# ARTICLE IN PRESS

Progress Report

# A "systems medicine" approach to the study of non-alcoholic fatty liver disease

Salvatore Petta[a], Luca Valenti[b], Elisabetta Bugianesi[c], Giovanni Targher[d], Stefano Bellentani[e,f], Ferruccio Bonino[g,*], the Special Interest Group on Personalised Hepatology of the Italian Association for the Study of the Liver (AISF)[1]

[a] Section of Gastroenterology, Di.Bi.M.I.S Policlinico Paolo Giaccone Hospital, University of Palermo, Italy
[b] Internal Medicine, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Department of Pathophysiology and Transplantation, University of Milan, Italy
[c] Gastroenterology and Hepatology, Department of Medical Sciences, Città della Salute e della Scienza di Torino Hospital, University of Turin, Italy
[d] Section of Endocrinology, Diabetes and Metabolism, Department of Medicine, University Hospital of Verona, Italy
[e] Shrewsbury and Telford NHS Trust, Department of Gastroenterology, Shrewsbury, UK
[f] Fondazione Italiana Fegato, Bassovizza, Trieste, Italy
[g] General Medicine 2, Department of Clinical and Experimental Medicine, University Hospital of Pisa, Italy

## ARTICLE INFO

## ABSTRACT

The prevalence of fatty liver (steatosis) in the general population is rapidly increasing worldwide. The progress of knowledge in the physiopathology of fatty liver is based on the systems biology approach to studying the complex interactions among different physiological systems. Similarly, translational and clinical research should address the complex interplay between these systems impacting on fatty liver. The clinical needs drive the applications of systems medicine to re-define clinical phenotypes, assessing the multiple nature of disease susceptibility and progression (e.g. the definition of risk, prognosis, diagnosis criteria, and new endpoints of clinical trials). Based on this premise and in light of recent findings, the complex mechanisms involved in the pathology of fatty liver and their impact on the short- and long-term clinical outcomes of cardiovascular, metabolic liver diseases associated with steatosis are presented in this review using a new "systems medicine" approach. A new data set is proposed for studying the impairments of different physiological systems that have an impact on fatty liver in different subsets of subjects and patients.

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## 1. Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) in the general population is growing worldwide: 44% in the USA, 33% in Europe and 25% in Italy [1–6]; for non-alcoholic steatohepatitis (NASH), the progressive form of NAFLD, the estimate is 2–3% [3,5]. A large cohort study reported that NAFLD was associated with 26% higher 5-year overall health-care costs, mainly from cardiometabolic diseases [6].

Progress in the knowledge of NAFLD/NASH pathophysiology was driven by the "systems biology" approach, i.e. the interdisciplinary study of complex interactions within different biological systems. "Systems pathophysiology" studies the complex interactions between major human vital systems and their interplay. "Systems medicine" combines systems biology and pathophysiological approaches to translational research, integrating various bio-medical tools and using the power of computational and mathematical modelling. This enables the personalization of diagnosis, prognosis and treatment. Systems medicine helps to re-define clinical phenotypes using molecular and dynamic parameters to discover new diagnostic and prognostic biomarkers and to guide the design of new clinical trials. Thus, a systems medicine approach appears mandatory for a "patient-based" classification of the complex interactions between different biological systems and physiological functions involved in NAFLD/NASH, now grouped under the general definition of "metabolic syndrome" (MetS; Table 1). A special interest group (SIG) of the Italian Association for the Study of the Liver (AISF) assembled after the 2013 Single Topic Conference on Personalized Hepatology, held in Pisa

* Corresponding author at: General Medicine 2, Department of Clinical and Experimental Medicine, University of Pisa, Lungarno Bruno Buozzi n. 13, 56125 Pisa, Italy. Tel.: +39 050 543858; fax: +39 050 995457.
  E-mail address: ferruccio.bonino@unipi.it (F. Bonino).
[1] See Appendix A.

**Table 1**
(a) Definition of the metabolic syndrome, according to recent classifications. (b) Quantitative score to estimate the impact of metabolic factors on nonalcoholic fatty liver disease.

**(a)**

| Feature | National Cholesterol Education Program, ATP-III | International Diabetes Federation | Joint statement of IDF, NHLBI, AHA, WHF, IAS, IASO |
|---|---|---|---|
| Visceral obesity | >102 cm (males), >88 cm (females) | ≥94 cm (males), ≥80 cm (females)(ethnic differences) | ≥94 cm (males), ≥80 cm (females)(ethnic differences) |
| Lipid levels | TG ≥ 150 mg/dL or treated for dyslipidemia HDL-Chol <40 mg/dL (males); <50 mg/dL (females) | TG ≥ 150 mg/dL or treated for dyslipidemia HDL-Chol <40 mg/dL (males); <50 mg/dL (females) | TG ≥ 150 mg/dL or treated for dyslipidemia HDL-Chol <40 mg/dL (males); <50 mg/dL (females) |
| Arterial pressure | ≥130/85 mmHg or treated for Htx | ≥130/85 mmHg or treated for Htx | ≥130/85 mmHg or treated for Htx |
| Blood glucose | ≥110 mg/dL or treated for DM | ≥100 mg/dL or treated for DM | ≥100 mg/dL or treated for DM |
| Notes | 3 of the above | Visceral obesity + 2 of the above | 3 of the above |

**(b)**

| Grade | |
|---|---|
| 0 | No abdominal adiposity and no other features of MetS |
| 1 | Abdominal adiposity |
| 2 | Abdominal adiposity + 1 feature of MetS (i.e. atherogenic dyslipidemia, low HDL cholesterol and/or high TG, hypertension or fasting hyperglycemia/glucose intolerance/diabetes) |
| 3 | Abdominal adiposity + 2 features of MetS |
| 4 | Abdominal adiposity + 3 features of MetS |

*Abbreviations*: ATPIII, Adult Treatment Panel-III; IDF, International Diabetes Federation; NHBLI, National Heart, Blood and Lung Institute; AHA, American Heart Association; WHF, World Heart Federation; IAS, International Atherosclerosis Society; IASO, International Association for the Study of Obesity; DM, diabetes mellitus; TG, triglycerides; Htx, hypertension; HDL, High Density Lipoprotein; MetS, Metabolic Syndrome.
*Notes*:
The 10-year risk of having a cardiovascular disease (CVD) can be estimated by the Framingham risk score equation inserting major CVD risk factors (i.e., age, sex, total cholesterol, HDL-cholesterol, smoking history, systolic blood pressure and current use of anti-hypertensive drugs) of the individual patient in the NIH web site: http://cvdrisk.nhlbi.nih.gov/.
All patients included in either score 0 or score 1 are in primary prevention of CVD.
Traditional CVD risk factors considered are as follows: family history of premature CVD, age, male sex, cigarette smoking, hypertension (i.e., blood pressure ≥140/90 mmHg or on treatment), LDL-C ≥130 mg/dl (or on treatment), atherogenic dyslipidemia (i.e., triglycerides ≥150 mg/dl and/or HDL-C <50 mg/dl in women <40 mg/dl in men or on treatment), obesity (BMI ≥30 kg/m$^2$) and CKD (estimated glomerular filtration rate <60 ml/min/1.73 m$^2$).

in October 2013. The steering and writing committees of this work included all the experts of the NAFLD/NASH session. Using a rigorous, evidence-based approach, the experts identified and synthesized the literature that forms the evidence regarding specific topics; using their expertise to interpret the evidence, they compiled the specific chapters. The final version of the manuscript was assembled using a step-wise editing process conducted via web-based communication. The steering and writing committees sought additional opinions from an external group of experts who offered their input on an individual basis. The proposed systems medicine approach for the clinico-pathological assessment of the complex abnormalities that have an impact on NAFLD was used as a basis for launching a large prospective cohort study supported by the AISF.

## 2. Pathophysiology

Obesity and insulin resistance (IR) lead to intrahepatic triglyceride storage, considered the 'first hit', that ultimately leads, after a 'second hit' [7], to hepatocyte necrosis, inflammation and fibrosis (NASH). More recently, this view was challenged by a 'multiple hits' hypothesis, where multiple extra- and intra-hepatic signals are implicated [8–11]. Lipotoxicity is not necessarily linked to triglycerides accumulating within the liver, as some free fatty acids (FFA; palmitate and other lipotoxic intermediates) were shown to be more hepatoxic than triglycerides [8–11]. Moreover, the liver is targeted by signals from other tissues, including adipose tissue, the gut and its microbiota.

### 2.1. Lipid partitioning, lipotoxicity and insulin resistance

The increased flux of FFA from an enlarged and insulin-resistant adipose tissue to the liver is considered the major determinant,

with smaller contributions from dietary fat and de novo lipogenesis [12–15]. In the liver, the bulk of absorbed FFA is re-esterified, packaged into very-low density lipoproteins (VLDL) and exported into the bloodstream as triglyceride-rich particles for supplying peripheral tissues. If the ability of insulin to suppress VLDL secretion is impaired, the result is atherogenic dyslipidaemia [13]. Liver steatosis per se does not cause liver necro-inflammation, and could even be seen as a mechanism for preventing lipotoxicity, allowing the storage of FFA in a less harmful form [14,15]. Lipotoxic intermediates and diacylglycerol (DAG) were implicated in hepatic IR, which, in turn, directly contributes to systemic IR and worsens both glucose and lipid metabolism [15].

### 2.2. Hepatic consequences of deranged metabolism

Different hepatic cell types and intracellular pathways determine the amount of damage and likelihood of progression to advanced fibrosis [16,17]. Kupffer cells play a key role in the pathogenesis of NASH; in mice, depletion of these cells ameliorates steatosis, inflammation, hepatic injury and fibrosis [17]. Kupffer cell activation is mainly dependent on danger signals from steatotic hepatocytes and the accumulation of toxic lipids and bacterial products, and is a critical contributor to the recruitment of monocytes into the liver.

Different signalling pathways regulate intra-hepatic inflammation. Inflammasome was indicated as a pivotal regulator of the interactions with the gut microbiota implicated in the progression of NAFLD and obesity [16–18]. NASH is characterized by hepatocyte apoptosis and apoptotic bodies are pro-inflammatory and pro-fibrogenic [19]; lipotoxic apoptosis is mediated by oxidative stress, considered a 'second hit', causing progression to NASH [8,20]. Autophagy is another relevant pathway for NAFLD pathogenesis; its inhibition increases triglyceride storage, but activation favours the progression of fibrosis [21].

## 2.3. Adipose tissue and NASH

Adipose tissue is a critical site for the development of systemic IR and an altered pattern of adipokine secretion is pivotal for adipose tissue dysfunction [22–25]. Leptin has pro-fibrogenic effects on the liver, via activation of several biological functions of hepatic stellate and Kupffer cells [23]. Adiponectin increases insulin sensitivity and has anti-inflammatory and anti-fibrogenic effects in the liver and in adipose tissue [22,24]. The renin–angiotensin system is another major modulator of IR, critical for liver inflammation and fibrogenesis [25].

## 2.4. Diet and gut microbiota

The gut microbiota contributes to obesity and fatty liver [26] and the intestinal immune system is critically involved in the regulation of gut microbiota and NALP3 for experimental NASH [18]. The severity of NAFLD histology was associated with higher carbohydrate intake (fructose and sucrose) [27] and accumulating evidence suggests that coffee might be protective on progression of NAFLD and fibrosis [28].

## 3. Diagnosis

The clinico-epidemiological features of high-risk groups play a major role in guiding the physician to suspect NAFLD and NASH in the single patient (age, sex, ethnicity, body weight and metabolic status) [1–6], whereas serum transaminases do not qualify as markers of NAFLD or NASH [29]. Liver biopsy is a "gold standard" for the diagnosis of NAFLD, even if the number of fat-containing hepatocytes does not correspond exactly to the quantification obtained by magnetic resonance proton spectroscopy ($^1$H MRS) fat-fraction [30–46]. Its major limitation is the tissue sample size, which corresponds only to 1/50,000th of the liver compared with 1/150th for MRS. Fat accumulation is spatially heterogeneous; a single biopsy may not adequately represent the overall fat content of the liver [31,32]. In addition, liver biopsy is invasive and impractical in monitoring persons at risk of fatty liver disease.

Ultrasound is the most commonly used technique for diagnosis [33,34], based on qualitative features, including echogenicity, echo-texture, beam attenuation, diaphragm and vessel visibility. Fat accentuates scattering; therefore, fatty liver appears hyper-echogenic. Because there is no absolute echogenicity for fat infiltration, a comparison of echogenicity of the kidney is required. These ultrasonographic criteria have a sensitivity range of 60–95% and specificity of 84–100%; in obese patients the sensitivity and specificity are reduced to 49% and 75% respectively [38]. Semi-quantitative ultrasound criteria (mild, moderate or severe steatosis) are affected by subjective interpretation, with poor reproducibility and a low sensitivity for mild steatosis [35]. Recently, several methods were proposed for the quantitative assessment of liver fat content using a combination of computer-assisted measures [39–43]. The computer-aided measurement of the ultrasound hepatic/renal echo intensity ratio and the hepatic echo intensity attenuation rate strongly correlated with liver fat content according to either histology or $^1$H MRS [38–43]. Therefore, the combination of ultrasound-based quantitative methods could be used to measure liver fat in clinical practice; however, prospective studies are needed to assess their accuracy and reproducibility.

$^1$H MRS techniques can measure liver fat, decomposing the liver signal into its fat and water components; the measure is not influenced by fibrosis or obesity and was proven to be highly reliable [38–43]. Its limitations are mainly costs and small spatial coverage with subjective positioning of the volume of interest that may affect accuracy. $^1$H MRS is now considered the gold standard – replacing liver biopsy – as the non-invasive modality for fat liver quantification. Chemical shift imaging (CSI) acquired routinely in liver MRI is also used for liver fat quantification and showed very good correlation with hepatic fat at histology [44].

## 4. Rationale and methodology of a cohort study

A better understanding of the multiple risk and pathogenetic factors of NAFLD/NASH is mandatory for new diagnostic strategies, and individualized prevention and management. We analysed herein the major pathophysiological systems, factors and co-factors involved in NAFLD/NASH and, using a "systems medicine" approach, we propose new algorithms to stage and/or grade their involvement in the individual.

### 4.1. Genetics

Heritability plays a major role in the progression of NAFLD towards fibrosing NASH and genome-wide association studies have identified common genetic determinants of steatosis [45–53]. Patatin-like phospholipase-domain-containing-3 (PNPLA3) rs738409 C>G single nucleotide polymorphism, encoding for I148M protein variant is a major determinant [54–59]. The I148M allele frequency explains the inter-ethnic variability of NAFLD: higher in Hispanics (minor allele frequency [MAF] 0.49) than in Europeans (MAF 0.23), and less common in Afro-Americans (MAF 0.17). The PNPLA3-I148M variant hampers triglyceride esterase causing reduced remodelling of lipid droplets in association with excessive intake of carbohydrates or saturated fatty acids and other genetic factors [60–63]. The association between the I148M variant and NAFLD holds true both in adults and in adolescents [64–68]; I148M allele homozygosity predisposes to NASH and hepatic fibrosis [56–58]. The association between the I148M variant and fibrosis is also evident in chronic viral hepatitis and genetic diseases, such as hereditary haemochromatosis [69–71]. In addition, the I148M variant predisposes to hepatocellular carcinoma (HCC), independently of the aetiology of chronic liver disease, and homozygous patients have a worse prognosis [72–76]. The TM6SF2-E167K variant was also associated with NAFLD [53]; it favours progression to NASH and fibrosis by impairing the secretion of very low density lipoproteins in hepatocytes, but protecting from atherosclerosis [59,77]. Therefore, both PNPLA3-I148M and TM6SF2E167K may be useful in identifying NAFLD patients at a higher risk of hepatic than cardiovascular complications and the PNPLA3-based categorisation of NAFLD may have therapeutic implications. Preliminary data suggest that I148M-homozygous subjects might benefit from weight loss after a short-term low-carbohydrate diet [68,78]; however, disease progression is modulated by multiple environmental and genetic factors [48,79–82]. Thus, PNPLA3 I148M and TM6SF2 variants plus/minus a family history for cirrhosis and/or HCC and ethnicity can be used to stratify patients at risk in clinical practice (Table 2).

### 4.2. Age and gender

#### 4.2.1. Childhood

In children, in whom alcohol abuse, drug consumption and co-morbidities are much less important than in adults, NAFLD is generally considered to be of primary origin [83]. Genetic background, epigenetic programming, intra-uterine environment and post-natal nutrition are major risk factors and single nucleotide polymorphisms (SNPs) may identify children who are at a higher risk of NAFLD [55,56,60,63,65]. In fact, the PNLA3 rs738409 C>G SNP polymorphism was associated with higher risks of fatty liver, NASH and fibrosis [64,67]. Intrauterine malnutrition causes a foetal

**Table 2**
Genetic factors score.

|  | Risk score |
|---|---|
| **Ethnicity** |  |
| Afro-American | 0 |
| Asiatic or European | 0.5 |
| Hispanic | 1 |
| **Family history for NAFLD/NASH** |  |
| Negative | 0 |
| Present in one parent | 1 |
| In more than one sibling | 1.5 |
| In both parents | 2 |
| **PNPLA3 I148M** |  |
| Normal | 0 |
| Heterozygous | 0.5 |
| Homozygous | 1 |
| **TM6SF2 E167K** |  |
| Absent | 0 |
| Present | 1 |

The overall score of genetic factors impact ranges from 0 to 5 resulting form the sum of the values of 3 different categories of genetic factors.
*Abbreviations*: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PNPLA3 I148M, patatin-like phosholipase-domain gene I148M variant; TM6SF2 E167K, trans-membrane 6 superfamily member 2 gene E167K variant.

**Table 3**
Hypothesis of a sex-related score for fatty liver disease.

|  | Females | Males |
|---|---|---|
| **(a) Effect of age** |  |  |
| <12 years | 0 | 0 |
| >12 < 50 years | 1 | 2 |
| >50 years | 3 | 3 |

| Type | Females | Males | Clinical features |
|---|---|---|---|
| **(b) Added effect of body weight and fat distribution** |  |  |  |
| A | 0 | 0 | Normal weight, female or male, without either gluteo-femoral or visceral fat accumulation pattern |
| B | 1 | 3 | Female overweight but without gluteo-femoral and or visceral fat accumulation or male overweight without visceral accumulation |
| C | 2 | 4 | Female overweight with gluteo-femoral and or visceral fat accumulation or male with visceral fat accumulation, independently of body weight |

adaptive response with a consequent permanent reprogramming of tissue structures and functions [84–86]. NASH and MetS were reported to be higher in children with intrauterine growth retardation, who were overfed after birth, possibly because of discordance between intrauterine and extrauterine environments, whereas breastfeeding seems to protect from NASH [87,88]. These studies suggest that prenatal and postnatal periods are critical for metabolic programming [89,90]. Also, physical inactivity and excessive caloric intake are responsible for the "obese and metabolically dysfunctional" phenotype; diets rich in sugar (soft drinks with fructose-based corn syrup), salt and saturated fats and poor in micronutrients are associated with obesity and NAFLD [91,92].

### 4.2.2. Gender

The prevalence of NAFLD is higher in men with an "inverted U shaped curve": it increases from young to middle-aged individuals and declines in the elderly [93]. The "protective" female cardio-metabolic phenotype, present in Caucasians and Asiatics, but not in Hispanics and Blacks, disappears with the menopause [94–98]. Accumulated experimental and clinical evidence suggests that estrogens might exert protective effects on the interplay among brain, endocrine and digestive systems and consequently morbidity and mortality [99–104]. However, the impact of oestrogen replacement in post-menopausal women with NAFLD is still debated [105]. Thus, gender and age-related hormonal changes should be considered for the re-definition of clinical phenotypes, both in a new "patient based" approach and in preventive educational programmes. On this basis, a tentative quali–quantitative score for grading the impact of gender on liver steatosis in relation to age, weight and fat distribution may be applied to stratify individuals with fatty liver disease (Tables 2 and 3).

### 4.3. Nutrition

The epidemiology of nutrition is a paradox: on the one hand, obesity has doubled in the last 30 years, reaching about one-third of the global population; on the other hand, malnutrition affects about 2 billion people worldwide. Fatty liver occurs in the most severe forms of protein calorie starvation, such as kwashiorkor anorexia, bulimia, cachexia, massive (rapid) weight loss, and uncontrolled inflammatory bowel diseases [106]. However, NASH and fibrosis are highly unusual in these conditions. A typical American diet

(100 g of fat daily) supplies the liver with ∼20 g of fat, equivalent to one-half of the total triglyceride (TG) content of an average liver, while the flux of FFA through the bloodstream amounts to ∼100 g/day, with 20% being extracted by the liver. Thus, the daily input of TG from the diet (∼20 g/day) and FFA from adipose tissue (∼20 g/day) approximates the entire TG content of the liver [107]. Under conditions of an acute intake–expenditure imbalance, metabolic tissues store excess nutrients for future use. With a chronic imbalance, the physiological storage capacity is exceeded, activating cellular stress signalling pathways that attempt to stem further nutrient influx by inhibiting insulin signalling and promoting inflammation. In obesity-induced metabolic diseases, the continued nutrient imbalance drives this process forward, leading to chronic inflammation and IR and, ultimately, to type 2 diabetes, cardiovascular disease (CVD) and other overtly pathological consequences. It has been reported that when IR is induced by excess nutrient intake, 59% of hepatic fat is derived from circulating FFA, with lesser contributions from de novo lipogenesis (26%) and diet (15%) [12]. Thus, overfeeding induces subcutaneous and visceral obesity; the latter directs an increased flux of FFA directly to the liver, thereby making a greater contribution to hepatic steatosis. Few studies have evaluated the role of food quantity and quality on NAFLD development and clinical outcomes [108]. The Mediterranean diet is associated with an improvement in health status, as indicated by a significant reduction in overall mortality (9%), mortality from CVD (9%), mortality from cancer (6%) and incidence of Parkinson's disease and Alzheimer's disease (13%) [109,110]. Concerning NAFLD and NASH, there are currently a few studies with low numbers of patients. Dietary habits, in particular saturated fat, may promote NASH by modulating hepatic triglyceride accumulation and antioxidant activity, and indirectly by affecting insulin sensitivity and postprandial triglyceride metabolism [111].

The American Heart Association recommends that added sugars should represent less than 5% of total calories (corresponding to 2.5% of calories from fructose), in spite of this the percentage of total energy from added sugars is still approximately 14–15% [112,113]. Substantial relationships have been demonstrated between increased fructose consumption and obesity, dyslipidemia and IR [114]. Compared with glucose, fructose enhances de novo lipogenesis, promotes postprandial hypertriglyceridaemia, induces hepatic and extrahepatic IR, reduces satiety and increases visceral adiposity. The mechanism behind the fructose-induced expansion of ectopic fat is still under intense investigation, but it is hypothesized that fructose might be a strong inducer of de novo lipogenesis, which only produces saturated fatty acids. Fructose consumption may induce hepatic lipid accumulation by activating lipogenic gene expression. Another explanation might be the direct flow of fructose carbon into the glycolytic pathway,

bypassing a key regulatory enzyme of glycolysis, phosphofructokinase. Through this route, a higher proportion of the carbon from ingested fructose, compared with glucose, is incorporated into triglycerides. Abdelmalek et al. studied the histological severity of NAFLD according to daily fructose intake in a large cohort of adults: although the steatosis grade was lower in those with increased fructose intake, the degree of fibrosis was higher [115]. The daily intake of industrial, not fruit, fructose is a risk factor for severe liver fibrosis, at least in patients with chronic hepatitis C (CHC) [116].

### 4.3.1. Alcohol intake in NAFLD

There is a great deal of uncertainty regarding the definition of the threshold of alcohol consumption and the duration of abstinence needed to rule out NAFLD [117]. A light-to-moderate alcohol consumption (defined as less than 20 g per day) was associated with a decreased prevalence of NASH and a lesser degree of hepatic fibrosis in a large survey of patients selected from a well-characterized population with biopsy-proven NAFLD, suggesting that light-to-moderate alcohol consumption might have a protective effect on NAFLD progression [118]. Such findings, however, should not encourage physicians to recommend alcohol drinking to teetotallers with NAFLD.

## 4.4. Metabolic and endocrine systems

### 4.4.1. Insulin resistance

IR is defined as a decreased sensitivity or responsiveness to the actions of insulin that promote glucose disposal. It plays a major role in type 2 diabetes, and is closely associated with the cluster of metabolic abnormalities that define MetS [119]. IR is a characteristic feature of NAFLD, even in non-obese, non-diabetic subjects [120], and affects both lipid and glucose metabolism in target organs (liver, skeletal muscle, adipose tissue and myocardial muscle). As IR is the hallmark of obesity and several metabolic complications, including NAFLD, quantifying insulin sensitivity/resistance in humans is of great importance. Several direct and indirect methods are currently employed, some of them relying on steady-state analysis of glucose and insulin concentrations, others – such as the gold standard hyperinsulinaemic–euglycaemic clamp – on dynamic testing. Simple surrogates, such as the homeostasis model assessment of IR (HOMA-IR) and the quantitative insulin sensitivity check index (QUICKI) are those most extensively used. Both indices are derived from fasting plasma insulin and glucose concentrations. HOMA-IR is calculated as {[fasting insulin (U/ml)] × [fasting glucose (mg/dl)]}/22.5 [121]. HOMA-IR $\geq 3$ generally defines a condition of IR, but the coefficient of variation of HOMA-IR varies considerably depending upon the number of samples obtained and the insulin assay used [122]. QUICKI can be calculated from a mathematical transformation of HOMA [123]. Although HOMA-IR and QUICKI are suited for use in large epidemiological or clinical research studies, their specificity in evaluating insulin sensitivity in individual patients is low.

### 4.4.2. Metabolic syndrome (MetS) and its clinical features

The MetS is a cluster of interrelated metabolic conditions that exponentially increase the risk of developing CVD, type 2 diabetes and NAFLD (Table 1).

The association between NAFLD and the features of MetS is well established. Prevalence of the MetS in NAFLD varies from 18% in normal-weight individuals to 67% in obese subjects [124]. In type 2 diabetes, NAFLD can be diagnosed by ultrasound scan in 69% of cases [125] and 87% of the patients with fatty infiltration who consented to biopsy have histological confirmation of NAFLD [126]. The prevalence of NAFLD in individuals with dyslipidaemia is estimated to be 50% [127].

In this complex interplay, obesity, which has reached epidemic proportions worldwide, is the most common and well-documented risk factor for NAFLD, visceral obesity playing a prominent role in the development of MetS features, including NAFLD [128]. A Chinese study enrolling 5562 subjects who were NAFLD free at baseline, reported that MetS was independently associated with the presence and development of NAFLD during a 5-year follow-up [129]. Along the same lines, a study in the USA reported that "lean NASH" was independently associated with Hispanic ethnicity, younger age and some features of MetS, such as hypertension [130]. Another study enrolling 29,994 adults reported that non-obese patients with NAFLD had a higher prevalence of MetS features than obese patients without NAFLD, especially among women [131]. A large Korean occupational cohort study, involving 2589 subjects who were NAFLD free at baseline, and who were re-examined after a mean of 4.4 years, reported that elevated triglycerides and fasting glucose levels and enlarged waist circumference were independently associated with incident NAFLD [132]. Furthermore, the same metabolic risk factors appear to be associated with its progression to NASH. In 109 NASH patients who underwent a second liver biopsy at least 3 years after the first, progression of liver fibrosis was found in approximately 30% of patients and was associated with increased waist circumference and BMI [133]. Severe liver damage was associated with decreased insulin sensitivity measured by the oral glucose tolerance test-derived oral glucose insulin sensitivity [134] and MetS [135]. Obesity and type 2 diabetes are also implicated in the development of HCC, even in the absence of severe fibrosis [136]. Of note, the relationship between NAFLD and MetS is bidirectional. Liver fat content, as detected by $^1$H MRS, is about 4-fold higher in those with than in those without MetS [107]. The risk of CVD mortality and morbidity greatly exceeds the risk of liver-related mortality; thus, NAFLD is not merely the hepatic manifestation of MetS, but rather a systemic pathogenic component (or precursor) of this syndrome [137,138]. Accordingly, we propose a score combining visceral obesity with one or more features of MetS to stratify NAFLD individuals (Tables 2–4).

### 4.4.3. Thyroid dysfunction

A consistent line of research has associated thyroid dysfunction with NAFLD [139–141]. A German study reported that low free thyroxine levels were associated with ultrasound-detected steatosis in 3661 participants with no history of thyroid or liver diseases [142]. Another study of 4648 health-check subjects (2324 subjects with hypothyroidism vs age- and sex-matched controls) reported that subclinical hypothyroidism was related to NAFLD in a dose-dependent manner [143].

## 4.5. Cardiovascular system

In the last decade, it became evident that the clinical burden of NAFLD is not restricted to liver-related morbidity or mortality, and that the majority of deaths among these patients are related to malignancy, coronary heart disease (CHD) and other cardiovascular complications [6]. The spectrum of cardiovascular complications associated with NAFLD spans from premature atherosclerosis to aortic valve sclerosis and left ventricular dysfunction/hypertrophy leading to congestive heart failure and cardiac arrhythmias (mainly atrial fibrillation) [138,144–147]. Growing evidence indicates that NAFLD may play a role in the development and progression of cardiovascular complications, not only through MetS, but also through multiple pathophysiological derangements, including chronic inflammation, hypercoagulation, chronic kidney disease, hyperuricaemia, hypovitaminosis D, hypoadiponectinaemia, and increased fetuin-A levels [138,146,147]. NAFLD exacerbates IR, causes atherogenic dyslipidaemia, and releases pro-inflammatory,

**Table 4**
Cardiovascular risk.

| Score | Degree of impairment of cardiovascular system | Patients | CVD risk factors and/or Framingham risk score[a] |
|---|---|---|---|
| 0 | Minimal | Asymptomatic[b] | Either <3 traditional CVD risk factors[c] or a low/intermediate Framingham risk score (i.e. a 10-year CHD risk <15%) |
| 1 | Intermediate | Asymptomatic[b] | Either a high Framingham risk score (i.e. a 10-year CHD risk >15%) or ≥3 traditional CVD risk factors[c] |
| 2 | Severe | | In secondary prevention of CVD or at very high CVD risk (e.g., angina, myocardial infarction, coronary revascularization), stroke or other clinical CVD complications such as peripheral artery disease, abdominal aortic aneurysm or carotid artery stenosis >60% or established diabetes |

[a] The 10-year risk of having a CVD can be estimated by the Framingham risk score equation inserting major CVD risk factors (i.e., age, sex, total cholesterol, HDL-cholesterol, smoking history, systolic blood pressure and current use of anti-hypertensive drugs) of the individual patient in the NIH web site: http://cvdrisk.nhlbi.nih.gov/.

[b] All patients included in either score 0 or score 1 are in primary prevention of CVD.

[c] Traditional CVD risk factors considered are as follows: family history of premature CVD, age, male sex, cigarette smoking, hypertension (i.e., blood pressure ≥140/90 mmHg or on treatment), LDL-C ≥130 mg/dl (or on treatment), atherogenic dyslipidemia (i.e., triglycerides ≥150 mg/dl and/or HDL-C <50 mg/dl in women <40 mg/dl in men or on treatment), obesity (BMI ≥30 kg/m²) and CKD (estimated glomerular filtration rate <60 ml/min/1.73 m²).

CVD, cardiovascular disease; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; CKD, chronic kidney disease.

pro-coagulant, pro-oxidant and pro-fibrogenic mediators of cardio-vascular pathophysiology [138,144–147].

Based on this evidence, scientific societies have suggested an assessment of the overall CVD risk in patients with NAFLD [1,148]; however, how such an assessment should be conducted is still poorly defined [146] and in general it follows the guidelines for the CVD risk of the general adult population [146,117,149]. Preliminary evidence suggests that the Framingham risk score might accurately predict the higher 10-year CHD risk in patients in the USA with NAFLD and identify patients expected to benefit from early interventions to prevent CHD events [149,150]. However, the accuracy of the Framingham risk score needs to be further validated in European patients with NAFLD, where this equation may overestimate the CHD risk compared with populations of Anglo-Saxon ancestry. Furthermore, future studies in larger cohorts of NAFLD patients with different ethnicities are needed to validate the Framingham or other risk score systems for predicting the global CVD risk, given that subclinical inflammation, IR and hypertriglyceri-daemia are not considered in any of the available risk score systems. In Table 4 we propose a grading scale for global CVD risk in NAFLD based on available evidence and guidelines.

### 4.6. Immune disorders, chronic viral infections, and gallstone disease

#### 4.6.1. Immune disorders

Both innate and adaptive immune response play a pivotal role in the pathogenesis and progression of NAFLD [9,151,152]. Experimental observations match clinical evidence on the association between immune disorders and the presence/severity of NAFLD [153,154]. Circulating autoantibodies are frequent in NAFLD in the absence of autoimmune hepatitis and associated with more advanced liver disease at histology [155]. Serum IgA levels are significantly associated with NASH and more advanced liver fibrosis in NAFLD [153,154]. An intriguing link between immune disorders and NAFLD is suggested by the prevalence of fatty liver in rheumatological disorders [156], primary biliary cirrhosis [157–159], coeliac and inflammatory bowel diseases (IBD) [160–163]. NAFLD is the third most common IBD-associated liver disease independent of classical risk factors such as obesity, IR or drug toxicity [161,162].

#### 4.6.2. Chronic viral infections

Fatty liver is common in patients with CHC, with prevalence ranging from 40 to 80%, higher than in chronic liver diseases of different aetiology [164,165]. The pathogenesis of steatosis in CHC is multifactorial, involving both host and viral factors. A direct steatogenic effect was shown for hepatitis C virus (HCV) genotype 3 [166–170], where steatosis is more frequent and severe according to viral load [167,168] and disappears after viral eradication

**Table 5**
Grading of the impact of chronic infections and/or immune disorders on fatty liver.

| Grade | Infections and/or immune disorders |
|---|---|
| 0 | Absence of clinical and pathological signs of immune disorders, circulating autoantibodies and chronic HBV or HCV infections |
| 1 | One of the following features: clinic-pathological signs of immune disorders or circulating auto-antibodies and chronic HBV or HCV infection without chronic active hepatitis (inactive HBsAg carrier or anti-HCV positive but HCV-RNA negative) |
| 2 | Both clinical or pathological signs of immune disorders or circulating auto-antibodies and chronic HBV or HCV infection without chronic hepatitis (inactive HBsAg carrier or anti-HCV positive but HCV-RNA negative) |
| 3 | Chronic hepatitis B |
| 4 | Chronic hepatitis C |

HBV, hepatitis B virus; HCV, hepatitis C virus; HBsAg, hepatitis B surface antigen.

[171]. Conversely, a combined viral and metabolic steatosis occurs in non-3 HCV genotype infections and correlates with age and IR [170]. Nevertheless, HCV genotype 1 can induce IR [171–175]. Interesting findings support the interplay between the host's genetics and fatty liver in HCV-infected individuals: the PNPLA3 l148M variant [176] is associated with steatosis, whereas the IL28B rs868 CC genotype is associated with a sustained response to peginterferon and ribavirin and lower prevalence of IR and steatosis [177]. Furthermore, steatosis has a relevant impact on the clinical history of HCV infection (liver disease progression and HCC) [170,178–185]. The association between chronic hepatitis B virus (HBV) infection and steatosis is less evident than in CHC; the prevalence of fatty liver is extremely variable (5–70%) [186–192]. Nevertheless, steatosis is more frequent in chronic hepatitis B (CHB) patients than in the general population in Western and Asian countries without the impact of ethnicity or viral genotypes [190–192]. In most studies of CHB patients steatosis was not associated with fibrosis; this raises the question whether NAFLD in HBsAg carriers might be linked to metabolic factors indirectly influenced by HBV. As in chronic HCV infection, the pathogenesis of steatosis is related to metabolic factors such as obesity and IR [187–189] and genetic background (PNPLA3 I148M variant) [70], but at variance with HCV a direct steatogenic impact of HBV has been reported in a few experimental studies [191–195]. Whatever the underlying mechanisms, steatosis in both CHB and CHC represents an important co-factor affecting the outcome of liver disease [192]. Thus, we proposed a scale for grading the combined impact of immune disorders and viral infections in NAFLD patients (Table 5).

#### 4.6.3. Gallstones or cholecystectomy

Studies of the association between NAFLD and gallstones provided conflicting results. In a large study NAFLD was associated with cholecystectomy, but not with gallstones, suggesting that

cholecystectomy might be a potential risk factor [196]. Another study found a high prevalence of gallstones in NAFLD patients with glucose intolerance/diabetes and advanced liver disease [197].

## 5. Future perspectives

With this approach, it is possible to characterize different NAFLD/NASH phenotypes within the definition of the general metabolic syndrome. Based on this premise we aim to launch a large observational cohort study supported by AISF and the Foundation for Research in Hepatology (FIRE). The goal is to study the clinical comorbidities and hepatic and extrahepatic outcomes associated with subsets of patients with NAFLD/NASH, to increase the understanding of the complex interplay among different physiological systems. A prospective cohort of consecutive asymptomatic individuals with fatty liver will be recruited in clinical centres distributed throughout Italy and followed up for at least five years. Novel statistical models such as non-linear and integrative epidemiological approaches [198], accounting for the patterns of interconnections between parameters affecting disease risk and their correlations, will be used to better understand the mechanisms involved. With this integrated approach we hope to identify new diagnostic and prognostic biomarkers and new targets for prevention and treatment.

## Conflict of interest
None declared.

## Appendix A. Collaborators

The following investigators contributed equally to the study as members of the writing committee:

Ele Ferrannini, National Research Council (CNR), Institute of Clinical Physiology, Pisa, Italy

Carmela Loguercio, Hepato-Gastroenterology, 2nd University of Naples, Italy

Amedeo Lonardo, Outpatient Liver Clinic and Internal Medicine NOCSAE, USL Modena, Italy

Fabio Marra, Department of Experimental and Clinical Medicine, University of Florence, Italy

Marcello Mancini, National Research Council (CNR), Institute of Biostructure and Bio-imaging, Naples, Italy

Luca Miele, Internal Medicine, Gastroenterology and Liver Diseases, Catholic University of Rome, Italy

Valerio Nobili, Metabolic Liver Diseases Research Unit, IRCCS Children Hospital "Bambino Gesù", Rome, Italy

Gianluca Svegliati Baroni, Department of Gastroenterology 1 and Obesity Center 2, Polytechnic University of Marche, Ancona, Italy

In addition to the above listed authors of the writing committee the following investigators contributed equally to the study as external advisors:

Federico Alessandro, Hepato-Gastroenterology, 2nd University of Naples, Italy

Stefano Ballestri, Medicine, Pavullo Hospital, Modena, Italy

Maurizia Rossana Brunetto and Barbara Coco, Hepatology Unit, University Hospital of Pisa, Italy

Antonio Grieco, Internal Medicine, Gastroenterology and Liver Diseases, Catholic University of Rome, Italy

Silvia Fargion, Internal Medicine, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Department of Pathophysiology and Transplantation, University of Milan, Italy

Loreta Kondili, Istituto Superiore di Sanità, Rome, Italy

Fabio Nascimbeni, Metabolic Medicine, NOCSAE, Baggiovara, Modena, Italy

Anna Prinster, National Research Council (CNR), Institute of Biostructure and Bioimaging, Naples, Italy

Dante Romagnoli, Outpatient Liver Clinic, Medicine NOCSAE, Baggiovara, Modena, Italy

Stefano Taddei, General Medicine 1, Department of Clinical and Experimental Medicine, University Hospital of Pisa, Italy

Ester Vanni, Gastroenterology and Hepatology, Department of Medical Sciences, Città della Salute e della Scienza di Torino Hospital, University of Turin, Italy

Stefano Vella, Istituto Superiore di Sanità, Rome, Italy

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