle [70,73]. Body weight mirrors dietary habits and physical exercise and is a major determinant of NAFLD. Body weight changes of as little as 2–3 kg are associated with either increased risk of developing NAFLD or its reversal [74]. Finally, various drugs may contribute to reversing NAFLD [5].

#### 3.2.2. NASH

Steatosis plus mild inflammatory changes and "ballooning" hepatocyte degeneration define NASH, which accounts for up to 30% of NAFLD cases [73].

The strongest predictors of NASH are: older age, male sex, and various genetic and metabolic risk factors [75–81].

Simple steatosis can certainly progress to NASH [82–84]. The time to progress to cirrhosis from early, disease would probably take as long as approximately 57years for steatosis compared to 24 years for NASH [3]. The rates of disease progression are slower than those observed in HCV infection [85].

Glitazones do not significantly reverse hepatocyte ballooning; this finding supports the view that hepatic/systemic IR plays a key role in triggering the early phases of NAFLD development rather than sustaining its subsequent progression [86].

#### 3.2.3. Advanced fibrosis/cirrhosis

NASH strongly predicts the development of advanced fibrosis together with age, body mass index (BMI), sex, genetic and hormonal/metabolic factors [87–94]. Fibrosis, in its turn, strongly predicts liver-related mortality in NAFLD [95]. Statins seem also to exert some beneficial effect on liver fibrosis progression in patients with and without T2DM [96,97].

NASH-cirrhosis histologically exhibits advanced fibrosis and nodular changes; however, fatty changes may typically disappear over time[98].

Obesity, T2DM and steatosis, together with the presence of carotid atherosclerotic plaques and increased intima-media thickness, are strongly associated with advanced fibrosis/cirrhosis [99–101]. In particular, the coexistence of T2DM and steatosis

strongly predicts the development of clinically significant hepatic fibrosis [100].

#### 3.2.4. HCC

Compared to other etiologies of HCC, NAFLD-HCC is associated with a lower male/female ratio and may occur in non-cirrhotic livers [102–104]. NAFLD-HCC is often diagnosed late as a result of its escaping surveillance protocols, which translates into curtailed chances for radical treatment and accounts for a worse prognosis compared with HCC caused by viral hepatitis [104–106]. The PNPLA3 I148M polymorphism, age, BMI, T2DM, dietary habits and drugs may bi-directionally modulate the risk of developing NAFLD-HCC [92,107–113] and T2DM aggravates HCC outcome [114].

### 3.3. Natural history of NAFLD: cardiovascular, arrhythmic and diabetes risks

Cardiovascular disease (CVD) is the leading cause of mortality in patients with NAFLD [95,115–117]. A recent systematic review and meta-analysis showed that patients with NAFLD had a higher risk of fatal and non-fatal CVD events than those without NAFLD [66]. Patients with more 'severe' NAFLD were also more likely to develop fatal and non-fatal CVD events [66]. Although the results of this updated meta-analysis provide robust evidence of the association between NAFLD (and NAFLD severity) and risk of major CVD events, it is important to highlight that causality remains to be further proven in high-quality intervention studies. Given that CVD complications frequently dictate the outcome of NAFLD, a screening of the cardiovascular system is mandatory in all patients with NAFLD, at least by detailed risk factor assessment [11].

NAFLD is also linked with subclinical myocardial remodeling and dysfunction (i.e., functional and structural cardiomyopathy), valvular heart diseases (i.e., aortic-valve sclerosis or mitral annulus calcification), and increased prevalence and incidence of permanent atrial fibrillation [118–124]. Moreover, NAFLD is strongly and independently associated with heart rate-corrected QT interval prolongation [125,126] and an increased prevalence of ventricular arrhythmias on 24-h ECG-Holter monitoring [127]. Finally, preliminary evidence also suggests that NAFLD is associated with higher 1-year re-hospitalization rates in patients hospitalized for acute heart failure [128].

For many years, NAFLD has been considered as the simple "*hepatic manifestation of the MetS*" [63,129]. However, a large number of retrospective and prospective observational studies have recently demonstrated that NAFLD is an early predictor of and a determinant for the development of new-onset T2DM and MetS [4,130]. A recent systematic review and meta-analysis confirmed that NAFLD (as diagnosed either by abnormal serum liver enzymes or by ultrasonography) is indeed associated with almost two-fold increased incidence rates of both T2DM and MetS over a median 5-year follow-up period [7]. Consistently, recent observational studies have shown that there is a strong and independent association between improvement of NAFLD and decreased incidence of T2DM [131,132]. Future controlled intervention trials are, therefore, needed to determine whether treating NAFLD leads to T2DM risk reduction.

### 3.4. Natural history of NAFLD: cancer risk

Indirect evidence for an association of NAFLD with cancer incidence and mortality came from large epidemiological studies, which have clearly demonstrated that obesity and T2DM contribute to increased incidence and mortality from several cancer types, including liver and colo-rectal cancers [133–135]. Consistently, longitudinal studies showed that NAFLD was associated with increased burden of hepatic and extra-hepatic cancers [136] and malignancy was a leading cause of mortality in patients with NAFLD, ranking second after CVD mortality [137] and accounting for up to one third of deaths in patients with NAFLD and T2DM [138].

Primary liver cancer is deemed to be causally associated with NAFLD. HCC incidence is steadily increasing over years in parallel with the burst of "*diabesity*". NAFLD is becoming a major cause of HCC and the second most common cause of HCC in patients listed for liver transplantation [106,139]. Worryingly, the whole histological spectrum of NAFLD can lead to HCC and as many as 50% of NAFLD-related HCC occur in patients without cirrhosis and are often detected late [104]. Cumulative HCC incidence or mortality rates seem to be lower in NASH-cirrhosis than in cirrhosis due to other etiologies; however, it is not possible to clearly gauge the actual HCC risk in the whole NAFLD population [140–142]. Apart from advanced fibrosis/cirrhosis, data only point to male sex, older age, alcohol abuse and MetS components as established clinical risk factors for HCC development in NAFLD [108,143,144]. For these reasons, except for patients with NASH-cirrhosis, specific surveillance/screening strategies cannot be currently recommended in NAFLD patients. Mounting evidence also suggests that MetS and NAFLD are involved in the development of other types of primary benign and malignant liver tumors, i.e. hepatocellular adenoma [145,146] and intra-hepatic cholangiocarcinoma [147–150]. Of note, the interplay between NAFLD and HCC seems to be more complex than the classic linear equation fibrosis-cirrhosis-dysplasia, and probably hepatocellular adenoma plays a role in this process [103,151,152].

Emerging evidence also suggests a link between NAFLD and some extra-hepatic cancers, typically those closely associated with "*diabesity*" [153]. In particular, colorectal adenoma and cancer have been associated with NAFLD by retrospective studies from Asia, USA and Europe [154–157]. There are also isolated reports of an increased risk of other gastrointestinal (esophagus, stomach and pancreas) and extra-gastrointestinal malignancies (kidney, prostate, lung and breast) [136,153,158–160]. However, all these data are preliminary, and their validity remains to be evaluated prospectively before specific surveillance/screening strategies may be issued.

## 4. Diagnosis and clinical approach to NAFLD

### 4.1. Diagnosis

Given that approximately one third of the adult Europe and USA populations have NAFLD and that invasive diagnostic techniques are not applicable in this impressive number of individuals, non-invasive biomarkers of steatosis and fibrosis are necessary. As shown in Fig. 2, the recently published European clinical practice guidelines for the management of NAFLD have proposed a diagnostic flow-chart to assess and monitor disease severity in the presence of suspected NAFLD and metabolic risk factors [11].

The diagnosis of NAFLD should rely on the following criteria: (i) hepatic steatosis on either imaging or histology, (ii) no excessive alcohol consumption (a threshold of 20 g/day for women and 30 g/day for men is conventionally adopted), and (iii) no competing causes of hepatic steatosis [11].

*Liver biopsy* remains the reference standard for diagnosing NASH and staging fibrosis in patients with NAFLD. However, this procedure is invasive, potentially risky, patient unfriendly and subject to sampling error; therefore, liver biopsy is not suitable for diagnosis in large cohorts of individuals or for patient monitoring [11].

*Imaging techniques*. The gold standard to diagnose hepatic steatosis is the magnetic resonance imaging (MRI) or spectroscopy (MRS) that can detect amount of fat as low as 1% [11]. However, the cost of MRI limits its routine clinical use. Ultrasonography

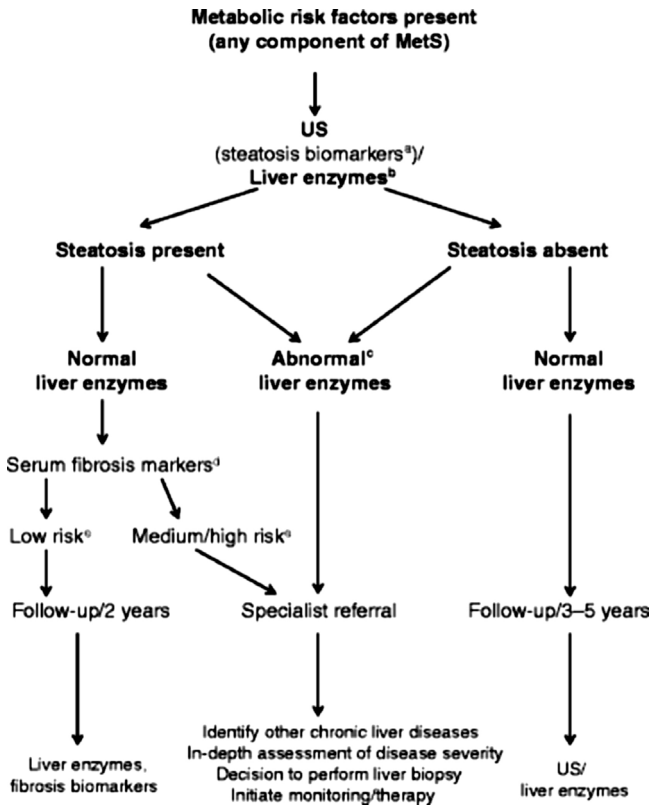


**Fig. 2.** Diagnostic flow-chart to assess and monitor disease severity in the presence of suspected NAFLD and metabolic risk factors. [1]Steatosis biomarkers: Fatty Liver Index, SteatoTest, NAFLD fat score. [2]Liver tests: ALT, AST orGGT. [3]Any increase in ALT, AST or GGT. [4]Serum fibrosis markers: NAFLD Fibrosis Score, FIB-4, Commercial tests (e.g., FibroTest, FibroMeter or ELF scores). [5]Low risk: indicative of no/mild fibrosis; medium/high risk: indicative of significant fibrosis or cirrhosis.
Reprinted, with permission, from Ref. [11].

remains the recommended first-line imaging modality in clinical practice, but its accuracy is much lower than that of MRI/MRS and its cost is also lower than that of MRI. Recently, with the development of new imaging sequences for MRI, time of screening is decreased (approximately 10–15 min/session) and thus the cost can become more affordable [161,162]. Other imaging techniques, e.g. ultrasonography-based transient elastography (Fibroscan), 2D acoustic radiation force impulse imaging or MR-elastography, can also be used to diagnose liver fibrosis [11]. The main limitation of ultrasonography-based transient elastography in clinical practice is its failure to obtain reliable liver stiffness measurements (~20% of cases), mainly in obese patients, which diminishes its application in NAFLD.

*Serum biomarkers*. Hepatic steatosis is often associated with mild-to-moderate elevations of serum aminotransferase and gamma-glutamyl transferase (GGT) levels, but, at best, liver enzymes only identify people who are at increased risk of NAFLD and who require further diagnostic tests. However, serum liver enzyme levels are inaccurate indicators for and, therefore, should not be used alone in clinical practice [11]. Among the best scores for steatosis there are the fatty liver index (FLI) [163], the NAFLD liver fat score [164] and the SteatoTest [165]. The first two scores can be easily calculated using serum triglycerides, GGT, insulin and aminotransferases, BMI, waist circumference and presence of MetS or T2DM [166]. The SteatoTest is a commercial kit.

A common clinical concern in patients with NAFLD is whether they have simple steatosis or NASH and, more importantly, what the stage of hepatic fibrosis is and whether the level of fibrosis (which dictates clinical outcome) has increased over time. There are various hepatic fibrosis non-invasive biomarkers: the NAFLD fibrosis score [167], the FIB-4 [168], the FibroTest, the Fibrometer, and the Enhanced Liver Fibrosis (ELF) score [165,169]. The first two scores can be calculated using platelet count, albumin, and aminotransferases. The FibroTest, Fibrometer and ELF scores are commercial tests. Although non-invasive methods require further validation, the various tests could be useful for selecting those patients with NAFLD who will require a liver biopsy. However, for the diagnosis of NASH biochemical tests or imaging techniques cannot distinguish NASH from simple steatosis and liver biopsy remains the reference standard [11].

### 4.2. Clinical approach

CVD complications are common in NAFLD patients and the risk of CVD and poor CVD outcomes is particularly increased in those NAFLD patients with severe liver damage [95,117], suggesting that NAFLD exerts a pro-atherogenic effect via a pro-inflammatory and pro-fibrogenic *milieu* that characterizes severe disease [170].

On these grounds, a careful assessment of cardiovascular risk (CVR) in all patients with NAFLD is warranted. It should include the assessment of traditional CVR factors and also indices of severity of liver disease, (serum sodium and albumin, the Model for end-stage liver disease (MELD) score and the NAFLD fibrosis score [171]). Whether PNPLA3 [172] and TM6SF2 [54] gene variants, which are associated with CVD alterations in NAFLD, may help to stratify the CVR needs further investigation.

Overall, both hepatic and extra-hepatic risk factors should be evaluated in all patients with NAFLD. Namely, besides the non-invasive assessment of liver damage (as described above), a careful search for cardio-metabolic comorbidities should also include the evaluation of family history for CVD/T2DM, the assessment of BMI and waist circumference, the measurement of plasma lipid values, estimated glomerular filtration rate and albuminuria and, in selected cases, the genetic assessment for storage disorders, such as the lysosomal acid lipase deficiency [116,173]. In nondiabetic patients, screening for T2DM by measuring fasting or random blood glucose or hemoglobin A1c levels is also mandatory. Similarly, repeated assessment of arterial pressure should be recommended to non-hypertensive patients. Finally, CVR assessment should rely not only on the available CVR scoring systems, such as the Framingham or "*Progetto cuore*" risk charts, but also on the assessment of the severity of liver disease [171]. In patients at high CVR or with established T2DM, referral to cardiologists should also be considered and further diagnostic tests performed whenever indicated. In nondiabetic patients at low CVR, re-assessment every 2–3 years may be suggested based on clinical judgment alone. Patients with advanced fibrosis/cirrhosis should enter appropriate follow-up schedules aimed at an early diagnosis of HCC and portal hypertension. However, a generalized HCC screening policy cannot be recommended in all non-cirrhotic NAFLD patients at present, although certain NAFLD individuals are at an increased risk.

Finally, in patients with dyslipidemia, hypertension or T2DM, the careful control of these cardiometabolic disorders will not only reduce the NAFLD-associated metabolic and CVR, but also slow liver disease progression. To achieve this goal statins, insulin-sensitizers, incretin mimetics and ACE-inhibitors/sartans, if necessary, can safely be used.

## 5. Treatment of NAFLD/NASH

### 5.1. Nutritional aspects and lifestyle changes

All the recommendations from the most important scientific societies indicate that body weight reduction obtained through

lifestyle changes is the most effective treatment for NAFLD. A large body of evidence indicates that unhealthy life-style is a relevant risk factor for NAFLD/NASH [17,174]. Accordingly, lifestyle changes focusing on weight loss, physical activity and healthy diet represent the best therapeutic approach [175].

A body weight reduction of 7–10% obtained with energy restriction and/or regular physical activity is associated with histological improvement/resolution of steatosis and inflammation and regression of fibrosis [176,177].

Energy restriction should be obtained with a low-calorie (about 1200–1600 kcal/day) low-fat low-carbohydrate diet [178]. The diet composition in NAFLD should consist of <30% fat, notably with no more than 10% of saturated fatty acid, and relatively low in carbohydrates, ~50% of total kcal, notably by reducing high-sugar food [179].

As for physical activity, both aerobic activities (150–200 min/week of moderate intensity aerobic exercise in about four sessions, i.e. brisk walking and/or stationery cycling) and resistance training effectively reduce hepatic fat content. Training should be tailored based on patients' preferences and maintained long-term [177,180,181].

Changes of diet composition, especially for non-obese patients, are a realistic and feasible approach even if based on less consistent evidence [182]. The gold standard option is a fiber-rich diet relatively low in carbohydrates (complex ones should be preferred) and fat content, with the avoidance of fructose [179], a moderate use of coffee and adherence to a Mediterranean dietary pattern [183,184]. Indeed, among all the proposed diets, the Mediterranean diet appears as the most effective dietary option for inducing a weight loss together with beneficial effects on all cardio-metabolic risk factors associated with NAFLD.

### 5.2. New drugs for NASH

Drug therapy should be reserved for NASH patients who are at maximal risk for disease progression. To date, there are very few high-quality, randomized, blinded, adequately powered, controlled studies of sufficient duration and with adequate histological outcomes. As a fact, currently there are no approved available drugs for the treatment of NASH. Moreover, drug treatment of NASH has been focused toward reducing steatosis, necro-inflammation and fibrosis.

Given that NASH is closely related to T2DM, hypoglycemic drugs have been largely evaluated. In patients with T2DM, incretin mimetics, which act by agonizing glucagon-like peptide 1 (GLP-1) receptor, have proven effective in reducing hepatic/systemic IR, serum liver enzymes and liver fat content. The LEAN trial performed on 52 obese patients showed that liraglutide was able to resolve histologic features of NASH in 39% of the treated patients [185]. These responders also registered a mean weight loss of 2.1 kg raising uncertainty as to whether the beneficial hepatic effect was due to liraglutide per se or combined with weight loss. Further long-term studies with liraglutide are needed to confirm its efficacy in patients with NASH.

Other insulin-sensitizers, such as PPAR-γ agonists have been evaluated in NASH [186]. The best level of evidence is available for pioglitazone in biopsy-proven NASH patients [187]. PPARs modulation results in transcriptional regulation of several genes involved in metabolic pathways [188] and the modulation of a dual PPAR-α/δ agonist through elafibranor is effective in improving plasma lipid profile, and hepatic/systemic IR [189]. In the phase IIb GOLDEN trial, elafibranor was tested with a primary histological end-point (NASH resolution without fibrosis worsening), which was achieved in 23% and 21% of patients treated with either 80 mg/day or 120 mg/day, respectively [190]. Post-hoc analysis showed a clear benefit in patients with more severe disease, prompting a phase III trial.

Another nuclear receptor to be targeted to obtain reduced IR and liver injury, is FXR, an intracellular bile acids receptor [191] capable of inhibiting bile acids synthesis, reducing de novo lipogenesis and steatosis by improving IR. In the FLINT trial, treatment with obeticholic acid, a potent FXR activator, achieved a primary end-point of improving the necro-inflammation without worsening of fibrosis in 46% of the treated patients. Moreover, compared to placebo, the NASH resolution was obtained in 22% of treated patients [192]. However, efficacy and long-term safety (e.g., pruritus and increased

**Table 1**
Ongoing clinical trials for NASH in adult subjects.

| Drug | Clinical trial number | Molecular target | Route of administration | Phase of clinical trial | Histological end-points |
|---|---|---|---|---|---|
| Drugs targeting metabolic homeostasis | | | | | |
| Elafibranor | NCT02704403 | Peroxisome proliferator-activator receptor α/δ agonist | Oral, once-daily | Phase 3 | Yes |
| Dapagliflozin | NCT02696941 | Sodium-glucose co-transporter 2 inhibitor | Oral, once-daily | Pilot | No |
| Empagliflozin | NCT02964715 and NCT02637973 | Sodium-glucose co-transporter 2 inhibitor | Oral, once-daily | Pilot | Yes |
| Tofogliflozin | NCT02649465 | Sodium-glucose co-transporter 2 inhibitor | Oral, once-daily | Pilot | Yes |
| Semaglutide | NCT02970942 | Glucagon-like peptide 1 analogue | Subcutaneous injection, once-daily | Phase 2 | Yes |
| Liraglutide | NCT02721888 | Glucagon-like peptide 1 analogue | Subcutaneous injection, once-daily | Pilot | No |
| MSDC-0602K | NCT02784444 | Mitochondrial target of thiazolidinediones modulator | Oral, once-daily | Phase 2 | Yes |
| Aramchol | NCT02279524 | Stearoyl CoA desaturase inhibitor | Oral, once-daily | Phase 2b | Yes, as secondary outcomes |
| GS-0976 | NCT02856555 | Acetyl-CoA carboxylase inhibitor | Oral, once-daily | Phase 2 | No |
| TVB-2640 | NCT02948569 | Fatty acid synthase inhibitor | Oral, once-daily | Phase 1/2[b] | No |
| ARI-3037MO | NCT02574325 | Niacin analogue | Oral, twice-daily | Phase 2 | No |
| AZD4076 | NCT02826525 | Micro RNA 103/107 antagonist | Subcutaneous injection | Phase 1/2a | No |

Table 1 (*Continued*)

| Drug | Clinical trial number | Molecular target | Route of administration | Phase of clinical trial | Histological end-points |
|---|---|---|---|---|---|
| MGL-3196 | NCT02912260 | Liver-targeted selective agonist for the thyroid hormone receptor-β | Oral, once-daily | Phase 2 | Yes, as secondary outcomes |
| VK2809 | NCT02927184 | Liver-targeted selective eagonist for the thyroid hormone receptor-β | Oral | Phase 2 | No |
| Obeticholic acid | NCT02548351 | Synthetic bile acid farnesoid X receptor agonist | Oral, once-daily | Phase 3 | Yes |
| LJN452 | NCT02855164 | Nonsteroidal farnesoid X receptor agonist | Oral | Phase 2 | No |
| GS-9674 | NCT02854605 | Nonsteroidal farnesoid X receptor agonist | Oral, once-daily | Phase 2 | No |
| EDP-305 | NCT02918929 | Nonsteroidal farnesoid X receptor agonist | Oral, once-daily | Phase 1 | No |
| BMS-986036 | NCT02413372 | Fibroblast growth factor 21 analogue | Subcutaneous injection, once-daily | Phase 2 | No |
| NGM282 | NCT02443116 | Fibroblast growth factor 19 analogue | Subcutaneous injection, once-daily | Phase 2 | No |
| Volixibat (SHP626) | NCT02787304 | Ileal apical sodium-dependent bile acid transporter inhibitor | Oral, once-daily | Phase 2 | Yes |
| MT-3995 | NCT02923154 | Nonsteroidal mineral corticoid receptor antagonist | Oral | Phase 2 | No |
| Testosterone undecanoate | NCT01919294 | Hypogonadism | Intramuscolar injection | Pilot | Yes |
| Somatropin | NCT02217345 | Growth hormone | Injection, once-daily | Pilot[b] | No |
| Tesamorelin | NCT02196831 | Synthetic analogue of growth hormone-releasing hormone | Subcutaneous injection, once-daily | Phase 2 (HIV only) | Yes, as secondary outcomes |
| Metreleptin | NCT01679197 and NCT02654977 | Recombinant-methionyl human leptin | Subcutaneous injection | Phase 2 (pts with partial lipodystrophy) | Yes |
| Drugs targeting oxidative stress, inflammation and apoptosis | | | | | |
| Vitamin E | NCT01792115 and NCT02962297 | Antioxidant | Oral | Phase 2-3[b] | Yes |
| Metadoxine | NCT02541045 | Antioxidant | Oral, twice-daily | Phase 3[b] | Yes |
| Emricasan | NCT02686762 and NCT02960204 | Caspase inhibitor | Oral, twice-daily | Phase 2[a] | Yes |
| GS-4997 | NCT02781584 and NCT02466516 | Apoptosis signal-regulating kinase 1 inhibitor | Oral, once-daily | Phase 2 | No |
| Cenicriviroc | NCT02330549 and NCT02217475 | Dual C-C chemokine receptor types 2 and 5 antagonist | Oral, once-daily | Phase 2 | Yes |
| JKB-121 | NCT02442687 | Toll-like receptor 4 antagonist | Oral, twice-daily | Phase 2 | No |
| Amlexanox | NCT01975935 and NCT01842282 | TANK-binding kinase 1 and IκB kinase ε inhibitor | Oral, thrice-daily | Phase 2 | No |
| CF102 | NCT02927314 | A3 adenosine receptor agonist | Oral, twice-daily | Phase 2 | No |
| MN-001 (tipelukast) | NCT02681055 | Multiple targets (leukotriene receptor antagonism, inhibition of phosphodiesterases 3 and 4, inhibition of 5-lipoxygenase) | Oral | Phase 2 | No |
| Drugs targeting fibrosis | | | | | |
| GR-MD-02 | NCT02421094 | Galectin-3 inhibitor | Intravenous infusion | Phase 2[a] | No |
| Simtuzumab | NCT01672866 and NCT01672879 | Lysyloxidase-like 2 antibody | Subcutaneous injection/intravenous infusion | Phase 2[a] | Yes |
| Other drugs | | | | | |
| Solithromycin | NCT02510599 | Antibiotic | Oral, once-daily | Phase 2 | Yes |
| IMM-124E | NCT02316717 | IgG-rich bovine colostrum | Oral, thrice-daily | Phase 2 | No |
| Fecal microbiota transplantation | NCT02469272 and NCT02868164 | Gutmicrobiota | Duodenal infusion | Pilot[a] | No |

From ClinicalTrials.gov accessed on 6th December, 2016 [Interventional Studies with Drugs/Biological Agents; Open Studies (recruiting; not yet recruiting; expanded access) or Closed Studies, but Active, Not Recruiting, without Results; Non Alcoholic Fatty Liver Disease/Non Alcoholic Steatohepatitis].

[a] Trials including patients with cirrhosis.

[b] Trials not including patients with type 2 diabetes.

LDL-cholesterol levels) associated with the use of this drug need to be addressed. And a phase III study is now ongoing to this end.

Another therapeutic option for NAFLD may be to decrease oxidative stress by administration of an antioxidant, such as vitamin E. In the PIVENS trial [187], in 247 nondiabetic NASH adults, vitamin E treatment (800 U/day for 96 weeks), was associated with significant improvements in serum liver enzymes and NASH histological features (steatosis, inflammation and ballooning) compared to placebo. However, before vitamin E can be recommended for the treatment of NAFLD, further studies are required to support efficacy and safety of this fat-soluble vitamin.

At today, only elafibranor and obeticholic acid are recruiting for phase III trials; results are expected in 2020. Moreover, several other phase II trials are in progress to evaluate the effect of newer drugs on different pathogenic pathways involved in NASH. Table 1 recapitulates ongoing clinical trials for NASH in adult subjects.

Promising results are expected for novel drugs, which improve metabolic disturbances such as Aramchol (phase II trial), a conjugate of cholic and arachidic acid, which partially reduces the activity of stearoyl-CoA desaturase and de novo lipogenesis [193,194]. Emricasan [195], an oral pan-caspase inhibitor acting on TNF-alpha driven inflammation, seems to be effective in reducing liver damage by modulating apoptosis. Cenicriviroc [196], an oral antagonist of chemokine CCR2/CCR5, has recently closed the phase II trial and results are expected. The possibility of reverting fibrosis and cirrhosis has also been explored by using simtuzumab, a monoclonal antibody against lysyloxidase-like-2, which is an extra-cellular amine oxidase involved in the post-translational modification of collagens and elastin in the extracellular matrix [197].

## 6. Conclusions and perspectives

A great deal of new knowledge in the physiopathology of NAFLD has been accumulating over the last decade, revealing the complexity of the mechanisms involved in the development and progression of this condition. The most recent guidelines/expert opinions for NAFLD management prompt a new “systems medicine” approach to the interplays between brain and nervous system, endocrine system, digestive system (gut, liver and microbiota) and immune system [11,198]. New concepts for patient stratification are needed to identify different clinical prototypes within the indistinct phenotype of the MetS [11,198].

The emerging and rapidly growing field of the molecular imaging will likely be providing an extraordinary new opportunity for non-invasive studies of in vivo physiopathology of NAFLD overcoming the inherent limitations of liver biopsy [161,199,200]. Such limitations have so far hampered the progress of the knowledge in key fields such as the impact and role of autophagy in the physiopathology of NAFLD [201]. Studies combining the new imaging techniques with liver and blood metabolomics and the analysis of the interplay between specific genes and the epigenetic factors conditioning their expression, will pave an attractive new way to targeting the dynamics of pathogenic processes involved in NAFLD and NASH. The results of these ongoing studies will identify novel risk factors, new diagnostic and prognostic biomarkers and therapy targets for a better prevention, outcome prediction and personalized treatment of NAFLD/NASH. In the meantime, to halt/reduce the tide of NAFLD, reference should be made to standard principles of good clinical practice for the management of this condition [11,15,202,203], which is expected to impact on the future global burden of disease worldwide.

## Conflict of interest

None to declare: Stefano Bellentani, Amedeo Lonardo, Gianluca Svegliati Baroni, Giovanni Targher, Luca Valenti Mauro Bernardi: CSL Behring GmbH (consultancy and speaker); Baxter Healthcare SA (consultancy and speaker); PPTA Europe, Gilead Sciences, AbbVie Italia (speaker).

Ferruccio Bonino: Adivsory Boards and/or Speakers Bureau for Abbott/Abbvie, Baldacci Laboratories, BMS, Fujirebio, GSK, Gilead, MSD, Novartis and Roche Elisabetta Bugianesi: Consultant for Genfit, IBSA, Innova, Boehriger Ingelheim, Intercept Alessandro Casini: Probios SrL: research contract Amalia Gastaldelli: Consultant for Roche, Eli-Lilly, Menarini, Sanofi and has received research support from Amylin-BMS AZ.

Giulio Marchesini: Consultant and/or speaker for: Eli Lilly, Astra Zeneca, Novartis, IBSA. Clinical studies: Novo Nordisk, Sanofi, Boehringer Ingelheim, Glaxo Smith Kline, Janssen, Gilead, Genfit.

Fabio Marra: Abbvie, AstraZeneca, Bayer, Menarini: consultant fees. Gilead, Bayer, ViiV Healthcare: speaker honoraria.

Luca Miele: Advisory Board: Synageva, MyGenomics, IBSA, MSD, Boehringer-Ingelheim, BMS Speaker fee: Rottapharm Madaus, MEDA Filomena Morisco: Nathura and IBI Lorenzini: research contract. BMS and Abbvie: speaker fee Salvo Petta: Advisory Boards and/or Speaker for Gilead, AbbVie, Janssen, Bristol-Myers Squibb, Merck Sharp and Dohme Fabio Piscaglia: Bayer (speaker fee and advisory board). Bracco (speaker fee). Esaote (research contract). Meda (speaker fee). Eisai (advisory board).

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