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"Non-invasive biomarkers for the prediction of disease progression in metabolic associated fatty liver disease"

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INTRODUCTION

Over the last century, significant changes in nutrition, lifestyle habits, and therapeutic successes against many infectious diseases have led to the emergence, on the world stage, of a series of non-communicable diseases. These conditions are responsible for most of the causes of mortality and morbidity not only in Western countries but also in the developing world.

The marked increase in the incidence of obesity, which has reached the levels of a real pandemic, has led to a parallel growth of all the pathologies that make up the so-called Metabolic Syndrome. Metabolic-dysfunction associated with fatty liver disease (MAFLD), formally known as non-alcoholic fatty liver disease (NAFLD), can be considered the liver manifestation of Reven Syndrome.

Currently, also thanks to the introduction of new drugs for the treatment of the hepatitis C infection, it appears to be the most common cause of chronic medical liver disease in the Western world and its prevalence is rapidly increasing also in Asian countries, following the changes in eating habits and a more sedentary lifestyle [1] [2].

According to current guidelines and consensus recommendations, NAFLD is characterized by the accumulation of hepatocytes fatty vacuoles, in subjects with no history of significant alcohol consumption or with no other identifiable secondary causes responsible for steatosis.

In the nineteenth century, Addison and subsequently Rokitansky highlighted the existence of a correlation between alcohol intake and the accumulation of fat in the hepatocyte.

In 1884, Pepper described the presence of fatty liver in a subject with a diabetes diagnosis, and in the following year, Bartholow suggested the potential association between this condition and obesity [3].

However, it was in the second half of the 1900s that the association between hepatic steatosis and altered glucose metabolism and insulin resistance (IR) was demonstrated, as it was first detected in animals and eventually also in human beings [4].

In 1962, Thaler postulated the existence of a pathogenetic relationship between fatty liver and the development of cirrhosis [5].

In 1980, Ludwig described 20 subjects whose liver biopsy presented histopathological characteristics typical of the picture that he defined as non-alcoholic steatohepatitis [6].

Currently, the NAFLD denomination has become an "umbrella term": it includes a wide spectrum of histological disorders ranging from simple liver steatosis (non-alcoholic fatty liver NAFL) to steatohepatitis (NASH), up to cirrhosis and hepatocellular carcinoma, observed in subjects with a daily alcohol consumption below 30 g and 20 g/day respectively in men and women, and in whom secondary causes of liver steatosis were excluded.

The gold standard for the diagnosis of steatosis and non-alcoholic steatohepatitis, according to the international societies' guidelines, is represented by liver biopsy, a procedure that is not devoid of risks and is sometimes rejected by the patients themselves.

For this reason, several authors have tried, over the years, to identify non-invasive tools (from imaging and radiological investigations to humoral markers) that can help the physician to recognize patients suffering from this pathology, staging NAFLD and predicting evolutive risk to define their better follow-up. For this purpose, different types of serum biomarkers have been proposed, such as cytokeratin 18 (CK-18), fibroblastic growth factor 21 (FGF-21) or a combined panel of soluble markers.

It has recently been observed that the levels of the immunocomplex resulting from the interaction between IgM and squamous cell carcinoma antigen (SCAA) correlate with the severity of the chronic medical liver disease, as they are higher in patients with fibrosing disease and hepatocellular carcinoma (HCC) compared to those with non-advanced pathology. However, it is not clear whether this is also applicable in patients with metabolic liver disease.

Moreover, in the last years robust evidence supports the role of genetic predisposition in NAFLD and HCC development; variants in the genes involved in the regulation of hepatic lipid

metabolisms, such as *PNPLA3*, *TM6SF2*, *MBOAT7*, and *GCKR*, are strongly associated with hepatic fat content (HFC) and progression of liver diseases.

The principal aim of this study is, therefore, to determine whether patients with histologically well-characterized cohorts of non-alcoholic fatty liver disease, in their different patterns of degrees of manifestation, have an autonomous production of SCCA-IgM/Serpin B3.

Secondly, we examine the impact of a polygenic risk score of hepatic fat content (PRS-HFC), based on well-characterized risk variants, on NAFLD-HCC in a cross-sectional cohort of at-risk individuals (NAFLD cross-sectional cohort), and we attempt to optimize it by adjusting for a protective variant in *HSD17B13* (PRS-5); finally, we identify the best diagnostic threshold.

DEFINITION

NAFLD refers to a broad spectrum of pathologies affecting liver parenchyma, which are observed in subjects without evidence of significant consumption of alcoholic beverages, ranging from simple hepatic steatosis to steatohepatitis and cirrhosis. The finding of more than 5% of steatosis affecting hepatocytes, in the absence of secondary causes that could justify it, can be detected through a radiological (spectroscopic or selective magnetic resonance for the study of the water/fat ratio) or histological investigation [7, 8].

The pathological evaluation of a biopsy sample in subjects with hepatic steatosis (NAFL) highlights the presence of fat vacuoles within more than 5% of hepatocytes. In the case of steatohepatitis (NASH), the presence of multifocal inflammation of the hepatic parenchyma, Mallory's hyaline bodies, phenomena of hepatocyte damage such as apoptosis and ballonization, in association or not with sinusoidal fibrosis is noted [7,9]. For the certain diagnosis of steatohepatitis, it is, therefore, necessary to perform a histological study [7]. It is estimated that about 10-30 % of NAFL patients develop liver cirrhosis and several papers suggest that over 70% of cases of cirrhosis classified as cryptogenic could be attributed to an unrecognized NAFLD.

In most patients, the presence of NAFLD is associated with the presence of metabolic risk factors, such as obesity, type II diabetes mellitus, or dyslipidaemia [8].

Furthermore, the concomitance of secondary causes of steatosis, the most frequent of which are listed in ***table 1***, should be excluded.

In the scientific community, there is no univocal consensus regarding the limits to be adopted concerning the level of hepatotoxicity secondary to alcohol consumption [10]. The European guidelines indicate the limit for the daily consumption of alcoholic beverages at 30 and 20 grams respectively for men and women [7]. The American ones consider 210/140 grams of alcohol per week as cut-offs for men and women respectively and recommend that candidates for clinical trials

should be abstinent for more than 2 years [8]. Finally, Asian standards apply even more restrictive limits referring to 140/70 grams of alcohol per week [11].

Nevertheless, some authors point out that these limits would be particularly arbitrary as the alcohol-induced liver damage does not depend only on the amount of alcohol consumed but also on the type, individual susceptibility or genetic predisposition, gender, sex, duration, and methods of exposure. In particular, subjects with metabolic risk factors, despite moderate alcohol consumption, still risk developing NAFLD, as it would seem that the weight of the former, on the risk of developing hepatic steatosis, is much greater than consumption of alcoholic beverages alone [12]. Finally, it should be remarked that some papers seem to describe a protective role played by the intake of moderate amounts of alcohol concerning the development of hepatic steatosis itself [13]. Epidemiological studies propose the existence of a J-shaped curve between mortality and alcohol intake [14] identifying as the turning point an average alcohol intake of about 25-30 g and 40 g respectively in women and men [15,16]. In 2017 Alberg et al. published the results of an epidemiological study conducted in Finland between 2000 and 2001 on a cohort of 6732 participants. Among the results of their study, it emerges that in the subgroup in which the subjects reported alcohol consumption within the limits recommended by the definition of NAFLD, alcohol intake, albeit in limited quantities, represents a significant risk factor for development of liver disease. This suggests that there may not be any safety limits regarding the use of alcoholic beverages concerning the risk of liver damage. It could be speculated that alcohol consumption and obesity synergistically increase the risk of liver disease progression [17].

During the first months of 2020, a consensus of international experts proposed the adoption of the acronym MAFLD (metabolic dysfunction associated fatty liver disease), as a more comprehensive term, instead of NAFLD [18,19], converting a “negative” definition in one based on positive criteria. The newly proposed nomenclature is applied to patients with fatty liver (detected by histology, imaging, or blood biomarker) and contemporary presence of at least one of the following conditions: type 2 diabetes, overweight or obesity, lean or normal weight with evidence of

metabolic abnormalities (presence of two or more of the following states: increased waist circumference, hypertension, hypertriglyceridemia, low levels of high-density lipoprotein (HDL) cholesterol, prediabetes, high levels of High sensitivity C-reactive protein (hs-PCR), high Homeostasis Model Assessment (HOMA)-insulin resistance score). This new definition better reflects the set of current knowledge relating to the mechanisms of metabolic disfunction that support the development of fatty liver disease. Alcohol consumption no longer represents a diagnostic criterion, according to the experts, MAFLD may occur with other liver diseases, including alcoholic ones, in this way authors attempt to avoid the stigmatization of patients. All these considerations enlarge the investigative scope and allow us to reach new therapy options. However, heterogeneity of this definition may make a better patient stratification and characterization in different subtypes necessary. Lin et al. [20] tested and evaluated in the real world, a population derived from the 1988-1994 NAHANES USA database, the new definition of MAFLD compared with the NAFLD one. They observed that the new definition was more practical to identify patient with fatty liver disease - in particular those at higher risk of disease progression. Patients with MAFLD results were older, with higher BMI, with higher metabolic comorbidities, present higher HOMA-IR index and higher levels of lipid and liver enzymes. Patients with alcohol consumption were younger, more frequently male, with higher levels of transaminase, and with more severe fibrosis. However, the two terms are not interchangeable: not all subjects with MAFLD have NAFLD and vice versa (nearly 15% discrepancy could be observed) [21]. Other authors, such as Younossi et al.[22] warn of a premature change in the nomenclature, which could prove counterproductive, and could inadequately describe NAFLD heterogeneity. The new definition does not address the main limitations in this field, particularly regarding pathophysiology, risk stratification, and molecular phenotyping, and management, thus maintaining a high degree of equivocation. Moreover, it must be kept in mind that nowadays there is a lack of consensus on the definition of “metabolic health”. The one used for the term “metabolic” does not resolve the ambiguity concerning the etiologic causes of the disease, many “metabolic” conditions support,

indeed, the development of the fatty liver disease. Finally, this definition does not include patients with liver steatosis in the absence of metabolic risk factors or significant alcohol consumption. These subjects seem to be younger and with more severe liver steatosis, which could represent the first expression of a metabolic syndrome [23]. The debate on which definition is the best applicable is still ongoing and further studies, research and validations will certainly be needed in order to decide on the basis of the evidence base medicine which definition between NAFLD and MAFLD is the best. Thanks to the evidence, it will therefore also be possible to overcome the clinical inertia that sometimes limits innovation and the application of changes in medicine.

EPIDEMIOLOGY

The NAFLD prevalence is progressively increased in recent decades, not only in Western countries, where it has doubled in the last forty years but also in Eastern ones [24–26].

Although over the years, there have been several attempts to precisely define the magnitude of this condition, it is widely believed that the data provided only describe the tip of the iceberg, being NAFLD an often under-diagnosed disease. Undoubtedly, hepatic steatosis and steatohepatitis are pathologies characterized by a "silent" nature. In a non-negligible percentage of subjects, the most used and common bio-humoral tests (for example hepatic cyto-necrosis index) results fall within a normal range, and diagnoses of NAFLD occur accidentally [27].

Worldwide, it is estimated that the median prevalence of NAFLD is around 20% (with a range between 6% and 35%), with wide variations within the different groups of subjects considered or according to the different diagnostic tool used [28]. Several studies have shown that higher prevalence (varying between 45 and 58%) is observed in subjects of Hispanic origin than in Caucasians (33-44%) and African-Americans (24-35%) [29,30]. In the United States of America, in the general population, the prevalence of NAFLD would be 27-34%, while in obese subjects it would be around 72-95% [27]. The prevalence rates among subjects with type II diabetes mellitus would eventually reach 60-70% [8]. Prevalence rates in Europe are also high and can vary from 8% to 35% (with a median of about 26%) [31]. Parallel to what can be observed for obesity, the presence of a North-South gradient is noted. This variability would not only be affected by the different diffusion of risk factors, but it could also be due to differences in the methods of diagnosis and definition of the disease in the various countries [25,27,31,32]. In Italy in particular it is assumed that NAFLD affects about 25% of the general population [33]. While in Greece, prevalence levels of around 45% have been established, while in Romania only 8% [27,34]. Similarly to the data in North American countries, in Europe there is a similar higher prevalence of the disease in patients with diabetes (42.6-69.7%) [24] and in those with metabolic syndrome (about 79%) [35]. In Asian countries, it is estimated that the

prevalence of hepatic steatosis is high (15-10%) and increasing in recent years [11,36]. In China, rates of about 20% (6-38%) have been reported, in Japan about 15%, while in India they vary from 8 to 30%, also based on the quality of the sources that are evaluated. Sri-Lanka, Hong Kong, Korea, and Indonesia show prevalence rates ranging from about 15 to 20% [37–40]. The prevalence rates seem to be generally increasing in the countries located further east than in those of the south of the Asian continent, where they have been stable in recent years.

Even regarding the incidence of NAFLD, there is a scarcity of data in the literature: it is estimated that it is around 10% and 5% respectively in the USA and Europe [41] and Asia [27].

Finally, it is estimated that about 2-5% of the general population is affected by NASH [42].

Most NAFLD diagnoses are made around the age of 40-50 [43], with higher prevalence rates found in older patients.

In the literature, on the other hand, there is no univocal prevalence in terms of gender: some studies report a greater diffusion in the female sex [6,44–47], others in the male one [29,30,48,49].

PATHOGENESIS

As previously mentioned, the term NAFLD describes a broad spectrum of histopathological conditions that include simple steatosis, steatohepatitis, and even liver cirrhosis. Data extrapolated from multiple epidemiological studies show how, while hepatic steatosis would be characterized by an indolent course, steatohepatitis would have a more aggressive one with a greater risk of evolving into cirrhosis and developing a primary liver tumor. However, it is still not completely clear and defined whether these two conditions (NAFL and NASH) represent a continuum in the context of the same pathology or if they are two different pathologies that are not correlated with each other [42]. Some authors recently suggested that NAFLD can be considered a dynamic disease, characterized by marked plasticity, in consideration of the possible fluctuations between steatosis and steatohepatitis over time [50].

In 1998, Day and James proposed what was called "two-hit hypothesis" to explain the pathogenesis of NAFLD, according to which steatosis would represent the first pathological event that would then predispose the hepatic parenchyma to an increased susceptibility to a second insult, which would induce the development of hepatocellular damage, followed by inflammation and finally fibrosis [51].

As more knowledge was gathered and analyzed, a new theory has emerged, according to which the development of steatohepatitis is the result of multiple etiological factors, which would act simultaneously and at several levels, and which would range from genetic predisposition to environmental factors, from dysmicrobism and overgrowth from the resident gut microbiota to the activity of the host's immune system[52–54].

Much remains to be understood and known about the concatenation of events that determine the evolution of simple hepatic steatosis into steatohepatitis, and the subsequent development of cirrhosis or hepatocellular carcinoma.

The presence of a predisposing polygenetic substrate is well established (epidemiological studies and case-control have in particular highlighted how *PLNPA3*, *TM6SF2*, *MBOAT7*, *GCKR*, *NCAN*, *LYPLAL1*, *SOD2*, *KLF6, HFE C282Y,* and *ATGR-1* are the most frequently associated with NASH) [27,55] on which various conditions are grafted to concur to induce and feed a picture of insulin resistance (IR) and lipotoxicity, which, at the level of hepatocytes, induce an altered functioning of the endothelial reticulum system (ER) and the mitochondria. The production of reactive oxygen species and pro-inflammatory cytokines, together with hepatocyte cytonecrosis, activate inflammatory cells and fibrogenesis, pathognomic elements of NASH itself.

An ever-increasing amount of data, obtained through genome-wide association studies (GWAS) conducted in large cohorts of extremely well-phenotyped subjects, has allowed us to demonstrate the pivotal role played by specific gene varieties in causing liver fat content, in the development and the progression of NAFLD [56].

The rs738409 C>G single nucleotide polymorphism (SNP), encoding for the I148M variant of *Patatin-like phospholipase domain-containing protein 3* (*PNPLA3)*, warrant the higher percentage of genetic predisposition to NAFLD, leading hepatic fat accumulation and increased susceptibility to hepatic injury, without determining direct consequences on IR, glucose homeostasis, lipoprotein metabolism, or whole-body adiposity [57,58]. In European populations, the frequency of this SNP allele is about 21%-28% [59]. Individuals carrying this variant are more susceptible to developing the wide spectrum of liver disease characterizing NAFLD and primary liver cancer (even those presenting an early stage of liver disease), moreover, they are also at greater risk of liver-related mortality with or without NAFLD presence [56,60–63]. The protein encoded by this gene, called Adiponutrin, is a 481 amino acid transmembrane bounded protein, localized in the endoplasmic reticulum and lipid membranes of hepatocytes and adipose tissue [59]. It is normally expressed under glycemic and insulin stimuli in the hepatocyte, hepatic stellate cells, retinal cells and, during IR conditions, even in adipocytes. This protein acts as a lipase, it is involved in the metabolism of phospholipids, in triglycerides remodeling and has a retinyl-palmitate esterase activity, thus

preventing fibrogenesis [64]. The enzymatic loss of function induced by the I148M variant causes fat accumulation, facilitating lipotoxicity and, thought intracellular retinol esters retention, inflammation and fibrogenesis [60].

The rs58542926 C>T SNP a missense mutation for the E167K variant of *transmembrane 6 superfamily member 2* (*TM6SF2)*, encoded on chromosome 19p13.11, has been reported to be associated with variations in plasma lipid profile, cholesterol metabolism disorders, liver steatosis, and risk of progression NAFLD-HCC, representing a strong modifier of hepatic fibrogenesis [58,65,66]. The *TM6SF2* gene encodes a 351-amino acid transmembrane protein expressed in the liver and intestine in humans [67]. The missense mutation is present in 7.2 % of Europeans, 4.7% Hispanics, and 3.4 % African Americans, resulting in a loss of function of the protein, that facilitates the reduction in the circulation of triglycerides-rich-lipoproteins and fat accumulation in the liver parenchyma, through compromised lipidation and maturation of chylomicrons in enterocytes and very-low-density lipoprotein (VLDL) in hepatocytes. [56,59,66,67]. Secondary to the reduction of circulating serum lipids, *TM6SF2* T alleles seem to confer protection against cardiovascular diseases [59,66].

Another significant polymorphism involved in NAFLD predisposition and evolution, particularly in patients with European ancestry, is the rs641738 variant of the membrane-bound *O-acyltransferase domain containing 7- Transmembranechannel-like4* (*MBOAT7-TMC4*) gene, localized on chromosome 19 [68,69]. It is expressed in hepatocytes, hepatic stellate cells, and hepatic sinusoidal cells [70]. *MBOAT7* encodes for a lysophospholipid acyl-transferase, a membrane-bound enzyme involved, thanks to its specificity for arachidonoyl-CoA as an acyl donor, in "Lands' Cycle" of phospholipid acyl-chain remodeling of membranes pathways. This enzyme represents a fine regulator of free arachidonic acid [58,69,71]. The rs641738 variant results in a lower protein expression and thus decreased enzyme activity and changes in plasma phosphatidylinositol species, which in turn result in increasing liver steatosis, inflammation, fibrosis, and development of HCC, even in patients without cirrhosis [56,67,69,72].

A consistent amount of data has demonstrated the association between the variant of *glucokinase regulator* (*GCKR*) gene locus and fatty liver disease. *GCKR* is involved in the regulation of the de novo-lipogenesis through the modulation of the influx of glucose in hepatocytes. The common missense P446L (rs1260326 C>T) variant encodes a loss-of-function of the proteins able to inhibit glucokinase in response to fructose-6-phosphate [67,71], which allow the uptake of glucose in the active hepatocytes and consequentially reduce the circulating fasting glucose and insulin levels [72]. In this condition, liver steatosis results as a consequence of the increased production of malonyl-CoA (involved in *de novo* lipogenesis) and by inhibition of fatty acid oxidation [58,67,68,72].

Recently, exome-wide sequencing studies have demonstrated that the loss-of-function of rs72613567:TA in *hydroxysteroid 17-beta dehydrogenase 13* (*HSD17B13*) confers protection against liver steatosis, and reduces the risk of devolving the whole spectrum of alcoholic non-alcoholic liver diseases [56,67,71]. The gene encodes for an enzyme, member of the short-chain dehydrogenase/reductase family, involved in the metabolism of fatty acids, sex hormones, and retinoids, expressed in hepatocytes and localized in the surface of lipid droplets [58,73]. Even if the specific protein function is under investigation, it has been hypothesized that defective enzymatic activity induces a lower production of several pro-inflammatory lipid species (e.g. leukotriene B4) into the liver [71]. Moreover, this SNP is associated with a reduction in *PNPLA3* messenger rRNA expression in an allele dose-dependent manner, mitigating in this way *PNPLA3* liver-damaging effects [58,74].

Pathogenesis and evolution of NAFLD can be thus characterized as an extremely complex process, secondary to gene-gene and gene-environmental interplay, as well as a still not completely understood inter-organ crosstalk involving adipose tissue, pancreas, gut, and liver.

The liver is an organ involved in the homeostasis of the entire organism by modulating not only glucose but also lipid metabolism. In obese subjects, the liver itself becomes a reservoir for lipids. Furthermore, in these subjects, the adipose tissue, which can be assimilated to a real organ with endocrine functions, undergoes processes characterized by inflammation and impaired functioning.

This is caused by excessive accumulation of lipids in the adipocytes, which determines a condition of hypertrophy and induces their proliferation, which in turn favor and contribute to a condition of hypoxia, which predisposes to cell death [75,76]. In this context, the altered adipocytes secrete pro-inflammatory cytokines, such as TNF (Tumor necrosis factor or tumor necrosis factor) and chemokines (for example CC chemokine ligand 2 or CCL2) which attract and favor infiltration by macrophages, that, in turn, support and maintain the inflammatory *milieu* and IR status [77–79]. Furthermore, there is a reduction in the production of adiponectin, an insulin-sensitizing and anti-inflammatory hormone (stimulating the production of IL-10, through the enzymatic cascade of NF-KB and inhibiting the release of TNFα, IL-6 and chemokines), and of visfatin (a central hormone in the biosynthesis of NAD)[80] against an increase in the production of leptin. The latter would be characterized by possessing a lipostatic function, and therefore would have a protective effect against the development of NAFLD, but in conditions of IR, there would also be a consensual leptin resistance which would instead favor the onset of hepatic steatosis itself [81]. IR also promotes the release into circulation by the adipose tissue of fatty acids, which are captured by the liver, thanks to transporters such as FATP5 and CD36, both up-regulated in conditions of obesity [27]. The gluconeogenesis induced by IR itself contributes to inducing the development of hepatic steatosis; while hyperinsulinism and hyperglycemia through the pathways mediated by SERBP1 and ChREBP [27] determine the *de novo* synthesis of lipids, as previously described.

The accumulation of lipid particles in the hepatocytes is a central event in determining a state of stress on the ER, which in turn favors and contributes to the maintenance of steatosis itself. This is possible on the one hand due to a reduction in the synthesis of apolipoproteins involved in the assembly and transport of VLDL (mediated by PERK or Protein Kinase RNA-like ER Kinase), and on the other following the induction of the SREBP1-SCAP complex and the activation of IRE1 (Inositol Requiring Enzyme 1) and XBP1 (X-box binding protein 1), both of which represent two important stimuli for lipogenic sequence transcription [27]. The alterations of the ER can also induce the death of the hepatocytes themselves.

It has recently been shown that autophagy plays an important role in the homeostasis of hepatocytes and adipocytes [82]. Autophagy refers to the process by which dysfunctional cell constituents are degraded and cellular energy deposits are maintained [27,82]. In hepatocytes, not particularly rich in lipases, this mechanism allows the metabolism of the lipids themselves. Sing et at. have shown that its dysfunction characterizes hepatic steatosis and promotes the death of the hepatocytes themselves [82].

A further factor favoring NAFLD is represented by the alteration of the signaling pathway mediated by FRX (Farnesoid X Receptor), a receptor with which bile acids interact, which in physiological conditions would inhibit lipogenesis and the synthesis of fatty acids [27].

As previously described, the dysmicrobism of the resident microbiota, induced by the characteristics of the constituents of the diet and by obesity itself, would favor not only the development of hepatic steatosis but also its progression. It was shown that patients who develop steatohepatitis presented a reduced proportion of Bacteroides and a higher ratio of Firmicutes-Bacteroides [83]. As previously described, this is associated with bacterial overgrowth, greater production of ethanol, reduced synthesis of choline, and an alteration of the intestinal mucosa that favor the passage of lipopolysaccharides into the circulation. All these factors contribute to determining and maintaining a pro-inflammatory state, with repercussions at the hepatocyte and systemic level.

The LPS interaction with the receptors of the TLRs family present on the hepatocytes induces the inflammatory cascade mediated by PAMPs and DAMPs, which through the pathway induced by NF-KB involves the production of TNFα, the activation of the "inflammasome" responsible for the activation of various pro-inflammatory cytokines, such as IL1, which in turn contribute to maintaining active and feeding the inflammasome and the production of pro-inflammatory chemokines [27,52,84]. This cascade can also be induced by the fatty acids themselves, through the interaction with the TLRs themselves but also through the induction of the inflammasome itself.

Kupffer cells in response to pro-inflammatory cytokines contribute in turn to develop and perpetuate cell damage and the intra-hepatocytic inflammatory state, also following differentiation into monocytes with a marked inflammatory phenotype, called M1[27].

Hepatocyte cytonecrosis also represents a further factor favoring inflammation and tissue damage, supported by multiple mechanisms, both receptor-dependent and independent. Among the former is caspase-induced cell apoptosis with consequent cell lysis, and necroptosis characterized by an inhibition of the caspase-mediated mechanism for which the signaling pathway that determines cell death is that secondary to PIP-PIP3-MLKL activation which induces hepatocyte membrane lysis. The receptor-independent mechanisms, on the other hand, are supported and induced by lipotoxicity, which determines a complex and extensive mitochondrial damage through the activation of JNK, which results in the production of ROS species, the depletion of ATP and therefore the activation of the caspases themselves. Last but not least, the activation of the inflammasome, again by fatty acids, can, as already mentioned, induce cell death through a mechanism "intrinsic" to the hepatocyte itself, also described as "pyroptosis" [27].

A condition of prolonged and sustained inflammation and hepatocyte damage involves the activation of stellate cells that differentiate into fibroblasts. Cell death represents a direct and indirect stimulus, mediated by the release of cytokines, towards the development of hepatic fibrosis. This condition is induced, on the one hand, by the differentiation of stellate cells into myofibroblasts and, on the other hand, by the presence of soluble factors that would favor the survival of the former. Leptin would also stimulate the stellate cells themselves to produce collagen. Conversely, the inhibiting action carried out physiologically by adipokine, resistin and visfatin would be ineffective given the low circulating levels.

Finally, it should be reported how recent studies have shown that hepatocytes that are characterized by ballooning phenomena, that is those hepatocytes that have started a process that ultimately results in the death of the cell itself, and that characterize the pictures of NASH, produce a protein that would favor tissue fibrosis, called "sonic hedgehog". This polypeptide would stimulate

cholangiocytes to produce osteopontin which would act in a paracrine manner on stellate cells favoring the deposition of collagen [85,86].

The cell death of hepatocytes triggers inflammation, fibrosis and even the onset of heteroplasia.

Certainly, further studies are needed, both in vivo and in vitro, to better define all the mechanisms involved in the development and progression of this complex pathological. Also, in order to be able to identify any treatments aimed at contrasting or antagonizing specific critical passages.

DIAGNOSIS

According to the last guidelines, the diagnosis of NAFLD is based on the presence of the following elements: (a) findings of hepatic steatosis through histological or radiological investigations; (b) the absence of alcohol consumption exceeding 20-30 g/day in women and men respectively; (c) the exclusion of conditions that can lead to the development of secondary steatosis [2,8,87,88].

In fact, given the lack of surrogate diagnostic markers of NAFLD, it is essential to perform a careful diagnostic work-up aimed at excluding other causes of liver disease: from viral to autoimmune forms, from hoarding to drug-induced or toxic forms. However, it should be remembered that hepatic steatosis is often associated with these pathologies, in particular, this has been shown in the course of HVC related hepatitis, where the concomitance of the two conditions has been shown to reduce the response to treatments involving the use of interferon [89].

The dosage of cytonecrosis indices is not useful for diagnostic purposes, since in 50-80% of patients with NAFLD the dosage of transaminases is within the normal range. The dosage of hyaluronic acid and other fibrosis markers is high in patients with a degree of fibrosis superior to F3, but these indicators are not routinely measurable in all laboratories. The finding of alterations in the platelet count, hypoalbuminemia, marked alteration of direct and indirect bilirubin or an increase in ammonia, are suggestive, however, of overt cirrhosis.

Liver biopsy

The gold standard for the diagnosis of NAFL or NASH is still represented by hepatic needle biopsy, as these two histopathological entities are different and not otherwise differentiated from each other. Nevertheless, this procedure is not free from risks and limitations. First of all, it is an invasive procedure, with possible complications such as bleedings, infections or bileo-puncture resulting in peritoneal resentment. Furthermore, it is also expensive. It may also no be representative, since the

sample taken represents only a small percentage of the entire organ, also in consideration of the fact that the disease does not affect the parenchyma uniformly. Moreover, the sampling may also be affected by sampling errors or the frustule may be unsatisfactory. Finally, the assessment is burdened by a wide operator-dependent variability, related to the experience levels of the different anatomopathologists conducting the examinations. It should be remembered that only a limited percentage of NAFLD patients have steatohepatitis, which means that, to diagnose this condition, the number of subjects to be biopsied is much higher than those who would need it. Therefore, it is possible to state that liver needle biopsy is a poorly suited tool as a diagnostic test for NASH. In light of all these considerations, a biopsy is currently indicated in patients considered most at risk of developing NASH with fibrosis or in those with concomitant chronic liver diseases [7,8].

The histopathological examination in the case of NAFL shows the presence of hepatic steatosis greater than 5%. In the case of NASH, on the other hand, in addition to steatosis, the presence of "balloon-like" degeneration and lobular inflammatory infiltrate is noted. Around the central veins, in the region known as zone 3, pathognomonic elements such as Mallory's hyaline bodies and the presence of peri-cellular fibrosis are observed. Some atypical elements have been described in morbidly obese subjects or children, such as periportal steatosis in zone 1, the absence of ballooning or Mallory's bodies, and greater degrees of fibrosis or portal inflammation [2,90–92].

Over the years, various histopathological classifications have been proposed for NAFLD: Matteoni's, Brunt's, the NAFLD activity score (NAS), and the SAF score [46,93–95]. In 1999, Matteoni et al. proposed a dichotomous classification that distinguished NASH and non-NASH, in particular, it identified: a type 1, or subjects with simple steatosis; a type 2, in which steatosis and lobular inflammation were noted; a type 3, who presented steatosis associated with hepatocellular ballooning; and a type 4, which in addition to the typical characteristics of the third group showed the presence of Mallory's bodies and fibrosis. Those who presented a picture compatible with the first and second types were characterized by an indolent and benign course compared to those who fell into the last two groups [46]. The latter was described as NASH, while the others, antithetically, as

non-NASH or NAFLD. This classification, however, presents a considerable bias as it does not provide an assessment of the severity or pattern of NASH: that is, it does not give information on the degree of steatosis, on inflammation, on the localization of these alterations (whether for example they are observed at the lobular or periportal level), as well as on the degree of fibrosis [2].

In the same year, a semi-quantitative system was proposed by Brunt and colleagues, to define the grading and staging of liver disease. This classification applied only to patients diagnosed with NASH [93].

Finally, in 2005, the NASH Clinical Research Network Pathology Committee developed and validated a histological scoring system based on the Brunt classification: the NAS. This semi-quantitative tool has been proposed as a reference classification for evaluating responses to any treatments or disease progression in clinical trials. It is characterized by the advantage of being independent of the experience of the pathologist who evaluates the liver frustule [96]. But it is not a tool to be used for diagnostic purposes. It was found that the presence of a typical histological picture for steatohepatitis may not coincide with a NAS score that could classify it within the NASH group, so it is recommended not to use it categorically for the diagnosis of NASH [97].

The NAS is addressed to the entire spectrum of NAFLD and it is applicable in both adults and pediatrics [2]. The score, between 0 and 8, is linked to the sum of the single values that describe steatosis (from 0 to 3), the presence of inflammatory lobular infiltrate (from 0 to 3) and ballooning-forms degeneration (from 0 to 2) (***figure 1***). A score above 5 is diagnostic of NASH, scores below 3 are "non-NASH", while those between 3 and 4 are defined as borderline. For what concerns fibrosis, stage 1 is described as the situation in which this affects the peri-sinusoidal region in zone 3. In particular, in the perivenular area, this can be mild 1A or dense 1B, while 1C is defined as the case of portal fibrosis without fibrosis perisinusoidal. Stage 2 is characterized by perisinusoidal and portal/periportal fibrosis. The presence of fibrin bridges is typical of stage 3, while the presence of cirrhosis identifies stage 4. Cohort studies have shown that the degree of fibrosis is an independent predictor of hepato-related mortality. Consequently, the precise quantification and determination of

the degree of fibrosis represent a prognostic element of fundamental importance [98–100] (***figure 2 and tables 2 and 3***).

Bardossa and co-workers proposed and validated a score and a diagnostic algorithm aimed at histopathologically categorizing hepatic lesions in the NAFLD [95]. The SAF score aims to overcome the main limitations attributed to the NAS score. It has been argued that it appears as an excessively artificial classification, that it describes borderline pictures poorly, and that it is characterized by a large gray area (values between 3 and 4) [92]. The SAF score, as a semi-quantitative model, evaluates the degree of steatosis, with a score ranging from 0 to 3, the degree of activity of the disease (combining the degree of lobular inflammation and ballooning, providing a result between 0 and 4) and the stage of fibrosis (0 to 4). Thanks to the separate description of the main histopathological characteristics, it is a useful tool for comparing biopsy samples and their evolution over time, both in the context of trials and in common clinical practice. Finally, it allows to identify those patients who have blurred pictures or not univocal categorization [95]. The evaluation, in patients with hepatic steatosis greater than 5% (i.e. with a degree of steatosis greater than 1), of the level of ballooning and lobular inflammation allows, thanks to the FLIP diagnostic algorithm proposed by Bedossa himself, to classify in a dichotomous manner the liver biopsies in steatosis and steatohepatitis [101] (***figure 3 and table 4***).

Many authors agree in recognizing the usefulness of all these classification tools but underline that these scores cannot replace the analytical description made by the pathologists themselves, who make a diagnosis based on the complex evaluation of each element found during the histological examination. Although these reports may appear subjective and less precise, the evaluation of the regression has shown that, if compared to the scores, these first appear to be the most powerful diagnostic tools [92,97].

Recently Nacimbeni et al. demonstrate, in a cohort of 140 consecutive patients, that FLIP/SAF classification allows to identify the different disease categories with a good match with entities of different clinical and biological severity. Furthermore, they observed the presence of a strong

association between the histological definition of steatohepatitis activity and the amount of fibrotic scarring [102].

Imaging

Ultrasound of the abdomen is the main method currently used for a qualitative assessment of the hepatic parenchyma and consequent identification of a steatosis picture. This is due to the characteristics of this method: non-invasive, easily accessible, inexpensive, and able to provide useful information on the characteristics of the hepatic parenchyma, the biliary tract, the size and vascularity of the organ.

The presence of hepatic steatosis is diagnosed ultrasonographically based on the finding of increased contrast following the comparison between the echogenic characteristics of the renal cortex and the hepatic parenchyma, the finding of a hyperechoic or "brighter" liver, posterior attenuation, and a sort of blurring of the main hepatic vessels [103,104]. Nevertheless, the diagnosis on an ultrasound basis has several limitations: first of all, ultrasound is operator dependent and, therefore, it is affected by the experience and subjective evaluation of the clinician or radiologist who performs the examination; it is also characterized by having a reduced sensitivity in identifying pictures of modest steatosis; finally, it is not able to quantify it [105,106].

Both computerized axial tomography (CT) and nuclear magnetic resonance (MRI) seem to offer greater degrees of objectivity and greater sensitivity in terms of quantification of steatosis. Both, however, are more expensive and not as universally, easily and readily accessible, especially MRI. Moreover, CT is burdened by the risk resulting from exposure to ionizing radiation and is characterized by low sensitivity in cases of mild/moderate steatosis, as well as of not being diagnostic in patients with hemochromatosis. The evaluation of hepatic steatosis by CT uses the determination of a hepato-splenic attenuation ratio lower than 0.9 [2,107,108].

It should also be remembered that in conditions of advanced fibrosis both ultrasound and CT see a further reduction in their diagnostic sensitivity, this following the interference caused by the fibrosis

itself [107]. Similarly, in the case of marked steatosis and obese patients, the ability to identify fibrosis is reduced with both methods [2].

Finally, it should be emphasized that none of these procedures allows us to identify NASH or distinguish it from NAFL.

Other non-invasive methods: Fibroscan®, CAP, ARFI e RET.

Over the years various non-invasive and easily repeatable methods have been proposed in order to obtain an estimate of hepatic fibrosis by evaluating the stiffness of the parenchyma itself; although severe obesity and the presence of marked tissue inflammation may affect the accuracy of the results thus obtained.

The FibroScan® (EchoSens, Paris, France) is an instrument similar to an ultrasound with a probe that functions as an ultrasound transducer and vibratory pulses. In particular, this transmits a vibration wave of medium amplitude and low frequency (50 Hz), from the body surface, at the level of the intercostal spaces, to the underlying hepatic parenchyma, in order to obtain an estimate of the elasticity of the latter, by the analysis carried out on a section of about 4 x 1 cm at a depth of about 2.5 cm. The vibration induces an elastic wave that propagates in the tissue at a speed that is proportional to its elasticity ($E = 3\rho V2$ where E represents the elasticity, ρ the density which is constant for a given tissue and for the liver is = 1, V the propagation speed of the wave). Therefore, the less elastic the parenchyma, the greater the speed measured. The stiffness, expressed in kPa, represents the median obtained following ten measurements. To be considered reliable, the data must have an IQR (variability of the measurements performed) which must not exceed 30% with respect to the median, and a "success rate", i.e. the number of useful measurements, which must be at least 60% with respect the total number of acquisitions made. The results are highly reproducible and can be repeated over time, without exposing the patient to peri or post-procedural risks, to invasive procedures or using ionizing radiation or contrast media [109]. Various studies, carried out with the standard probe M, have attempted to define the reference values for the different degrees of

fibrosis. These vary according to the etiopathogenesis of liver disease. For NAFLD values between 6.6-7.8 kPa fall within grade F2; 7.1-10.4 kPa describe a stage equal to F3, while 10.3-22.3 kPa correspond to an evolution now in terms of cirrhosis (F4) [110–112]. The cut-off values for each degree of fibrosis vary according to the studies examined [109]. Obesity, frequently present in patients with NAFLD, causes failures of between 5 and 9% in acquisitions by FibroScan® in the measure of stiffness [108,113], with a reduction in reliability of about 23% [111]. This is probably attributable to the interference caused by excess subcutaneous adipose tissue [114]. To overcome this drawback, a probe called XL has been developed, which can be used in obese subjects.
In the literature, conflicting data also emerged on the possible interference caused by steatosis and necro-inflammation on the reliability of the results provided by FibroScan® in this subset of patients [110,111,115].

An innovative and promising application of FibroScan® is based on the measurement of the degree of acoustic attenuation as the ultrasound passes through the parenchyma, allowing the calculation of the CAP (controlled attenuation parameter). This parameter, expressed in dB/m, seems to correlate with the degree of hepatic steatosis. However, the lack of defined reference levels and the influence of various variables such as the presence of diabetes mellitus, BMI and etiology, limits the use of CAP in daily clinical practice. Furthermore, in about 15% of patients the results obtained by CAP do not coincide with the histological data [116]. Although the supporters of the method underline that the CAP allows to analyze a tissue sample about 100 times higher than the histologically evaluable one and that therefore this discrepancy could be attributed to the intrinsic limits of the biopsy sample rather than to the CAP itself. Until now, however, no reliability criteria have been established for CAP measurements. Kars et al. have recently proposed as cut-offs: 248, 268 and 280 dB / m for S0, S1 and S2, respectively [116]. However, further studies are necessary to better investigate the underlying mechanisms, as well as to validate the method and the reference limits in larger populations.

The ARFI or Acoustic Radiation Force Impulse devised by Siemens is applied to Acuson S2000® (Siemens AG, Erlangen, Germany) [117], during the B-mode examination, it studies the elasticity of a portion of the liver parenchyma identified by a ROI (Region of interest). The tissue identified through the ROI is mechanically excited using acoustic pulses to induce localized tissue displacements, secondary to wave propagation. This in turn generates a wave, called shear wave or shear wave, which is followed using ultrasonic correlation methods. Its propagation speed is then estimated by evaluating the lateral displacement time in a defined spatial area. The study is performed on the right lobe of the liver, 2-3 cm below the hepatic capsule. The median of ten suitable acquisitions is expressed in terms of shear wave velocity (SWV) in m/s. SWV is considered proportional to the square root of the elasticity of the liver parenchyma [114]. Data with an IQR/mean ratio of over 0.3 are considered to be affected by excessive variability and not considered reliable.

The limit values of the various degrees of fibrotic evolution in NAFLD vary considerably in different papers [41–44]. The cut-off values reported for F2, F3 and F4 were 1.165, 1.48-2.06 and 1.635-1.9 m/s respectively. The limits proposed to discriminate NASH from NAFL are 1.105 m / s (F1) [44] or 1.3 m/s (identifying steatosis with inflammation, without fibrosis) [118]. From the literature data it would seem that obesity or various degrees of liver fibrosis or ballooning do not affect the results obtained. Yoneda et al. described how there is a variation in velocities detected between the groups with different inflammatory activity, even if this does not start gradually. Thus, it could be assumed that steatosis probably decreases SWV while inflammation increases it [119]. Comparing the diagnostic accuracy of the two procedures, no statistically significant differences were identified between ARFI and FibroScan® for the diagnosis of fibrosis, cirrhosis and steatohepatitis. So that, in the event that reliable data are purchased, both methods can be considered reliable.

On its ultrasound systems, the Hitachi company has developed real-time elastography (RTE), which provides information on the physical characteristics of tissues using the normal ultrasound probe. During the execution of the B-mode study, the elastic properties of a portion of the parenchyma are studied, identified through the ROI. The ROI consists of approximately 30,000 elements. During

the compression induced by the probe, the displacement of each element is measured. In rigid fabrics, the amount of displacement is low, while in stretch fabrics it is high. The calculation of the elasticity distribution of the fabrics is performed in real time and the results are displayed as colored images with the conventional image B in the background. The final result is based on data obtained from ten reproducible measurements. To date, there are few studies evaluating the applicability of RTE in subjects with NAFLD, so further research is needed to define the diagnostic cut-offs for NASH and for the various degrees of fibrosis [109].

Biomarkers

The term NAFLD, in general clinical practice, is appropriately used for the diagnosis and management of a wide variety of patients and, in this context, serum biomarkers, useful tools for identifying the degree of fibrosis, can be exploited to in turn for the identification of NAFLD in patients with fibrosis or cirrhosis [2].

CK-18, FGF-21, CBP.

Hepatic steatosis is frequently accompanied by a slight to moderate increase in the serum level of aminotransferases and changes in gamma-glutamyl transferases (γGT). These alterations, in the most favorable condition, allow to identify only those at greater risk of being affected by NAFLD, and who therefore require further diagnostic tests. However, as previously mentioned, they are imprecise indicators and therefore should not be the only tools used in clinical practice [120]. The dosage of hepatic cytonecrosis indices, in particular ALT, can be particularly misleading, appearing, in a not limited percentage of patients, even in the presence of a histological alteration, within the normal limits [121,122]. The diagnostic accuracy of ALT alteration, estimated in patients with NASH, is particularly low, around 40% [122,123].

In light of these limitations, in recent years many authors have pursued biomarkers able to predict the risk of evolution of NAFL or the presence of hepatic fibrosis. An ideal serum marker should certainly be easy to measure, accurate and reproducible, inexpensive, and immediately accessible and available. It should allow to favor discrimination between the various stages of the disease and to follow it over time and evaluate the effectiveness and response to a therapeutic treatment. Generally, while most biomarkers and scoring systems are similar in terms of accuracy for identifying advanced fibrosis conditions, their accuracy is limited in mild fibrosis cases [2,122]. The diagnostic accuracy of various proteins was studied, released into the circulation as a consequence of oxidative stress, inflammatory processes, hepatocyte apoptosis or in response to alterations in lipid metabolism. These, in a limited number of studies, were evaluated both individually and in combination with each other to evaluate their diagnostic accuracy.

Cytokeratin 18 (CK-18) is one of the major intermediate filament proteins contained in hepatocytes. Following apoptosis, fragments of CK-18, whether or not subjected to cleavage by caspases, called M30 and M65 respectively, are released into the circulation [122]. The assay of both fragments was found to be accurate in discriminating between NASH and NAFL, with AUROC values of 0.82 (95% CI, 0.79-0.85) for M30 and 0.80 (95% CI, 0.76-0.83) for M65 [122]. The normal limits identified in the various studies for M65 are between 243.8–790 U / L (0.62–1 sensitivity and 0.65–0.89 specificity), while for M30 they are 121.6–380.2 U / L (0.60–0.95 sensitivity and 0.60–0.97 specificity) [122].

The fibroblast growth factor 21 (FGF-21) is instead a hormone secreted by hepatocytes that has been found to have beneficial properties on lipid metabolism and hepatic steatosis [122]. Several studies have shown how the serum levels of this marker are associated with the hepatocyte content of lipids, especially in patients with moderate hepatic steatosis [122]. In the meta-analysis performed by He and co-workers, the standardized mean difference is greater in patients with NASH than in those with simple hepatic steatosis (MDS 1.47, 95% CI, 0.13–3.07 vs SMD 1.12, 95% CI 0.27–1.97), thus allowing for the assumption that this marker can be used in the diagnosis of

steatohepatitis. The authors underline that further ad hoc studies are needed to better define the sensitivity and specificity and evaluate the AUROC [122]. Some researchers, in consideration of the complexity of the pathophysiological process underlying the development and evolution of NAFLD, have suggested resorting to the use of a combination of two or more serum markers, to provide more precise information about the risk of evolution. of the pathology.

For this reason, the combined biomarker panel (CBP) has been proposed, which also includes the assay of CK-18 and FGF-21 among others. CBP would have greater diagnostic accuracy than that possessed by single markers with an AUROC equal to 0.94 (95% CI, 0.92-0.96), demonstrating a better discriminatory capacity between NASH and hepatic steatosis, with a sensitivity and specificity of 0.92 respectively (95% CI, 0.88-0.95) and 0.85 (95% CI, 0.72-0.92) [122].

In conclusion, the data available up to now in the literature would seem to show that increased serum levels of these markers are associated with an increased risk of developing NASH. The combination of the latter allows to optimize the ability to distinguish between simple hepatic steatosis and hepatitis.

Although many papers have evaluated the performance of these markers, there is no consensus on which of these has the best diagnostic power.

Furthermore, the marked heterogeneity that characterizes many of the published research makes it difficult to compare them, so that it is mandatory to expand the number of studies, their sample size, and make them homogeneous to optimize and improve research on this subject.

Tests were also proposed that combine clinical and other information extrapolated from biohumoral data to predict the risk of NAFLD/NASH of the individual patient, offer a stratification of the same and provide indications for any second-level assessment. These include the NAFLD fibrosis score [120,124], the FIB-4 [125], the FibroTest, the Fibrometer, and the Enhanced Liver Fibrosis (ELF) score [120,125,126]. The first two can be calculated about platelet counts, albumin levels and ALT. FibroTest, Fibrometer and ELF score are commercially available tests. Even these non-invasive methods require further studies and validation and can be useful for identifying patients deserving to undergo

liver needle biopsy which to date remains the only reference method to distinguish NASH from simple steatosis.

Squamous cell carcinoma antigen (SCCA)/SerpinB3

Serpine isoforms B3 and B4, also known as squamous cell carcinoma antigen 1 and 2 (SCCA1 and SCCA2) belong to the ovserpine/clade B serpin family. They are protease inhibitors implicating in many control processes of cellular homeostasis and as many biological functions [127].

More than 1500 members of serpins have been identified in plants, invertebrates, bacteria, and viruses [128,129]. Ovserpines are typical of vertebrates, fish, and mammals, and can be considered an evolution in two gene loci of a single ancestral gene. Serpins inhibit proteases by a suicidal inhibition mechanism. They possess a marked specificity for their target proteins, linked to the differences in the sequence of the site of action, which allows them to be adequately identified as proteases. These determine the cleavage of a specific domain inducing an alteration of the form of the ovserpine which therefore irreversibly inhibits the protease itself [127].

Isoforms B3 and B4 evolved by acquiring inhibitory activities against cysteine proteases [130,131]

The two genes encoding the two isoforms of serpin are located on chromosome 18, in the q21.3 region of 600 kb [132], together with at least four other genes encoding serpin variants. These two almost identical genes are distributed in tandem on the chromosome (head-tail pairing): the gene for SerpinB3 is located in the most proximal portion to the centromere, while that for SerpinB4 is located more distally near the telomere region. This peculiar arrangement suggests that they are the result of a duplication of a single common ancestral gene [133]. Even if characterized by a high homology of their amino acid sequences, they demonstrate similarities and different substrates [134]: SerpinB3 inhibits cysteine-proteases (papain, cathepsin S, K and L) [135], while SerpinB4 acts on both serine protease (cathepsin G) and cysteine proteases (Der p1 and Der f1) [129,131,136]. The peculiarities of the reaction site (RSL) of each serpin is responsible for the different specificities of action. Only 7 out of 13 amino acids coincide, or 54% [137].

In vivo, it is still not entirely clear what their role at the physiological level is, nor what the mechanisms underlying the regulation of their different expression are [129]; this is partly because they are co-expressed in both healthy and pathological tissues and that they have high percentages of homology both at the level of the messenger RNA sequence and when the amino acid sequences that constitute them are evaluated [138].

Both isoforms are expressed by the spiny and granular layers of the normal squamous epithelium in a variety of organs including tonsils, tongue, esophagus, cervix, vagina, major airways, and Hassall's corpuscles in the thymus [139].

Regarding their role in normal tissues, it has been postulated that the two isoforms play a protective role against bacterial and viral proteases [140], mast cell chymases [141] and that they can play a role in preventing the apoptosis of the stratum corneum cells. SerpinB3, in particular, is normally expressed in squamous epithelia such as the epidermis, cervix, bladder, esophagus, tonsils, airway epithelium, as well as in the prostate, testicle, or thymus [138].

In the course of chronic inflammatory processes involving the skin (as in the case of atopic dermatitis and psoriasis) and the respiratory tract (asthma, chronic bronchitis, and tuberculosis), marked levels of expression were detected [142–145], further supporting the hypothesis its involvement in cellular homeostasis and the modulation of inflammatory response [138]. Turato and colleagues have shown that chronic hepatocytic damage can induce the expression of SerpinB3 and TGF-β1. The same serpin-stimulated antiprotease activity would, in turn, be involved in the induction of the same TGF-β1, acting as a modulating protein. The combination of these two stimuli would favor the development of hepatic fibrosis [146].

A further stimulus capable of inducing the transcription, synthesis, and release of SerpinB3, mediated by HIF-2α and by the presence of reactive oxygen species, appears to be hypoxia, as described by Cannito et al. [147].

Novo and coworkers recently demonstrated, in an in vitro study, that during chronic liver diseases the hepatocytes release SerpinB3, which contributes to parenchymal fibrogenesis through the activation of myofibroblast-like hepatic stellate cells (HSC/MFs) [148].

The hyper-expression of SerpinB3 has also been observed in the course of various heteroplastic lesions: particularly in those of epithelial origin [139,149], in adenocarcinoma of the lung, breast, and pancreas, as well as in hepatocarcinoma [150–153]. The degree of expression seems to correlate with the development of tumor disease and is a useful predictor of the stage of the disease and its response to therapy [138,154,155]. SerpinB3 is involved in various stages of the oncogenic process, acting as an authentic oncoprotein [138]. It favors the survival of neoplastic cells by interfering with cellular apoptotic processes, it promotes cell proliferation and migration, and it is involved in the processes of intrinsic resistance to chemotherapy [138,156–158].

It is known that the loss of homeostasis between proteases and its inhibitors has repercussions on mobility, invasiveness, proliferation, and finally on cell death itself [159]. On the one hand, Serpin B3 plays a role in the regulation of proteolytic processes (important junctions in the development of the tumor phenotype), on the other hand it is able to protect the neoplastic cell from apoptosis induced by various stimuli [160,161] by inhibiting the activity of caspase-3 [160], and, finally, at the same time can promote cell proliferation [158].

Furthermore, it has been shown in vitro that SerpinB3 inhibits the release of cytochrome c from the mitochondrion, thus suggesting its influence at the level of a bid or bcl-2 activation [162].

SerpinB3 can induce the production of pro-inflammatory and pro-tumor cytokines such as interleukin 6 (IL-6) [157,163]. It is an important mediator of RAS-induced pro-inflammatory cytokine production, such as interleukin 8 (IL-8), granulocyte-macrophage colony-stimulating factor (Granulocyte-Macrophage Colony-Stimulating Factor or GM-CSF) and platelet factor 4 (PF4) also known as chemokine CXC motif ligand 4 or CXCL4 [138]. It also increases the expression of c-Myc [164]. These processes are involved in neoplastic invasiveness and the epithelium-mesenchymal transition (EMT) [165] (***figures 4 and 5***).

Another mechanism favoring tumor growth is linked to the ability of this serpin to block the intratumoral translocation of Natural Killer (NK) cells [166].

The altered pro-apoptotic pathway has also been similarly shown in hepatocarcinoma, where it is precisely inhibited [167], this could be at least partially linked to SerpinB3. The overexpression of this serine would negatively modulate programmed cell death, favoring the immortalization of neoplastic cells.

SerpinB3 has been detected in the cytoplasm and at the nuclear level of tumor cells, and in the serum of patients with squamous-cellular tumors. Moreover, concentrations of this serpin in the form of circulating immune complexes composed with immunoglobulins of the IgM isotype have also been recently detected in the serum of subjects affected by hepatocellular carcinoma. In this condition, an imbalance in the ratio of serpinB4-IgM and serpinB3-IgM concentrations was detected [129,150].

This complex was also detected in patients with chronic hepatitis and/or liver cirrhosis, but not in healthy controls, and any case at lower concentrations than those circulating in patients with HCC. These considerations have led to the hypothesis that SCCA-IgM may represent a useful diagnostic marker for cancer and advanced liver disease [129,168,169].

Martini and co-workers finally described how elevated plasma levels of SCCA-IgM in patients with HCV-related liver disease are significantly associated with the histological presence of a NASH picture.

Because the SCCA-IgM immune complex was mainly detected in the serum of HCV positive subjects, compared to HCV negative controls, it can be hypothesized that a viral infection causing cytolytic damage may induce the expression of serpin or that the presence of SCCA-IgM is linked to the presence of steatohepatitis itself, regardless of the etiology of chronic liver disease [170].

PROGNOSIS AND COMPLICATIONS

Simple steatosis can progress to steatohepatitis [43,46,54,171–173]. The greatest predictors of risk are represented, as previously mentioned, by advanced age, male sex, genetic and metabolic factors. The rates of disease progression are lower than those observed in other liver diseases such as in the case of HCV related [174]. It is estimated that progression from steatosis to cirrhosis may take about 57 years, while in the case of steatohepatitis about 24 years [173].

Fibrosis by itself is a strong predictor of hepato-related mortality in NAFLD patients [120,171]. Obesity, type II diabetes mellitus, hypertension, premature menopause, increase in intima-media thickness (IMT) at the carotid level, whether or not accompanied by atherosclerotic plaques, are all elements associated with fibrosis progression and cirrhosis development in subjects with liver steatosis [175–177]. In particular, the presence of type II diabetes mellitus and steatosis predicts the development of clinically significant hepatic fibrosis [178].

El-Serag et al. estimate that NAFLD accounts for the 30-40% of HCC worldwide cases [179]. Younossi and colleagues in a metanalysis published in 2016 calculated HCC incidence in subjects with NAFLD to be 0.44 per 1000 person-years. However, for subjects who develop NASH, the incidence of HCC increases to 5.29 per 1000 person years [26]. Pathogenesis of primary liver tumor in NAFLD is not completely understood, since multiple mechanisms are involved: as already mentioned, the presence of specific genetic polymorphisms plays a central role, together with environmental factors, obesity, and type II diabetes mellitus. Low-grade chronic inflammation, insulin resistance, hyperinsulinemia, increased levels of insulin growth factor, fat accumulation inducing lipotoxicity, mitochondrial dysfunction, stellate cell activation, increased levels of LPS due to gut microbiota alteration, all represent the main actors that interplay together to activate and produce inflammatory cytokines (i.e. TNFα, TGFβ, IL-6, and IL-17), reactivate oxygen species, deregulate phosphatidylinositol-3-kinase (PI3K) and Akt, and finally cause genomic instability through pro-oncogenic signaling activation (i.e. segregation defects, and alterations in the DNA-

damage-response pathways [180–182]. Individuals with NAFLD are at an increased risk of developing HCC even in the absence of significant cirrhosis or fibrosis. Even though in these individuals HCC is characterized by larger sizes and poorly differentiated [180,181], to date there is no possibility of accurately predicting the risk of developing hepatocellular carcinoma in patients who do not have a histological liver specimen characterize by a fibrosing evolution. Therefore, there are currently no strategies or protocols for surveillance or monitoring of these patients. It follows that the disease is often diagnosed late, often when eradicating treatments are no longer possible, with negative consequences in terms of prognosis and survival [183–185].

However, the main cause of mortality in these patients remains cardiovascular diseases. A recent meta-analysis demonstrates that subjects with NAFLD have a greater risk of developing fatal and non-cardiovascular events than those not affected [186]. This according to a gradient that proceeds parallel to the severity of NAFLD itself. Thriving literature has shown how this condition is also associated with myocardial remodeling, favoring the development of functional and structural myocardiopathy, correlated on the one hand with the development of valvulopathies (from aortosclerosis to calcification of the mitral annulus) and on the other, to the increase in the incidence and prevalence of permanent atrial fibrillation [187–192]. NAFLD is also associated with QTc elongation [193,194] and with an increased prevalence of detection of ventricular arrhythmias with Holter electrocardiographic monitoring, [195]. Moreover, preliminary data would seem to suggest an association between NAFLD and an increase in the risk of re-hospitalization one year after an episode of heart failure [196,197].

Finally, it should be noted that extensive epidemiological studies reveal the existence of an association between this condition and the onset of other types of heteroplasias, such as colorectal cancer [198,199]. So much so that mortality from neoplastic diseases ranks second among the causes of death in subjects with NAFLD. Other associations have been reported such as cancers of the pancreas, esophagus, stomach, kidney, prostate, lungs, and breast [198,200–202]. These data are all

preliminary and while noting a close association with the condition called "diabesity", further studies deserve observations to validate any correlation with NAFLD [120].

AIMS OF THE STUDY

NAFLD, over the last two decades, has become the most common liver disease affecting about 25% of the general population worldwide [182]. At the same time, it has also become the main and emerging cause of HCC in patients with and without cirrhosis, favored by the obesity pandemic and the increased incidence of diabetes and other characteristic elements of the metabolic syndrome (MetS).

The lack of NAFLD awareness, due to the absence of specific symptoms and signals, exposes unknowingly affected patients to a high risk of disease progression and late diagnosis of its complications.

Current guidelines [7] recommend screening for features of MetS in all individuals with steatosis, independently of liver enzymes levels, and screening all subject with persistently abnormal liver enzymes (level A1) for NAFLD. Furthermore, for individuals with obesity or MetS, an evaluation of liver enzymes and/or an ultrasound study of the liver (level A2) is recommended as part of routine work-up screening for NAFLD. For patients with age> 50, T2DM and MetS (which qualify them as high risk) it is advisable to investigate the presence of advanced disease (NASH with fibrosis) (level A2) [7].

Liver biopsy, despite sampling variability limitations and procedural risks, still represents the gold standard for the diagnosis of NASH, and it is the only tool currently available to distinguish between pure fatty liver and steatohepatitis.

Over the years, many authors have searched for surrogate markers of hepatocyte damage and fibrosing evolution to identify patients at greater risk of disease evolution, to be subjected to second-level investigations or a closer clinical follow-up. However, there is no unique consensus on thresholds and/or strategies that physicians ought to use in clinical practice whit the aim of avoiding liver biopsy [7,203].

The major unmet needs, despite all the medical progress in the NAFLD field, is represented by the lack of non-invasive means allowing clinicians to identify subjects at risk of disease evolution. One branch of research has actively focused on soluble bio-markers, another one has moved towards genetic variables.

In the last years, many authors have described increasing value levels of serum SCCA-IgM in patients suffering from chronic hepatitis (secondary to alcoholic and viral etiologies), liver cirrhosis, and HCC, but not in healthy controls [129,168,169], leading to the hypothesis that SCCA-IgM can represent a useful diagnostic marker for the presence of neoplasia and advanced hepatopathy. Martini et al. described how elevated plasma SCCA-IgM levels in patients with relative HCV liver disease are significantly associated with the presence of NASH at the histological level, but it is not clear if the presence of the immunocomplex is related to the presence of viral infection or steatohepatitis itself [204]. There is a lack of data on the association between SCCA-IgM and fatty liver disease in the literature, so the first aim of this study is to investigate whether patients with NAFLD, in their different patterns of disease manifestation, present an autonomous production of SCCA-IgM/serpin B3 and whether their levels are predictive of liver illness evolution risk.

Moreover, recently acquired robust evidence supports the role of genetic predisposition and heritability of NAFLD and HCC development; variants in the gene involved in the regulation of hepatic lipid metabolism, such as in *PNPLA3*, *TM6SF2*, *MBOAT7*, and *GCKR*, are strongly associated with hepatic fat content (HFC) and progression of liver diseases.

Recently, Dongiovanni and co-workers [205] proposed the use of a weighted polygenic risk score (PRS) for hepatic fat accumulation based on SNC variants to stratify the risk of HCC development. As a secondary aim, we examined the impact of a polygenic risk score of hepatic fat content (PRS-HFC), based on well-characterized risk genetic variants, on NAFLD-HCC in a cross-sectional cohort of at-risk individuals (NAFLD cross-sectional cohort). We tried to optimize it by adjusting for a protective variant in *HSD17B13* (PRS-5). Finally, we aimed to identify the best diagnostic threshold.

MATERIALS and METHODS

Study participants

- SCCA-IgM

Patients with NAFLD (NAFL and NASH) diagnosed through clinical criteria (increased transaminases, exclusion of alcohol abuse, exclusion of other chronic liver diseases) and confirmed by histological examination were selected.

84 patients with NAFLD were enlisted. 39 patients presented a histological diagnosis of liver steatosis and 45 of steatohepatitis. These patients were followed at the Liver Unit of the Hospitals of Udine (Medical Clinic), Trieste (Pathologic Liver Clinic), and Milan (Internal Medicine and Metabolic Diseases).

The inclusion criteria were as follows: over 18 years of age, both sexes, confirmed clinical diagnosis of NASH or NAFL by a histological investigation. Patients who met the following exclusion criteria (condition inducing liver steatosis or increasing levels of the immunocomplex studied) were not enrolled in the study: a histological examination of a picture compatible with chronic liver disease evolved into cirrhosis; the presence of HCC; concomitance of HCV or HBV related infections; the presence of autoimmune liver disease, alpha1antitripsy deficiency, celiac sprue, hemochromatosis or Wilson's disease; daily alcohol consumption exceeding 30 g and 20 g for men and women respectively; exposure to hepatotoxic drugs or drugs inducing hepatic steatosis; a history of other types of cancer of epithelial origin; psoriasis, atopic dermatitis, allergic asthma and squamous skin carcinomas. The patients expressed their informed consent to grant access to their medical records and to participate in the study.

The enrolled cohort for evaluation of SCCA-IgM was characterized through the evaluation of biochemical and anthropometric parameters of cardiometabolic risk. Weight, height, and waist circumference were measured. The BMI was calculated using the ratio between weight express in

kilograms and the square of height express in meters. At fasting, blood samples were taken to determine: blood count with formula, phlogosis indices (C-reactive protein - CRP - and Erythrocyte Sedimentation Rate -ESR-), renal function (creatininemia and azotemia), electrolyte dosage (sodium, potassium, magnesium, calcium, phosphorus, chlorine), cytonecrosis and colostasis index (aspartate aminotranferases –AST-, alanine aminotransferases –ALT-, gamma-glutamyl transpeptidase –γGT- , alkaline phosphatase –ALP-, total and direct bilirubin), hepatic synthesis indices (international normalized ratio –INR-, albumin), pseudo-cholinesterase, glycaemia, insulinemia, c-peptide, glycated hemoglobin (HbA1c%), lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides), Vitamin B12, folate, thyroid stimulating hormone (TSH), triiodiothyronine (fT3), thyroxine (fT4), cortisolemia, growth hormone (GH), insulin-like growth factor or somatomedin (IGF-1), homocysteinemia, lipotrotein(a) (Lp(a)), plasminogen activator inhibitor-1 (PAI-1), tissue plasminogen activator (t-PA), iron profile (sideremia, ferritin, transferrin, transferrin saturation).

In non-diabetic patients, a glucose tolerance test was performed with 75 g of glucose and determination of blood glucose, insulin, and c-peptide levels at 0-60-90-120-180 minutes.

The circulating SCCA-IgM complexes, dosed with the ELISA method, were also determined through a serum sampling in the morning, after an overnight fast, with the HepaIC kit (Xeptagen S.p.A., Venice).

The HOMA-Index (insulin resistance index) was calculated: blood sugar (expressed in mg/100ml) multiplied by insulinemia (expressed in mUI / L) divided by 405 [206,207].

For each histological report, related to liver biopsy, the NAS score was defined, the SAF score and the patients were categorized into NAFL and NASH also based on the FLIP algorithm [92,208].

The thickness of the subcutaneous (7.5 Mhz linear probe) and visceral (3.5 Mhz convex probe) fat was also measured.

- PRS-HFC and PRS-5

In the NAFLD/MAFLD case-control cross-sectional cohort, 1,699 patients with a diagnosis of NAFLD were enlisted (as defined in the guidelines[7], thusly based on clinical, radiological, or histological characteristics, in which secondary causes of liver steatosis were ruled out).

These subjects, with European ancestry, were followed from 2008 until 2019, in several Italian (Milan, Udine, Trieste, Varese, Rome, Naples, and Palermo) and an English center (Newcastle upon Tyne), and were affected from different stages of liver disease: NAFL, NASH, cirrhosis, and HCC with or without severe degrees of fibrosis. Some of them underwent diagnostic biopsy during bariatric surgery.

82 patients were enlisted in the Liver Unit of the Hospital of Udine (at Medical Clinic): 33 of which presented NAFLD with a low degree of fibrosis (F0-F2), 19 NAFLD with severe fibrosis (F3-F4) and lastly 30 with HCC related to NAFLD.

Clinical (including sex, age, BMI, diagnosis of type 2 diabetes, histological features, NAS score) and biochemical data were collected and included in the analysis, when available.

Moreover, 865 healthy subjects were enlisted as controls, matched for age and sex, without clinical and biochemical evidence of liver disease. Informed written consent was obtained from each participant.

Genotyping

Participants of both studies were genotyped for the rs738409 *PNPLA3* I148M variant, rs58542926 *TM6SF2* E167K variant, rs641738 C>T *MBOAT7* variant, rs1260326 *GCKR* P446L variant, and rs72613567 *HSD17B13:TA* variant. DNA was extracted from peripheral blood mononuclear cells. Genotyping has been performed in nuclease assays duplicated by TaqMan 5’ at the Translational Medicine and Metabolic Liver Disease lab of the University of Milan.

The genetic risk scores

The genetic risk score (GRS), developed by Dongiovanni and colleagues [205], is based on the evaluation of cohorts of at-risk subjects and individuals from the general population enlisted in "Liver Biopsy Cohort" (LBC) [209], "Swedish Obese Subjects Study" (SOS) [210], and in particular in sub-groups of "Dallas Heart Study" (DHS) [211]. For each patient enlisted the authors compiled information about genetic characteristic, referred to the major risk alleles involved in NAFLD predisposition (*PNPLA3, TM6SF2, MBOAT7* and *GCKR*), and hepatic liver content obtained through biopsies (in LBC or SOS) or with proton magnetic resonance spectroscopy (in DHS). Mendelian randomization analysis demonstrated a specific impact of genetic variants on liver damage, that results proportional to their effect on hepatic fat accumulation. The GRS represents the sum of the steatosis predisposing alleles in four genes (*PNPLA3, TM6SF2, MBOAT7* and *GCKR*), weighted by their effect size on hepatic steatosis quantified by reference standard in the general population: [(0.266 x number of G alleles of PNPLA3) + (0.264 x number of T alleles of TM6SF2) + (0.063 x number of T alleles of MBOAT7) + (0.065 x number of T alleles of GCKR)]. In our study, we refer to it as PRS-HFC.

Subsequently, a modified score of NAFLD was developed and adjusted for the rs72613567 *HSD17B13* variant [74], which we called PRS-5 (PRS-5: available in 2,532, 98.7%, coefficient: -0.361). We reported the association of both instruments with phenotypes throughout the study since PRS-HFC is a proxy for genetic predisposition to accumulate liver fat, while PRS-5 considers all variants strongly associated with NAFLD at the time of study design [56].

Statistical analysis

- SCCA-IgM

The data were analyzed using linear regression analysis for continuous variables and the Spearman Rank test for categorical ones. Besides, the Student "t" test, in case of parametric distribution, or Mann-Whitney test, in case of non-parametric distribution, was performed to compare the two groups of patients with hepatic steatosis and steatohepatitis. Finally, multiple regression analyzes and Chi-square tests were performed. P values <0.05 (two-tailed) were considered significant.

- PRS-HFC and PRS-5

For descriptive statistic, categorical variables are expressed as number and proportion, while continuous ones as mean and standard deviation (SD) or median and interquartile range (IQR), as appropriate. Observational associations were performed by fitting data to generalized linear models. Logistic regression models were employed to examine binary traits, and the association between PRS and liver disease was adjusted for age, sex, body mass index (BMI), type 2 diabetes (T2D), with or without further adjustment for the presence of severe fibrosis stage (F3-F4), the major risk factors for FLD and HCC, which were available for all individuals in the NAFLD cohort.

To estimate the causal relationship between genetically determined predisposition to accumulate liver fat and HCC, Mendelian randomization analysis was used. Mendelian randomization analysis is an epidemiological method that used measured variations in human genes with a known function to examine the presence of a causal effect of a modifiable exposure on disease or trait of interest in observational studies. It allows to obtain fair estimates of the outcomes of a putative causal variable when conducting a traditional randomized controlled trial, i.e. the ‘gold standard’ for empirically performing hypotheses in clinical research, is not practicable. This analysis is based on the concept that, since the distribution of alleles during gamete formation is random - independently of any

other confounding factor, and interpersonal covariates are balanced among people with theoretically different polymorphisms, then genetic variation influencing a trait can be used to assess causality against another condition or characteristic of interest. For causal effects to be consistently evaluated, each instrumental variable (IV) used in this analysis must satisfy three key assumptions: (a) relevance assumption: the variable is associated with the exposure; (b) independence assumption: the variable shares no common cause with the outcome; (c) exclusion restriction assumption: the variable does not affect the outcome except through the risk factor [212–215]. In Mendelian randomization analysis, the well-known HFC genetic risk variants expressed in PRS were employed as an instrument variable. The causal effect of hepatic fat on HCC was estimated by examining the PRS for association with liver steatosis and with HCC using a triangular approach: (a) the observational association between hepatic fat and HCC was examined in a traditional cross-sectional study design. These observational associations can occur from both directions and can be biased due to confounding. (b) PRS are confirmed to be associated with fatty liver. (c) The association between PRS and HCC is tested. The genetic effect on HCC is assumed to be mediated by hepatic fat. Since genetic variants are inherited randomly at conception, the transmission of the effects may be assumed independent of other confounders. Moreover, genetic variation cannot be modified by phenotype, therefore ruling out reverse causation.

The causal effect of genetic predisposition to fatty liver content on HCC was estimated by instrumental variable regression analysis in two-sample Mendelian randomization approached by a two-stage least squares regression procedure (using the 'ivreg' command in the AER package in R), which was adjusted for age, sex, BMI, and T2D. We assumed a lesser relevance or neutral impact of horizontal pleiotropic effects, that is an impact of the genetic variants on HCC risk independent of liver steatosis, that can invalidate the Mendelian randomization analysis. This was supported by the direct relationship between the risk conferred towards fatty liver content and HCC independently of the specific mechanism underlying the association with liver disease. The F statistics of the model was 107, thereby excluding weak instrument bias. Wu-Hausman p=0.09,

suggesting that the causal estimate was consistent with the observational association – the test examines the difference between the instrumental variant (PRS) and the observational (fatty liver disease-FLD) association with the outcome (HCC).

To further account for the possible pleiotropy of the genetic variants considered, was also included robust Mendelian randomization approaches via the Mendelian Randomization R package in our sensitivity analyses [215]. The inverse-variance weighted (IVW) method is the equivalent to the standard instrumental method using individual-level data (the two-stage least squares method reported above) but can be performed on summarized data. The robust option uses robust regression rather than standard regression in the analyses, and the penalized option down-weights the contribution to the analyses of genetic variants with outlying (heterogeneous) causal estimates. The median- and mode-based methods calculate respectively a median or mode of the variant-specific causal estimates from the ratio method for each genetic variant individually. The MR-Egger method can assess whether genetic variants have pleiotropic effects on the outcome that differ on average from zero (directional pleiotropy), as well as to provide a consistent estimate of the causal effect, under a weaker assumption-the InSIDE (Instrument Strength Independent of Direct Effect) assumption. The intercept from the MR-Egger analysis can be interpreted as the average pleiotropic effect of a genetic variant included in the analysis. The maximum likelihood involves maximizing a likelihood that has one parameter for each genetic variant, plus a causal effect parameter. The heterogeneity-penalized method uses the same consistency criterion as the mode-based estimation method but evaluates the modal estimate by assessing weights for all subsets of genetic variants.

Mediation analysis was conducted to estimate the fraction of the effect of hepatic fat accumulation – FLD on HCC predisposition, which is mediated through the development of severe fibrosis. Analyses were conducted by the "mediation" package in R (http://CRAN.R-project.org/ package=mediation). We used model-based causal mediation analysis ("mediate" function), calculating quasi-Bayesian confidence estimated with 1,000 simulations. In a Mendelian

randomization framework, a positive PRS score, indicating increased genetic predisposition, was treated as active treatment/exposure. The analysis was adjusted for age, sex, BMI, and T2D.

Furthermore, we determined the thresholds in the PRS able to identify individuals at higher genetic risk of HCC. Diagnostic accuracy of PRS was evaluated by receiver operating characteristic (ROC) curves, and the best cut-off identified as the value that maximizes the difference between true positives and false positives (sensitivity+specificity-1).

Statistical analysis was carried out using the JMP Pro 14.0 Statistical Analysis Software (SAS Institute, Cary, NC), and R statistical analysis software version 3.5.2 (http://www.R-project.org/). P values <0.05 (two-tailed) were considered significant.

RESULTS

- SCCA-IgM

84 patients were enrolled, 39 of which with histological diagnosis of NAFL (46,4%) and 45 with a liver biopsy of NASH (53,6%), whose anthropometric characteristics, the main bio-humoral indices, and the levels of SCCA-IgM are shown in ***table 5***.

The analysis of the collected data showed that patients with NASH were significantly younger (50.7 ± 2.8 years vs NAFL 57.4 ± 1.6 years; p = 0.03) and characterized by a higher body weight (97.2 ± 4.7 kg vs NAFL 85.9 ± 2.9 kg; p = 0.02) compared to those with a histological picture of steatosis alone.

A statistically significant difference (p = 0.001) was even observed in terms of BMI. Patients with NASH have higher BMIs (34.9 ± 1.7 kg/m^2) than the group of patients with NAFL (29.1 ± 0.6 kg/m^2). While no significant differences were found comparing values of the waist circumference (101.8 ± 3.2 cm and 102.2 ± 2.1 cm respectively for NASH and NAFL), thickness of the subcutaneous fat (24 ± 3 mm and 23 ± 1 mm respectively for NASH and NAFL), and thickness of visceral fat (64 ± 5 mm and 69 ± 3 mm for NASH and NAFL respectively) measured by ultrasound. In the two groups there were no statistically significant differences in terms of glycemia (100 ± 3.7 mg/dL and 103.9 ± 4 mg/dL respectively for NASH and NAFL), glycated hemoglobin (6.5 ± 0.2 % and 6.2 ± 0.1% respectively for NASH and NAFL), insulin (49.5 ± 29.7 mUI/L and 23.9 ± 6.2 mUI/L respectively for NASH and NAFL) and HOMA-index (5.2 ± 1.1 and 4.1 ± 0.4 respectively for NASH and NAFL). However, we have to underling that patients with NASH presented higher insulin values and higher HOMA-index compared with whom present only steatosis, levels this that are compatible with an IR condition.

The evaluation of the lipid profile, while not showing statistically significant differences, between the two groups, in terms of HDL cholesterol (55 ± 2 mg/dL and 56 ± 3 rmg/dL respectively for

NASH and NAFL) and triglycerides, shows that the latter are higher in patients with NASH than in patients with NAFL (158 ± 18 mg/dL and 143 ± 10 mg/dL or NASH and NAFL respectively). The dosage of total cholesterol (222 ± 8 mg/dL and 200 ± 5 mg/dL respectively for NASH and NAFL; p = 0.02) and LDL cholesterol (138 ± 7 mg/dL and 116 ± 5 mg/dL respectively for NASH and NAFL; p = 0.01) instead result significantly higher in subjects witch NASH.

Considering the cholostasisi indices, the two groups of patients are homogeneous (γGT 65 ± 9 U/L and 93 ± 31 U/L respectively for NASH and NAFL; total bilirubin 0.7 ± 0.1 mg/dL and 0.7 ± 0.1mg/dL respectively for NASH and NAFL).

The AST dosage was not significantly dissimilar in the two groups (28 ± 2 U/L and 30 ± 2 U/L for NASH and NAFL, respectively). The ALT value is significantly lower in patients with steatohepatitis than in patients with steatosis alone (31 ± 4 UI/L and 39 ± 2 U/L respectively for NASH and NAFL; p = 0.05), furthermore the AST/ALT ratio results statistically significantly lower in patients with NASH compared to NAFL patients (1.1 ± 0.1 and 1.4 ± 0.1 for NASH and NAFL, respectively; p = 0.01).

No statistically significant differences were observed between the two groups in terms of C reactive protein (CRP) level (3.7 ± 1.2 mg/L and 13.4 ± 0.8 mg/L respectively for NASH and NAFL), PAI (14 ± 2 U/mL and 19.8 ± 4.8 U/mL for NASH and NAFL respectively), tPA (7.5 ± 0.8 ng/mL and 10.3 ± 1 ng/mL for NASH and NAFL respectively), Lp (a) (21 ± 6 mg/dL and 25 ± 6 mg/dL for NASH and NAFL respectively) and ferritin (179 ± 41 mcg/L and 213 ± 32 mcg/L for NASH and NAFL respectively). While patients with NASH were shown to have statistically significant higher TSH levels (2.2 ± 0.2 mU/L and 1.4 ± 0.1 mU/L for NASH and NAFL, respectively; p = 0.001).

Furthermore, in patients with NASH higher IGF-1 values were detected than in patients with NAFL, at the limits of statistical sensitivity (122 ± 15 ng/mL and 97 ± 7 ng/mL, respectively for NASH and NAFL; p = 0.09), while no differences are detectable when plasma cortisol levels were compared (323 ± 44 mcg/L and 361 ± 22 mcg/L for NASH and NAFL, respectively).

Finally, we observed the presence of significantly higher levels of SCAA-IgM in patient with NASH compared with levels detected in those presenting only NAFL (31.7 ± 7.2 IU / mL vs 9.2 ± 1.8 IU/mL respectively) (***figure 6***).

Statistical regression analysis found a statistical correlation between SCCA-IgM levels, age ($p < 0.05$; $r = 0.269$), BMI ($p = 0.01$; $r = 0.3$), homocysteinemia ($p = 0.02$; $r = 0.33$), and SAF-F score ($p < 0.05$; $r = 0.29$). No correlation was found with the degree of steatosis, inflammation and ballooning detected on liver biopsy, with the average-intimal thickness of visceral and subcutaneous fat, nor with the alteration of the indices of cytonecrosis (***table 6***).

By dividing the levels of SCCA-IgM into quartiles, four different groups were identified on the basis of plasma concentrations: the first quartile between 0.06 IU/mL and 3.01 IU/mL with mean SCCA-IgM of 1.98 ± 0.67 IU/mL; the second quartile included concentrations ranging from 3.18 to 5.6 IU/mL with a mean of 4.11 ± 0.67 IU/mL; the third between 6.8 and 10.23 IU/mL, with an average concentration of 7.95 ± 1.33 UI/mL; the fourth with values between 12.2 and 201.92 IU/mL with an average concentration of 35.31 ± 46.39 IU/mL. Thusly, we demonstrated that patients with higher SerpinB3 concentrations presented histological pictures characterized by more marked fibrosis, significant in statistical terms ($p < 0.05$) (***figure 7***).

Finally, we dosed free-Serpin3/SCCA, testing the hypothesis that it could increase the sensibility of the biomarker and aiming to evaluate whether the ratio between the free form and IgM-linked one could better identify NASH and eventually replace biopsy. Although the dosage of Free-SCCA was higher in patients with NASH compared to those with NAFL (1.7±0.4 ng/mL vs 1.4±0.1ng/mL, respectively), it still did not reach statistical significance. Free-SCCA/SCCA-IgM ratio turned out to be statistically significant in patients with NAFL (22.3±3.7 ng/mL in subjects with NASH vs 36.6±5.9ng/mL in NAFL ones, $p = 0.039$) (***figure 8***).

- PRS-HFC and PRS-5

1699 patients with different stages of NAFLD and 865 healthy subjects, all with European ancestry, were enlisted in the cross-sectional NAFLD cohort study. Demographic and PRS characteristic are shown in ***table 7.***

We observed that patients with severe fibrosis (F3 -F4) and HCC were older (58±14 years and 69±9 years NAFLD F3-F4 and HCC respectively compared with 42±16 years and 44±6 years in NAFLD F0-F2 and control group respectively; $p<0.0001$) and had higher prevalence of type II diabetes in NAFDL with F3-F4 degree and HCC respectively, compared with patients prevalence observed in subjects with low fibrosis degree (F0-F2) and with controls (56.9% and 64.2% NAFLD F3-F4 and HCC respectively compared with 20.2% and 0.9% in NAFLD F0-F2 and control group respectively; $p<0.0001$).

HCC patients were more frequently male: we observed that male prevalence in the evaluated groups is respectively 78.8% in HCC, 57.6% in NAFLD with F3-F4, 57.6 in NAFLD with F0-F2 and 52.6% in healthy individuals ($p<0.0001$).

PRS-HCF increased progressively according to the severity of the liver disease (0.266, 0.392, 0.457, and 0.459 respectively in controls, NAFLD F0-F2, NAFLD F3-F4 and HCC; $p<0.0001$). Whereas PRS-5 result higher in patients with severe fibrosis than in subjects with HCC (patients with NAFLD F3-f4 present PSR-5 median equal to 0.421 compared with 0.233 observed in controls, 0.329 in NAFLD F0-F2 and 0.33 in NAFLD-HCC; $p<0.0001$).

Through Mendelian randomization we have examined the relationship between the impact of genetic risk variants on liver steatosis and that on severe liver fibrosis and HCC (***figure 9***). The increase in the risk of HCC conferred by risk genetic polymorphisms was proportional to the increase in the risk of NAFLD ($p=0.02$). Furthermore, the analysis revealed the presence of a direct relationship between the risk conferred to NAFLD and severe fibrosis ($p=0.0001$), and between severe fibrosis and HCC ($p=0.002$).

Using PRS as instrument in Mendelian analysis, we demonstrate that HFC was causally associated with HCC (beta +0.30±0.06, OR 1.35, 1.18-1.58, $p=1*10^{-5}$ for PRS-HFC, and beta +0.29±0.07, OR 1.27, 1.10-1.45, $p=1*10^{-5}$ for PRS-5) independently of age, sex, BMI, and presence of T2D. With the aim of estimating the weight of the effect of fat accumulation on HCC development predisposition, mediated by the progression of the fibrosis, we used mediation analysis. The association coefficient was attenuated by 37-41%, but remained statistically significant, after further correction for severe liver fibrosis ($p<0.05$).

We estimated causality using a wide range of modern Mendelian randomization approaches, which considered the possible pleiotropy of the effects of the genetic instruments (a direct impact on HCC not mediated by HFC). Other sensitivity analyses were generally consistent with a causal effect of FLD on HCC (***table 8***).

By assimilating the score to a continuous variable, linear regression analysis highlighted the existence of a statistically significant association between PRSs and the entire spectrum of NAFLD. The impact of both PRSs on the full spectrum of liver disease in the NAFLD cohort is reported in ***figure 10.*** PRS result associated with an about 12-fold increased OR of severe fibrosis ($p<10^{-27}$ for both) and an about 9-fold increased OR of HCC (OR=9.2, 5.2-16.3, $p=2.7*10^{-14}$ and OR=9.1, 5.2-16.0, $p=1.6*10^{-14}$, respectively for PRS-HCF and PRS-5). The association was independent of age, sex, BMI, and T2D ($p<0.01$ for both PRSs), but not of severe fibrosis ($p>0.1$). In the NAFLD cohort, there was no significant association between PRS and BMI, T2D or HOMA-IR in determining HCC risk ($p>0.1$). These results are consistent with a causative effect of genetic predisposition to hepatic fat accumulation on carcinogenesis, partially mediated by severe fibrosis, but independent on T2D presence.

The AUROC of PRS-HFC for HCC diagnosis was 0.64 and for PRS-5 was 0.65 (***table 9 and figure 11***).

The best single cut-off value for PRS-HCF was ≥0.532, with 43% sensitivity and 80% specificity. As regards to PRS-5, the corresponding cut-off (43% sensitivity and 79% specificity) was ≥0.495

(***table 9 and figure 11***). Therefore, we defined PRS-HFC ≥0.532 and PRS-5 ≥0.495 as "positive" tests. A positive PRS-HFC was associated with a 3-fold higher risk of HCC, and a positive PSR-5 with a 2.9-fold risk ($p<10^{-12}$ for both). In the NAFLD cohort, prevalence of positive tests was 22.2% for PRS-HFC and 22.9% for PRS-5.

Both PRS were able to predict the risk of HCC development more robustly than single variants, with PRS-5 conferring a slight improvement over PRS-HFC (***table 10***).

The sensitive analysis demonstrated that positive PRSs is associated with HCC increased risk even in individuals without severe fibroses (OR>2.0, 1.1-3.8, $p=3.3*10^{-2}$ for PRS-HFC and OR=2.3, 1.2-4.5, $p=1.2*10^{-2}$ for PRS-5; ***figure 12***). Furthermore, positive tests improved HCC detection in subjects over 40 years independently of severe fibrosis (OR=1.5, 1.1-2.2, $p=1.0*10^{-2}$ for PRS-HFC and OR=1.5, 1.1-2.1, $p=2.4*10^{-2}$ for PRS-5). Finally, PRS-5 can predict HCC risk even in non-obese subjects (OR=3.5, $p=7.2*10^{-9}$)

The results of analysis for the 82 patients enlisted in Udine were consistent with what was observed in the completely NAFLD cohort. In particular, the patients with HCC were significantly older than those with NAFLD and controls (69±9 years, 59±9 years, 57±9 years, and 45±6 years respectively for HCC, NAFDL F3-F4, NAFLD F0-F2 and controls; p=5,00E-94). No significant differences were observed in regard to gender. Patient with NAFLD (F3-F4) presented significantly higher BMIs (20.3±6.7 kg, 31±4.1 kg, 28.2±4 kg, and 24.1±1.9 years respectively for HCC, NAFDL F3-F4, NAFLD F0-F2 and controls; p=2,50E-64). Individuals with HCC were more frequently affected from type II diabetes (63.3%, 31.6%, 15.1% respectively in HCC, NAFLD F3-F4 and NAFLD F0-F2; p= 2,40E-36). Subjects with HCC and NAFLD-F0-F2 presented PRS-HCF median higher levels (0.266, 0.459, 0.331, and 0.459 respectively in controls, NAFLD F0-F2, NAFLD F3-F4 and HCC; p= 4,03E-09), NAFDL F0-F2 group were characterize by median higher levels of PRS-5 (0.223, 0.426, 0.224, and 0.396 respectively in controls, NAFLD F0-F2, NAFLD F3-F4 and HCC; p=4,79E-09). Baseline characteristic are reported in ***table 11***.

Even in the Udine subgroup, both PRSs results were able to predict the full spectrum of liver disease at univariate analysis. After adjusting for clinical variables, only PRS-5 can predict NAFLD risk, PRSs do not result independently from age, sex, BMI, presence of type II diabetes and advanced fibrosis (***table 12***). Logistic regression analysis shows that PRSs predict HCC risk development better than single genetic variable (p=2.20 E-05 for PSR-HFC and p=4.50E-04 for PRS-5). Among the latter, only *PNPLA3 I148M,* demonstrates significant power (p=1,80E-03) (***table 13***). However, significance is lost when we adjust the analysis on the basis of the main clinical variables. Finally, the performance test shows for both PRSs a AUROC equal to 07. The prevalence of patients with PRS-HCF equal or higher than 0.532 were observed in 14.1% of the individuals enrolled, and a similar prevalence resulted for cut-off equal or higher than 0.495 for PRS-5. Both the scores were characterized by 40% of sensitivity and 86% of specificity (***table 14***).

We did not find association between PRSs and SCCA-IgM levels in a subgroup of patients, for whom both the non-invasive tools were analyzed. Evaluating the single variants, only GCKR P446L carrier presents a significant association with SCCA-IgM (p=4,07e-2) (***table 15***).

DICUSSION

- SCAA-IgM

Our first study aim is to evaluate whether, in a selected group of patients with histological characterized purely metabolic liver disease, the dosage of SCCA-IgM/SerpinB3 is significantly higher in patients with steatohepatitis than in those suffering from steatosis alone.

In 2015, Martini et al. demonstrated the existence of an association between the levels of SCCA-IgM and the presence of steatohepatitis at histological examination in a group of patients with chronic HVC infection. The SCCA-IgM immunocomplex was more often detected in subjects with related HCV infection than in patients with negative serology (34.9% vs 4.3% respectively; P <0.0001) [170]. Moreover, about one third of the patients with chronic HCV-related liver disease had elevated levels of SCCA-IgM. These concentrations correlated with the presence of a histological picture of NASH and with the genotype 3 of HCV, which is usually associated with more marked insulin resistance and hepatic steatosis [216] .

The dosage of the SCCA-IgM immunocomplex in our population turned out to be inferior from limits so far proposed in the literature by the studies of Turato and colleagues, equal to 160 U / mL [217], or used by Martini and coworkers, equal to 200 U / mL [170], or by Cagnig et al. equal to 120 U/mL [218] . In only one case we found a value equal to 201.92 U / mL, relating to a patient suffering from severe obesity. Overall, although the distribution from the concentrations is characterized by a wide dispersion and variability, we found that, in patients diagnosed with steatohepatitis, the dosages of the immunocomplex were almost thrice as high as those of patients with simple hepatic steatosis, thus reaching statistical significance.

Interesting individuals with higher degrees of SAF-F score shown at histological liver evaluation presented a significantly higher concentration of SCCA-IgM. This is consistent with what has been

reported so far in the literature: the existence of an association between conditions characterized by fibrosing evolution, advanced or worsening liver disease, and an increase in the biomarker [217,218].

Unlike what has been observed in the literature, we can explain the lower levels or SCCA-IgM detected by considering the characteristics of our patients: they present an initial picture of NASH, without high degrees of inflammation, ballooning or, in particular, fibrosis, resulting far from cirrhosis and obviously being free from heteroplastic pathology. This last condition in particular showed higher levels of the immunocomplex in the previously mentioned studies.

Martini et al. also showed that patients not affected by HCV-related chronic infection had lower levels of circulating immunocomplex. From a purely speculative point of view, we can therefore hypothesize that the concomitant presence of the hepatotropic virus has favored and amplified, through the continuous cytotoxic damage, the induction of SCCA-IgM/SerpinB3 production.

Recently, Bettini et al. evaluated SCCA-IgM as a non-invasive biomarker in 56 patients with metabolic complicated obesity before and up to 12 months after laparoscopic sleeve gastrectomy [219]. Presence of liver steatosis was evaluated only through ultrasound scans (16 without liver steatosis and 40 with it). It should be noted that the average starting BMI in subjects was found to be 44 kg/m^2 and 46 kg/m^2 for those without and with ultrasound observed liver steatosis respectively. Only one patient had a histological diagnosis of NASH, however the author did not share data about the presence of fibrosis or inflammation, however the reported immunocomplex concentration fell within the normal range. Even in this cohort, only 3 subjects presented immunocomplex levels above the “normal cut-off”. The author did not observe a significant difference in SCCA-IgM levels between patients with and without liver steatosis. After gastrectomy a reduction in the levels of SCCA-IgM in both groups was observed, however they did not reach statistical significance. No association between the decrease of immunocomplex concentrations and the reduction in inflammation maskers (as IL-6, leptin and hsCRP) was found [219]. This study has several limitations, first of all subject with severe obesity were categorized as either having steatosis or not only on the basis of an abdominal ultrasound study, which is extremely operator dependent

and hardly provides information on any regressions/improvement of the pathology. No histological information and stratification according to a pathological scoring system was available. Even the use of transaminase as a tool to identify NAFLD disease presents several limitations, as previously discussed. Moreover, the author did not exclude patients with concomitant autoimmunity disease, which explains the increased levels of SCCA-IgM detected in 3 subjects. Considering the characteristics of SCCA-IgM, we can expect higher levels, regardless of the cut-offs proposed so far, in individuals with liver fibrosis and inflammation. Obviously, according to current literature, the higher the titers the more pronounced these phenomena are (such as during viral infections, alcohol abuse, overt cirrhosis, and HCC).

Our initial proposal was furthermore to test the possible uses of this immunocomplex in providing the definition of NAFLD disease evolution risk, following patient according to anthropometric, ARFI, and biohumoral data. Unfortunately, the bankruptcy of the manufacturer of the kit and the absence of similar products on the world market prevented the prospective development of the research.

However, further studies evaluating the kinetics of production and disposal of SCCA-IgM and determining whether different cut-offs can be defined in the context of the different etiopathologies and degrees of evolution of chronic liver disease will be necessary.

To date, no studies have been published in the literature relating to the dosage of SCCA-IgM/SerpnB3 in a population with the characteristics of the one we selected, so the evaluations we can make here are purely speculative.

Just as we will be able to explain the reduced levels of free-SCCA found in patients with simple liver statosis, referring to the fact that in NAFL there are reduced levels of inflammation compared to those with NASH. Consequently, the free-SCCA/SCCA-IgM ratio is higher in patients with steatosis alone.

Regression analysis showed the existence of a correlation between SCAA-IgM and age, BMI, homocysteinemia and SAF-F score.

The association with age and BMI could be intuitive. In the literature, several studies have observed that patients with non-alcoholic fatty liver disease are characterized by having an average age of about 40-50 years and a high BMI [27]. In particular, the prevalence of NAFLD increases with increasing age, and aging itself rapresent, as already described above, an independent risk factor for the development of fibrosis and cirrhosis [88].

Although liver disease progression may represent the result of a complex sum and interplay of metabolic events, risk factor exposure, and long-lasting metabolic disease [171], some longitudinal studies do not seem to consistently indicate that age itself has an impact on the rate of progression of hepatic fibrosis [173].

In our study, patients diagnosed with steatohepatitis were on average younger than those with hepatic steatosis alone. Although our sample was affected by a bias related to the presence of younger patients, as they were candidates for bariatric surgery; SCCA-IgM levels correlate with age, suggesting that, on average, a higher age is associated with a more prolonged exposure to pathogenic *noxa* and a pabulum of pro-inflammatory cytokines favoring the production and release of the immunocomplex by the hepatocytes.

Over the years, a growing and consolidated literature has shown how obesity, as a constituent element of the metabolic syndrome, is a risk factor for the development of NAFLD. In some populations of patients undergoing bariatric surgery, about 90% had NAFLD, and about 5% were affected by unrecognized cirrhosis [220]. Pang et al. described how every one-point increase in BMI increases the risk of developing NAFLD by 0.25 [221]. Although the population studies are limited and difficult to perform, since hepatic needle biopsy is an invasive diagnostic procedure not free from the risk of complications, some authors have described, in selected groups of patients, how the degree of hepatic steatosis appears to correlate with BMI [222]. In particular, this could be linked to the increased thickness of visceral fat observed in patients with (i) progressively increasing stages of obesity, (ii) increasing de novo lipogenesis – an altered secretion and sensitivity of peripheral tissues to adipokines (adiponectin, resistin, leptin) -, and (iii) a low, but chronic, degree of

inflammation, with the presence of a circulation of pro-inflammatory cytokines (IL-6, IL-1β, TNF-β, TNF-α) which, in their complex, favor the development and progression of liver disease on a metabolic basis [223]. Unfortunately we do not have information about pro-inflammatory cytokines in our cohort. The levels of SCCA-IgM in our study correlate with the BMI, which is consistent with our hypothesis that subjects with higher BMI have a greater degree of obesity and are characterized by a greater thickness of visceral fat, which in turn contributes to oxidative stress and the production of pro-inflammatory cytokines favors the development of hepatocyte damage and, therefore, the release of SerpinB3. In patients with higher BMI there is a higher prevalence of NASH and therefore greater induction and release of SCCA-IgM. Unfortunately, compared to what we expected, we did not identify statistically significant differences in terms of subcutaneous fat thickness, measured by ultrasound, between patients with steatosis and steatohepatitis.

Although we were not able to demonstrate the existence of a correlation between SCCA-IgM concentrations and the degree of inflammation and ballooning described on the biopsy, a correlation was nevertheless observed with the degree of fibrosis expressed by SAF-F score. This is consistent with the detection of a statistically significant association between fibrosis and the highest concentrations of SCCA-IgM, thus perhaps suggesting that fibrosis may have a greater weight in the induction of this molecule. Turato and coworkers described the presence of high concentrations of a variant of SerpinB3, called SCCA-1 (SCCA-PD), resulting from a single mutation at the level of Gly351Ala in subjects affected by liver cirrhosis and hepatocellular carcinoma. In this case the serpin levels were higher in patients with advanced liver disease than in healthy controls or in patients with chronic liver disease [217]. This finding can therefore lead to the hypothesis that elevated stages of hepatic fibrosis are one of the elements that contribute to the production, disposal, and formation of the immunocomplex. Our NASH patients had mild degrees of inflammation and ballooning (with NAS score equal to or just above the diagnostic limit for NASH) as well as mild degrees of fibrosis, so could be categorized as early NASH [7]. These conditions could explain why

the SCCA-IgM levels do not show the marked increased we expected from the perusal of other antecedent studies observed in the literature.

The correlation detected with homocysteinemia is less immediate and could appear to be purely coincidental. There are not many works in the literature relating to the association between homocysteinemia and NAFLD. Most of the methionine introduced through the diet is metabolized in the liver, so we can say that this organ plays a central role in the synthesis and metabolism of homocysteine. In the literature, it has been reported that patients affected by alcohol-based hepatitis or liver cirrhosis have reduced homocysteine levels compared to the general population [224]. Some authors have highlighted how hyperhomocysteinemia seems to be associated with NAFLD, unlike what is observed in patients with viral hepatitis or in the general population [224,225]. The data relating to the levels of homocysteinemia during NASH are not univocal: cases have been reported in which an increase in levels was found [224] and others in which the levels are lower than in controls with steatosis alone [226]. Homocysteine would favor the development of IR, inducing the synthesis of glycogen and insulin through its metabolite, homocysteine thiolactone. High levels of the latter hormone would regulate the levels of homocysteine itself, thus favoring the establishment of a vicious circle between IR and hyperhomocysteinemia [227–229]. It has also been described how conditions of hyperhomocysteinemia are responsible for alterations in the composition of plasma lipids and favor their tissue accumulation, thus inducing the development of hepatic steatosis [230,231]. Several authors have also highlighted, mainly on mouse models, how homocysteinemia can induce stress at the ER level, thus causing an altered regulation of the sterol pathway [232–235]. Finally, it is now a consolidated fact that hyperhomocysteine plays an active role in endothelial damage by causing a reduction in nitric oxide levels and favoring thrombosis of the microcirculation [236–238]. If these phenomena also affect the hepatic sinusoids, this condition could account for of a further pathogenetic mechanism that would explain its role in the context of NAFLD. Polyzos et al. demonstrated that homocysteinemia levels in NASH patients are lower than those found in NAFL patients, identifying the existence of a correlation between homocysteine levels and degree of

steatosis, inflammation and portal fibrosis, and going so far as suggesting the use of this hormone as an independent predictor of NASH [239]. In fact, it has been hypothesized that a reduction in homocysteine levels may be secondary to an alteration in the equilibrium of homocysteine metabolism, both through the remethylation pathway and the transulfuration pathway. Through this first, and then using the available methyl groups, homocysteine would be consumed for the constitution and release of VLDL from the liver into the circulation. The increasing degree of oxidative stress involved in the most advanced stages of the disease would require a progressive and continuous consumption of glutathione reductase, which is then regenerated through the transulfuration pathway, always starting from homocysteine. Furthermore, a reduced availability of methyl groups would result in a reduced synthesis of phosphatidylcholine, which plays a key role in the assembly and release of VLDL from the liver, thus favoring the further accumulation of lipids at the hepatocyte level and thus helping to support the steatosis itself [239]. The data deriving from our work are consistent and agree with what was found in previous research, in fact in our patients with NASH the homocysteine levels are 12.8 ± 0.6 μmol/L while in those with NAFL of 13.4 ± 0.8 μmol/L. It can therefore be hypothesized that high homocysteine levels favor the development of hepatic steatosis through various mechanisms ranging from the maintenance of IR to the alterations of the lipid profile, from the accumulation of fatty vacuoles in hepatocytes to the induction of thrombosis of the microcirculation, from the reduction of release of nitric oxide from endothelial cells to the production of inflammatory cytokines by monocyte/macrophage cells. Over time, the progression of the disease and the perpetuation of oxidative stress would induce a drop-in homocysteine levels which could participate in the development of steatohepatitis, through a reduction in the levels of antioxidants and favoring the accumulation of hepatocyte lipids. Perhaps precisely because of this reduction in concentrations observed in patients with NASH, the studies that attempted to verify the effectiveness of a treatment by administering B vitamins and folic acid did not yield positive results [240,241]. In light of these findings, the correlation between SCCA-IgM and homocysteine levels, which emerged in our population, may be consistent with an

etiopathological hypothesis and with the data collected in the literature. Both of these phenomena are associated or are the result of a marked oxidative stress affecting the hepatocytes. Further studies and investigations must be carried out to verify the validity of this hypothesis and these findings. However, it should be emphasized that, with regard to the population under examination, we have no information regarding the genetic characteristics of the state of methylene-tetraidofolate-reductase (MTHRF) or any vitamin B6 deficiency. While, all patients had concentrations of vitamin B12 or folic acid within the normal limits.

From the comparison between the two groups of patients, it emerged that subjects with a histological diagnosis of NASH had a statistically lower age than patients with hepatic steatosis, as well as a higher weight and BMI.

The average age of the two groups is comparable to that reported in the literature in which the greater prevalence of NAFLD is observed. The fact that the NASH patients in our study were on average younger than those with NAFL can be explained by the fact that of the 45 patients enrolled with a histological diagnosis of hepatic steatosis, 10 (23%) were candidates for bariatric surgery. This same element can also account for the data collected regarding the fact that patients with NASH had on average a higher weight and a higher BMI. Weight and BMI are known risk factors for the development of metabolic syndrome, fatty liver disease, its evolution into steatohepatitis and the progression of fibrosing damage, as already extensively discussed above. BMI consistent with literature data can be considered a predictor of the severity of NAFLD [242].

The glycemic profile and glycated hemoglobin are comparable in the two groups, not detecting statistically significant differences. The liver itself is an organ involved in the development of diabetes mellitus: it is in fact closely linked with IR, in turn suffering complications but also feeding and lying to this condition. Patients with type II diabetes mellitus have an approximately 40% risk of developing NASH, and have greater degrees of hepatocyte damage than those who do not have this disease [243–245]. The same NAFLD fibrosis score, devised by Bazik and co-workers, on the basis of these observations, is aimed at identifying patients at greater risk of presenting NASH and

advanced fibrosis in diabetic patients with radiological evidence of hepatic steatosis [246]. In the population examined in this study, glycated hemoglobin levels were significantly higher in patients with steatohepatitis than in those who were not affected. Barros et al. also found elevated levels of glycated hemoglobin in their severely obese and NAFLD patients, but did not check whether there were any differences between patients with steatosis and steatohepatitis [247]. In our "population" in both the two groups average values were found at the limits of the diagnosis of impaired fasting glycaemia. Glycated hemoglobin was on average within normal limits, an expression of adequate glycometabolic compensation in both groups of patients, even in those with histological pictures compatible with greater hepatocyte damage and fibrosis. There was no difference in the prevalence of type II diabetes mellitus in the two groups under review. In part this can be explained by the limited sample size, in part by an adequate management of diabetes mellitus expressed in terms of good glycometabolic control through adequate pharmacological therapy or guaranteed by a marked inulinemia. A discrepancy can be observed between the insulin-concentration and HOMA-index means in the two groups of patients can be observed, albeit one that does not reach statistical significance. In both groups there is a marked increase in insulin levels, more marked in patients with NASH, in which there is also a more marked dispersion of the values. This last element in particular could account for the failure to achieve significance in statistical terms. The finding of hyperisulinemia, in addition to being consistent with the literature and with the etiopathogenetic mechanisms underlying NAFLD, is a direct consequence of the degree of obesity, expression of excess weight, and high BMI. Subjects in both groups have a diagnostic HOMA-I by IR, with values in both cases higher than the diagnostic cut-off (equal to 2.5), although it is higher in patients with steatohepatitis. These data confirm the high level of IR that characterizes these patients and is consistent with the data collected in the literature. The role of IR in the etiopathogenesis of NAFLD has also been extensively investigated. Park et al. in the biopsies of a group of pediatric patients, recently found that those who were characterized by a more pronounced IR presented more severe

pictures of lobular inflammation and fibrosis, suggesting its possible use to identify NASH patients in lieu of a liver biopsy [248].

The dosage of total cholesterol and LDL cholesterol was significantly higher in patients with NASH than in those with steatosis alone. This is also consistent with the current literature, in fact obese patients with NAFLD have higher concentrations of LDL cholesterol, compared to those who are not suffering from fatty liver disease [247]. Siddiqui and colleagues demonstrated in their case control study that NAFLD patients had higher levels of free fatty acids and LDL cholesterol than patients who did not have fatty liver disease, regardless of whether or not obesity was present [249]. NAFLD is characterized by having alterations in the lipid profile with a prevalence of pro-atherogenic lipoproteins, an increase in LDL, triglycerides and a reduction in HDL cholesterol levels [250,251]. However, it has been suggested that this different lipid profile is a direct consequence of the liver lipid content and IR, and that it does not appear to be linked or worsened by the degree of obesity or the severity of the disease in terms of progression to steatohepatitis [252]. In our study, triglyceride levels were higher in patients with NASH, although they did not reach statistical significance.

In the subjects evaluated in our study, on average, both in patients with fatty liver and in those with a history of steatohepatitis, the levels of the indices of cytonecrosis and colostasis were within normal limits. Even if we observed that patient with NASH were characterize by statistical significant lower levels of ALT compared to subjects with simple steatosis. These results are consistent with the finding that in 50-80% of NAFLD patients no alteration of hepatic cytolysis indices are observed [253]. In the literature, it has been reported that patients with NAFLD tend to have ALT levels higher than those of AST, this finding is more marked especially when compared with the values found in patients with alcoholic fatty liver disease. A retrospective analysis performed on a patient with NAFLD found mean ALT values of 83 IU/mL, and AST of 63 IU/mL [254]. Some authors have also postulated how a reduction in the levels of alanine amino-transferases can predict a progressive evolution of the pathology in fibrotic terms. This could also by coherent with our data. Assessment of serum ALT levels is frequently used to screen for unrecognized liver

disease. In the context of NASH, however, the true value of this measurement is discussed. Mofrad et al. described patients with ALT included within the normal range and who could present any of the histological manifestations that make up the broad spectrum of NAFLD. An increase in the indices of cytonecrosis has not been shown to be associated with a more severe histological picture, in the same way that normal transaminases or reduced ALT levels cannot exclude the concomitant presence of hepatic fibrosis [121]. Other studies underline how the presence of ALT at the upper limits of the norm must in any case be considered as an element of suspicion of an underlying liver disease, such as to make the patient who presents them deserving of further diagnostic investigations and close clinical and instrumental monitoring [255,256]. Kim and co-workers therefore proposed, on the basis of the data extrapolated in their work, cut-offs capable of predicting the risk of liver disease: ALT values equal to 31 IU/L and 30 IU/L respectively for male and female [256]. In consideration of the wide variability of the data, measurement methods, available laboratory assays, as well as in the light of the absence of a precise and specific definition of high ALT, Neuschwander-Tetri et al. note that to date it is not possible to use the value of alanine amino-transferase as a sufficiently sensitive and specific index to identify those with steatohepatitis among patients with NAFLD [257]. In our study, patients with NASH had significantly lower AST/ALT ratios compared with individuals with NAFL. Many studies in the literature have suggested that an AST/ALT ratio above the value of 1 may be suggestive of NAFLD in the absence of significant fibrosis. Cichoż-Lach et al. found that patients with progressively higher degrees of fibrosis found on histological evaluation presented a progressive increase in the AST/ALT ratio. On the basis of these findings, the BAARD score has been elaborated. It incorporates, in addition to the relationship between hepatic cytolysis indices, BMI and the presence of diabetes mellitus [258]. The same NAFLD fibrosis score also uses this first parameter, together with other biohumoral and clinical indices, to estimate the degree of hepatocyte fibrosis. Both of these scores have been shown to have a high negative predictive value, capable of identifying patients with a low degree of fibrosis and directing the others to the biopsy analysis. Our findings also differ from what theoretically expected in the

light of the available literature data. However, several authors highlight how the dosage of transaminases is characterized by a wide variability, even within the normal limits, which does not correlate with histological severity, and that there are no threshold limits defined in the different populations [259]. Finally, it should always be remembered that these enzymes can also be disposed of by other tissues and that, in our case, even the low sample size may have influenced the results.

In our patients, the cholestasis indices were on average within the normal range, thus not showing statistically significant differences between the two groups of subjects evaluated. Cholestasis indices were also elevated in patients with NAFLD, so much so that some authors suggest that such finding in patients with risk factors for the development of NAFLD should be carefully evaluated to verify the presence of this pathology [260].

The hepatic synthesis indices (INR, albumin, Cholinesterase) as well as the bilirubin levels were within the normal limits, and this element is easily explained in the light that none of the patients evaluated has an advanced degree of liver disease.

The finding of an increase in ferritin levels, with transferrin saturation values in the normal range, is frequently observed in patients with hepatic steatosis. Kodely et al. described histological pictures of greater severity in patients with NAFLD and an increase in ferritin up to 1.5 times the normal limits (e.g. over 450 ng/mL in men and 300 ng/mL in women). to present with greater degrees of steatosis, ballooning, fibrosis and therefore characterized by the presence of NASH. The multivariate regression analysis revealed that this ferritin level is independently associated with the diagnosis of steatohepatitis but also with hepatic hemosiderosis, with a greater degree of inflammation and hepatocyte damage, as well as with a more advanced degree of fibrosis [261]. In light of these data, its use as a non-invasive test for the detection of hepatocyte damage has been proposed. However, in our cohort we did not observe significant differences in ferritin levels between the two groups, perhaps due to the lower degree of inflammation and hepatocyte damage.

Even in terms of C-reactive protein (PCR) we did not observe statistical significant differences between patient with NASH and NAFL. This is an opsonin belonging to the pentasserin family, an

textbook acute phase protein. It is produced by the liver during inflammatory states, following various stimuli induced by adipose tissue. Higher plasma CRP levels have been reported in subjects with type II DM and metabolic syndrome, and higher CRP concentrations have been described in cases of hepatic steatosis and steatohepatitis. It still remains a matter of discussion whether this index can be used as a marker capable of distinguishing between these last two conditions, also in consideration of the few and controversial data in the literature on the subject, which do not seem to demonstrate the existence of a correlation between PCR levels and the histological presence of NASH [262,263].

The haemostatic factors associated with an increased cardiovascular risk are represented by the inhibitor of the plasminogen activator (PAI-1), the tissue plasminogen activator (t-PA), the von Willebrand factor and fibrinogen. Wild et al. have shown that patients with metabolic syndrome have significantly higher values of t-PA, CRP, e-selectin, uricemia and IL-6 than patients without this syndrome [264]. On a group of healthy non-smoking volunteers, Tarher et al. found that the plasma levels of PCR, fibrinogen, von Willebrand factor, and PAI-1 were higher in patients with fatty liver than in those who they did not have this condition, regardless of gender, age, BMI, blood pressure, IR, and triglyceridemia [265]. Concentrations of IL-6 and CRP appear to correlate with elevated levels of fibrosis and inflammation in patients with hepatic steatosis, as observed in steatohepatitis [266]. NAFL and NASH as conditions characterized by chronic inflammation and the presence of pro-inflammatory cytokines represents an additional stimulus for the development and progression of atherosclerotic disease. Our data revealed the absence of statistically significant differences in the levels of PAI-1 and t-PA in the two groups of patients. This finding differs from the data available in the literature, perhaps due to the limited sample size. From the work of Verrijken and co-workers, it emerges that the levels of PAI-1 are associated with the severity of the histological picture of the NAFLD patients. In the multivariate analysis it emerged that the degree of steatosis, in particular, appears to be an independent predictor of the levels of PAI-1. However, only 12% of the variability could be explained on the basis of the histological findings, and this is

probably due to the ubiquitous production of expression of this serine protease inhibitor. The hypothesis that the increase in PAI-1 levels may derive, at least in part, from hepatocytes, would seem to be supported by the detection of higher concentrations of this protein in patients with stetohepatitis than in those who were not affected by this condition [267]. Moreover, it should be taken into consideration first of all that the conditions associated with NAFLD and the metabolic syndrome, such as IR, obesity, diabetes mellitus, and sedentary lifestyle favor the induction of PAI-1 and, secondly, that PAI-1 itself can play a pro-fibrotic role in the liver parenchyma. Further studies are needed in order to deepen the clinical picture.

Patients with NASH demonstrated higher levels of TSH than patients with steatosis alone. This finding is consistent if evaluated in the context of the characteristics of these subgroups of patients, characterized by a higher body weight, a higher BMI and a more marked IR. The data relating to the association between thyroid hormones and NAFLD are still controversial. The former is involved in the regulation of body weight, in lipid and energy metabolism, in adipogenesis and in IR. In clinical practice, subclinical hypothyroidism has been associated with metabolic syndrome, cardiovascular mortality, and alterations in lipid metabolism [268]. A growing literature is also highlighting how there is a high prevalence of subclinical hypothyroidism, between 15% and 36%, in patients with NAFLD. Several studies have shown that hypothyroidism appears to be an independent risk factor for the development of NAFLD, suggesting that hypothyroidism can directly determine the onset of NAFLD regardless of the presence of other metabolic risk factors. Considering the results of these studies, hypothyroidism can be added to the risk factors of which can induce the development of NAFLD [269]. The mechanisms by which hypothyroidism would be able to favor the development of NAFLD would be mediated by the induction of reactive oxygen species, IR (through an increase in FGF-21 and leptin), and alteration of lipid metabolism (increase in triglycerides and cholesterol). It is still debated whether there is an association between hypothyroidism and the degree of severity of hepatocyte damage, i.e. the presence of steatohepatitis. Chung et al. evaluated a large number of healthy patients and found that an increased prevalence of NAFLD and increased serum ALT levels

could be observed in patients with hypothyroidism [270]. An increase in alanine aminotransferase concentrations, in this study, appears to be a surrogate biomarker of the presence of NAFLD in the absence of other causes of liver disease, and can also be an indicator of risk for the development of diabetes, cardiovascular disease, and long-term complications of metabolic syndrome. The work in question is however limited by the lack of histological data, an element evaluated in the study carried out by Pagada et al. The latter found a higher prevalence of hypothyroidism in patients with NASH than in those with steatosis alone. This figure was statistically significant even after the correction for variables such as age, diabetes, dyslipidemia, and hypertension [271]. Contrary to these results, other studies, including those by Mazo et al. have not shown the existence of a statistically significant association between hypothyroidism, NAFL, and NASH [272–274]. Furthermore, the hypothesis has been raised that the alterations in the levels of thyroid hormones can be attributed to the so-called euthyroid sick syndrome, or to the presence of concomitant systemic pathologies in a subject with normal thyroid functioning [269]. It seems reasonable, in consideration of the existing association between impaired thyroid function, dyslipidemia, IR, and NAFLD, to evaluate the thyroid profile in patients with hepatic steatosis and possibly correct any dystyroidism, as this is a modifiable risk factor. Further studies will be performed to evaluate the existence of a possible association between NASH and hypothyroidism.

IGF-1 or somatomedin is produced mainly in the hepatocyte as a result of the stimulus induced by growth hormone or GH, but other tissues are also able to produce it given the paracrine and autocrine activities of this hormone. It is carried into the circulation by various IGF-1 binding proteins, which modulate its activity and release at the tissue level, as well as guaranteeing an increase in its half-life. Its activity is mediated by the interaction with its specific receptor (IGF-1R), which in consideration of its autocrine, paracrine, and endocrine effects appears to be ubiquitously expressed in the various tissues of the body. Its effects on fetal growth and development, normal bone growth, ovarian follicogenesis, testicular integrity and function, cardiovascular development, its cardio-protective effects, and its effects on neuronal development

and muscle growth have been long since defined and proven [275]. In patients with visceral obesity, the existence of an altered functioning of the GH/IGF-1 axis has been demonstrated, although the mechanisms underlying this phenomenon have not been fully elucidated. It has been postulated that at the base there may be an imbalance of the hypothalamic axis with consequently reduced secretion of GHRH and/or with an excessive tone of somatostatin. Furthermore, the elevated contractions of circulating FFAs can inhibit GH secretion in the pituitary. Even leptin seems to be involved in the regulation of GH secretion, albeit in a not fully understood way, but probably acting at the level of GHRH and somatostatin [276]. The circulating levels of IGF-1 are mainly induced by the secretion of GH and through a negative feedback mechanism regulate its production at the hypothalamic level. Obese patients tend to have normal or increased levels of total IGF-1 and elevated levels of free IGF-1. This finding suggests that other factors besides GH alone can influence circulating levels of IGF-1. In particular, excessive caloric intake and a state of hyperinsulinemia could contribute to this condition. Furthermore, IR, by inducing a decrease in the production of IGF-1 binding proteins (IGF-1BP), contributes to an increase in the levels of free circulating IGF-1, which could therefore inhibit the release of GH at the hypothalamic level and favor the development of hyposomatostatinemia. In mouse models, it has been shown that IGF-1 deficiency is related to IR, alterations in lipid metabolism, damage related to oxidative stress, and alteration of the neuro-hormonal axis [275]. Some authors have found that there is an inverse relationship between the circulating levels of IGF-1 and the incidence of the metabolic syndrome, with hepatic steatosis, IR, dyslipidemia, and visceral obesity and cardiovascular risk [276–280]. All these results suggest a possible role of IGF-1 not only in the development of the metabolic syndrome, but also in the etiopathognesis of NAFLD itself. This could be attributed to the permissive role towards the induction of IR, oxidative stress, and altered lipid metabolism. Being a condition found in both NAFLD and metabolic syndrome, it can also be considered that this deficiency represents a common passage in the pathways that determine the development of these pathologies. In vivo studies have shown how a deficiency in IGF-1 levels is associated with alterations in the cellular

architecture of the hepatic parenchyma, suggesting how this deficiency may be implicated from the earliest stages of hepatocyte damage. A partial deficiency of this hormone could also lead to an altered expression at the level of the hepatocytes of the genes encoding the IGF-1 receptor (IGF-1R) and the proteins involved in the acute phase and in inflammation, thus favoring the hepatic oxidative damage [275]. Chishima et al. have shown that elevated GH levels and reduced concentrations of circulating IGF-1 can contribute to the progression of metabolic-based liver disease [281]. In their study, they observed that patients with cirrhosis had low levels of IGF-1, and higher levels of GH, compared to those who had lower degrees of fibrosis. Moreover, patients with higher degrees of steatosis had reduced GH levels and increased IGF-1 levels. Zhuravlyova et al. identified 143.9 ± 4.92 ng/ml as a cut-off value below which there would be an increase in the risk of liver disease evolution, suggesting that a reduction in serum IGF-1 concentrations is associated with an increase in liver cytolysis , triglycerides, and resistin values [282]. The patients involved in our study presented on average reduced IGF-1 values, unlike what might be expected in the light of the above considerations. Subjects with NASH dosed higher levels of IGF-1 than patients with steatosis alone. This finding could be attributable to the small sample considered, which is characterized by the absence of high degrees of fibrosis on histological investigation (condition in which the levels of IGF-1 should be markedly lower), or on the basis of the characteristics of the subjects included in this subpopulation: patients on average younger (it is known that IGF-1 levels decrease with increasing age), suffering from higher degrees of obesity (in obesity conditions increased or normal levels are observed of IGF-1 and a greater quantity of free circulating IGF-1 [276]) and a state of more marked hyperinsulinemia. The finding of higher mean values in patients with NASH compared to those detected in subjects with steatosis, may also lead us to reflect on the greater risk incurred by this population of developing a hetoroplastic pathology. In fact, in patients with hepatocarcinoma (HCC), the existence of the alteration of the GH/IGF-1 axis and the expression of IGF-1R has been demonstrated. SerpnB3 itself would induce an increase in the expression of IGF-1R, through the inhibition of miR-122 [283], thus promoting oncogenesis. Alterations typically found in HCC and

hepatoma cell lines include increased expression of IGF-2 and IGF-1 (IGF-IR), crucial elements involved in malignant transformation and tumor growth. Alterations in the production and degradation of IGF-1 binding proteins and the proteolytic degradation of IGF-BP results in an excess of IGF, as well as impaired function of the IGF-2 / mannose 6-phosphate receptor (IGF-2 / M6PR), and can further enhance the mitogenic activity of IGFs in favoring the development of HCC [284–287]. This mechanism could help to favor the development of primary neoplastic pathology on non-cirrhotic liver in this subset of patients. The growing interest in this path has led to the development of pharmacological research lines for the treatment of HCC based on the inhibition of IGF-1R itself [288].

The limitations of our research are represented above all by the low number of the analyzed sample, which was certainly influenced by the strict inclusion criteria.

Furthermore, patients enrolled with a diagnosis of steatohepatitis are characterized by presenting low degrees of firbosis or inflammation on histological examination in images compatible with early stages of NASH. Therefore, in the analyzed cohort the broad spectrum of histological pictures that characterize a dynamic pathology such as NAFLD (in which the factors contributing to the progression of the disease have not yet been fully understood and described) was not fully represented. The identification of one or more control groups could be useful to better understand, contextualize, and compare the data obtained.

The use of hepatic needle biopsy as a tool to define the degree and stage of the disease, although considered the gold standard by the guidelines for the diagnosis of NASH or NAFL, is limited by the fact that this investigation is subject to sampling errors, the biopsy frustule is not representative of the entire hepatic parenchyma since the pathologies are heterogeneously distributed throughout, and the histological diagnosis is strongly operator-dependent. The elements collected so far allow us to describe the baseline characteristics of the population evaluated. To define whether the SCCA-IgM is able to predict the risk of any progression of liver disease, it will be necessary to continue monitoring the enrolled patients in the long term.

Furthermore, it should be emphasized that there are no studies in the literature that specifically evaluate the levels of this immune complex in the histologically characterized population of subjects affected by NAFLD. Therefore, there are no ad hoc cut-offs validated for this context.

In consideration of the fact that NAFLD is a very widespread and often misunderstood, the search for soluble markers that can be used in order to make a diagnosis or to stratify the patient's risk of presenting a more aggressive and/or advanced disease, deserving of greater diagnostic investigations or a more stringent clinical or instrumental monitoring, is the object of fervent interest.

From this perspective, it could therefore be interesting to evaluate in further and prospective studies whether SCCA-IgM, alone or in association with other biohumoral and/or clinical indices, for example in the context of a score, could play a role in identifying the most at risk patients.

- PRS-HFC and PRS-5

Consistently epidemiologic data has shown, in the last years, a progressive increase in the incidence and prevalence of NAFLD/MAFLD due to radical changes in lifestyle, eating habits, and the discovery and dissemination of therapy for the treatment of viral hepatitis. This has led to NAFLD becoming a major cause of chronic liver disease. It should be noted that it is a subtle pathology that is often diagnosed accidentally, given the absence of specific signs or symptoms, except in advanced stages in which the typical stigmata of cirrogenic evolution or the presence of HCC are appreciable. The gold standard for diagnosis, which at the same time also provides prognostic information, giving indications relating to the degree of fibrosis, remains the liver biopsy, an investigation that is not free from risks and limitations, certainly not practicable in an extensive manner to all patients. Certainly, the greatest unmet need is therefore linked to the lack, in this context, of non-invasive markers with diagnostic and prognostic value.

Due to its wide diffusion, the lack of non-invasive and widespread diagnostic tools, the inadequacy of oncological surveillance strategies, even those economically viable, and the absence of ad hoc therapies, often only a small percentage of patients with already overt liver disease are adequately monitored in order to intercept the possible onset of HCC. It follows that a large percentage of subjects at risk escape surveillance programs with the risk of a late diagnosis and in already advanced stages of cancer.

In recent years, increasingly widespread and extensive studies deriving from candidate genes, genome-wide association studies and exome-level association studies have made it possible to identify SNPs associated with genetic susceptibility and risk of evolution of NAFLD. In particular, on the basis of the different designs of the studies developed, the populations and ethnic groups considered and the methodology applied, it is estimated that genetic susceptibility plays a role ranging from 20 to 70% [289–291].

Having identified the individual genetic variants and genes involved in predisposition to disease onset, many authors have sought to investigate their combined effect on NAFLD susceptibility. Approximately from 2009, a lot of research groups have developed studies to examine how genetic variants either on their own or combined with each other or other clinical variables can predict the presence of liver steatosis, steatohepatitis, and cirrhosis [68,291,292].

More recently, the genetic impact on NAFLD-HCC development has been intensely researched. In 2017, Donati et al. developed a genetic and clinical score that combine rs738409 *PNPLA3* variant, rs58542926 *TM6SF2* variant and rs641738 *MBOAT7*, age, sex, presence of obesity, type 2 diabetes, and severe fibrosis in a group of Italian NAFLD-HCC patients. The score as created identifies patients with HCC with an AUROC of 0.96 ± 0.04 (96% sensitivity, 89% specificity) [293]. Pelusi and coworkers aimed to stratify and discriminate NAFLD-HCC risk by investigating a wide inherited pathogenic variant in candidate genes and elaborating a genetic risk score, in a cohort of metabolic related HCC, NAFLD with advanced fibroses, and a control group of European, non-Finnish forefathers. The best threshold resulted 0.22 with an AUROC of 0.74 (61% sensitivity, 76%

specificity). The genetic risk score result has shown to be better at discriminating the risk of developing liver tumor than the single risk variants, even the most common ones (i.e. PNPLA3) or the combination of some of them (i.e. *PNPLA3* I148M and *TM6SF2* E167K). Furthermore, the score proved to be associated with the risk of HCC regardless of the classical risk factors. Finally, the combination of this score and clinical data allows to optimize the diagnostic yield [294]. Recently, Gellert-Kristensen et al. published a study in which they examined whether a genetic risk score (GRS) based on a 0 to 6 evaluation of risk-increasing alleles for the three principal genetic variants involved in fatty liver disease determination (PNPLA3 I148M, TM6SF2 E167K, HSD17B13 rs72613567) is able to predict the risk of cirrhosis and hepatocellular carcinoma (HCC) development. The data were collect relying on two studies of the Danish General population and from the UK Biobank. Data about the diagnosis of cirrhosis or HCC were based on International Classification of Diseases (ICD) inferred by the patients and causes of death registers. The genetic risk score results showed an up to 12-fold higher risk of cirrhosis and an up to 29-fold higher risk of HCC. Furthermore, a high GRS causes a 19-fold increased risk of progression from cirrhosis to HCC [295].

In this landscape and bearing in mind that hepatic fat content is at least 50% genetically determined [57,68,296–298], we took part in a multicenter cross-sectional cohort study, with the main purpose of investigating whether a weighted PRS, based on principal risk alleles affecting genetic predisposition to liver fat accumulation and consequent NAFLD development, can predict the risk of HCC development in at-risk subject and in the general population.

We observed that the PRSs values vary according to the severity of liver disease, which was particularly well represented for PSR-HCF, reaching statistically significant higher levels in patients with HCC. On the other hand, PRS-5 results are higher in patient with severe fibrosis compared with those affected from liver tumor. This difference may in part be attributed to the effect of *HSD17B13*, since it has been shown to have a protective effect against the risk of liver disease

progression [299,300], specifically by protecting against the development and recurrence of HCC, not only direct but also through mitigation of the effect of other genetic variants [299–302] .

Thought Mendelian Randomization we demonstrated that genetic risk variants confer a risk of HCC development that is proportional to the increase of NAFLD risk. Furthermore, a direct connection was noticed between the risk of NAFLD and severe fibrosis, and between the latter and HCC. We should note that the only exception to the direct relationship between the risk of NAFLD and that of HCC was related to the *GCKR* variant (i.e. the genetic variant with the major known pleiotropic effect) which decreases type II diabetes risk, and may thus result in a decreased causal link between liver fat and HCC [56] .

Our results are consistent with the presence of a causal association between hepatic fat content and NAFLD-HCC, independent of major clinical risk factors (i.e. sex, age, presence of type II diabetes, or obesity), partly due to severe fibroses, even when accounting for pleiotropic effects of the genetic instrument. We have estimated that fibrosis has a weight of 65% on the onset of HCC, the remaining percentage could be attributable to the HFC share, that's genetically determinate. However, it is not known, and unfortunately our analysis is not able to discriminate, how much the risk of HCC is affected by qualitative or quantitative alterations of the hepatic fat content.

However, these data are consistent with the fact that, as mentioned above, hepatic fat content is partially genetically determined and has been shown to influence the development of HCC. This consideration can account for cases in which primary liver cancer develops in individuals without cirrhosis or high degree of fibrosis [180–182,185,303–306].

Consistent with previous studies on genetic risk scores, we demonstrated that the PRSs can more accurately foretell the risk of developing HCC than single variants. The grade of accuracy is moderate, even with an AUROC equal to 0.65 in NAFLD cohort.

This could represent the exceeding of the current guidelines [7] that considers the evaluation of a single genetic variant and, given its stability over a person's lifespan independently of

environmental triggers, could be applied to a selected portion of patients and thus could improve risk stratification (in term of cirrhosis and HCC development) and help tailor the follow-up.

We thereafter identified the thresholds for each PRSs (i.e. ≥0.532 and 0.495 for PRS-HFC and PRS-5, respectively) with the best predictive value with the aim to pick out NAFLD-HCC in the NAFLD cohort. We observed that subjects with a PRS over this cut-off present a > 3-fold higher adjusted OR of HCC.

Interestingly, PRS-HCF has been shown to be able to identify patients at risk of developing HCC even considering subjects over the age of 40 and diabetics, regardless of the degree of hepatic fibrosis, and PRS-5 demonstrated good performance in non-obesity individuals.

We could therefore hope to use these scores, after their validation in larger populations, alone or in combination with other clinical data or biomarkers, to intercept patients with metabolic liver diseases at risk of evolution/progression in selected subgroups, including those with no evidence of cirrhosis or severe fibroses.

The deviation and lack of significance obtained from the data analysis applied to the subgroup of patients enrolled in our Center, compared to the complete cohort, is attributable to the reduced sample size.

Finally, we did not observe a significant relationship between SCCA-IgM and PRSs. This can be explained by considering the pathophysiological basis of the two mechanisms studied: the gene variants are mainly associated with the hepatic fat content, while SCCA-IgM induced by oxidative stress and chronic inflammatory processes has been shown to be associated with hetrosplastic evolution and development of fibrosis. Another limitation could be represented by the low number of patients in whom we evaluated both non-invasive biomarkers.

Similarly, the statistically significant association observed between SCCA-IgM and the P446L polymorphism of the *GCKR* gene is likely to be random. In literature and from a pathophysiological point of view at the moment there are no data correlating the constitutive activation of the glucose up-take at the hepatocyte level, with consequent increase in the production of malonyl-COA and

induction of lipogenesis (elements observed in carriers of the variant of the rs1260326 mutation of the *GCKR* gene) [72] with the induction of SCCA-IgM, secondary to chronic inflammation and oxidative stress [129,138,165,307].

Our study is at the moment unique in literature. Compared to previous similar studies [205,293–295], we used a robust instrument estimating inherent predisposition of HFC to develop a polygenic risk score, based on the principal genetic variants weighted by their effect size on HFC. A consistent inference emerged on the causal relationship between the role played by the hepatic fat content and the development of HCC, only partially mediated by the presence of fibrosis, a major independent risk factor for the development of primary liver cancer. Moreover, we evaluated the score on a well characterized population of at risk-subjects and in the general one, even stratifying the analysis according to fibroses severity, finding a threshold able to predict the risk of HCC devilment even in subjects without significant fibrosis, obesity, or relatively young age.

However, the study is not without limitations: first of all, the cross-sectional design. In fact, this type of study provides a snapshot of the analyzed cohort, in which exposure and outcome are assessed simultaneously at the time of enrollment. It should also be considered that there are no data relating to the average duration of the disease in the various groups of patients analyzed.

A further limitation, indeed, is represented by the fact that the subjects enrolled in the control group and those with NAFLD and low degree of firbosis are characterized by a significantly lower average age than patients with more advanced degrees of firbosis and HCC (as we have seen according to literature data, the age and duration of the disease influence the risk of evolution of NAFLD).

Furthermore, the results apply only at subjects with a European (non-Finnish) origins, the only ancestry studied. It will be interesting to extend the study to other ethical groups or to specific genetic isolates present in Italy or Europe.

Finally, information on eating habits or lifestyle was not collected in an extensive and uniform manner. More generally, there are no data on environmental risk factors that may interact with the

patient's genetic substrate variants and contribute to to, trigger, or favor the development and progression of NAFLD.

Prospective studies will therefore be necessary to assess the magnitude of the increase in HCC risk conferred by PRS more accurately. In the future, the integration in the PRS of other SNP (e.g. *HFE* and *SERPINA1* mutations observed for Europeans[308], or ADIPOQ, COL13A1, and SAMM50 genes detected in Mexican populations[309]) involved in the risk of developing HCC or with protective action could improve the diagnostic accuracy of the test and possibly extend it to other populations/ethnic groups.

CONCLUSION

NAFLD has become the main chronic liver disease in the world and includes a broad histological spectrum that extends from simple steatosis to steatohepatitis, up to cirrhosis and HCC.

In the population, the percentage of patients with steatohepatitis is much lower than that with steatosis alone; the prevalence of both conditions is difficult to define, and the data in our possession probably underestimate its actual scope, if we consider how widespread the metabolic syndrome and the pathologies that constitute it are in the population.

A growing literature shows how, in patients with NAFLD, cases of hepatocarcinoma are not infrequently observed in the absence of overt liver cirrhosis. Such heteroplasias are often more aggressive, diagnosed late and therefore burdened with high mortality rates and low therapeutic responses.

Liver biopsy is the gold standard for making a diagnosis, and it is also the only tool that currently allows to distinguish between NASH and NAFL.

Given the vast prevalence of the disease and the disadvantages associated with carrying out this diagnostic procedure, great interest has been placed on the search for non-invasive markers for the diagnosis of these two conditions.

Over the years, various soluble markers, radiological tools and scores have been proposed resulting from the association of clinical and biohumoral data that could at least serve as a tool for selecting patients to be subjected to level II or more invasive investigations and assessments.

The objective of our study is to evaluate non-invasive biomarkers to stratify the risk of disease evolution of NAFLD subject. First of all, in the light of the data described by Martini et al. we studied the role of SCCA-IgM, in particular we try to verify whether the serum levels of this immunocomplex were associated with the progression of histological damage, in particular the presence of NASH. We observed that patient with histological diagnosis of NASH present significantly higher concentrations of the immunocomplex. The levels of SCCA-IgM, on linear

correlation analysis, are associated with the BMI, the age of the patients and the levels of homocysteinemia.

The continuation of the study, with the clinical and laboratory follow-up of the selected patients will allow us to evaluate whether the SCCA-IgM immunocomplex may be a marker of evolutionary metabolic liver disease towards liver cirrhosis and primary liver cancer. Moreover, higher levels of SCCA-IgM were detecting in whom present higher degree of fibrosis at the histology exam.

According to the linear correlation analysis, SCCA-IgM levels correlated with BMI, age, homocystein levels, and with the histological grading of fibrosis. We have to point out that there are no studies in the available literature evaluating the role of this immuno-complex in a cohort of NAFLD histologically characterized. No ad-hoc cut-offs are available for this population.

The clinical and laboratory follow-up of our patients will allow us to further evaluate whether SCCA-IgM (alone or included in a novel clinical and biochemical score), might represent a marker of progression to cirrhosis and primary liver tumour. Recruitment of new cases across different stages of NAFLD spectrum will be crucial to further confirm the present data.

The second non-invasive biomarker that we chose to examine are the principal genetic variables involved in lipid metabolism pathways and in hepatic stellate cells function, combined in a had hoc score defined by adding the predisposing alleles weighted by their effects size.

The results of the study are consistent with a causal role of hepatic fat accumulation in liver carcinogenesis. PRSs may be useful to non-invasively predict the risk of HCC in subjects with NAFLD, independently of the presence of severe liver fibrosis, even in non-obese and young individuals. We demonstrated that a positive PRS allow clinicians to identify a subset of individuals with dysmetabolism at high genetic risk of HCC development. Therefore, PRSs may be useful to stratify NAFLD-HCC risk and guide and improve cancer surveillance strategies

Large studies integrating genetic and other biomarkers are necessary to further improve the risk stratification and promote the clinical and wide application of these results.

FIGURES AND TABLES

Figure 1

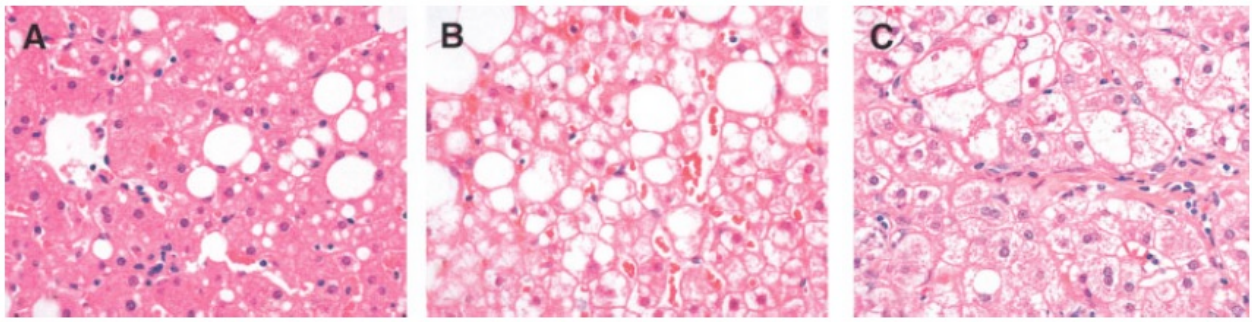


Figure 1. Three different histological pictures characterized by a progressive increase degree in ballooning (from A to C) [94].

Figure 2

Table 1. NASH Clinical Research Network Scoring System Definitions and Scores in Study Set

Item	Definition	Score/Code	% Responses in Category for Study Set Cases	
			Adult (n = 576)	Pediatric (n = 162)
Steatosis				
Grade	Low- to medium-power evaluation of parenchymal involvement by steatosis			
	<5%	0	10%	3%
	5%-33%	1	34%	29%
	>33%-66%	2	31%	31%
	>66%	3	26%	36%
Location	Predominant distribution pattern			
	Zone 3	0	31%	14%
	Zone 1	1	1%	12%
	Azonal	2	37%	22%
	Panacinar	3	31%	52%
Microvesicular steatosis*	Contiguous patches			
	Not present	0	90%	96%
	Present	1	10%	4%
Fibrosis				
Stage				
	None	0	40%	29%
	Perisinusoidal or periportal	1		
	Mild, zone 3, perisinusoidal	1A	15%	4%
	Moderate, zone 3, perisinusoidal	1B	6%	5%
	Portal/periportal	1C	6%	27%
	Perisinusoidal and portal/periportal	2	12%	10%
	Bridging fibrosis	3	15%	17%
	Cirrhosis	4	6%	9%
Inflammation				
Lobular inflammation	Overall assessment of all inflammatory foci			
	No foci	0	14%	15%
	<2 foci per 200× field	1	53%	60%
	2-4 foci per 200× field	2	27%	22%
	>4 foci per 200× field	3	6%	3%
Microgranulomas	Small aggregates of macrophages			
	Absent	0	57%	53%
	Present	1	43%	47%
Large lipogranulomas	Usually in portal areas or adjacent to central veins			
	Absent	0	86%	99%
	Present	1	14%	1%
Portal inflammation	Assessed from low magnification			
	None to minimal	0	67%	67%
	Greater than minimal	1	33%	33%
Liver cell injury				
Ballooning*				
	None	0	33%	49%
	Few balloon cells	1	38%	36%
	Many cells/prominent ballooning	2	29%	15%
Acidophil bodies				
	None to rare†	0	89%	90%
	Many	1	11%	10%
Pigmented macrophages				
	None to rare†	0	87%	82%
	Many	1	13%	18%
Megamitochondria*				
	None to rare†	0	86%	95%
	Many	1	14%	5%
Other findings				
Mallory's hyaline	Visible on routine stains			
	None to rare†	0	80%	94%
	Many	1	20%	6%
Glycogenated nuclei	Contiguous patches			
	None to rare†	0	57%	71%
	Many	1	43%	29%
Diagnostic classification‡				
Not steatohepatitis		0	31%	32%
Possible/borderline		1	26%	33%
Definite steatohepatitis		2	43%	35%

*Ballooning classification: few indicates rare but definite ballooned hepatocytes as well as case that are diagnostically borderline; examples are shown in Fig. 1. Examples of patches of microvesicular steatosis and megamitochondria are shown in Fig. 2.

†The "None to rare" category is meant to alleviate the need for time-consuming searches for rare examples or deliberation over diagnostically borderline changes. If the feature is identified after a reasonable search, it should be coded as "many."

‡Diagnostic classification was not available on 2 sets of adult biopsy observations, reducing the total of such observations to 512.

Figure 2. NASH Clinical Research Network Histologic Scoring System definition and scores in study according to Kleiner et al. [94].

Figure 3

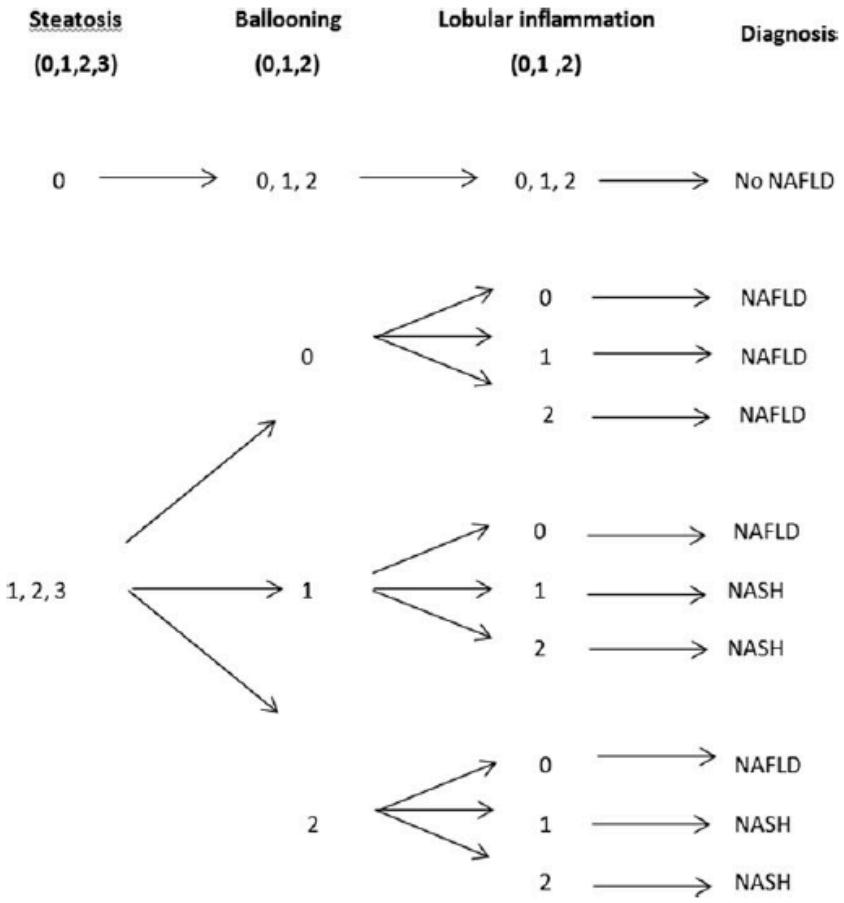


Fig. 2. Diagnostic algorithm for NASH.

Figure 3. FLIP diagnostic algorithm for categorization in NASH and steatosis [95].

Figure 4

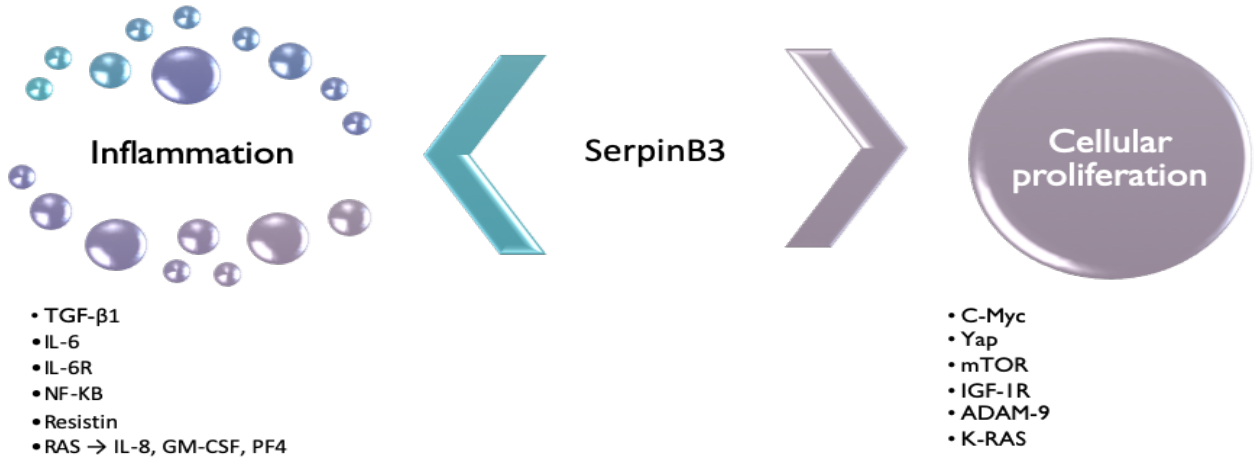


Figure 4. SerpinB3, cistein-proteinasi inibhitor, represents a turning point between inflammation and cellular proliferation [138,307,310].

Figure 5

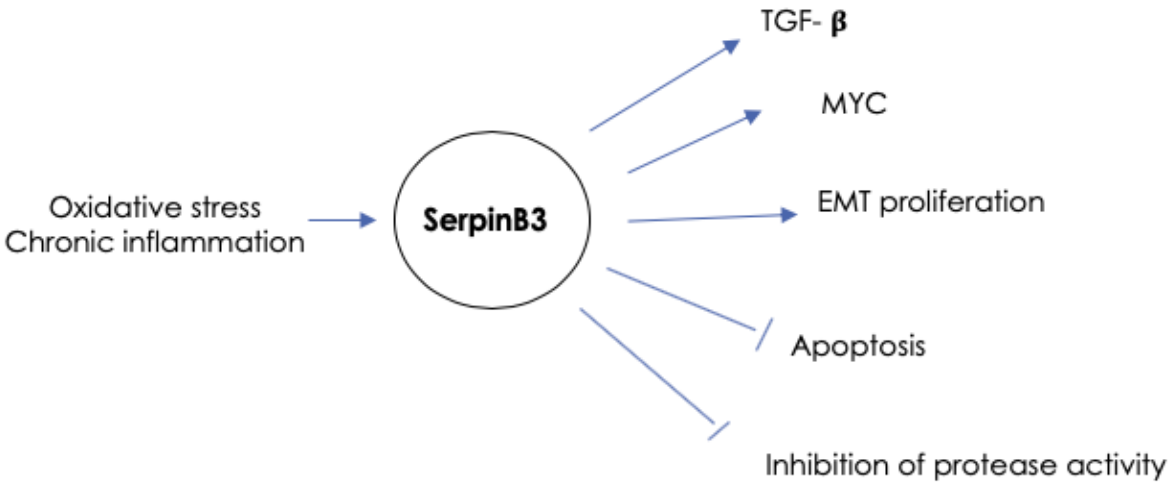


Figure 5. SerpinB3, cistein-proteasi inhibitor, involved in cellular proliferation process and in the development and maintenance of fibrosis, inflammation, and tissue transformation [129,148,157,311].

Figure 6

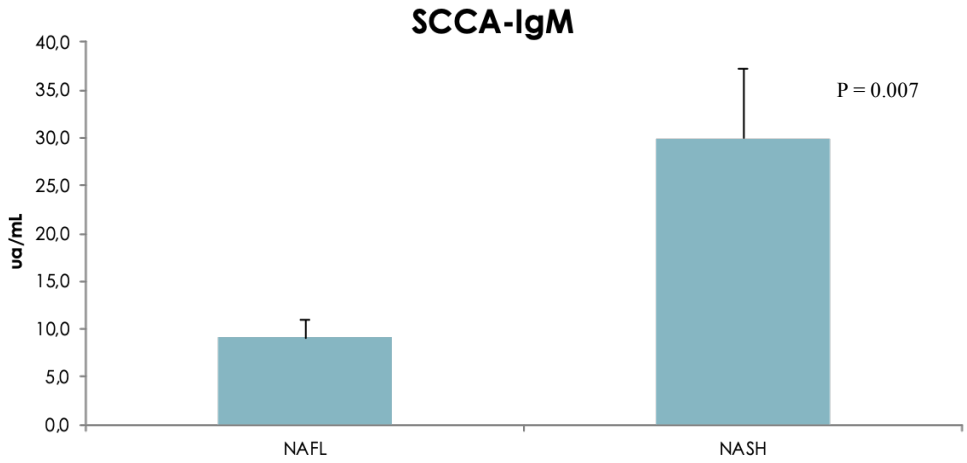


Figure 6. SCCA-IgM levels in NASH and NAFL. SCCA-IgM levels were significantly higher in NASH patients compared to NAFL patients (31.0 ± 7.2 ua/mL vs 9.2 ± 1.8 ua/mL respectively; p=0.007).

Figure 7

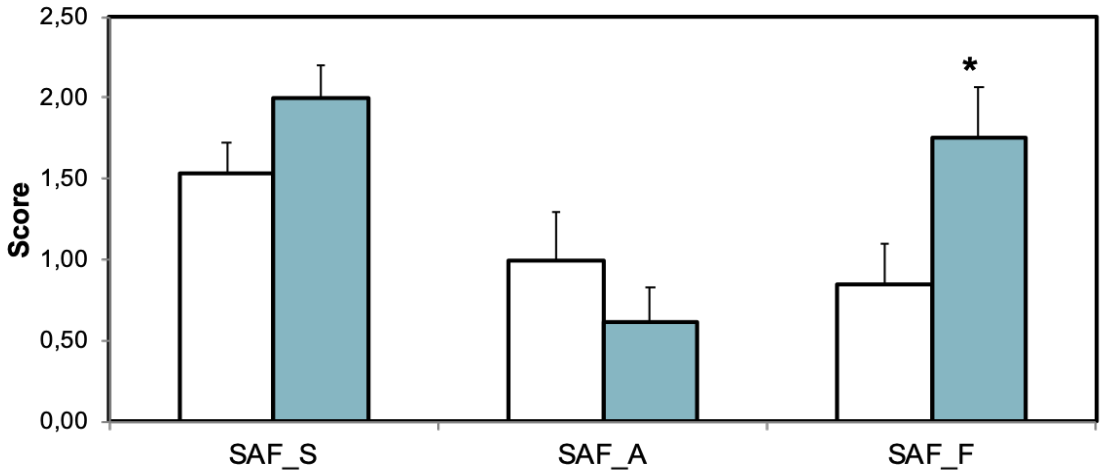
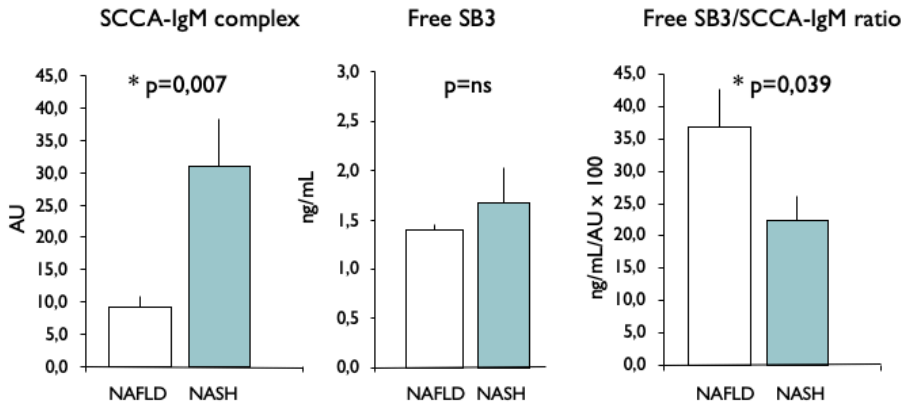


Figure 7. Comparison of SAF score according to I and IV quartiles of SCCA-IgM concentrations (p<0.05). I, II, III, IV quartile medium concentrations of SCCA-IgM: 1.98 ua/mL, 4.11 ua/mL, 7.95 ua/mL, 35.31 ua/ml. In white are represents I quartile, in light blue IV quartile.

Figure 8



	SCCA-IgM	sem	free SB3	sem	free/complex ratio	sem
NAFLD	9,2	1,8	1,4	0,1	36,6	5,9
NASH	31,0	7,2	1,7	0,4	22,3	3,7

Figure 8. Dosage of Free SerpinB3, SCCA-IgM, and Free SerpinB3/SCCA-IgM ratio.

Figure 9

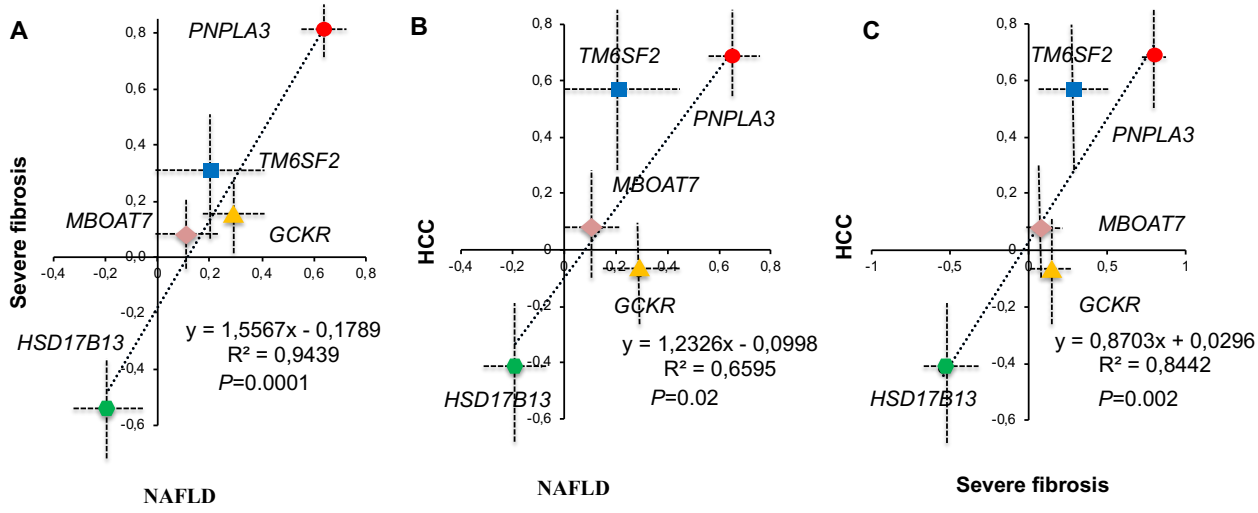


Figure 9. Mendenlian Rondomization. Genetic risk variants confer a risk of HCC development that is proportional to the increase in the risk of NAFLD (figure B). A direct relationship is observed between the risk of NAFLD and severe fibrosis, and between severe fibrosis and HCC.

Figure 10

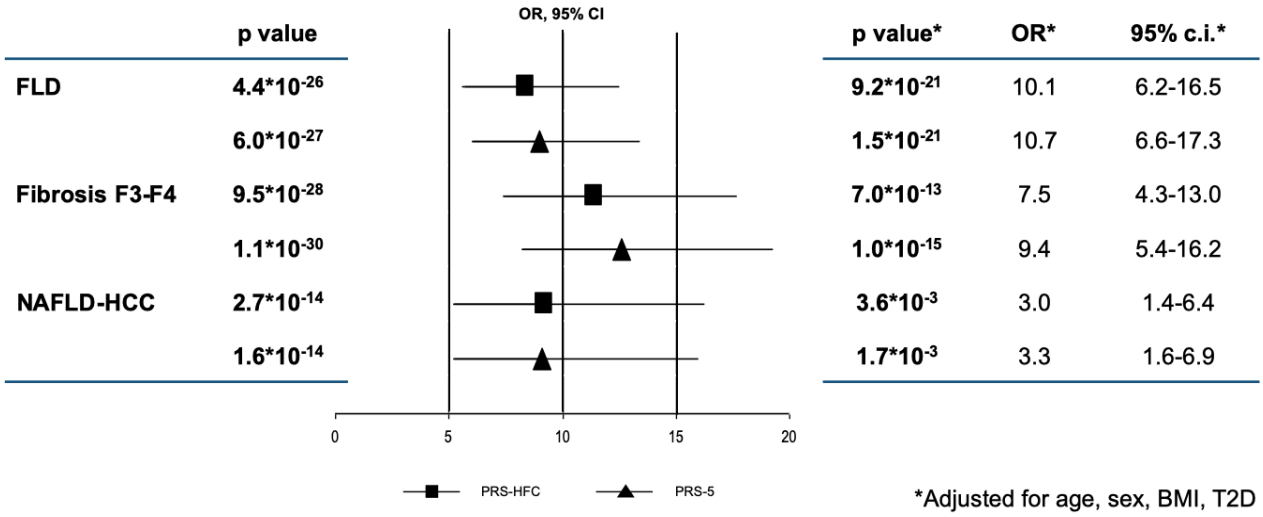


Figure 10. Association between PRSs and the full spectrum of liver disease

Figure 11

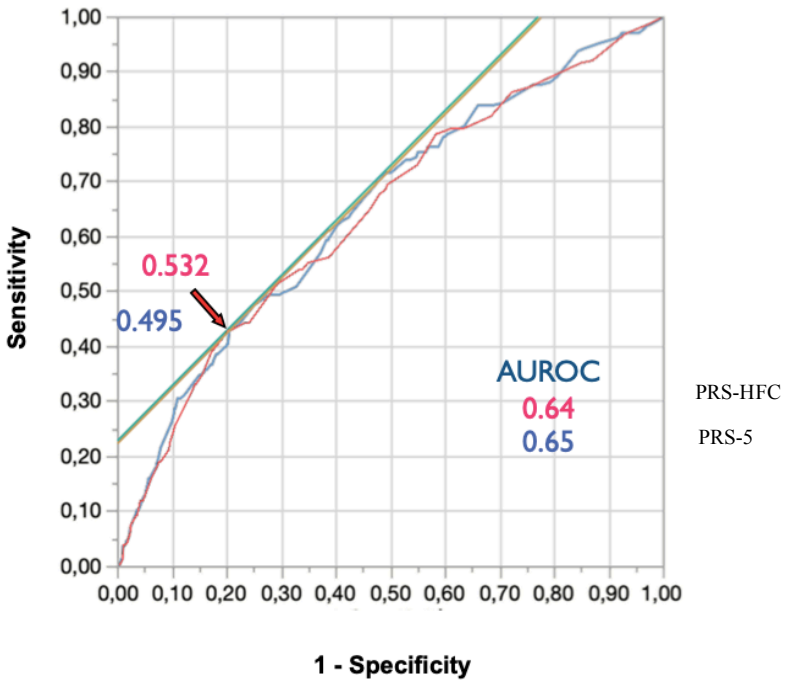


Figure 11. Performance test: comparison of the diagnostic accuracy of the PRS-HFC and the PRS-5 for NAFLD-HCC in the NAFLD cohort. The AUROC of the two PRSs to predict NAFLD-HCC and the optimal diagnostic thresholds are shown (the best cut-off for PRS-HFC result 0.532, with an AUROC 0.64; for PRS-5 the best threshold results 0.495 with an AUROC equal to 0.65)

Figure 12

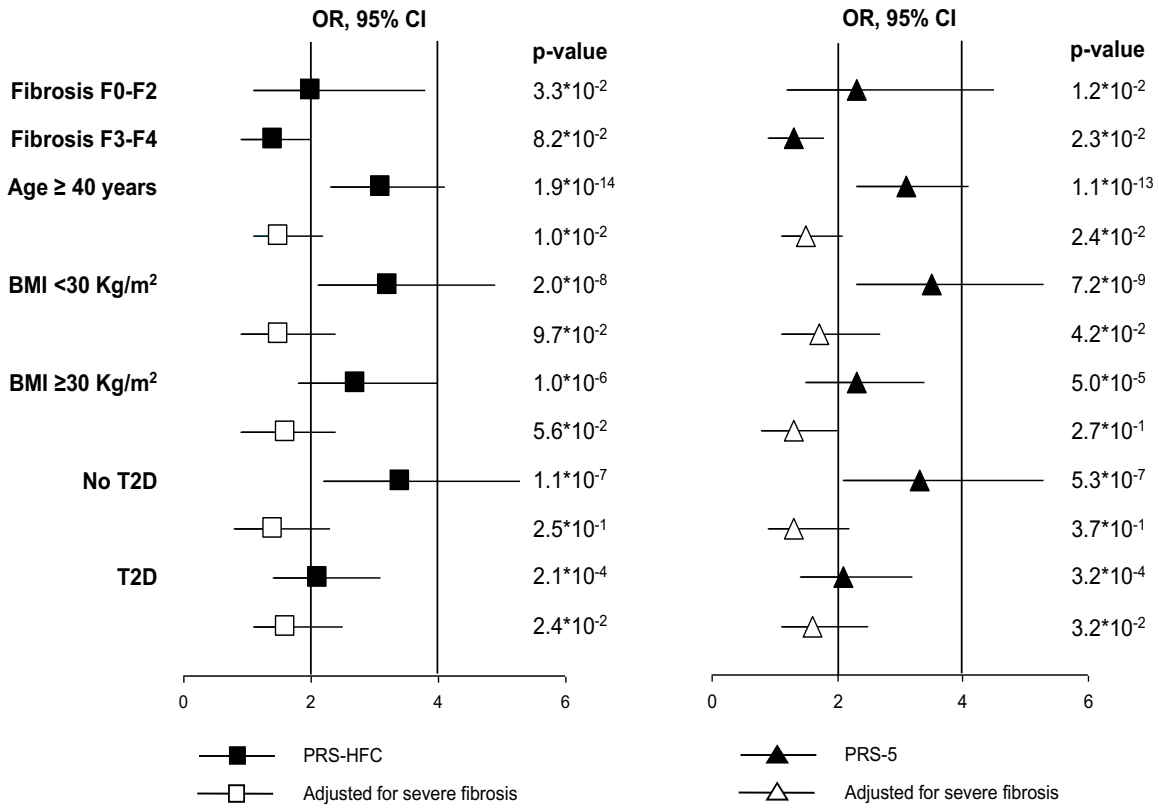


Figure 12. Sensitive analysis. Ability of PRSs to predict the risk of developing NAFLD-HCC in different subgroups at risk

Table 1

Macrovesicular steatosis	Microvesicular steatosis
Excessive alcohol consumption	Reye's syndrome
Hepatitis C (genotype 3)	Medications (valproate, anti-retroviral medicines)
Wilson's disease	Acute fatty liver of pregnancy
Lipodystrophy	HELLP syndrome
Starvation	Inborn errors of metabolism (e.g., LCAT deficiency, cholesterol ester storage disease, Wolman disease)
Parenteral nutrition	
Abetalipoproteinemia	
Medications (e.g., amiodarone, methotrexate, tamoxifen, corticosteroids)	
Non-tropical sprue	
Tesaurismosis (galactosemia, glycogenosis, tyrosinemia, fructose intolerance)	

Table 1. Common Causes of Secondary Hepatic Steatosis (modify by [8]).

Table 2

Histological figure	***Score***	***Criterion***
Steatosis	0 1 2 3	< 5% 5-33% 34-66% >66%
Ballooning	0 1 2	None Some ballooning-form cells Many cells / prominent ballooning
Lobular inflammation	0 1 2 3	No foci <2 foci/20x 2-4 foci/20x >4 foci/20 x

Table 2. NAS score, evaluation of the degree of activity proposed by the Nonalcoholic Steatohepatitis Clinical Research Network Histologic Scoring System [94]

Table 3

Stage	*Criterion*
0	None
1	Perisinusoidal or periportal a. Mild, zone 3, perisinusoidal b. Moderate, zone 3, perisinusoidal c. Portal/periportal
2	Perisinusoidal and portal/periportal
3	Bridging fibrosis
4	Cirrhosis

Table 3. NAS score, evaluation of the stage of fibrosis proposed by the Nonalcoholic Steatohepatitis Clinical Research Network Histologic Scoring System [94]

Table 4

Histological figure	*Score*	*Criterion*
Degree of steatosis (S)	**0-3**	
Steatosis	0 1 2 3	< 5% 5-33% 34-66% >66%
Degree of activity (A)	**0-4**	
Ballooning	0 1 2	None Some ballooning-form cells Many cells / prominent ballooning
Lobular inflammation	0 1 2	None <2 foci/20x >2 foci/20x
Degree of fibrosis (F)	**0-4**	
Fibrosis	0 1a 1b 1c 2 3 4	None Mild, zone 3 perisinusoidal/pericellular Moderate, zone 3 perisinusoidal/pericellular Portal/periportal Perisisusoidal/periportal e portal/periportal Bridging fibrosis Cirrhosis

Table 4. Steatosis, Activity and Fibrosis (SAF) histological score for NAFLD [95]

Table 5

	NAFL (n=39)	**NASH (n=45)**	**p**
Age (years)	57.4±1.6	50.7±2.8	0.03
Weight (kg)	85.9±2.5	97.2±4.7	0.02
BMI (kg/m^2)	29.1±0.6	34.9±1.7	0.001
Weist (cm)	102.2±2.1	101.8±3.2	Ns
Subcutaneous fat. (mm)	23±1	24±3	Ns
Visceral fat (mm)	69±3	64±5	Ns
Glycemia (mg/dL)	103.9±4.0	100±3.7	Ns
Insulin (mUI/L)	23.9±6.2	49.5±29.7	Ns
HOMA-I	4.1±0.4	5.2±1.1	Ns
HbA1c (%)	6.2±0.1	6.5±0.2	Ns
Total Cholesterol (mg/dL)	200±5	222±8	0.02
HDL Cholesterol (mg/dL)	56±3	55±2	Ns
LDL Cholesterol (mg/dL)	116±5	138±7	0.01
Tryglicerids (mg/dL)	143±10	158±18	Ns
AST (U/L)	30±2	28±2	Ns
ALT (U/L)	39±2	31±4	0.05
AST/ALT	1.4±0.1	1.1±0.1	0.01
γ-GT (UI/L)	65±9	93±31	Ns
Total Bilirubin (mg/dL)	0.7±0.1	0.7±0.1	Ns
Pseudocholinesterase (UI/L)	8818±236	9322±404	Ns
Homocysteine (μmol/L)	13.4±0.8	12.8±0.6	Ns
PCR (mg/L)	9.9±0.3	3.7±1.2	Ns
PAI	19.8±4.8	14±2.2	Ns
tPA	10.3±1.0	7.5±0.8	Ns
Lp(a)	25±6	21±6	Ns

Ferritin (ng/ml)	213±32	179±41	Ns
TSH (mU/L)	1.4±0.1	2.2±0.2	0.001
IGF-1 (ng/ml)	97±7	122±15	Ns
Cortisol	361±22	323±44	Ns
SCCA-IgM (ua/mL)	9.2±1.8	31.7±7.2	0.007

Table 5. Anthropometric, biohumoral, and ultrasound baseline data characteristics in patients with NASH and NAFL.

Table 6

SCCA-IgM vs	**r**	**n**	**p**
Age (years)	0.269	64	**<0.05**
Waist (cm)	0.08	54	Ns
BMI (kg/m^2)	0.30	64	**0.01**
Visceral fat (mm)	0.16	54	Ns
Subcutaneous fat (mm)	0.06	54	Ns
Homocysteine (µmol/L)	0.33	54	**0.02**
AST (U/L)	0.12	54	Ns
ALT (U/L)	0.06	54	Ns
AST/ALT	0.15	54	Ns
NAS score*	0.18	54	Ns
Steatosis grade*	0.17	54	Ns
SAF-S*	0.17	54	Ns
SAF-A*	-0.14	54	Ns
SAF-F*	0.29	54	**<0.05**

Table 6. Correlation between SCCA-IgM and demographic, biochemical, and histological variables in NAFLD patients.

Table 7

	No liver disease (n=865, 33.7%)	**NAFLD F0-F2 (n=1,176, 45.8%)**	**NAFLD F3-F4 (n=297, 11.6%)**	**NAFLD HCC (n=226, 8.9%)**	**p value**
Age	44 ± 6	42 ± 16	58 ± 14	69 ± 9	**<.0001**
Sex, M	455 (52.6)	677 (57.6)	171 (57.6)	178 (78.8)	**<.0001**
T2D, yes	8 (0.9)	238 (20.2)	169 (56.9)	145 (64.2)	**<.0001**
BMI, Kg/m^2	25.3 ± 5.0	32.7 ± 8.6	30.7 ± 5.1	30.2 ± 5.6	**<.0001**
PRS-HFC	0.266 (0.128-0.402)	0.392 (0.130-0.522)	0.457 (0.329-0.631)	0.459 (0.329-0.662)	**<.0001**
PRS-5	0.233 (0.065-0.394)	0.329 (0.128-0.459)	0.421 (0.256-0.597)	0.399 (0.266-0.660)	**<.0001**

Table 7. Demographic and PRSs baseline characteristics of patients enlisted in the NAFLD cohort study.

Table 8

Method	**Estimate**	**SE**	**95% c.i.**	**p value**
Weighted median	1.018	0.188	0.650 - 1.387	<0.001
Robust IVW	0.969	0.226	0.526 - 1.412	<0.001
Robust MR-Egger	0.998	0.190	0.627 - 1.370	<0.001

Table 8. Comparison of causality estimates of HFC on HCC by different Mendelian randomization approaches taking into consideration possible horizontal pleiotropic effect of the variants under consideration in the NAFLD cohort

Table 9

	PRS-HFC	**PRS-5**
AUROC	0.64	0.65
Diagnostic threshold	0.532	0.495
Prevalence (%)	569 (22.2)	580 (22.9)
OR (95% c.i.)	3.0 (2.2-3.9)	2.9 (2.1-3.8)
p value*	$3.7*10^{-14}$	$8.1*10^{-13}$
Sensitivity (95% c.i.)	0.43 (0.37-0.49)	0.43 (0.37-0.50)
Specificity (95% c.i.)	0.80 (0.78-0.81)	0.79 (0.77-0.81)
PPV (95% c.i.)	0.17 (0.14-0.20)	0.16 (0.13-0.19)
NPV (95% c.i.)	0.93 (0.92-0.94)	0.94 (0.93-0.95)
LR+ (95% c.i.)	2.13 (1.79-2.52)	2.06 (1.74-2.54)
LR- (95% c.i.)	0.71 (0.64-0.80)	0.72 (0.64-0.81)

Table 9. Diagnostic accuracy of PRS-HFC and PRS-5 for HCC in the NAFLD cohort.

Table 10

	p value*	**OR**	**95% c.i.**
PRS-HFC ≥ 0.532	**$6.5*10^{-4}$**	1.9	1.3-2.8
PNPLA3	**$3.4*10^{-2}$**	1.5	1.1-2.3
TM6SF2	$8.4*10^{-2}$	1.5	0.9-2.4
MBOAT7	$8.6*10^{-1}$	1.0	0.7-1.5
GCKR	$5.8*10^{-1}$	0.8	0.6-1.4
HSD17B13	**$3.8*10^{-2}$**	0.5	0.2-1.0
PRS-5 ≥ 0.495	**$4.3*10^{-4}$**	2.0	1.3-2.9

Table 10. Both PRS resulted superior to single variants for predicting NAFLD-HCC (*Adjusted for age, sex, BMI, T2D).

Table 11

	No liver disease (n=773)	**MAFLD F0-F2 (n=33)**	**MAFLD F3-F4 (n=19)**	**HCC (n=30)**	**p-value**
Age, years	45 ± 6	57 ± 9	59 ± 9	65 ± 9	**5,00E-94**
Sex, M	432 (55.9%)	22 (66.6%)	8 (42.1%)	21 (70.0%)	1,40E-01
BMI, Kg/m2	24.1 ± 1.9	28.2 ± 4.0	31.0 ± 4.1	29.3 ± 6.7	**2,50E-64**
T2D, yes	0 (0%)	5 (15.1%)	6 (31.6%)	19 (63.3%)	**2,40E-36**
PRS-HFC	0.266 (0.128-0.402)	0.459 (0.191-0.668)	0.331 (0.191-0.595)	0.459 (0.379-0.663)	**4,03E-09**
PRS-5	0.223 (0.065-0.394)	0.426 (0.191-0.640)	0.224 (0.191-0.595)	0.396 (0.295-0.666)	**4,79E-09**

Table 11. Baseline characteristics of 82 patients enlisted from Udine in the NAFLD study cohort.

Table 12

		Univariate analysis			**Model 1**			**Model 2**		
		p-value	OR	95% c.i.	p-value	OR	95% c.i.	p-value	OR	95% c.i.
PRS-HFC	MAFLD	**6,40E-09**	21.3	7.6-59.9	8,00E-02	5.7	0.8-40.9	-	-	-
	Fibrosis F3-F4	**4,20E-04**	10.3	2.8-37.9	4,00E-01	2.4	0.3-19.2	-	-	-
	HCC	**1,60E-05**	29.8	6.4-139.5	3,20E-01	3.3	0.3-36.5	2,90E-01	4.6	0.3-79.4
PRS-5	MAFLD	**6,80E-09**	19.9	7.2-53.9	**3,00E-02**	8.8	1.2-63.2	-	-	-
	Fibrosis F3-F4	**5,90E-04**	9.2	2.6-32.6	4,80E-01	0.5	0.1-3.6	-	-	-
	HCC	**6,70E-05**	20.5	4.6-90.8	3,60E-01	2.9	0.3-27.9	3,80E-01	3.3	0.2-50.2

Table 12. PRS-HFC and PRS-5 as predictors of liver disease in Udine subgroup (Model 1: adjusted for age, sex, BMI, T2D. Model 2: further adjustment for advanced fibrosis).

Table 13

	p-value°	OR	95% c.i.	p-value*	OR	95% c.i.
PRS-HFC ≥0.532	**2,20E-05**	5.1	2.4-10.7	3,70E-01	1.7	0.5-5.4
PRS-5 ≥0.495	**4,50E-04**	4.1	1.9-8.9	5,30E-01	1.5	0.4-4.7
PNPLA3 I148M, carrier	**1,80E-03**	3..9	1.7-9.2	4,30E-01	1.6	0.5-5.0
TM6SF2 E167K, carrier	3,00E-01	1.6	0.6-4.1	2,20E-01	2.2	0.6-8.3
MBOAT7 rs641738 C>T, carrier	7,20E-01	1.2	0.5-2.6	1,40E-01	0.4	0.1-1.3
GCKR P446L, carrier	4,30E-01	1.4	0.6-3.6	7,80E-01	0.8	0.2-2.9
HSD17B13 rs72613567:TA, carrier	3,70E-01	0.7	0.3-1.5	5,60E-01	0.7	0.2-2.3

Table 13. Comparison of PRSs vs. single variants for the prediction of HCC (° At logistic regression, unadjusted; * At logistic regression adjusted for age, sex, BMI, T2D).

Table 14

	PRS-HFC	PRS-5	PRS-HFC°	PRS-5°	PRS-HFC*	PRS-5*
AUROC	0.72	0.70				
	PRS-HFC ≥0.532	PRS-5 ≥0.495	PRS-HFC ≥0.532	PRS-5 ≥0.495	PRS-HFC ≥0.532	PRS-5 ≥0.495
Positive PRS prevalence	121 (14.1%)	124 (14.6%)				
OR (95% c.i.)	5.1 (2.4-10.7)	4.1 (1.9-8.9)	1.7 (0.5-5.4)	1.5 (0.4-4.7)	2.9 (0.9-9.0)	2.5 (0.8-8.0)
p-value	2,17E-05	4,54E-04	3,69E-01	5,32E-01	6,32E-02	1,18E-01
Sensitivity (95% c.i.)	0.43 (0.27-0.61)	0.39 (0.24-0.58)				
Specificity (95% c.i.)	0.87 (0.84-0.89)	0.86 (0.84-0.88)				
PPV (95% c.i.)	0.11 (0.06-0.17)	0.09 (0.05-0.15)				
NPV (95% c.i.)	0.98 (0.96-0.98)	0.98 (0.96-0.98)				
LR+ (95% c.i.)	3.31 (2.12-5.17)	2.86 (1.75-4.68)				
LR- (95% c.i.)	0.65 (0.48-0.89)	0.70 (0.52-0.95)				

Table 14. Diagnostic accuracy of PRS-HFC and PRS-5 for HCC in subgroup of Udine patients (° adjusted for age, sex, BMI, T2D; * adjusted for severe fibrosis).

Table 15

	Estimate	**SE**	**95% c.i.**	**p-value**
PNPLA3 I148M, carrier	-0.02	0.07	-0.16 - 0.11	7,22e-1
TM6SF2 E167K, carrier	0.01	0.08	-0.16 - 0.16	9,9e-1
MBOAT7 rs641738 C>T, carrier	-0.05	0.09	-0,23 - 0.13	6,03e-1
GCKR P446L, carrier	-0.16	0.08	-0.32 - - 0.01	**4,07e-2**
HSD17B13 rs72613567:TA, carrier	0.05	0.07	-0.08 - 0.19	4,29e-1
PRS-HFC ≥0.532	-0.06	2.14	-0.35 - 4.21	9,75e-1
PRS-5 ≥0.495	0.16	2.19	-4.23 - 4.55	9,43e-1

Table 15. Association between genetic variants/PRS and SCCA-IgM levels.

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