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Risk Assessment in Non-Alcoholic Fatty Liver Disease: From Human Genetics to Novel Blood-Based Biomarkers

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“Your work is going to fill a large part of your life, and the only way to be truly satisfied is to do what you believe is great work. And the only way to do great work is to love what you do. If you haven't found it yet, keep looking. Don't settle. As with all matters of the heart, you'll know when you find it.”

Steve Jobs

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ABSTRACT

Paralleling the epidemics of obesity and type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) is now the leading cause of chronic liver disease worldwide. Most individuals remain asymptomatic for long periods of time with slowly progressive disease, but a minority progress to cirrhosis, liver failure, and hepatocellular carcinoma. In this scenario, the identification of risk factors for liver disease progression is crucial. Additionally, given the huge number of individuals at high risk for NAFLD and the invasiveness of liver biopsy, accurate and affordable non-invasive strategies to screen for liver disease are urgently needed.

In **Paper I**, we investigated the individual contribution of inborn and acquired risk factors for the development of severe liver disease (cirrhosis, decompensated liver disease, hepatocellular carcinoma, liver transplantation) in 22,812 Europeans with type 2 diabetes from the prospective UK Biobank study. Abnormal AST, decrease in serum albumin and platelet count, cardiovascular disease, microalbuminuria, and genetic variants in *PNPLA3* and *TM6SF2* genes were found to be the major independent risk factors for incident severe liver disease.

In **Paper II**, we developed and validated the Fibrotic NASH Index (FNI), an accurate, simple, and affordable non-invasive score based on routine laboratory tests (AST, HDL cholesterol, HbA1c) to screen for fibrotic NASH in individuals with metabolic risk factors in primary healthcare and diabetology/endocrinology clinics. The derivation cohort included 264 morbidly obese individuals undergoing intraoperative liver biopsy in Rome, Italy. External validation was assessed in three independent European cohorts (Finland, n=370; Italy n=947; England n=5,368) of individuals at high risk for NAFLD. The model was developed using a bootstrapping stepwise logistic regression analysis. Performance was satisfactory in both derivation and external validation cohorts (AUROCs 0.78 and 0.80-0.95, respectively).

In conclusion, these findings may contribute in clinical care to identify individuals at risk for severe liver disease, in turn leading to personalised risk prediction and prevention strategies.

Keywords: non-alcoholic fatty liver disease, NAFLD; human genetics; non-invasive tests; biomarkers; risk stratification.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. Tavaglione F*, De Vincentis A*, Jamialahmadi O, Pujia R, Spagnuolo R, Picardi A, Morano S, Valenti L, Romeo S, Vespasiani-Gentilucci U. *Co-first author. Inborn and acquired risk factors for severe liver disease in Europeans with type 2 diabetes from the UK Biobank. *JHEP Rep.* 2021 Mar 2;3(3):100262.

II. Tavaglione F*, Jamialahmadi O*, De Vincentis A*, Qadri S, Mowlaei ME, Mancina RM, Ciociola E, Carotti S, Perrone G, Bruni V, Gallo IF, Tuccinardi D, Bianco C, Prati D, Manfrini S, Pozzilli P, Picardi A, Caricato M, Yki-Järvinen H, Valenti L, Vespasiani-Gentilucci U, Romeo S. *Co-first author. Development and Validation of a Score for Fibrotic Nonalcoholic Steatohepatitis. *Clin Gastroenterol Hepatol*. 2022 Apr 11:S1542-3565(22)00385-8.

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ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APOE	Apolipoprotein E
AST	Aspartate aminotransferase
AUROC	Area under the receiver operating characteristic curve
BMI	Body mass index
CAP	Controlled attenuation parameter
CI	Confidence interval
cT1	Iron-corrected T1
eGFR	Estimated glomerular filtration rate
FAST	FibroScan-AST
FIB-4	Fibrosis-4 index
FLD	Fatty liver disease
FNI	Fibrotic NASH index
GGT	Gamma glutamyltransferase
GCKR	Glucokinase regulator
GPAM	Glycerol-3-phosphate acyltransferase 1, mitochondrial
GWAS	Genome-wide association studies
HbA1c	Hemoglobin a1c
HDL	High-density lipoprotein
HR	Hazard ratio
HSD17B13	Hydroxysteroid 17-beta dehydrogenase 13
ICD-10	International Classification of Diseases 10th edition
INR	International normalized ratio
LDL	Low-density lipoprotein
LSM	Liver stiffness measurement
MARC1	Mitochondrial Amidoxime Reducing Component 1
MBOAT7	Membrane-bound O-acyltransferase domain-containing 7
MRE	Magnetic resonance elastography
MRI	Magnetic resonance imaging

MRS	Magnetic resonance spectroscopy
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NPV	Negative predictive value
PDFF	Proton density fat fraction
PNPLA3	Patatin-like phospholipase domain-containing 3
PPV	Positive predictive value
PRO-C3	Pro-collagen III
PRS	Polygenic risk score
SLD	Severe liver disease
TIMP1	Tissue inhibitor of metalloproteinase 1
TM6SF2	Transmembrane 6 superfamily member 2
VCTE	Vibration-controlled transient elastography

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

Paralleling the epidemics of obesity and type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) is now the leading cause of chronic liver disease worldwide, affecting up to 30% of the global population [1]. NAFLD encompasses a broad spectrum of conditions, from isolated hepatic fat accumulation (non-alcoholic fatty liver, NAFL) to the development of hepatocellular damage and inflammation (non-alcoholic steatohepatitis, NASH), leading to fibrosis and adverse liver-related outcomes, namely cirrhosis and hepatocellular carcinoma [2]. Over the past two decades, NASH has become one of the major causes of cirrhosis in adults, and NASH-related cirrhosis is currently the second leading indication for liver transplantation in the United States [3].

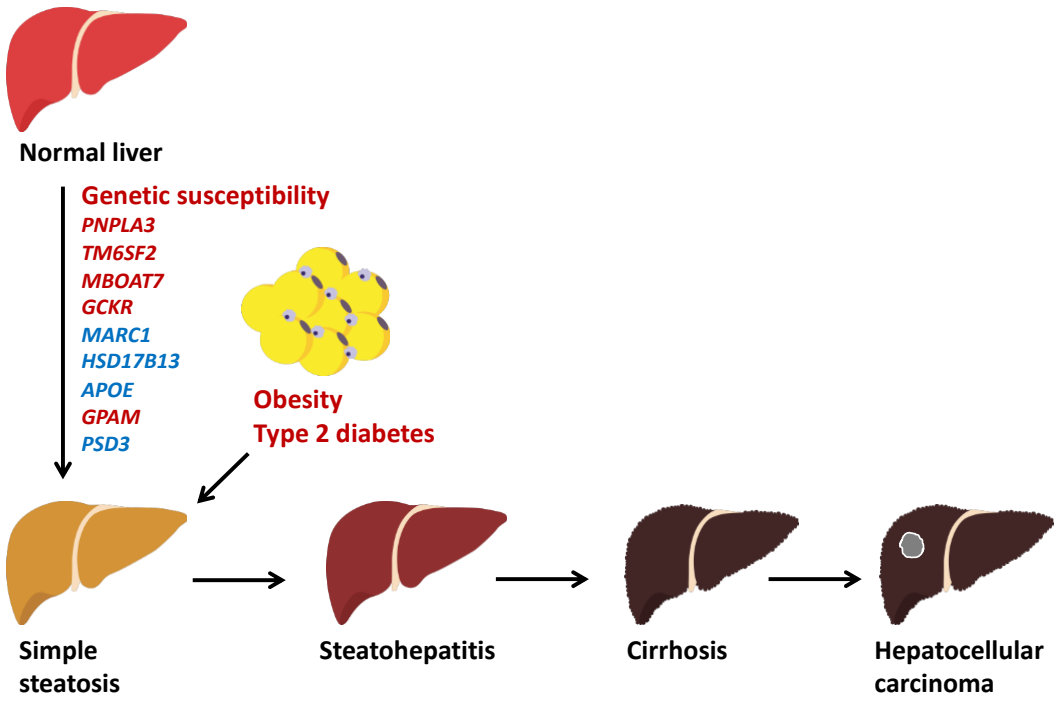
Most individuals remain asymptomatic for long periods of time (years/decades) with slowly progressive disease, but a minority progress to cirrhosis, liver failure, and hepatocellular carcinoma [4].

INBORN AND ACQUIRED RISK FACTORS FOR NAFLD

NAFLD is a complex and heterogenous trait deriving from the interaction between genetic, epigenetic, and environmental factors [5, 6] (Figure 1). However, the exact contribution of each component is unknown and may be influenced by ethnicity, geography and the interplay between these components (gene-environment interactions) [6-8]. NAFLD inheritance is substantially varying between 20% and 70% [9]. In the past few years, genome-wide association studies (GWAS) and candidate-gene studies have identified several genetic *loci* implicated in hepatic lipid handling and contributing to NAFLD, including *PNPLA3*, *TM6SF2*, *MBOAT7*, *GCKR*, and *HSD17B13* [6] (Figure 2). To date, the single nucleotide polymorphism rs738409 in *PNPLA3* (p.I148M), encoding for a substitution of an isoleucine with a methionine at position 148 of the protein, is the strongest genetic determinant of NAFLD and the entire spectrum of its adverse clinical outcomes [10]. Within this context, a recent GWAS including more than 400,000 Europeans identified a *locus* in *MARC1* as a novel protective factor for fatty liver and all-cause cirrhosis [11]. Along this line, novel genetic common variants in *GPAM* and *APOE* have been recently associated with steatosis and liver damage at an exome-wide level in

European participants from the UK Biobank [12]. Finally, the rs71519934 at the Pleckstrin and Sec7 domain-containing 3 (*PSD3*) gene, resulting in a leucine to threonine substitution at position 186 of the protein (L186T), was recently found to reduce susceptibility to the entire spectrum of NAFLD in at-risk individuals [13].

Figure 1. Inborn and acquired risk factors for NAFLD.



Adapted from Tavaglione F et al. Endocrinol Diabetes Metab. 2020

Obesity and type 2 diabetes are the strongest environmental risk factors for developing NAFLD. Indeed, up to 65% of individuals with type 2 diabetes have NAFLD and this rate becomes even higher in individuals with morbid obesity [14]. However, despite the very large number of individuals with NAFLD, only a minority progress to cirrhosis and hepatocellular carcinoma [2]. Acquired risk factors and environmental triggers predisposing to NAFLD also include alcohol consumption, dietary habits, lack of exercise, and familial disorders (e.g., hypobetalipoproteinemia or lipodystrophy) [2, 6, 7, 15]. Finally, the crosstalk between liver and gut may also contribute to metabolic aberrations occurring in NAFLD, as evidenced by studies reporting changes in gut microbiota compositions in individuals with NAFLD [16-18].

Figure 2. Metabolic pathways of common genetic determinants of NAFLD.

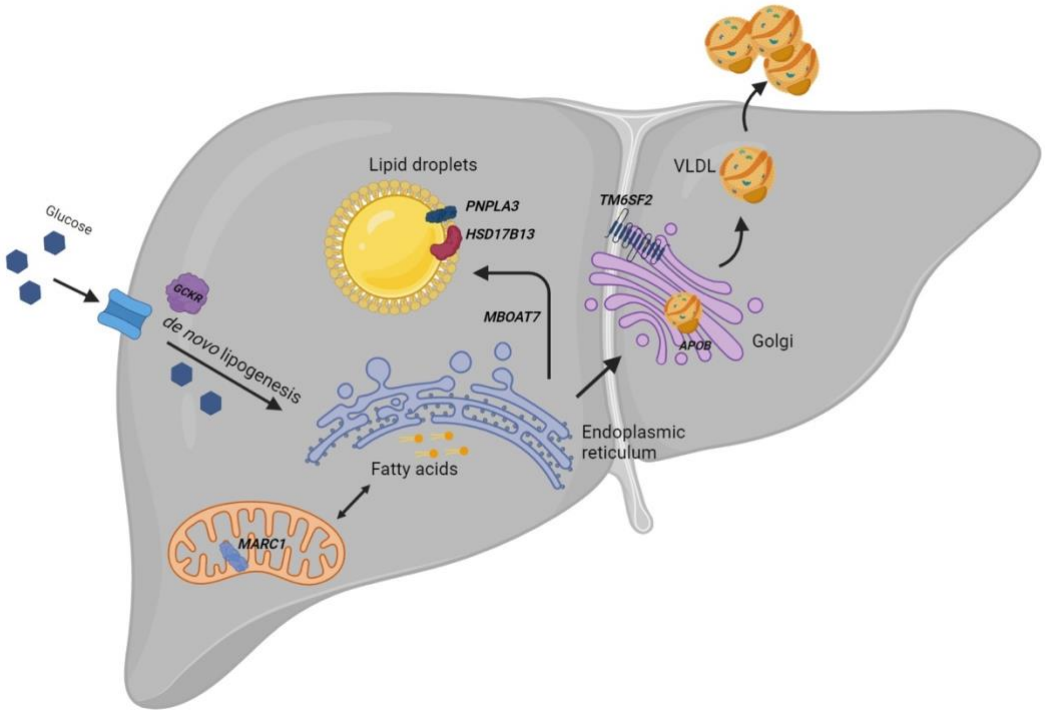


Image is courtesy of Dr. Oveis Jamialahmadi, University of Gothenburg, Sweden.

INVASIVE ASSESSMENT OF NAFLD

Currently, the gold standard for diagnosing NASH and liver fibrosis is still a histological assessment by liver biopsy, an invasive and costly procedure which is not devoid of complications [19, 20]. Indeed, liver biopsy carries potential risks of pain, bleeding, infection, and extremely rarely, even death [21]. Additionally, liver biopsy is able to assess a small proportion of the liver, and interpretation and scoring of liver specimen are affected by significant inter- and intra-observer variability. Thus, liver biopsy is not suitable for screening at the general population level, and it's recommended to be performed only in selected individuals [19, 20].

NON-INVASIVE ASSESSMENT OF NAFLD

Blood-based biomarkers

The presence of advanced fibrosis is considered a clinically relevant milestone in the natural history of NAFLD, being positively correlated with the occurrence of liver-related complications and mortality [22, 23]. Therefore, much attention has been focused on

developing non-invasive biomarkers able to detect advanced fibrosis [24]. Currently, the fibrosis-4 index (FIB-4, non-patented) and the enhanced liver fibrosis test (ELF, patented) are the most widely adopted serum biomarkers. Overall, non-invasive scores are highly effective at ruling out advanced fibrosis, showing high negative predictive values (NPVs >90%). However, their positive predictive values (PPVs) are often modest, and they are unable to accurately discriminate the different fibrosis stages [25]. The FIB-4 score (age, AST, ALT, platelet count) was originally developed in individuals with HCV/HIV co-infection with an AUROC of 0.74 and 0.77 in the derivation and validation cohorts, respectively [26]. Subsequently, FIB-4 was found to be one of the best performing and simple score for advanced fibrosis in individuals undergoing liver biopsy for suspected NAFLD, with an AUROC of 0.86 (0.78–0.94) [27]. Values <1.3 effectively rule out advanced fibrosis in NAFLD, while those >2.67 (or >3.25 in some studies) identify individuals with advanced fibrosis. In clinical practice, the lower (high-sensitivity) threshold is the most used given that the NPV is most robust [25].

In addition to FIB-4, the NAFLD fibrosis score (NFS: age, type 2 diabetes, BMI, AST/ALT, albumin, platelet count) was found to be the other best-performing simple biomarker for advanced fibrosis. NFS was developed in an international cohort of individuals with biopsy-proven NAFLD with an AUROC of 0.81 (0.71–0.91) [28]. Values <-1.455 exclude advanced fibrosis with high accuracy and values >0.676 diagnose advance fibrosis with improved PPV. This score has been independently validated in several studies [25].

Other non-invasive scores for liver fibrosis include the AST/ALT ratio (AAR) [29], AST-to-platelet ratio index (APRI: AST expressed as ratio of the upper limit of normal, platelet count) [30], BARD score (BMI, AST/ALT, type 2 diabetes) [31], and FORNS (age, cholesterol, GGT, platelet count) [32].

The ELF test is a panel of direct markers of fibrosis and matrix turnover, namely hyaluronic acid, pro-collagen III (PRO-C3), and tissue inhibitor of metalloproteinase 1 (TIMP1). It was first developed in individuals with HCV, and subsequentially validated in NAFLD with an AUROC of 0.90 (0.84-0.96) [33]. The ELF threshold <7.7 (low threshold recommended by the manufacturer) demonstrated high sensitivity of 0.93 (0.82-0.98) but low specificity of 0.34 (0.13-0.65), whereas the ELF threshold >9.8 (high threshold recommended by the manufacturer) showed a specificity of 0.86 (0.77-0.92)

and sensitivity of 0.65 (0.49-0.77). A specificity of 0.93 (0.85-0.96) and sensitivity of 0.51 (0.31-0.70) were observed at the ELF threshold of >10.51 (high threshold recommended by the UK NICE guideline). Importantly, the NPV was good when the prevalence of advanced fibrosis ranged from 5-10%, while the PPV was less strong [34]. Additional scores that comprise direct biomarkers of liver fibrosis are FibroTest (total bilirubin, GGT, α2-macroglobulin, apolipoprotein A1, and haptoglobin, corrected for age and gender) [35], FIBC3 (PRO-C3, platelet count, age, BMI, type 2 diabetes) [36], and ADAPT (PRO-C3, platelet count, age, type 2 diabetes) [37].

Alongside with liver fibrosis, a body of evidence shows that fibrotic NASH, the inflammatory form of NAFLD associated with significant activity (NAS ≥4) and fibrosis (F ≥2) (also classified as "at risk" NASH), is a key driver for developing advanced liver disease [8]. Up to date, three non-invasive scores have been generated to assess fibrotic NASH, namely the blood-based MACK-3 (hoMa, Ast, CK18) [38] and NIS4 (miR-34a-5p, alpha-2 macroglobulin, YKL-40, HbA1c) [39], and the transient elastography-based FibroScan-AST (FAST) score (AST, controlled attenuation parameter [CAP], liver stiffness measurement [LSM]) [40].

Notably, polygenic risk scores (PRSs) are emerging as promising non-invasive clinical tools to estimate the risk of NAFLD development and progression [41]. In this scenario, the PRS of hepatic fat (PRS-HFC) was developed by combining four common steatogenic risk *loci* in *PNPLA3-TM6SF2-MBOAT7-GCKR* weighted by their effect size on hepatic fat [42]. The PRS-HFC was found to predict the full spectrum of histological liver damage in patients with NAFLD [42]. Additionally, PRS-HFC and PRS-5 (i.e., PRS-HFC integrated with the *HSD17B13 locus*) predicted hepatocellular carcinoma better than single genetic risk variants either in at-risk individuals or in Europeans from the general population [43]. Specifically, positive PRS (defined as PRS-HFC ≥0.532 and PRS-5 ≥0.495, prevalence ~10%) conferred ~3-fold increased risk of hepatocellular carcinoma. Despite a limited sensitivity, PRSs showed a good specificity (~90%) to discriminate hepatocellular carcinoma in the general population and performed better in the subset of individuals with dysmetabolism [43]. Gellert-Kristensen *et al.* [44] proposed an unweighted PRS based on the number of at-risk alleles in *PNPLA3-TM6SF2-HSD17B13* and demonstrated that higher score values were associated with a higher risk of non-viral liver disease progression in large cohorts of Europeans. Specifically, individuals with the

highest PRS had ~12-fold and ~29-fold increased risk of cirrhosis and hepatocellular carcinoma, respectively, as compared to those with the lowest score in a population-based cohort in Europeans. Advantages of this approach are the easy score calculation, the ease of applicability and the use of variants robustly associated with FLD. On the other hand, PRSs have a normal distribution with the majority of individuals in the general population bearing an average to low risk, thereby limiting their predictive ability [41].

Finally, lipidomics, proteomics, metabolomics, metagenomics, and genomics technologies have been developed to identify novel biomarkers for NAFLD. However, currently none of the available biomarkers are able to diagnose and stage NAFLD with high sensitivity and specificity [45].

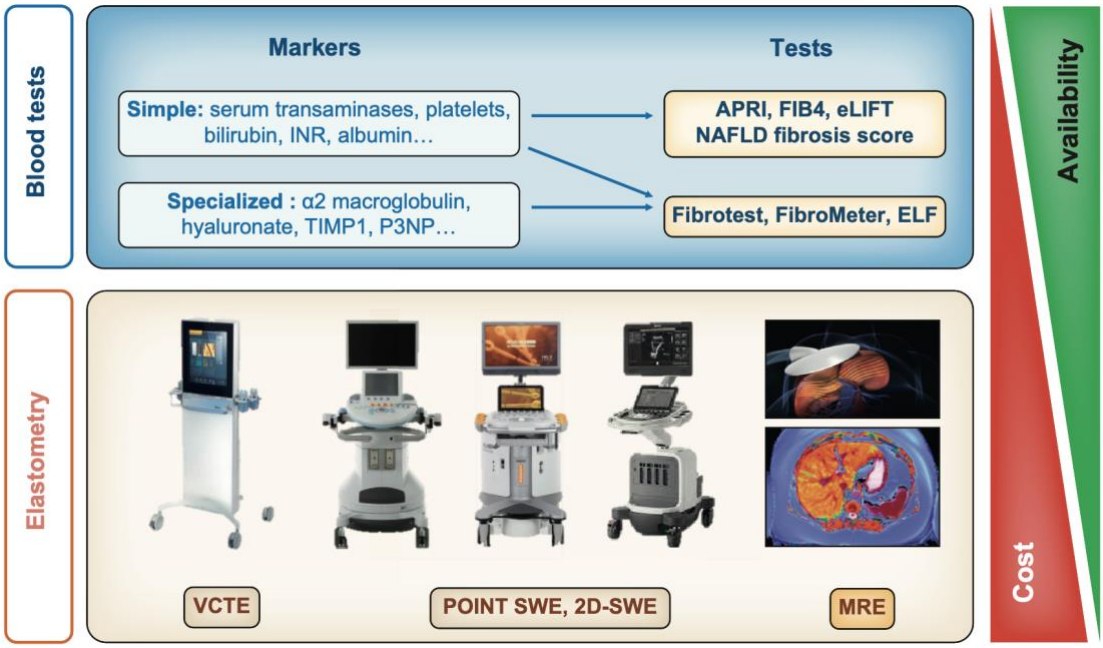
Imaging-based biomarkers

Several non-invasive imaging procedures have been developed to assess liver fibrosis by measuring liver stiffness as a surrogate, including ultrasound-based elastography techniques (e.g., point-shear wave elastography [pSWE] and bidimensional shear wave elastography [2D-SWE]), vibration-controlled transient elastography (VCTE) by FibroScan® (Echosens, Paris, France), and more recently, magnetic resonance elastography (MRE) [46] (Figure 3).

Ultrasound-based elastography techniques use high-frequency ultrasound impulses to generate sheer waves and require the operator to obtain a series of liver stiffness measurements in the region of interest. There are limited studies demonstrating good diagnostic accuracy of these techniques for detecting advanced fibrosis in individuals with NAFLD. Moreover, additional data on the optimal cut-offs and quality criteria are needed [47].

VCTE is the most widely adopted and validated elastography technique to estimate liver fibrosis in clinical care [25]. VCTE measures the speed of a mechanically induced shear wave in liver tissue using ultrasound and provides an estimate of the degree of liver fibrosis with LSM [48]. Examinations with at least 10 valid measurements of which >60% should be valid and with an interquartile range/median of LSM ≤30% are deemed valid [24].

Figure 3. Non-invasive tests for liver fibrosis.



Boursier J and Tsochatzis EA. JHEP Rep. 2020

The diagnostic performance of VCTE has been extensively validated in individuals with NAFLD [48, 49]. Limitations of VCTE in NAFLD include unclear optimal cut-offs, invalid or unreliable exams particularly in morbidly obese individuals or with inexperienced operators, and limited diagnostic accuracy for diagnosing earlier stages of fibrosis [47]. In addition to obesity, results of LSM can overestimate fibrosis in case of congestive heart failure, inflammation, and recent food ingestion [25]. According to the latest EASL guidelines, values ≥8 kPa might be used to identify individuals at intermediate-high risk for advanced fibrosis, requiring a referral to liver clinic for further investigation and follow-up [50]. More recently, VCTE by FibroScan® equipment has been implemented by the ability to quantify hepatic steatosis by measuring the CAP, an ultrasound-based tool which measures the ultrasound attenuation of the echo wave and is obtained simultaneously to LSM [48, 51]. As previously detailed regarding LSM, there are no consensual CAP cut-offs for identifying hepatic steatosis. According to the latest EASL guidelines, values above 275 dB/m might be used to diagnose steatosis at a sensitivity >90% [50].

VCTE-based biomarkers have also been combined with blood-based biomarkers to non-invasively identify individuals with fibrotic NASH and advanced fibrosis. The FAST

score (LSM, CAP, AST) showed satisfactory diagnostic accuracy for detecting fibrotic NASH in individuals with NAFLD from secondary/tertiary international care centers with an AUROC of 0.80 (0.76-0.85) and 0.85 (0.83-0.87) in derivation and external validation cohorts, respectively. A FAST score ≤0.35 (rule-out threshold) is associated with a lower likelihood of having fibrotic NASH, while a FAST score ≥0.67 (rule-in threshold) is associated with a higher likelihood of having fibrotic NASH [40].

Along this line, two new transient elastography-based scores, Agile 4 and Agile 3+ (LSM, AST/ALT ratio, platelet counts, age, sex, diabetes status), were recently developed to identify cirrhosis and advanced fibrosis, respectively, in individuals with NAFLD in secondary/tertiary care liver clinics. Substantially, both Agile 4 and Agile 3+ showed a higher performance than FIB-4 and LSM for cirrhosis or advanced fibrosis with an AUROC of 0.91 (0.89-0.92) and 0.90 (0.88-0.91), respectively, in the training cohorts [52].

MRE has been shown to be the most accurate technique to quantify and stage liver fibrosis in NAFLD. By assessing a larger proportion of the liver than ultrasound-based elastography techniques, MRE is less prone to sampling error and is less affected by obesity with excellent interobserver agreement [47, 50]. MRE cut-off of 3.63 kPa showed excellent diagnostic accuracy for advanced fibrosis (c-statistic >0.90) [53]. However, given its cost and limited availability, MRE is more suited for use in tertiary referral centers and for research purposes [50].

Conventional ultrasound and computed tomography can be used in clinical practice for assessing hepatic steatosis. However, these methods are not quantitative and have poor sensitivity for detecting mild grades of steatosis in NAFLD. Additionally, computed tomography exposes subjects to ionizing radiations, thereby limiting its use to assess repeated measurements of liver fat over time [47].

Within this context, the gold-standard technique for the non-invasive quantification of liver fat content is magnetic resonance spectroscopy (MRS). However, MRS requires specialized expertise and is not easily available in clinical settings [47]. Conversely, magnetic resonance imaging-derived proton-density-fat-fraction (MRI-PDFF) is emerging as an accurate and reproducible biomarker of liver fat content which is widely available in commercial MRI systems. PDFF estimates fat content over the entire liver

and represents a suitable surrogate to liver biopsy for assessing longitudinal changes in liver fat content, especially in NASH clinical trials [47, 54].

AIMS

The overall aims of this thesis were:

1) To elucidate risk factors associated with NAFLD progression in at-risk individuals.

2) To develop novel and reliable non-invasive biomarkers for NAFLD screening in at-risk individuals.

The aim of **Paper I** was to investigate the independent contribution of the main inborn and acquired risk factors for NAFLD to the development of severe liver disease in individuals with type 2 diabetes from the large UK Biobank.

The aim of **Paper II** was to generate a simple and affordable blood-based biomarker for fibrotic NASH to screen for liver disease in high-risk individuals (obesity, type 2 diabetes, metabolic syndrome) in primary care and general population settings.

PAPER I

Research article JHEP|Reports

Inborn and acquired risk factors for severe liver disease in Europeans with type 2 diabetes from the UK Biobank

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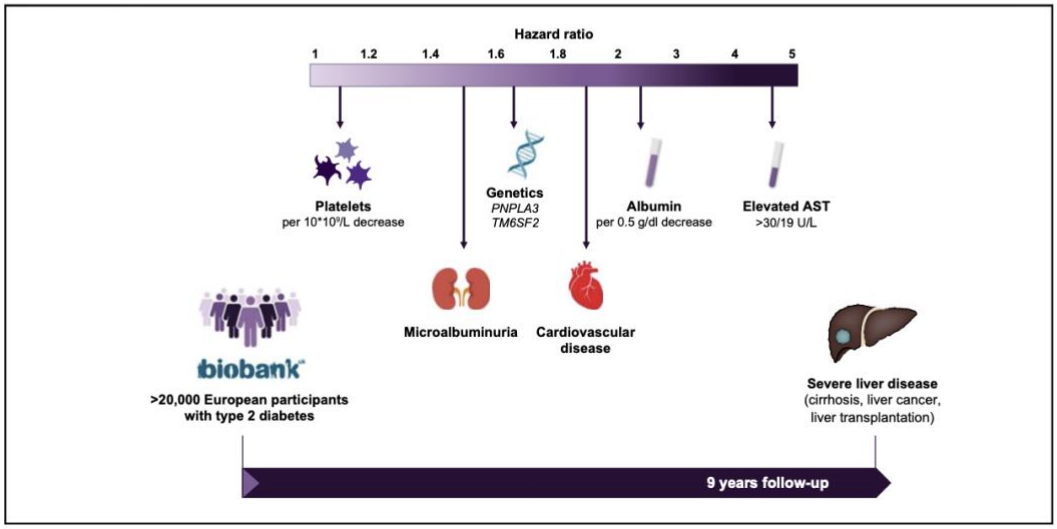
Graphical abstract



ABSTRACT

Background & Aims: Type 2 diabetes is a major driver of fatty liver disease and its long-term complications. Aim of this study was to investigate the individual contribution of inborn and acquired risk factors for severe liver disease in individuals with type 2 diabetes from the UK Biobank study.

Methods: A total of 22,812 UK Biobank participants of European descent without clinical history of liver disease and liver cancer were prospectively followed for the development of severe liver disease, defined as a composite diagnosis of cirrhosis, decompensated liver disease, hepatocellular carcinoma and/or liver transplantation from the National Health Service records. The contribution of inborn and acquired risk factors to the risk of incident severe liver disease was assessed by Cox proportional hazards models.

Results: During a median follow-up of 8.9 years (interquartile range 8.1-9.6), there were 279 individuals with severe liver disease, including 255 with cirrhosis and/or decompensated liver disease, 47 with hepatocellular carcinoma and 5 with liver transplantation; death from severe liver disease occurred in 83 individuals. Risk factors independently associated with increased risk of incident severe liver disease included abnormal aspartate aminotransferase (adjusted hazard ratio [aHR] 4.85, 95%CI 2.76-8.54), decrease in serum albumin (aHR 2.39, 95%CI 1.76-3.24) and platelet count (aHR 1.12, 95%CI 1.09-1.16), cardiovascular disease (aHR 1.86, 95%CI 1.23-2.79), microalbuminuria (aHR 1.55, 95%CI 1.04-2.30), *PNPLA3* rs738409 (aHR 1.67, 95%CI 1.27-2.18) and *TM6SF2* rs58542926 (aHR 1.63, 95%CI 1.12-2.39), while the net effect of male gender was protective (aHR 0.49, 95%CI 0.26-0.94).

Conclusions: These findings may help in clinical care to identify individuals with type 2 diabetes at risk of severe liver disease, in turn leading to personalized risk prediction and prevention strategies.

INTRODUCTION

The growing epidemic of diabetes is a serious global public health issue with nearly half a billion individuals living with diabetes and approximately 90% of them suffering from type 2 diabetes [1]. An overwhelming body of evidence supports that type 2 diabetes is a key risk factor for fatty liver disease (FLD) that is the most common chronic liver disease worldwide [2, 3]. The global prevalence of FLD in individuals with type 2 diabetes is more than 2-fold higher than in the general population (55% vs 25%, respectively), with the highest rate reported in Europe (68%) [2, 4].

Strikingly, among FLD comorbidities (e.g. obesity, hypertension and dyslipidemia), type 2 diabetes seems to be the strongest risk factor for the progression of liver disease to its long-term complications, namely cirrhosis and hepatocellular carcinoma, and for mortality [2]. The risk of life-threatening liver-related complications increases with the increase in the number of features of metabolic syndrome [5]. Harmful alcohol consumption is the other major cause of non-viral cirrhosis and hepatocellular carcinoma in Europe and worldwide [6-8]. In addition to the well-established metabolic and environmental risk factors, in recent years common genetic variants in several genes were found to robustly contribute to FLD and the entire spectrum of its complications [9, 10].

Within this context, to identify and closely monitor those who are at risk of progressive liver disease, it will be key to identify drivers and predictors of liver damage and fibrosis in individuals with type 2 diabetes. Björkström et al. have recently examined the contribution of clinical risk factors for developing severe liver disease in a very large cohort of individuals with type 2 diabetes from the Swedish National Diabetes Register [11]. They found older age, male gender, higher body mass index (BMI), hypertension, lower kidney function, microalbuminuria and smoking as independent risk factors while statins were protective against severe liver disease. However, the predictive value of biochemical proxies of liver damage and function was not examined. Moreover, the contribution of human genetics and alcohol use in this context remains to be investigated.

Therefore, aim of this study was to examine the major inborn and acquired independent risk factors contributing to severe liver disease among participants with type 2 diabetes from the prospective UK Biobank study.

METHODS

Study population and data collection

Study design and methods of the UK Biobank have been described in detail previously [12]. Briefly, the UK Biobank is a large prospective cohort study with approximately 500,000 participants aged 40-69 years, recruited between 2006-2010 from 22 assessment centers across the UK. The UK Biobank study has been approved by the North West Multicenter Research Ethics Committee (reference number 11/NW/0382). All participants provided informed consent to the study.

Potential participants were identified from the National Health Service patient registers and invited to attend the local assessment center. At the baseline assessment visit, they completed a touch-screen self-administered questionnaire and a computer-assisted interview regarding medical history, current pharmacological therapy, sociodemographic characteristics, smoking status, alcohol consumption, dietary habits, physical activity and family history of major diseases. Baseline anthropometric measures (e.g. height, weight and waist circumference) were assessed by trained staff using standardized procedures. Blood samples were collected for genome-wide genotyping and biochemical analyses, including glycated hemoglobin (HbA1c) (VARIANT II TURBO Hemoglobin Testing System, Bio-Rad), serum glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin and creatinine (AU5800, Beckman Coulter), and urine albumin (AU5400, Randox Bioscience) and creatinine (AU5400, Beckman Coulter). The protocol for samples collecting, processing and storage was developed using a highly automated and validated approach [13]. Further information about the study protocol and methods is available in the UK Biobank website (https://www.ukbiobank.ac.uk/).

Definition of baseline exclusion criteria

Baseline exclusion criteria were as follows: 1) self-reported history of liver disease; 2) hospital diagnosis of chronic viral hepatitis, severe liver disease (SLD, see definition later in this article) and/or other causes of liver disease occurred before the baseline assessment visit and defined according to the International Classification of Diseases 10th edition (ICD-10 B18, B19, C22.0, E83.0, E83.1, I85.0, I85.9, K70.3, K70.4, K70.9, K71, K72.1, K72.9, K74.1, K74.2, K74.3, K74.4, K74.5, K74.6, K75.2, K75.3, K75.4, K75.8, K75.9, K76.6, K76.7, K76.8, K76.9, R18, Z94.4); 3) self-reported history of liver cancer; 4)

diagnosis of liver cancer based on cancer register occurred before the baseline assessment visit (ICD-10 C22); 5) self-reported non-European ancestry (i.e. all ethnic groups other than white British, white Irish and any other white background); 6) participants with withdrawn consent. A total of 466,783 participants were included for the final analyses. Details of baseline exclusion criteria have been provided in supplementary material (Supplementary Tables S1-S3).

Definition of baseline type 2 diabetes

Baseline previously diagnosed type 2 diabetes was defined by at least one of the following criteria: 1) self-reported history of type 2 or unspecified diabetes; 2) hospital diagnosis of type 2 or unspecified diabetes occurred before the baseline assessment visit (ICD-10 E11, E14); 3) current insulin treatment and/or use of oral hypoglycemic drugs. Among individuals without a prior diagnosis of diabetes, undiagnosed type 2 diabetes was defined by at least one of the following criteria: 1) serum glucose level ≥ 11.1 mmol/L (200 mg/dL); 2) HbA1c ≥ 48 mmol/mol (6.5%). The threshold of 11.1 mmol/L (200 mg/dL) for serum glucose was chosen to avoid false positives, since blood samples were collected not necessarily fasting. The final baseline population included 22,812 participants with type 2 diabetes.

Definition of covariates and comorbidities

Baseline anthropometric measures were assessed by trained staff using standardized procedures. Height and weight were measured using the Seca 202 height measure (Seca, Hamburg, Germany) and the Tanita BC-418 MA body composition analyser (Tanita Europe, Amsterdam, Netherlands), respectively. BMI was calculated by dividing the weight (kg) by the square of the height (m^2). Waist circumference was measured at the umbilicus level using the Wessex non-stretchable sprung tape measure (Wessex, UK).

Socioeconomic status was defined using the Townsend deprivation index [14]. Data on family history of diabetes, smoking, alcohol consumption and physical activity were collected through a baseline touch-screen questionnaire. A positive family history of diabetes was defined as participants who had one or more first-degree relatives (i.e. parents and/or siblings) diagnosed as having diabetes. Smoking status was categorized into two groups: current smoking and never/former smoking. Frequency of daily alcohol

consumption (g/day) was quantified based on the average weekly alcohol intake or on the average monthly alcohol intake (when the average weekly data was missing). Alcohol grams for each type of drink (i.e. red wine, white wine or champagne, beer or cider, spirits, fortified wine and other alcoholic drinks) were derived from the corresponding reference alcohol content reported in the National Health Service UK guidelines (1 unit of alcohol = 8 g of alcohol, https://www.nhs.uk/live-well/alcohol-support/calculating-alcohol-units/. Accessed on January 2020). Excessive alcohol consumption was defined when daily alcohol intake was ≥ 30 g and ≥ 20 g for men and women, respectively [15]. A detailed explanation of alcohol consumption pipeline has been provided in supplementary material. Regarding physical activity, participants were categorized into two groups if they underwent or not physical exercise according to the UK physical activity recommendations (i.e. ≥ 150 minutes/week and ≥ 75 minutes/week for moderate and vigorous physical activity, respectively; UK Biobank data-field 22035).

Estimated glomerular filtration rate (eGFR) was calculated from serum creatinine using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [16]. Albuminuria categories were defined based on a single sample spot urinary albumin-to-creatinine ratio (UACR) (i.e. 3-29 mg/mmol and ≥ 30 mg/mmol for micro- and macro-albuminuria, respectively) [17].

Baseline dyslipidemia was defined as self-reported history of high cholesterol or use of lipid-lowering drugs. Similarly, baseline hypertension was defined as self-reported history of hypertension or use of anti-hypertensive drugs. Baseline cardiovascular disease was defined as self-reported history or hospital diagnosis of angina, myocardial infarction, stroke or transient ischemic attack (ICD-10 I20–I25, I60–I64, I69, G45).

Genotyping

Detailed information about genotyping and arrays used in the UK Biobank study has been provided elsewhere [18]. Genotype data were available for approximately 490,000 participants. *PNPLA3* rs738409 C>G (p.I148M), *TM6SF2* rs58542926 C>T (p.E167K), *MBOAT7* rs641738 C>T, *GCKR* rs1260326 C>T (p.P446L) and *HSD17B13* rs72613567:TA were assayed using two similar genotyping arrays (i.e. Affymetrix UK BiLEVE and UK Biobank Axiom arrays) and coded as 0, 1 or 2 for non-carriers, heterozygous carriers and homozygous carriers of the minor allele, respectively. For

PNPLA3 rs738409, *TM6SF2* rs58542926, *MBOAT7* rs641738 and *GCKR* rs1260326 the minor allele (i.e. G allele, T allele, T allele and T allele, respectively) was the risk-increasing allele, while for *HSD17B13* rs72613567 the minor TA allele had a protective effect [19-23].

Follow-up outcome

Follow-up data on health-related events and mortality were obtained through linkage of the National Health Service records, including in-hospital admissions, death register and cancer register (UK Biobank data-fields 41270, 40001, 40002 and 40006). Detailed information regarding the linkage procedure is available in the UK Biobank website (https://www.ukbiobank.ac.uk/wp-content/uploads/2011/11/UK-Biobank-Protocol.pdf. Accessed on January 2020). The study outcome was incident SLD, defined as a composite diagnosis of cirrhosis, decompensated liver disease (i.e. esophageal varices with or without bleeding, portal hypertension, hepatorenal syndrome, liver failure), hepatocellular carcinoma and/or liver transplantation (ICD-10 C22.0, I85.0, I85.9, K70.3, K70.4, K72.1, K72.9, K74.1, K74.2, K74.6, K76.6, K76.7, Z94.4) in any of the aforementioned records. A list of all the diagnoses used to define SLD is presented in supplementary material (Supplementary Table S3). Among those with SLD, individuals were excluded if they received hospital diagnosis of chronic viral hepatitis or other causes of liver disease (ICD-10 B18, B19, E83.0, E83.1, K71, K74.3, K74.4, K74.5, K75.2, K75.3, K75.4, K75.8, K75.9) before the diagnosis of the outcome of interest. The length of follow-up for each participant was calculated from the date of baseline assessment visit up to the first date of SLD diagnosis, the date of death or the date of end of follow-up for the assessment center attended (31 January 2018), whichever occurred first. The study flowchart has been provided in supplementary material (Supplementary Figure S1).

Statistical analysis

Continuous variables were shown as mean ± standard deviation if normally distributed or median (interquartile range) if skewed. Categorical variables were shown as number (percentage).

In UK Biobank participants with type 2 diabetes, the following risk factors for SLD were tested: age (continuous), gender, family history of diabetes, duration of diabetes

(continuous), hypertension, dyslipidemia, BMI ≥ 30 kg/m^2, waist circumference ≥ 94 cm and ≥ 80 cm (for men and women, respectively [24]), alcohol consumption (complete abstinence, low-moderate intake and excessive intake [i.e. ≥ 30 g/day and ≥ 20 g/day for men and women, respectively [15]]), current smoking status, physical activity ≥ 150 minutes/week and ≥ 75 minutes/week (for moderate and vigorous physical activity, respectively), HbA1c (continuous), ALT > 30 U/L and > 19 U/L (for men and women, respectively [25]), AST > 30 U/L and > 19 U/L (for men and women, respectively), serum albumin (continuous), platelet count (continuous), eGFR < 60 mL/min/1.73 m^2, micro- and macro-albuminuria (i.e. UACR 3-29 mg/mmol and ≥ 30 mg/mmol, respectively [17]), *PNPLA3* rs738409, *TM6SF2* rs58542926, *MBOAT7* rs641738, *GCKR* rs1260326, *HSD17B13* rs72613567, use of oral hypoglycemic drugs, insulin treatment and use of statins. The association of the above-mentioned risk factors with incident SLD was assessed by Cox proportional hazards models, including age, gender, BMI, duration of diabetes, alcohol intake and all predictor variables with a P value < 0.05 in the univariate model. The contribution of genetic factors was estimated by assuming additive or recessive model, separately. Missing data for any of the covariates were removed from the analyses.

Two sensitivity analyses were performed: 1) excluding participants with excessive alcohol consumption at baseline; 2) stratifying by gender.

Cumulative incidence curves were computed using Aalen-Johansen estimator, with mortality and liver diagnoses other than FLD entered as the competing events for SLD and analyzed according to the different genetic variants. Comparisons were carried out by means of the log-rank test.

All analyses were performed using R statistical software, version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Baseline characteristics, genotyping and incidence of severe liver disease

A total of 22,812 participants of European descent from the UK Biobank with type 2 diabetes was included in the analyses (see Supplementary Figure S1 for selection criteria). We defined SLD as diagnosis of cirrhosis and its complications, namely hepatic decompensation, hepatocellular carcinoma and liver transplantation. The baseline characteristics of the study participants stratified by incident SLD status are shown in Table 1 (see Supplementary Table S4 for baseline characteristics of the entire UK Biobank population stratified by type 2 diabetes status). In the overall cohort, two out of three participants were men and approximately half of them had a positive family history of diabetes. The mean ± standard deviation age was 60.1 ± 7 years and BMI was 31.6 ± 5.9 kg/m^2, indicating that a large number of individuals with type 2 diabetes are obese and overweight. The median (interquartile range) duration of diabetes was 4.5 (2.5-9.4) years while HbA1c was 50.3 (43.2-59.7) mmol/mol.

Individuals with development of SLD during follow-up were older, had higher BMI and waist circumference, higher alcohol intake, higher transaminases, lower serum albumin, lower platelet count and higher prevalence of cardiovascular disease compared to those without. Moreover, they were more likely to be treated with metformin, thiazolidinediones and sulfonylureas. There were no differences in glycemic control, duration of diabetes, family history of diabetes and use of insulin therapy between the two groups.

Minor allele frequencies (MAF) of known genetic variants associated with SLD in the general population were consistent with previous reports in Europeans [19, 21, 23, 26, 27] and genotype frequency distribution of these variants was in Hardy-Weinberg Equilibrium. Genotype frequency stratified by incident SLD status is shown in Table 2. As expected, there was an enrichment of the minor allele for the *PNPLA3* rs738409 and the *TM6SF2* rs58542926 variants in individuals who developed SLD compared to those without, while the *HSD17B13* rs72613567 variant was less common in this group.

During a median (interquartile range) follow-up of 8.9 (8.1-9.6) years, there were 279 individuals with SLD, including 255 with cirrhosis and/or decompensated liver disease, 47 with hepatocellular carcinoma and 5 that underwent liver transplantation; death from SLD occurred in 83 individuals.

Cumulative incidence of SLD for the different genetic variants across genotypes in the entire cohort is displayed in Figure 1.

Risk factors for severe liver disease

Risk factors independently associated with increased risk of incident SLD in European participants with type 2 diabetes are shown in Table 3 and included: AST > 30/19 U/L (adjusted hazard ratio [HR] 4.85, 95% confidence interval [CI] 2.76-8.54), decrease in serum albumin (adjusted HR 2.39, 95% CI 1.76-3.24) and platelet count (adjusted HR 1.12, 95% CI 1.09-1.16), cardiovascular disease (adjusted HR 1.86, 95% CI 1.23-2.79), microalbuminuria (adjusted HR 1.55, 95% CI 1.04-2.30), *PNPLA3* rs738409 (adjusted HR 1.67, 95% CI 1.27-2.18 and adjusted HR 2.32, 95% CI 1.36-3.96 for additive and recessive model, respectively) and *TM6SF2* rs58542926 (adjusted HR 1.63, 95% CI 1.12-2.39 and adjusted HR 4.33, 95% CI 1.74-10.80 for additive and recessive model, respectively), while the net effect of male gender was protective (adjusted HR 0.49, 95% CI 0.26-0.94). In sensitivity analyses, after excluding participants with excessive alcohol consumption and stratifying by gender, results were substantially similar to the main model except that in women higher BMI was positively correlated with SLD (adjusted HR 1.41, 95% CI 1.03-1.93) (Supplementary Tables S5-S6).

Table 1. Baseline characteristics of UK Biobank participants of European descent with type 2 diabetes stratified by incident sever liver disease status

	Total (n = 22,812)	No Severe Liver Disease (n = 22,533)	Severe Liver Disease (n = 279)	P value
Age, years	60.1 ± 7	60.1 ± 7	61.5 ± 6.2	0.002
Men, n (%)	14,273 (63%)	14,066 (62%)	207 (74%)	<0.001
Townsend deprivation index	-1.5 (-3.3-1.7)	-1.5 (-3.3-1.7)	-0.6 (-2.8-2.4)	<0.001
Family history of diabetes, n (%)	9,692 (43%)	9,591 (43%)	101 (37%)	0.11
Duration of diabetes, years	4.5 (2.5-9.4)	4.5 (2.5-9.4)	6.3 (2.5-9.5)	0.49
BMI, kg/m^2	31.6 ± 5.9	31.6 ± 5.9	33.7 ± 6	<0.001
Waist circumference, cm	103.4 ± 14.6	103.4 ± 14.6	110.3 ± 13.7	<0.001
Lifestyle				
Current smoking, n (%)	2,505 (11%)	2,472 (11%)	33 (12%)	0.61
Alcohol intake, g/day	5.8 (0-19.4)	5.7 (0-19.4)	9.7 (0-30.9)	<0.001
Alcohol intake ≥30/20 g/day, n (%)	3,566 (16%)	3,491 (15%)	75 (27%)	<0.001
Physical activity ≥ 150/75 min/week, n (%)	7,980 (45%)	7,883 (45%)	97 (45%)	0.99
Clinical chemistry				
HbA1c, mmol/mol	50.3 (43.2-59.7)	50.3 (43.2-59.7)	50 (42.5-60.1)	0.59
ALT, U/L	25.3 (18.8-34.7)	25.2 (18.7-34.5)	36.4 (26-53.5)	<0.001
AST, U/L	25.2 (21-31.1)	25.1 (21-30.9)	41.8 (29.5-57.7)	<0.001
Albumin, g/dL	4.5 ± 0.3	4.5 ± 0.3	4.3 ± 0.3	<0.001
Platelet count, $*10^9/L$	247.4 ± 65	248 ± 64.7	198.2 ± 71	<0.001
eGFR, mL/min/1.73 m^2	88.3 ± 16.5	88.3 ± 16.5	87.5 ± 19	0.48
Microalbuminuria, n (%)	3,110 (27%)	3,052 (26%)	58 (37%)	0.004
Macroalbuminuria, n (%)	488 (4%)	480 (4%)	8 (5%)	0.64
Comorbidities				
Hypertension, n (%)	16,958 (74%)	16,734 (74%)	224 (80%)	0.18
Dyslipidemia, n (%)	18,213 (80%)	17,996 (80%)	217 (78%)	0.082
Cardiovascular disease, n (%)	5,068 (22%)	4,972 (22%)	96 (34%)	<0.001
Drugs				
Metformin, n (%)	12,278 (54%)	12,105 (54%)	173 (62%)	0.005
Thiazolidinediones, n (%)	1,726 (8%)	1,695 (8%)	31 (11%)	0.042
Sulfonylureas, n (%)	4,584 (20%)	4,512 (20%)	72 (26%)	0.043
Insulin, n (%)	4,530 (20%)	4,483 (20%)	47 (17%)	0.46
Statins, n (%)	16,500 (72%)	16,304 (72%)	196 (70%)	0.13

Continuous variables are shown as mean ± SD or median and (IQR) if normally distributed or skewed, respectively. Categorical variables are shown as number and (proportion).
P values are from generalized linear models adjusted for age, gender and assessment center.

P values <0.05 were considered statistically significant.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin.

Table 2. Genotype frequency of *PNPLA3* rs738409, *TM6SF2* rs58542926, *MBOAT7* rs641738, *GCKR* rs1260326 and *HSD17B13* rs72613567 in UK Biobank participants of European descent with type 2 diabetes stratified by incident severe liver disease status

	Total (n = 22,812)	No Severe Liver Disease (n =22,533)	Severe Liver Disease (n = 279)	P value
PNPLA3 rs738409				
CC, n (%)	13,436 (61%)†	13,318 (61%)	118 (45%)	
CG, n (%)	7,540 (34%)	7,435 (34%)	105 (40%)	<0.001
GG, n (%)	1,133 (5%)	1,092 (5%)	41 (15%)	
TM6SF2 rs58542926				
CC, n (%)	18,626 (84%)†	18,425 (84%)	201 (76%)	
CT, n (%)	3,274 (15%)	3,219 (15%)	55 (21%)	<0.001
TT, n (%)	167 (1%)	160 (1%)	7 (3%)	
MBOAT7 rs641738				
CC, n (%)	6,869 (31%)†	6,797 (31%)	72 (28%)	
CT, n (%)	10,765 (49%)	10,629 (49%)	136 (52%)	0.30
TT, n (%)	4,279 (20%)	4,226 (20%)	53 (20%)	
GCKR rs1260326				
CC, n (%)	8,556 (39%)†	8,455 (39%)	101 (38%)	
CT, n (%)	10,320 (47%)	10,200 (47%)	120 (46%)	0.61
TT, n (%)	3,150 (14%)	3,109 (14%)	41 (16%)	
HSD17B13 rs72613567				
T/T, n (%)	11,525 (52%)†	11,365 (52%)	160 (61%)	
T/TA, n (%)	8,770 (40%)	8,682 (40%)	88 (34%)	0.004
TA/TA, n (%)	1,738 (8%)	1,724 (8%)	14 (5%)	

P values are from generalized linear models adjusted for age, gender and assessment center.
P values <0.05 were considered statistically significant.

†Genotype distribution is in Hardy-Weinberg equilibrium.

Table 3. Risk factors for severe liver disease in UK Biobank participants of European descent with type 2 diabetes (n = 22,812)

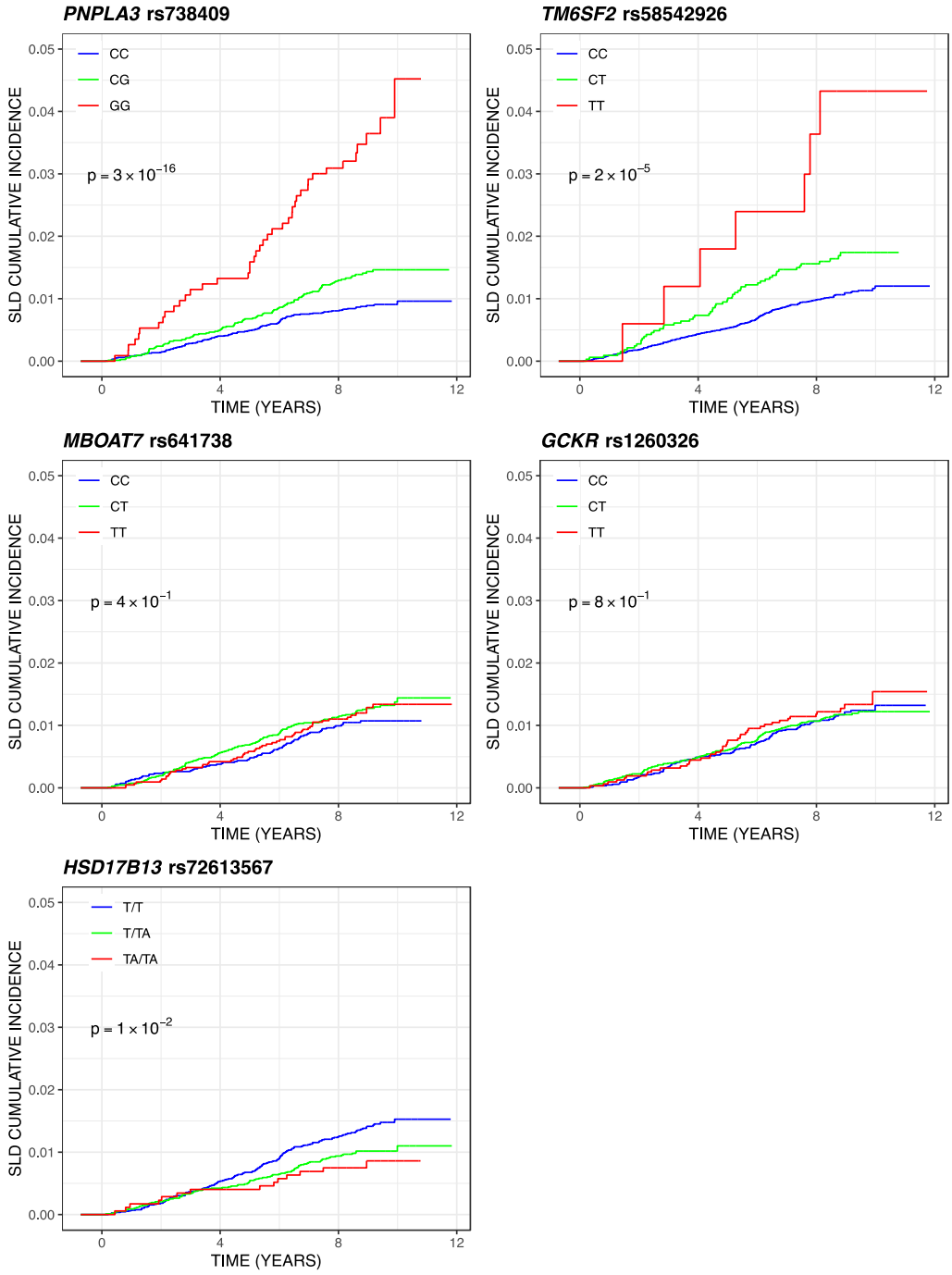
Variable	HR (95% CI)	P value	aHR (95% CI)	P value
Age, years	1.03 (1.02-1.05)	<0.001	1.01 (0.98-1.05)	0.39
Male gender	1.76 (1.35-2.31)	<0.001	0.49 (0.26-0.94)	0.031
Family history of diabetes	0.76 (0.60-0.98)	0.031	0.74 (0.50-1.10)	0.13
Duration of diabetes, years	1.01 (0.99-1.03)	0.18	1.00 (0.97-1.03)	0.97
Comorbidities				
Hypertension	1.44 (1.07-1.93)	0.016	0.70 (0.42-1.18)	0.18
Dyslipidemia	0.89 (0.67-1.18)	0.42		
BMI ≥ 30 kg/m^2	1.84 (1.42-2.38)	<0.001	1.02 (0.64-1.64)	0.93
Waist circumference ≥ 94/80 cm	2.80 (1.69-4.64)	<0.001	1.19 (0.49-2.92)	0.70
Cardiovascular disease	1.94 (1.52-2.49)	<0.001	1.86 (1.23-2.79)	0.003
Lifestyle				
Low-moderate alcohol intake*	0.71 (0.53-0.94)	0.018	0.80 (0.49-1.31)	0.38
Excessive alcohol intake*	1.63 (1.19-2.23)	0.003	1.59 (0.93-2.71)	0.091
Current smoking status	1.13 (0.79-1.63)	0.51		
Physical activity ≥ 150/75 min/week	1.00 (0.83-1.21)	0.99		
Clinical chemistry				
HbA1c, mmol/mol	1.00 (0.99-1.01)	0.74		
ALT > 30/19 U/L	2.26 (1.76-2.90)	<0.001	1.37 (0.83-2.24)	0.22
AST > 30/19 U/L	5.18 (4.09-6.55)	<0.001	4.85 (2.76-8.54)	<0.001
Albumin, per 0.5 g/dL decrease	2.45 (2.01-2.99)	<0.001	2.39 (1.76-3.24)	<0.001
Platelet count, per 10*10^9/L decrease	1.16 (1.13-1.18)	<0.001	1.12 (1.09-1.16)	<0.001
eGFR < 60 mL/min/1.73 m^2	1.68 (1.12-2.51)	0.012	1.22 (0.66-2.25)	0.52
Microalbuminuria	1.70 (1.23-2.35)	0.001	1.55 (1.04-2.30)	0.03
Macroalbuminuria	1.37 (0.67-2.79)	0.38		
Genetic risk factors				
PNPLA3 rs738409 genotype				
Additive model	1.92 (1.61-2.30)	<0.001	1.67 (1.27-2.18)	<0.001
Recessive model	3.47 (2.49-4.84)	<0.001	2.32 (1.36-3.96)†	0.002
TM6SF2 rs58542926 genotype				
Additive model	1.69 (1.32-2.17)	<0.001	1.63 (1.12-2.39)	0.011
Recessive model	3.59 (1.70-7.62)	<0.001	4.33 (1.74-10.80)†	0.002
MBOAT7 rs641738 genotype				
Additive model	1.09 (0.92-1.30)	0.30		
Recessive model	1.05 (0.78-1.42)	0.75		
GCKR rs1260326 genotype				
Additive model	1.04 (0.87-1.24)	0.67		
Recessive model	1.12 (0.80-1.56)	0.52		
HSD17B13 rs72613567 genotype				

Additive model	0.74 (0.60-0.91)	0.004	0.74 (0.54-1.03)	0.076
Recessive model	0.66 (0.38-1.13)	0.13		
Drugs				
Metformin	1.40 (1.10-1.78)	0.006	1.46 (0.96-2.23)	0.076
Thiazolidinediones	1.51 (1.04-2.20)	0.03	1.48 (0.82-2.67)	0.19
Sulfonylureas	1.40 (1.07-1.83)	0.014	1.32 (0.87-1.99)	0.19
Insulin	0.83 (0.61-1.14)	0.26		
Statins	0.91 (0.70-1.17)	0.45		

HRs with 95% CIs were calculated by Cox proportional hazards models.
Age, gender, BMI, alcohol intake, duration of diabetes and all predictor variables with a P value < 0.05 in the univariate model were included in the multivariate model.
*Low-moderate (<20/30 g/day) and excessive (≥20/30 g/day) alcohol intake tested against abstainers.
†aHR calculated assuming recessive model instead of additive model.
Abbreviations: aHR, adjusted HR; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HR, hazard ratio.

Figure 1. Cumulative incidence of severe liver disease for *PNPLA3* rs738409, *TM6SF2* rs58542926, *MBOAT7* rs641738, *GCKR* rs1260326 and *HSD17B13* rs7261356 across genotypes in the entire cohort with type 2 diabetes Blue, green and red lines represent non-carriers, heterozygous carriers and homozygous carriers of the minor allele, respectively. P values are from log-rank test for trend.

Abbreviations: SLD, severe liver disease.



DISCUSSION

In this work we investigate for the first time in the UK Biobank the acquired and inborn independent risk factors for SLD among Europeans with type 2 diabetes. Among the acquired, we demonstrate that abnormal AST levels, decrease in serum albumin and platelet count, cardiovascular disease and microalbuminuria are independent markers of SLD. Among the inborn, genetic variants in *PNPLA3* and *TM6SF2* genes increase the risk of SLD in this population.

In our analyses, we started by: a) selecting individuals with type 2 diabetes as those with diagnosis of type 2 or unspecified diabetes and/or self-reported history of these conditions and b) excluding at baseline those with diagnosis of all major causes of liver disease and liver cancer and/or self-reported history of these conditions. Then, we prospectively examined the incidence of SLD, defined as a composite diagnosis of severe chronic liver disease including non-viral cirrhosis, decompensated liver disease, hepatocellular carcinoma and liver transplantation.

Our results provide several clues regarding the risk prediction of SLD in individuals with type 2 diabetes. Indeed, biochemical proxies of hepatocellular damage, i.e. transaminases, are the strongest predictor of SLD with an elevation above the upper normal limit associated with an approximately five-fold increased risk. Interestingly, abnormal AST seems to predict adverse liver outcomes more accurately than abnormal ALT. High AST levels may indicate mitochondrial damage due to alcohol abuse and they correlate with liver fibrosis better than ALT [28]. As a result, transaminases remain, also in individuals with type 2 diabetes, a main screening test to define the risk level of developing life-threatening liver-related complications. However, it should be born in mind that chronic liver damage and advanced fibrosis may develop even with normal liver enzymes [29].

Biochemical proxies of reduced liver function (low albumin) and portal hypertension (low platelet count) are strong markers of SLD in individuals with type 2 diabetes. This is likely due to the fact that they mirror the presence of an underlying and unknown advanced liver disease. Consistent with the present study, where we have excluded at baseline only those with self-reported or diagnosed SLD, FLD may progress to advanced fibrosis without having being diagnosed [30, 31]. This may suggest that, during follow-up of individuals with type 2 diabetes, special attention is required towards lowering of

platelet count and albumin levels. Indeed, in diabetic individuals low albumin levels may be more frequently attributed to proteinuria due to advanced chronic kidney disease.

Excess in alcohol intake is well-known to cause liver damage and to exacerbate liver injury induced by other causes. Moreover, it is also associated with development of type 2 diabetes and with worse glycemic control [32]. Here we find an almost doubling of the risk of SLD in individuals with type 2 diabetes and excessive alcohol consumption even if not statistically significant, which is consistent with the risk observed in the general population [33]. Notably, low-moderate alcohol intake appears not to increase the risk of SLD. Future studies are warranted to prove if a complete abstinence is not required to prevent liver disease progression among individuals with type 2 diabetes.

Cardiovascular disease represents the first cause of death in individuals suffering from FLD [5]. Consistently, cardiovascular disease resulted as a strong risk factor for developing SLD in our cohort, as well as the presence of microalbuminuria. These data support the notion that individuals with type 2 diabetes and cardiovascular/kidney complications should be screened for liver disease. Taking all this together, liver disease may be considered among diabetes-related complications.

Unfavorable genetics is a robust independent risk factor for SLD. Of note, this is the first prospective study specifically evaluating the impact of genetic risk variants on the risk of developing advanced liver disease in individuals of European descent with type 2 diabetes. In particular, our data demonstrate that *PNPLA3* rs738409 and *TM6SF2* rs58542926, the two strongest genetic variants increasing the risk of SLD in the general population [19, 20], confer also a strong susceptibility to SLD in those with type 2 diabetes. Notably, unlike traditional risk factors that may vary over time, the risk conferred by genetic variants is very stable and constitutes a lifetime burden. As a consequence, a genetic testing for *PNPLA3* rs738409 and *TM6SF2* rs58542926 might be useful to identify individuals with type 2 diabetes at high-risk for progressive liver disease, thus requiring more intensive follow-up strategies or specific lifestyle changes (e.g. reduction in alcohol and fructose intake).

Surprisingly, increased BMI was not associated with increase in the risk of SLD in the overall cohort. This may be due to the fact that the mean BMI of the cohort was in the range of class I obesity and the net contribution of obesity to SLD is likely diluted by the absence of normal weight individuals. Notably, obesity had a greater impact on SLD risk

in women than in men, supporting the presence of sexual dysmorphism underlying human disease. Indeed, this could be due to the complex interaction between genetic factors, gender and its related biological components. In agreement, data in literature report a stronger association between increased BMI and diabetes risk in women than in men [34]. However, tailored studies with larger sample size, specifically focusing on gender differences in liver disease, are needed to ascertain this issue.

To date, there is a lack of prospective cohort studies evaluating the contribution of multiple risk factors for advanced liver disease in type 2 diabetes. In a large prospective cohort study of over 400,000 Swedish subjects with type 2 diabetes, Björkström et al. have recently shown that risk factors independently associated with SLD were older age, male gender, higher BMI, hypertension, lower eGFR, microalbuminuria and smoking, while use of statins conferred decreased risk [11]. However, in this study the contribution of biochemical proxies of liver function and damage, alcohol consumption and genetic variations was not investigated.

We confirm the role of microalbuminuria as independent risk factor for SLD. Additionally, we show that microalbuminuria is correlated with increased risk of SLD especially in men, in line with the well-documented higher frequency of albuminuric renal impairment in men with type 2 diabetes compared to women [35]. Notwithstanding the significantly higher incidence of SLD in men, we find that male gender is protective against SLD. This is likely because we included the main mediators of the association between male gender and SLD in the multivariate Cox regression analysis. Further studies specifically aimed at evaluating the effect of gender on the risk of SLD are required to verify this finding. We do not find age, high BMI, hypertension, low eGFR and smoking associated with SLD. These results might be explained by the fact that we included in the multivariate model additional stronger risk factors for SLD, such as biochemical proxies of liver damage, harmful alcohol consumption and genetic variants. Alternatively, these data might be due to the different category of variables included in the multivariate model (binary vs continuous) or to the relatively lower sample size of our study compared to that by Björkström et al.

Another major difference between the study by Björkström et al. and our study is that we included alcoholic-related diagnoses in the definition of SLD. This is because metabolic and alcoholic liver disease share similar molecular pathways [10, 19-21, 27, 36] and also

because a clear distinction is often difficult to assess in the real life. Moreover, the sensitivity analysis excluding participants with excessive alcohol consumption showed similar results to the main model.

Additionally, in both studies HbA1c was found to be not related to increased risk of SLD. Since the well-established association of HbA1c with higher risk of chronic micro- and macro-vascular complications of type 2 diabetes [37], this finding might be explained by the fact that HbA1c does not accurately reflect glycemic control in individuals with diabetes and cirrhosis [38-40].

The major strengths of our study are the following: 1) the large sample size, including more than 20,000 individuals with type 2 diabetes from the general population; 2) the prospective study design; 3) the use of standardized procedures and centrally validated protocol for blood samples collecting, processing and storage of the UK Biobank. Furthermore, this is the first prospective study collectively examining the impact of indices of liver function and damage, alcohol consumption and genetic variants on the risk of developing SLD in European individuals with type 2 diabetes.

This study also has limitations. First, some cases of asymptomatic liver disease (e.g. compensated cirrhosis or early stages of hepatocellular carcinoma) may have been underdiagnosed. Similarly, some cases of chronic viral hepatitis may be unknown or sometimes the viral etiology of cirrhosis may be not specified in hospital records. However, we tried to reduce this bias by linking liver-related diagnoses from multiple registers (i.e. hospital records, death register and cancer register). Second, we included participants with previous history and/or hospital diagnosis of unspecified diabetes, those treated with insulin and those with undiagnosed diabetes based on circulating glucose and/or HbA1c tests. As a result, although type 2 diabetes accounts for approximately 90% of diabetes cases [1], few participants may be affected by type 1 diabetes or latent autoimmune diabetes of adulthood. Third, since we had only one baseline urine sample to establish albuminuria categories, the diagnosis of moderately and severely increased albuminuria could be overestimated, although an Italian multicenter prospective cohort study of over 15,000 subjects with type 2 diabetes reported similar rates [41]. Finally, results were obtained in Europeans and further studies are needed to validate them in other ethnic groups.

In conclusion, we demonstrate that in Europeans with type 2 diabetes: a) abnormal AST levels, decrease in serum albumin and platelet count, cardiovascular disease and microalbuminuria are independent markers of SLD; b) genetic variants in *PNPLA3* and *TM6SF2* genes increase the risk of SLD. Our findings may help to identify individuals with type 2 diabetes at-risk for SLD by identifying acquired and inborn risk factors. This may contribute to estimate a personalized risk prediction and to implement strategies to prevent SLD.

SUPPLEMENTARY MATERIAL

Daily alcohol consumption extraction pipeline

To generate a continuous variable for alcohol consumption rate in UK Biobank, the following data-fields only at baseline (initial assessment visit (2006-2010), i.e. instance 0) were employed:

1. Alcohol intake frequency (data-field 1558)

2. Average weekly alcohol consumption intake:
   2.1. Average weekly red wine intake (data-field 1568)
   2.2. Average weekly champagne plus white wine intake (data-field 1578)
   2.3. Average weekly beer plus cider intake (data-field 1588)
   2.4. Average weekly spirits intake (data-field 1598)
   2.5. Average weekly fortified wine intake (data-field 1608)
   2.6. Average weekly intake of other alcoholic drinks (data-field 5364)

3. Average monthly alcohol consumption intake:
   3.1. Average monthly red wine intake (data-field 4407)
   3.2. Average monthly champagne plus white wine intake (data-field 4418)
   3.3. Average monthly beer plus cider intake (data-field 4429)
   3.4. Average monthly spirits intake (data-field 4440)
   3.5. Average monthly fortified wine intake (data-field 4451)
   3.6. Average monthly intake of other alcoholic drinks (data-field 4462)

Below are the steps used for this purpose:

1. Average weekly alcohol consumption intake was preferentially used and when data was missing (unknown, ‘do not know’ or ‘prefer not to answer’), average monthly alcohol consumption intake was used. The following table was used to convert the extracted values to grams per day (after dividing by 7 and 30.4375, for weekly and monthly data, respectively). Alcohol consumption guidelines from United Kingdom National Health Service were used to derive the Table below (United Kingdom

National Health Service. Alcohol units. https://www.nhs.uk/live- well/alcohol-support/calculating-alcohol-units/. Accessed on January 2020).

Type of drink	**Alcohol units**	**Alcohol grams**
Red wine, 1 glass	1.5	12
White wine or champagne, 1 glass	1.5	12
Beer/cider, 1 pint	2	16
Spirits, 1 measure	1	8
Fortified wine, 1 glass	1.5	12
Other alcoholic drinks, 1 glass	1.5	12

2. Missing values for individuals with alcohol intake frequency of "Never" (N = 40,641) or "Special occasions only" (N = 36,820), were replaced with 0 g/day (https://www.nature.com/articles/s41467-019-12424-x; https://doi.org/10.1093/ije/dyz064).

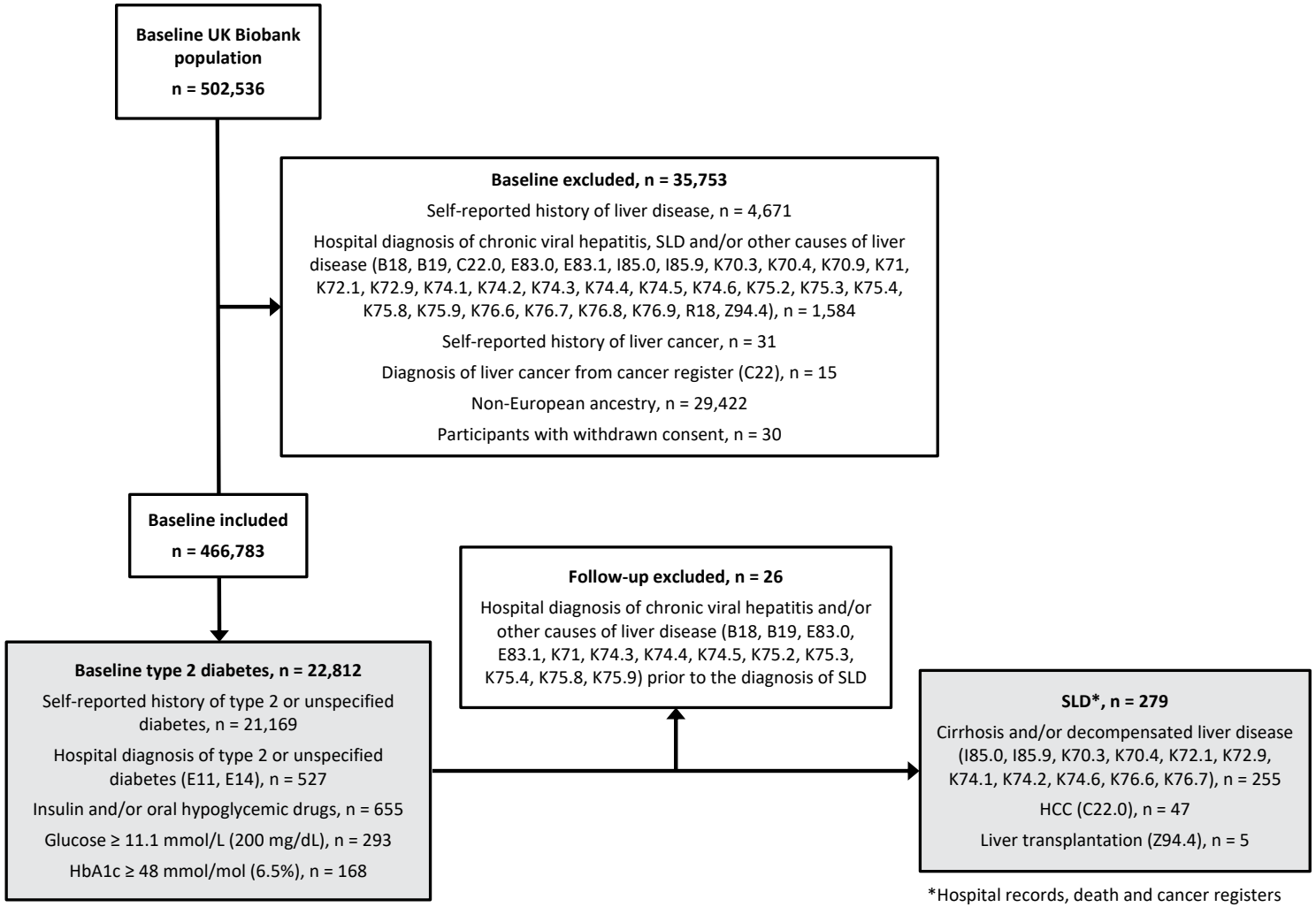
3. Individuals who reported an alcohol intake frequency of "Once or twice a week", "Daily or almost daily", "Three or four times a week", but reported 0 g/day of alcohol intake (for all types of alcoholic drinks) were excluded (N = 1,044, 365 and 340, respectively).

4. Individuals with an alcohol consumption value deviating from 5 s.d. from their gender-specific mean were excluded. For women and men these values were 70.86 and 127.76 g/day, respectively (N = 930 and 610, for women and men, respectively) (https://www.nature.com/articles/mp2017153).

5. For participants who had unknown g/day of alcohol but who reported one of these categories of overall alcohol intake frequency: "Once or twice a week" (N = 66), "One to three times a month" (N = 36,318), "Daily or almost daily" (N = 46), "Three or four times a week" (N = 35), the median value (g/day) from their category was assigned (https://doi.org/10.1093/ije/dyz064).

6. Finally, all the remaining individuals with missing values were excluded and the continuous variable of alcohol consumption g/day was created (N = 497,717).

Supplementary Figure S1. Study flowchart HbA1c, glycated hemoglobin; HCC, hepatocellular carcinoma; SLD, severe liver disease.



Supplementary Table S1. Definition of self-reported history of liver disease (data-field 20002)

Code	Description
1136	liver/biliary/pancreas problem
1141	oesophageal varices
1155	hepatitis
1156	infective/viral hepatitis
1157	non-infective hepatitis
1158	liver failure/cirrhosis
1159	bile duct disease
1408	alcohol dependency
1506	primary biliary cirrhosis
1507	haemochromatosis
1578	hepatitis a
1579	hepatitis b
1580	hepatitis c
1581	hepatitis d
1582	hepatitis e
1604	alcoholic liver disease / alcoholic cirrhosis

Supplementary Table S2. ICD-10 codes used to define baseline liver disease other than severe liver disease

Diagnosis	ICD-10
Viral hepatitis, chronic	B18
Viral hepatitis, unspecified	B19
Disorders of copper metabolism	E83.0
Disorders of iron metabolism	E83.1
Alcoholic liver disease, unspecified	K70.9
Toxic liver disease	K71
Primary biliary cirrhosis	K74.3
Secondary biliary cirrhosis	K74.4
Biliary cirrhosis, unspecified	K74.5
Nonspecific reactive hepatitis	K75.2
Granulomatous hepatitis, not elsewhere classified	K75.3
Autoimmune hepatitis	K75.4
Other specified inflammatory liver diseases	K75.8
Inflammatory liver disease, unspecified	K75.9
Other specified diseases of liver	K76.8
Liver disease, unspecified	K76.9
Ascites	R18

Supplementary Table S3. ICD-10 codes used to define severe liver disease endpoint

Diagnosis	ICD-10
Hepatocellular carcinoma	C22.0
Esophageal varices, bleeding	I85.0
Esophageal varices, not bleeding	I85.9
Alcoholic liver cirrhosis	K70.3
Alcoholic liver failure	K70.4
Liver failure, chronic	K72.1
Liver failure, unspecified	K72.9
Hepatic sclerosis	K74.1
Hepatic fibrosis with hepatic sclerosis	K74.2
Liver cirrhosis, other and unspecified	K74.6
Portal hypertension	K76.6
Hepatorenal syndrome	K76.7
Liver transplant status	Z94.4

Supplementary Table S4. Baseline characteristics of the overall UK Biobank cohort stratified by type 2 diabetes status

	Total (n = 466,783)	No Type 2 Diabetes (n = 443,971)	Type 2 Diabetes (n = 22,812)	P value
Age, years	56.7 ± 8	56.6 ± 8	60.1 ± 7	<0.001
Men, n (%)	212,105 (45%)	197,832 (45%)	14,273 (63%)	<0.001
Townsend deprivation index	-2.3 (-3.7-0.3)	-2.3 (-3.7-0.2)	-1.5 (-3.3-1.7)	<0.001
Family history of diabetes, n (%)	98,463 (21%)	88,771 (20%)	9,692 (43%)	<0.001
BMI, kg/m^2	27.4 ± 4.8	27.2 ± 4.6	31.6 ± 5.9	<0.001
Waist circumference, cm	90.2 ± 13.5	89.6 ± 13.1	103.4 ± 14.6	<0.001
Lifestyle				
Current smoking, n (%)	48,330 (10%)	45,825 (10%)	2,505 (11%)	<0.001
Alcohol intake, g/day	10.3 (2-21.1)	10.3 (2-21.1)	5.8 (0-19.4)	<0.001
Alcohol intake ≥30/20 g/day, n (%)	91,902 (20%)	88,336 (20%)	3,566 (16%)	<0.001
Physical activity ≥ 150/75 min/week, n (%)	204,514 (54%)	196,534 (55%)	7,980 (45%)	<0.001
Clinical chemistry				
HbA1c, mmol/mol	35.1 (32.7-37.7)	35 (32.6-37.3)	50.3 (43.2-59.7)	<0.001
ALT, U/L	20.1 (15.4-27.3)	19.9 (15.3-26.9)	25.3 (18.8-34.7)	<0.001
AST, U/L	24.3 (21-28.8)	24.3 (21-28.7)	25.2 (21-31.1)	<0.001
Albumin, g/dL	4.5 ± 0.3	4.5 ± 0.3	4.5 ± 0.3	<0.001
Platelet count, *10^9/L	253.4 ± 59.7	253.7 ± 59.4	247.4 ± 65	0.30
eGFR, mL/min/1.73 m^2	90.5 ± 13.2	90.7 ± 13	88.3 ± 16.5	<0.001
Microalbuminuria, n (%)	20,906 (15%)	17,796 (14%)	3,110 (27%)	<0.001
Macroalbuminuria, n (%)	1,927 (1%)	1,439 (1%)	488 (4%)	<0.001
Comorbidities				
Hypertension, n (%)	138,878 (30%)	121,920 (27%)	16,958 (74%)	<0.001
Dyslipidemia, n (%)	101,914 (22%)	83,701 (19%)	18,213 (80%)	<0.001
Cardiovascular disease, n (%)	32,000 (7%)	26,932 (6%)	5,068 (22%)	<0.001
Incident severe liver disease, n (%)	1,336 (0.29%)	1,057 (0.24%)	279 (1.22%)	<0.001
Follow-up time, years	9 (8.3-9.7)	9 (8.3-9.7)	8.9 (8.1-9.6)	<0.001

Continuous variables are shown as mean ± SD or median and (IQR) if normally distributed or skewed, respectively. Categorical variables are shown as number and (proportion).
P values are from generalized linear models adjusted for age, gender and assessment center.
P values <0.05 were considered statistically significant.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin.

Supplementary Table S5. Risk factors for severe liver disease in UK Biobank participants of European descent with type 2 diabetes excluding those with excessive alcohol consumption at baseline (n = 19,246)

Variable	HR (95% CI)	P value	aHR (95% CI)	P value
Age, years	1.03 (1.01-1.05)	0.007	1.01 (0.98-1.05)	0.44
Male gender	1.49 (1.11-2.00)	0.008	0.37 (0.18-0.76)	0.007
Family history of diabetes	0.83 (0.62-1.10)	0.19		
Duration of diabetes, years	1.01 (0.99-1.04)	0.28	1.00 (0.96-1.03)	0.85
Comorbidities				
Hypertension	1.42 (1.01-2.00)	0.044	0.60 (0.33-1.08)	0.087
Dyslipidemia	0.90 (0.65-1.25)	0.53		
BMI ≥ 30 kg/m^2	1.74 (1.29-2.34)	<0.001	1.09 (0.62-1.91)	0.77
Waist circumference ≥ 94/80 cm	2.47 (1.41-4.34)	0.002	0.96 (0.38-2.43)	0.93
Cardiovascular disease	1.88 (1.41-2.50)	<0.001	1.96 (1.21-3.18)	0.006
Lifestyle				
Current smoking status	0.90 (0.56-1.45)	0.68		
Physical activity ≥ 150/75 min/week	1.02 (0.82-1.28)	0.84		
Clinical chemistry				
HbA1c, mmol/mol	1.00 (0.99-1.01)	0.98		
ALT > 30/19 U/L	2.22 (1.66-2.97)	<0.001	1.13 (0.64-1.98)	0.68
AST > 30/19 U/L	4.85 (3.68-6.38)	<0.001	7.74 (3.93-15.26)	<0.001
Albumin, per 0.5 g/dL decrease	1.08 (1.06-1.11)	<0.001	1.07 (1.03-1.11)	<0.001
Platelet count, per 10*10^9/L decrease	1.13 (1.10-1.16)	<0.001	1.10 (1.06-1.15)	<0.001
eGFR < 60 mL/min/1.73 m^2	1.95 (1.26-3.01)	0.003	1.78 (0.95-3.33)	0.07
Microalbuminuria	1.82 (1.24-2.67)	0.002	1.77 (1.11-2.82)	0.017
Macroalbuminuria	1.45 (0.64-3.31)	0.37		
Genetic risk factors				
PNPLA3 rs738409 genotype				
Additive model	1.79 (1.45-2.21)	<0.001	1.66 (1.19-2.30)	0.003
Recessive model	3.00 (1.98-4.53)	<0.001	2.20 (1.12-4.32)†	0.022
TM6SF2 rs58542926 genotype				
Additive model	1.54 (1.14-2.09)	0.006	1.54 (0.96-2.49)	0.075
Recessive model	2.06 (0.66-6.45)	0.21		
MBOAT7 rs641738 genotype				
Additive model	1.05 (0.86-1.29)	0.61		
Recessive model	0.91 (0.63-1.31)	0.61		
GCKR rs1260326 genotype				
Additive model	1.03 (0.84-1.27)	0.75		
Recessive model	1.10 (0.75-1.63)	0.63		
HSD17B13 rs72613567 genotype				

Additive model	0.78 (0.61-0.99)	0.038	0.60 (0.40-0.91)	0.016
Recessive model	0.52 (0.26-1.06)	0.073		
Drugs				
Metformin	1.26 (0.95-1.67)	0.11		
Thiazolidinediones	1.46 (0.95-2.25)	0.087		
Sulfonylureas	1.36 (0.99-1.85)	0.056		
Insulin	0.97 (0.69-1.37)	0.87		
Statins	0.97 (0.72-1.32)	0.86		

HRs with 95% CIs were calculated by Cox proportional hazards models.
Age, gender, BMI, duration of diabetes and all predictor variables with a P value < 0.05 in the univariate model were included in the multivariate model.

†aHR calculated assuming recessive model instead of additive model.

Abbreviations: aHR, adjusted HR; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HR, hazard ratio.

Supplementary Table S6. Risk factors for severe liver disease in UK Biobank participants of European descent with type 2 diabetes stratified by gender

	Men (n = 14,273)				Women (n = 8,539)			
Variable	**HR (95% CI)**	**P value**	**aHR (95% CI)**	**P value**	**HR (95% CI)**	**P value**	**aHR (95% CI)**	**P value**
Age, years	1.03 (1.01-1.05)	0.007	1.02 (0.99-1.05)	0.23	1.04 (1.00-1.08)	0.029	1.03 (1.00-1.05)	0.024
Family history of diabetes	0.83 (0.62-1.10)	0.20			0.69 (0.42-1.11)	0.12		
Duration of diabetes, years	1.01 (0.99-1.03)	0.42	1.00 (0.97-1.03)	0.89	1.02 (0.98-1.06)	0.31	1.01 (0.98-1.03)	0.56
Comorbidities								
Hypertension	1.32 (0.94-1.87)	0.11			1.58 (0.89-2.78)	0.12		
Dyslipidemia	0.89 (0.64-1.24)	0.50			0.81 (0.48-1.37)	0.44		
BMI ≥ 30 kg/m2	1.89 (1.41-2.54)	<0.001	1.01 (0.64-1.59)	0.98	1.95 (1.14-3.32)	0.015	1.41 (1.03-1.93)	0.031
Waist circumference ≥ 94/80 cm	3.28 (1.83-5.87)	<0.001	1.28 (0.53-3.12)	0.58	2.09 (0.76-5.73)	0.15		
Cardiovascular disease	1.92 (1.45-2.54)	<0.001	1.59 (1.08-2.34)	0.019	1.54 (0.88-2.68)	0.13		
Lifestyle								
Low-moderate alcohol intake*	0.73 (0.51-1.07)	0.11	0.79 (0.49-1.28)	0.34	0.48 (0.29-0.80)	0.005	0.74 (0.52-1.05)	0.091
Excessive alcohol intake*	1.63 (1.10-2.42)	0.015	1.46 (0.87-2.45)	0.15	0.80 (0.36-1.78)	0.58	1.29 (0.87-1.90)	0.20
Current smoking status	1.26 (0.85-1.87)	0.25			0.58 (0.21-1.58)	0.28		
Physical activity ≥ 150/75 min/week	1.01 (0.81-1.26)	0.92			0.96 (0.65-1.42)	0.83		
Clinical chemistry								
HbA1c, mmol/mol	1.00 (0.99-1.01)	0.61			1.01 (1.00-1.03)	0.13		
ALT > 30/19 U/L	2.76 (2.08-3.66)	<0.001	1.66 (1.04-2.66)	0.035	2.03 (1.19-3.46)	0.009	1.64 (1.17-2.29)	0.004
AST > 30/19 U/L	5.60 (4.16-7.54)	<0.001	3.53 (2.26-5.50)	<0.001	11.43 (4.96-26.38)	<0.001	3.56 (2.58-4.90)	<0.001
Albumin, per 0.5 g/dL decrease	2.42 (1.94-3.02)	<0.001	2.46 (1.85-3.28)	<0.001	3.61 (2.36-5.54)	<0.001	2.53 (2.06-3.09)	<0.001
Platelet count, per $10*10^9$/L decrease	1.16 (1.13-1.19)	<0.001	1.12 (1.09-1.16)	<0.001	1.14 (1.10-1.19)	<0.001	1.13 (1.10-1.16)	<0.001
eGFR < 60 mL/min/1.73 m^2	1.95 (1.24-3.07)	0.004	1.29 (0.71-2.33)	0.41	1.12 (0.45-2.79)	0.80		

Microalbuminuria	2.04 (1.42-2.92)	<0.001	1.48 (1.01-2.17)	0.046	0.75 (0.33-1.71)	0.50		
Macroalbuminuria	1.16 (0.51-2.65)	0.72			2.11 (0.51-8.79)	0.30		
Genetic risk factors								
PNPLA3 rs738409 genotype								
Additive model	1.90 (1.55-2.34)	<0.001	1.66 (1.28-2.16)	<0.001	1.96 (1.38-2.78)	<0.001	1.57 (1.28-1.91)	<0.001
Recessive model	3.44 (2.34-5.05)	<0.001	2.32 (1.38-3.89)	0.001	3.44 (1.76-6.73)	<0.001	2.21 (1.50-3.25)†	<0.001
TM6SF2 rs58542926 genotype								
Additive model	1.96 (1.49-2.57)	<0.001	1.72 (1.20-2.47)	<0.001	0.85 (0.43-1.67)	0.64		
Recessive model	4.43 (2.08-9.42)	<0.001	4.87 (2.11-11.25)	<0.001	NA‡	NA‡		
MBOAT7 rs641738 genotype								
Additive model	1.04 (0.85-1.27)	0.69			1.25 (0.89-1.75)	0.19		
Recessive model	1.03 (0.73-1.46)	0.87			1.09 (0.61-1.96)	0.77		
GCKR rs1260326 genotype								
Additive model	0.95 (0.77-1.16)	0.60			1.37 (0.97-1.93)	0.071		
Recessive model	0.84 (0.55-1.29)	0.43			2.05 (1.18-3.56)	0.011	1.29 (0.89-1.88)	0.18
HSD17B13 rs72613567 genotype								
Additive model	0.71 (0.56-0.90)	<0.001	0.73 (0.53-1.00)	0.05	0.83 (0.56-1.23)	0.36		
Recessive model	0.61 (0.32-1.15)	0.13			0.78 (0.29-2.15)	0.64		
Drugs								
Metformin	1.44 (1.09-1.91)	0.011	1.44 (0.96-2.15)	0.077	1.25 (0.78-2.00)	0.35		
Thiazolidinediones	1.50 (0.98-2.29)	0.064			1.45 (0.66-3.15)	0.35		
Sulfonylureas	1.35 (1.00-1.84)	0.052			1.32 (0.75-2.33)	0.34		
Insulin	0.72 (0.48-1.06)	0.095			1.24 (0.73-2.12)	0.43		
Statins	0.89 (0.66-1.20)	0.44			0.87 (0.53-1.42)	0.58		

HRs with 95% CIs were calculated by Cox proportional hazards models.
Age, BMI, alcohol intake, duration of diabetes and all predictor variables with a P value < 0.05 in the univariate model were included in the multivariate model.
*Low-moderate (<20/30 g/day) and excessive (≥20/30 g/day) alcohol intake tested against abstainers.

†aHR calculated assuming recessive model instead of additive model.

‡Model failed to converge.

Abbreviations: aHR, adjusted HR; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HR, hazard ratio; NA, not applicable.

PAPER II

Clinical Gastroenterology and Hepatology 2022;■:■–■

Development and Validation of a Score for Fibrotic Nonalcoholic Steatohepatitis

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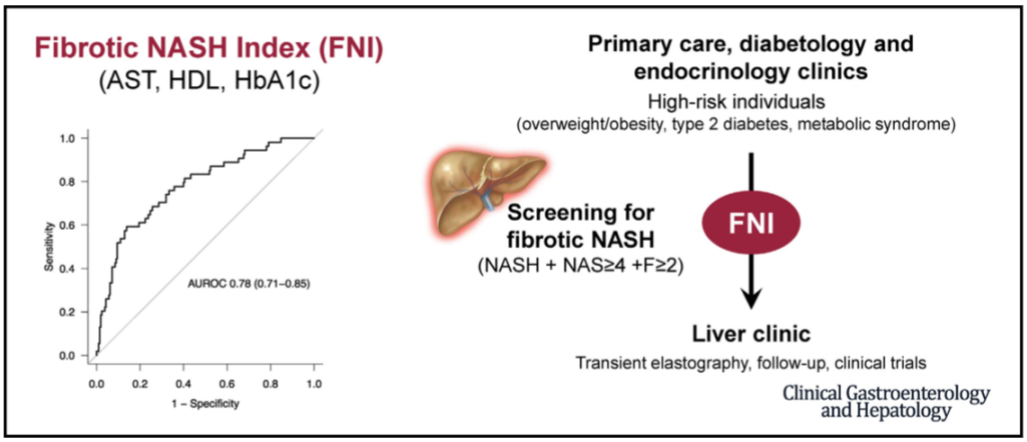


ABSTRACT

Background and aims: Non-invasive assessment of histological features of non-alcoholic fatty liver disease (NAFLD) has been an intensive research area over the last decade. Herein, we aimed to develop a simple non-invasive score using routine laboratory tests to identify, among individuals at high risk for NAFLD, those with fibrotic non-alcoholic steatohepatitis (NASH) defined as NASH, NAFLD activity score (NAS) ≥4, and fibrosis stage ≥2.

Methods: The derivation cohort included 264 morbidly obese individuals undergoing intraoperative liver biopsy in Rome, Italy. The best predictive model was developed and internally validated using a bootstrapping stepwise logistic regression analysis (2000 bootstrap samples). Performance was estimated by the area under the receiver operating characteristic curve (AUROC). External validation was assessed in three independent European cohorts (Finland, n=370; Italy n=947; England n=5,368) of individuals at high risk for NAFLD.

Results: The final predictive model, designated as Fibrotic NASH Index (FNI), combined aspartate aminotransferase (AST), high-density lipoprotein (HDL) cholesterol, and hemoglobin A1c (HbA1c). The performance of FNI for fibrotic NASH was satisfactory in both derivation and external validation cohorts (AUROCs 0.78 and 0.80-0.95, respectively). In the derivation cohort, rule-out and rule-in cut-offs were 0.10 for sensitivity ≥0.89 (negative predictive value [NPV] 0.93) and 0.33 for specificity ≥0.90 (positive predictive value [PPV] 0.57), respectively. In the external validation cohorts, sensitivity ranged from 0.87 to 1 (NPV 0.99-1) and specificity from 0.73 to 0.94 (PPV 0.12-0.49) for rule-out and rule-in cut-off, respectively.

Conclusion: FNI is an accurate, simple, and affordable non-invasive score which can be used in primary healthcare to screen for fibrotic NASH individuals with dysmetabolism.

INTRODUCTION

Following the global burden of obesity and type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) is now the major cause of chronic liver disease worldwide.[2] NAFLD encompasses a broad spectrum of conditions, from isolated hepatic fat accumulation to hepatocellular damage and inflammation (non-alcoholic steatohepatitis, NASH), leading to fibrosis and end-stage liver disease, namely cirrhosis and hepatocellular carcinoma.[5, 6] Obesity and type 2 diabetes are the strongest environmental factors increasing the risk of NAFLD.[14] However, despite the very large number of individuals with NAFLD, only a minority progress to cirrhosis and hepatocellular carcinoma.[2]

A body of evidence shows that individuals with fibrotic NASH, the inflammatory form of NAFLD associated with significant activity and fibrosis, are at risk of developing advanced liver disease.[8] The gold standard for diagnosing NASH and liver fibrosis is still a histological assessment by liver biopsy, an invasive and costly procedure which is not devoid of complications.[19, 20]

The identification of individuals with fibrotic NASH in primary healthcare is crucial because these individuals will benefit the most from a referral to liver clinic for further investigation and follow-up. Moreover, these individuals are the ideal candidates for inclusion in NASH clinical trials.[55, 56]

Therefore, due to the large number of individuals with NAFLD and the invasiveness of liver biopsy, non-invasive screening scores for fibrotic NASH are urgently needed. Indeed, existing scores are mainly focused on the assessment of liver fibrosis, the most relevant prognostic factor in NAFLD.[22, 24] Up to date, three non-invasive scores have been specifically generated to assess fibrotic NASH, namely MACK-3 (hoMa, Ast, CK18),[38] NIS4,[39] and FibroScan-AST (FAST) score.[40] However, these scores are based on blood tests available only in highly specialized liver clinics or require instrumental evaluation by vibration-controlled transient elastography.

In this study, we aimed to develop a simple non-invasive score based on routine laboratory tests to screen for and identify fibrotic NASH in individuals at high risk for NAFLD in primary healthcare.

METHODS

Derivation cohort

MAFALDA cohort. A total of 264 participants from the "Molecular Architecture of FAtty Liver Disease in individuals with obesity undergoing bAriatric surgery (MAFALDA)" were included in the analyses.[57] Briefly, consecutive individuals with morbid obesity eligible for bariatric surgery, without history of alcohol abuse (≥30/20 g/day in men/women), chronic viral hepatitis, and other causes of liver disease, were recruited from May 2020 to June 2021 at Campus Bio-Medico University Hospital, Rome, Italy. Preoperative clinical and laboratory data were collected using standardized procedures. Intraoperative liver biopsy was obtained and scored according to NAS classification.[58] NASH was diagnosed with at least grade one for steatosis, ballooning, and lobular inflammation.[59] Fibrotic NASH was defined as NASH, NAS≥4, and fibrosis stage ≥2. The MAFALDA study has been approved by the Local Research Ethics Committee (no. 16/20) and it was conducted in accordance with the principles of the Declaration of Helsinki. All participants gave written informed consent to the study.

External validation cohorts

Helsinki cohort. A total of 328 consecutive individuals with morbid obesity eligible for bariatric surgery and 42 consecutive individuals with body mass index (BMI) ≥25 kg/m^2 undergoing liver biopsy for suspected NASH were recruited between 2006 and 2018 at Helsinki University Hospital, Helsinki, Finland. All participants were 18-75 years old, without history of alcohol abuse (≥30/20 g/day in men/women), chronic viral hepatitis, and other causes of liver disease. A week before liver biopsy, participants underwent clinical examination and blood sampling as previously described.[60] Liver biopsies were scored according to NAS classification.[58] NASH was diagnosed when steatosis, lobular inflammation, and ballooning each had at least one grade.[61] Fibrotic NASH was defined as NASH, NAS≥4, and fibrosis stage ≥2. The study was approved by the Local Research Ethics Committee at Helsinki University Hospital. All participants gave written informed consent to the study.

Liver Bible cohort. A total of 947 consecutive individuals with dysmetabolism (at least three criteria among overweight [BMI >25 kg/m^2], hypertension [>130/85 mmHg or use of medication], hyperglycemia [>100 mg/dL], low high-density lipoprotein [HDL]

cholesterol [<45/55 mg/dL in men/women], and increased triglycerides [>150 mg/dL]) were recruited from July 2019 to July 2021 at the Transfusion Center, Fondazione Ca' Granda Hospital, Milan, Italy.[43, 62] All participants were 18-65 years old, without history of alcohol abuse (≥30/20 g/day in men/women), chronic viral hepatitis, and other causes of liver disease, and were enrolled as part of a preventive medicine program among blood donors. Liver steatosis and fibrosis were non-invasively assessed by vibration-controlled transient elastography and controlled attenuation parameter (CAP) with FibroScan® (Echosens, Paris, France), which was performed at the time of biochemical tests. Individuals at-risk of fibrotic NASH were defined as those with FAST score >0.35.[40] The study was approved by the Local Research Ethics Committee at the Fondazione IRCCS Ca' Granda. All participants gave written informed consent to the study.

UK Biobank cohort. The UK Biobank is a large prospective cohort study recruiting approximately 500,000 participants (age 40-69 years) between 2006-2010 throughout the UK.[63] The UK Biobank study has been approved by the North West Multicenter Research Ethics Committee (no. 11/NW/0274). All participants gave written informed consent to the study.

First, we selected unrelated UK Biobank participants of European ancestry based on our quality control pipeline which has been described in detail previously.[12, 43] Next, we included in our analyses only individuals with BMI ≥25 kg/m^2 and/or with type 2 diabetes as defined elsewhere.[64]

Then, to assess the performance of our score for fibrotic NASH, we selected 5,368 individuals without chronic viral hepatitis and with liver magnetic resonance imaging (MRI) proton density fat fraction (PDFF) and iron-corrected T1 (cT1) measurements available.[65, 66] Fibrotic NASH was defined as steatosis by PDFF >5.5%,[65] NASH by cT1 >800 msec,[67] and significant fibrosis by Fibrosis-4 (FIB-4) index ≥1.3.[26]

Finally, to assess the performance of our score for incident severe liver disease (SLD),[64] after excluding participants with MRI data available, we selected 305,745 individuals without liver disease at baseline and estimated those who developed SLD prospectively. Detailed information about the UK Biobank methods is provided in supplementary material.

Statistical analyses

The score was developed based on 264 morbidly obese individuals in the derivation cohort and internally validated using a bootstrapping stepwise logistic regression model (2000 bootstrap samples). A total of 15 predictors were included in the model: age, gender, BMI, waist circumference, glucose, hemoglobin A1c (HbA1c), total cholesterol, HDL cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), platelet count, albumin, and total bilirubin. Logarithmic transformation was considered for continuous variables to improve the normality of distribution. Two (0.8%) individuals were removed from the analysis due to missing values. The score was derived based on the final predictors and the corresponding regression coefficients. Performance for fibrotic NASH was assessed by the area under the receiver operating characteristic curve (AUROC) in the derivation and validation cohorts. Rule-out and rule-in cut-offs were derived in the derivation cohort based on sensitivity ≥0.89 and specificity ≥0.90, respectively. Cut-off based on the maximal sum of sensitivity and specificity (Youden index) was also determined. At each cut-off, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were computed together with 95% confidence interval (CI). AUROCs were compared using the DeLong test. Calibration was assessed in the derivation cohort using Hosmer-Lemeshow goodness of fit test and calibration plot. Performance for incident SLD in the UK Biobank was estimated by AUROC of Cox proportional hazards models. Statistical analyses were performed using the software R, version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Clinical characteristics of derivation and external validation cohorts

Clinical characteristics of derivation and external validation cohorts are shown in Table 1. The two histological cohorts (MAFALDA and Helsinki cohorts) were well matched for age and gender, while the Liver Bible and UK Biobank cohorts had higher mean age and higher rate of men. Biochemical parameters were similar across the cohorts. The Liver Bible cohort had the highest rate of hypertension (74% vs 41-63%), whereas the Helsinki cohort had the highest rate of type 2 diabetes (38% vs 4-16%). Biopsy-proven NASH was diagnosed in 42% individuals of the derivation cohort and in 12% individuals of the Helsinki cohort. Fibrotic NASH was reported in 20% individuals of the derivation cohort and in 2-5% individuals of the external validation cohorts.

Development of a prediction model for fibrotic NASH

Bootstrapping stepwise logistic regression analysis identified three final independent predictors of fibrotic NASH: AST, HDL cholesterol, and HbA1c. Based on the corresponding regression coefficients, the following index–the Fibrotic NASH Index (FNI)–was derived:

$$FNI = \frac{e^{(-10.33+2.54\times\ln AST\ [U/L]\ +3.86\times\ln \text{HbA1c}\ [\%]-1.66\times\ln \text{HDL}\ [mg/dL])}}{1+e^{(-10.33+2.54\times\ln AST\ [U/L]\ +3.86\times\ln \text{HbA1c}\ [\%]-1.66\times\ln \text{HDL}[mg/dL])}}$$

The FNI is a predicted probability score and ranges from 0 to 1. As an example, an individual with a FNI of 0.10 would have a 10% predicted probability of fibrotic NASH (NASH + NAS≥4 + F≥2). The FNI can be easily calculated on the following website: https://fniscore.github.io/.

In the derivation cohort, the performance of FNI for fibrotic NASH estimated by AUROC was 0.78 (95% CI 0.71-0.85) with satisfactory calibration of predicted probabilities (Figure 1). In the external validation cohorts, AUROCs ranged from 0.80 to 0.95 (Table 2). In the derivation cohort, cut-off for sensitivity ≥0.89 (rule-out zone) was 0.10, with a NPV of 0.93. Cut-off for specificity ≥0.90 (rule-in zone) was 0.33, with a PPV of 0.57 (Table 2). When applying these cut-offs to the external validation cohorts, at the rule-out cut-off of 0.10, sensitivity ranged from 0.87 to 1, with a NPV between 0.99 and 1; at the

rule-in cut-off of 0.33, specificity ranged from 0.73 to 0.98, with a PPV between 0.12 and 0.49 (Table 2).

The performance of FNI and FIB-4 for fibrotic NASH was compared in derivation and two external validation cohorts (Figure 2, Table 2). Corresponding AUROCs were higher for FNI in the derivation and Liver Bible cohorts (p=0.001 and $3.08x10^{-08}$, respectively), whereas no difference was found between the two scores in the Helsinki cohort (p=0.85).

Performance for incident severe liver disease

During a median (interquartile range) follow-up of 9.0 (8.3-9.7) years, there were 1,054 individuals who developed SLD, including 928 with cirrhosis and/or decompensated liver disease, 126 with hepatocellular carcinoma, and 18 that underwent liver transplantation. Death from SLD occurred in 542 individuals.

The AUROC of FNI for incident SLD was 0.77 (95% CI 0.75-0.79) which was higher than the AUROC of FIB-4 (0.75, 95% CI 0.73-0.77; p=0.03) (Figure 3). At the FNI cut-off of 0.10 (rule-out zone), sensitivity was 0.81 *vs* 0.75 of FIB-4 cut-off of 1.3, with a NPV of 1 for both scores (Table 3). A FNI >0.10 conferred a nearly four-fold increased risk of incident SLD (adjusted hazard ratio [HR] 3.55, 95% CI 2.96-4.25; p<0.001), which was higher than the increase in risk conferred by a FIB-4 ≥1.3 (adjusted HR 3.0, 95% CI 2.54-3.54; p<0.001) (Table 3).

Table 1. Clinical characteristics of derivation and external validation cohorts.

	MAFALDA	Helsinki	Liver Bible	MRI UK Biobank
n	264	370	947	5,368
Clinical data				
Age, years	43.4 (10.1)	49.1 (9.5)	53.9 (6.3)	55.3 (7.3)
Women, n (%)	195 (74%)	262 (71%)	157 (17%)	2,406 (45%)
BMI, kg/m^2	41.6 (4.4)	42.3 (7.7)	28.5 (3.1)	28.8 (3.4)
Metabolic profile				
Glucose, mg/dL	98 (92-106)	105 (96-114)	94 (87-103)	88 (83-95)
HbA1c, %	5.5 (5.3-5.9)	5.7 (5.4-6.2)	5.4 (5.2-5.6)	5.3 (5.1-5.6)
Cholesterol, mg/dL	179.1 (31.2)	163.8 (41.6)	202.1 (32.3)	224 (43)
HDL cholesterol, mg/dL	45.8 (9.8)	46.2 (12.1)	45.3 (10.1)	54 (12)
LDL cholesterol, mg/dL	121.3 (30.1)	99.1 (35.1)	123.3 (28.9)	143 (35)
Triglycerides, mg/dL	122 (90.8-164.2)	108 (80-145)	159 (114-199)	142 (106-204)
Liver function tests				
ALT, U/L	30.5 (20-41)	32 (22-46)	26 (21-35)	22.1 (16.7-30)
AST, U/L	26 (22-32)	29 (24-36)	23 (19-27)	24.8 (21.3-29.2)
GGT, U/L	25 (17.5-34)	31 (20-52)	23 (17-32)	28.2 (19.9-42.8)
Bilirubin, mg/dL	0.5 (0.4-0.7)	-	-	0.5 (0.4-0.6)
Albumin, g/dL	4.2 (0.3)	3.8 (0.4)	-	4.5 (0.3)
Platelets, 10e3/uL	282.7 (63.4)	252.7 (63.0)	234.7 (51.5)	250.8 (56.6)
Comorbidities				
Hypertension, n (%)	109 (41%)	232 (63%)	699 (74%)	2,236 (42%)
Type 2 diabetes, n (%)	41 (16%)	141 (38%)	35 (4%)	405 (8%)
Liver histology				
Steatosis grade, n (%)			NA	NA

0	88 (33%)	135 (37%)
1	93 (35%)	153 (41%)
2	48 (18%)	51 (14%)
3	35 (13%)	31 (8%)
Lobular inflammation grade, n (%)		
0	108 (41%)	312 (84%)
1	143 (54%)	48 (13%)
2	13 (5%)	10 (3%)
3	0 (0%)	0 (0%)
Ballooning grade, n (%)		
0	22 (8%)	318 (86%)
1	176 (67%)	39 (11%)
2	66 (25%)	13 (4%)
NASH, n (%)	110 (42%)	45 (12%)
NAS ≥4, n (%)	109 (41%)	42 (11%)
NASH + NAS≥4 +F≥2, n (%)	54 (20%)	17 (5%)
Fibrosis staging, n (%)		
0	80 (30%)	215 (58%)
1	117 (44%)	121 (33%)
2	59 (22%)	18 (5%)
3	7 (3%)	10 (3%)
4	1 (0%)	6 (2%)

Continuous variables are shown as mean (SD) or median (IQR) as appropriate. Categorical variables are shown as number (percentage).

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma glutamyltransferase; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MRI, magnetic resonance imaging; NA, not available; NAS, NAFLD Activity Score; NASH, non-alcoholic steatohepatitis.

Table 2. Diagnostic performance of FNI and FIB-4 for fibrotic NASH and cut-off values in derivation and external validation cohorts.

	MAFALDA	Helsinki	Liver Bible	MRI UK Biobank
N	264	370	947	5,368
Fibrotic NASH definition	NASH + NAS≥4 + F≥2	NASH + NAS≥4 + F≥2	FAST score >0.35	PDFF>5.5% + cT1>800 msec + FIB-4≥1.3
Fibrotic NASH, n (%)	54 (20%)	17 (5%)	37 (4%)	118 (2%)
FNI AUROC (95% CI)	0.78 (0.71-0.85)	0.83 (0.72-0.95)	0.95 (0.92-0.98)	0.80 (0.75-0.83)
FIB-4 AUROC (95% CI)	0.63 (0.54-0.71)	0.82 (0.72-0.92)	0.68 (0.58-0.78)	NA
FNI ≥0.30 (Youden index)				
n (%)	59 (22.3%)	124 (33.5%)	52 (5.5%)	433 (8.1%)
Sensitivity	0.57	0.88	0.62	0.39
Specificity	0.87	0.69	0.97	0.93
PPV	0.53	0.12	0.44	0.11
NPV	0.89	0.99	0.98	0.99
FNI ≤0.10 (Rule-out zone)				
n (%)	83 (31.4%)	77 (20.8%)	464 (50%)	2,526 (47.1%)
Sensitivity	0.89	0.94	1	0.87
Specificity	0.37	0.22	0.51	0.54
PPV	0.27	0.06	0.08	0.04
NPV	0.93	0.99	1	0.99
FNI ≥0.33 (Rule-in zone)				
n (%)	49 (18.6%)	109 (29.4%)	41 (4.3%)	337 (6.3%)
Sensitivity	0.52	0.82	0.54	0.34
Specificity	0.90	0.73	0.98	0.94
PPV	0.57	0.13	0.49	0.12
NPV	0.88	0.99	0.98	0.98
FIB-4 ≥1.3				
n (%)	21 (8.0%)	111 (30.0%)	216 (22.8%)	NA
Sensitivity	0.11	0.76	0.53	NA
Specificity	0.93	0.72	0.78	NA
PPV	0.29	0.12	0.09	NA

NPV	0.80	0.98	0.98	NA

Optimal cut-offs for fibrotic NASH were obtained in the derivation cohort based on the maximal sum of sensitivity and specificity (Youden index), on sensitivity ≥89% (rule-out zone), and on specificity ≥90% (rule-in zone).

Abbreviations: AUROC, area under the receiver operating characteristic curve; CI, confidence interval; cT1, iron-corrected T1; FAST, FibroScan-AST score; FIB-4, fibrosis-4 index; FNI, fibrotic NASH index; MRI, magnetic resonance imaging; NA, not applicable; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NPV, negative predictive value; PDFF, proton density fat fraction; PPV, positive predictive value; NA, not applicable.

Table 3. Diagnostic performance of FNI and FIB-4 for incident severe liver disease in the UK Biobank (n=305,745).

	FNI	FIB-4
AUROC (95% CI)	0.77 (0.75-0.79)	0.75 (0.73-0.77)
Cut-off	>0.10	≥1.3
n (%)	127,460 (51.1%)	119,658 (43.0%)
HR (95% CI)	4.21 (3.55-5.01)*	4.10 (3.54-4.75)[#]
aHR (95% CI)	3.55 (2.96-4.25)*	3.0 (2.54-3.54)[#]
Sensitivity	0.81	0.75
Specificity	0.49	0.57
PPV	0.01	0.01
NPV	1	1

HRs with 95% CIs were calculated by Cox proportional hazards models.
Age, gender, and alcohol intake (g/day) were included in the multivariable models.

*p<0.001 vs FNI ≤0.10
#p<0.001 vs FIB-4 <1.3

Abbreviations: aHR, adjusted HR; AUROC, area under the receiver operating characteristic curve; CI, confidence interval; FIB-4, fibrosis-4 index; FNI, fibrotic NASH index; HR, hazard ratio; PPV, positive predictive value.

Figure 1. Diagnostic performance of FNI for fibrotic NASH in the MAFALDA cohort (n=264). (A) ROC curve. Numbers in brackets are 95% CI. **(B) Calibration plot.** The solid line represents the ideal calibration. The dashed line represents the calibration estimated using locally estimated scatterplot smoothing (Loess). The shaded area indicates 95% CI. Triangles represent sextiles of participants grouped by similar predicted risk. P value is calculated using Hosmer-Lemeshow goodness of fit test. *Abbreviations:* AUROC, area under the receiver operating characteristic curve; FIB-4, Fibrosis-4 index; FNI, fibrotic NASH index.

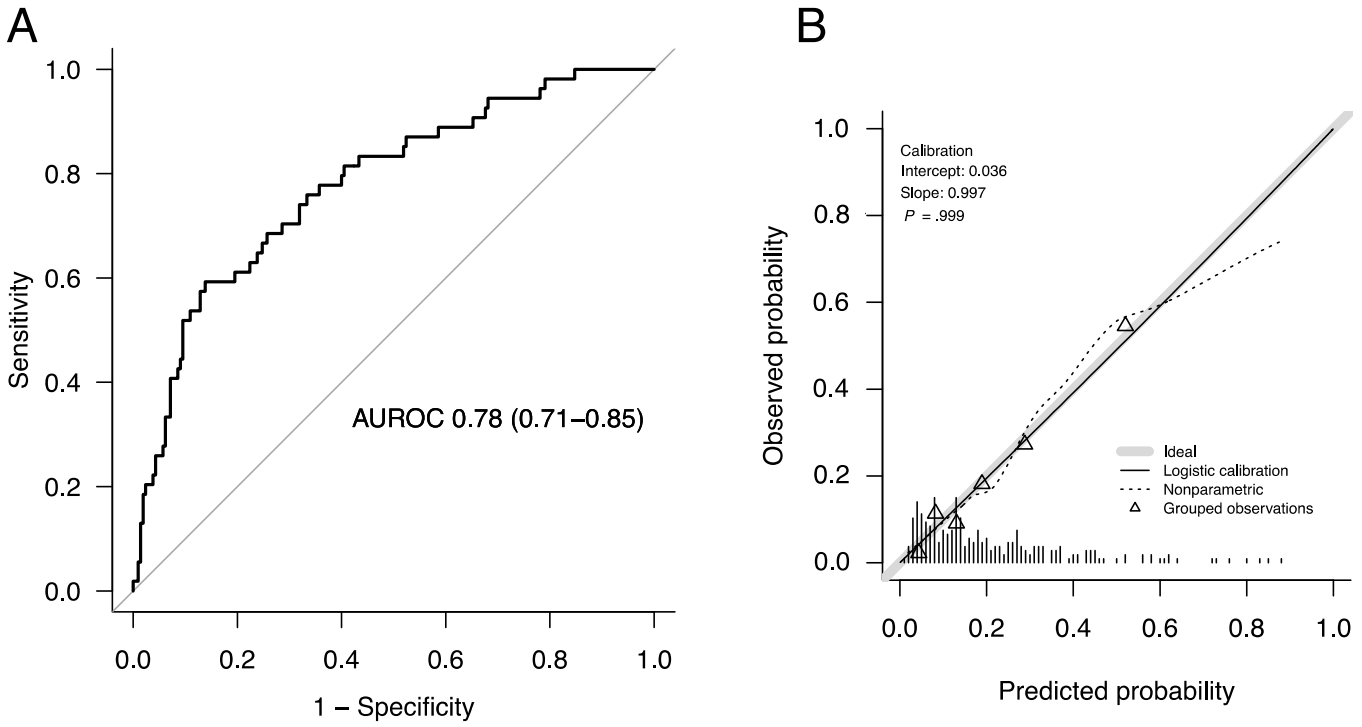


Figure 2. ROC curves for fibrotic NASH by FNI and FIB-4 in the (A) MAFALDA cohort (n=264), (B) Helsinki cohort (n=370), and (C) Liver Bible cohort (n=947). Numbers in brackets are 95% CI. P values are calculated using the DeLong test. P values <0.05 are considered statistically significant. *Abbreviations:* AUROC, area under the receiver operating characteristic curve; FIB-4, Fibrosis-4 index; FNI, fibrotic NASH index.

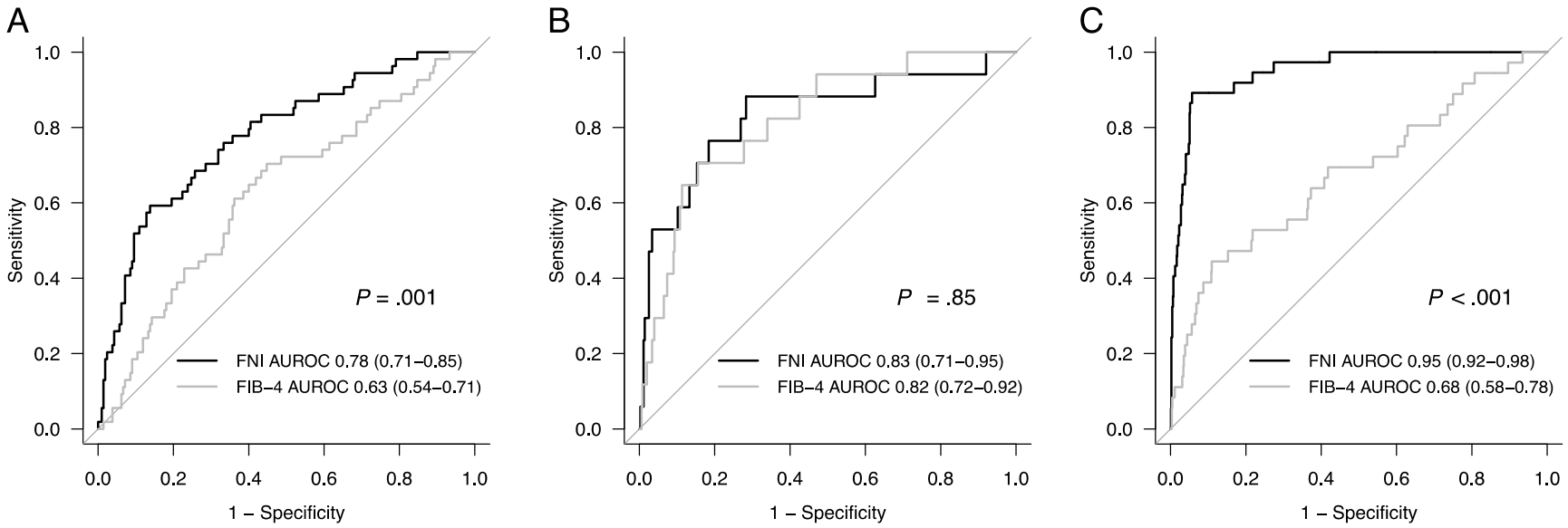
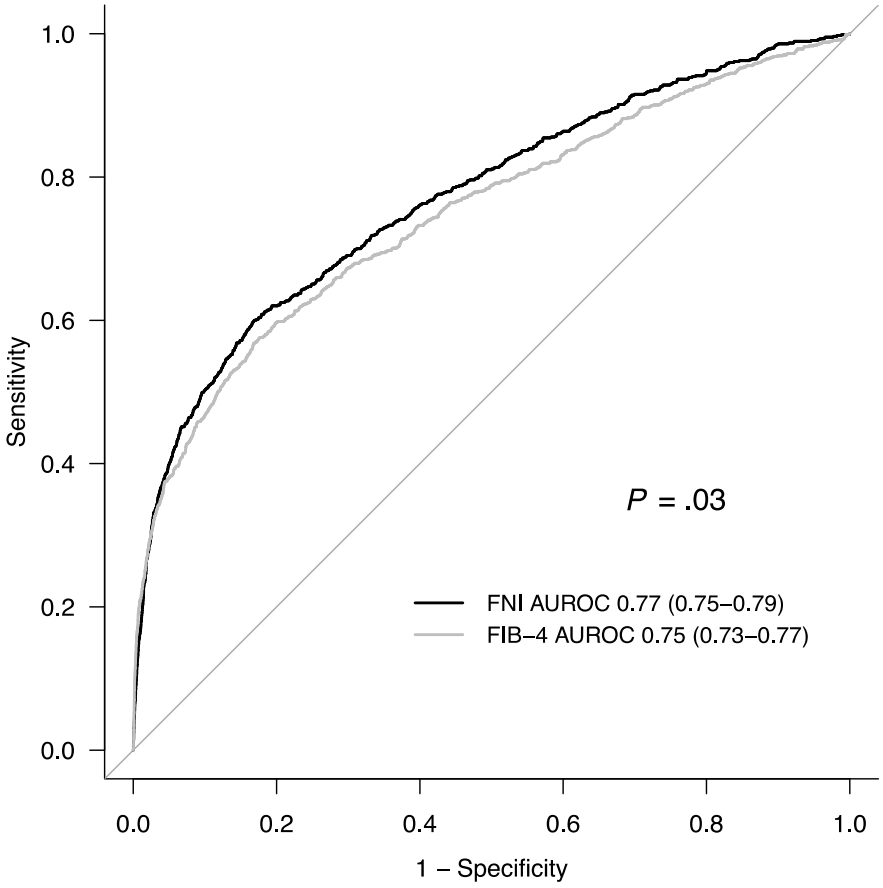
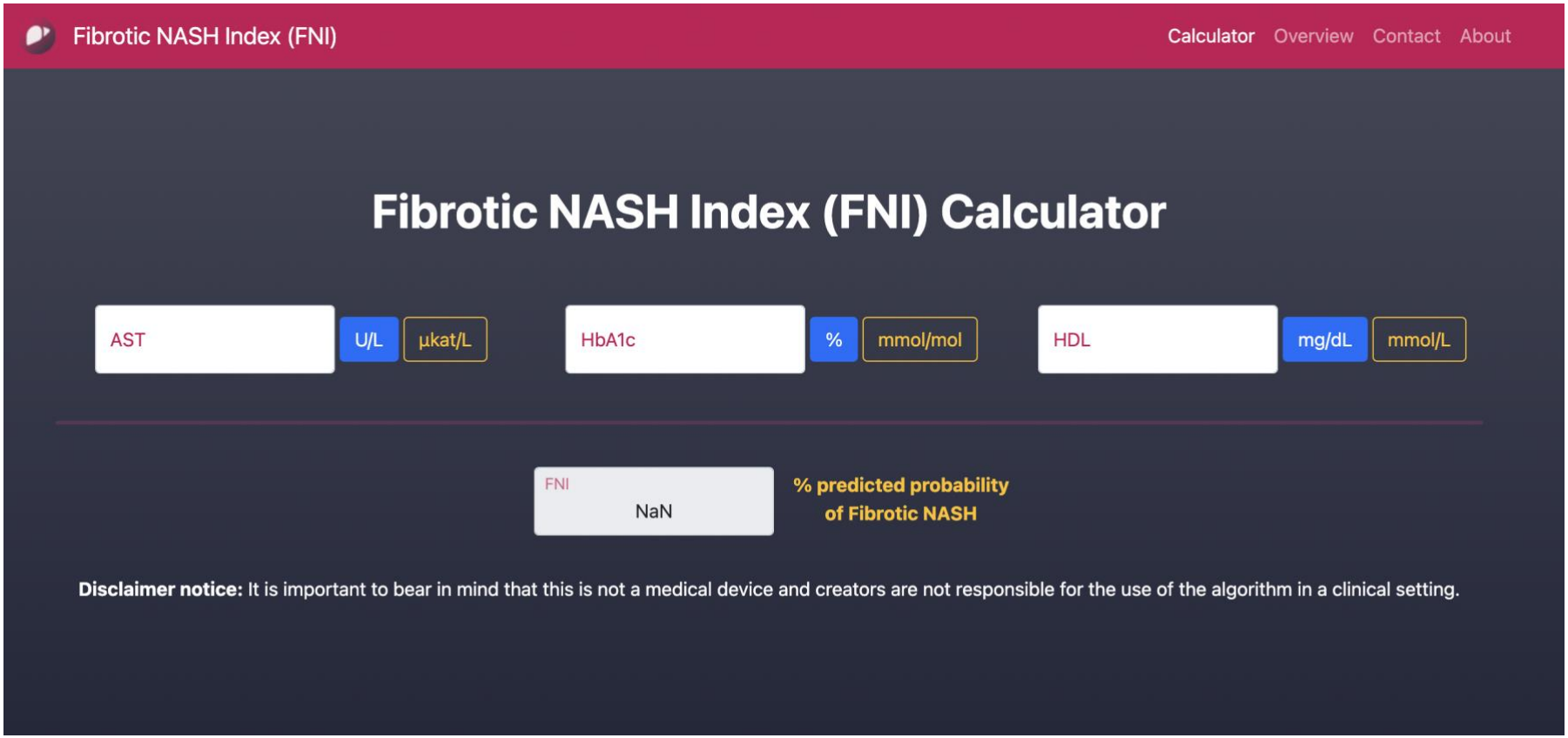


Figure 3. ROC curves for incident severe liver disease by FNI and FIB-4 in the UK Biobank (n=305,745). Numbers in brackets are 95% CI. P values are calculated using the DeLong test. P values <0.05 are considered statistically significant.

Abbreviations: AUROC, area under the receiver operating characteristic curve; FIB-4, Fibrosis-4 index; FNI, fibrotic NASH index.



FNI website: https://fniscore.github.io/



DISCUSSION

In this study, we develop and validate the FNI, a novel and simple non-invasive score for detecting fibrotic NASH among individuals at high risk for NAFLD, namely those with overweight/obesity, type 2 diabetes, and metabolic syndrome. Notably, this is the first score tailored for fibrotic NASH based on routine laboratory tests, namely AST, HDL cholesterol, and HbA1c.

We started by examining the MAFALDA, a cross-sectional cohort of morbidly obese individuals in whom the diagnosis of fibrotic NASH was assessed by histology. In MAFALDA, we generated and internally validated a prediction model for fibrotic NASH by using a bootstrapping stepwise regression analysis. We found that AST, HDL cholesterol, and HbA1c were the best independent predictors of this condition. Consistently, elevated AST is a well-known biomarker of liver fibrosis,[68] whereas HbA1c and HDL cholesterol are both flagging the presence of dysmetabolism, given their correlation with insulin resistance and impaired glucose tolerance.[69, 70] In the derivation cohort, this model showed good success in predicting fibrotic NASH with an AUROC of 0.78 (0.71-0.85).

Next, we validated our prediction model in three independent external cohorts comprising individuals with overweight/obesity, type 2 diabetes, and metabolic syndrome. In these cohorts, irrespective of the methodology used to assess fibrotic NASH (liver biopsy, vibration-controlled transient elastography including CAP, or liver MRI), the performance of our score was very good with an AUROC range of 0.80-0.95. Notably, one of the external validation cohorts included more than 5,000 high-risk individuals from the UK Biobank.

Existing non-invasive clinical scores are focused on detecting advanced fibrosis, the most relevant predictor of mortality in NAFLD.[22] However, the degree of liver inflammation is a crucial driver of liver damage.[71] In this scenario, the presence of NASH with significant activity (NAS≥4) has been identified as an essential condition for enrollment in NAFLD clinical trials.[56] This is mainly due to two reasons: 1) the histological response to drug therapy is higher in individuals with an active disease,[72] and 2) the inclusion of individuals with fibrotic NASH is more likely to ensure that the estimated number of clinical events will occur during the study observation period. Along this line, the presence of an active liver disease is expected to be included among the prescribing

criteria of new emerging pharmacotherapies once they become available. Within this context, FNI may also be used as a longitudinal biomarker to non-invasively monitor the effectiveness of interventional strategies for NASH.

Very recently, three non-invasive scores have been generated to detect fibrotic NASH: two blood-based, MACK-3[38] (AST, glucose, insulin, cytokeratin 18) and NIS4[39] (miR-34a-5p, alpha-2 macroglobulin, YKL-40, HbA1c), and the transient elastography-based FAST score (AST, CAP, liver stiffness measurement).[40] The accuracy of these scores for fibrotic NASH was good and comparable to that of FNI, with AUROCs ranging from 0.80 to 0.85. However, these scores are based on blood/instrumental tests relatively expensive and/or not widely available in primary care. Consequently, although FibroScan® is increasingly used worldwide, the screening for fibrotic NASH in large at-risk populations in primary care using these scores appears to be impractical and costly.

Would the FNI score be a viable option to screen for fibrotic NASH in large at-risk populations? Within this context, the risk stratification pathway recently proposed by the European Association for the Study of the Liver (EASL) recommended a FIB-4 cut-off <1.3 to rule out those not needing a referral to the liver specialist.[50] In individuals with metabolic risk factors from the general population, a FNI value ≤0.10 (rule-out zone) would exclude the presence of fibrotic NASH with high sensitivity and high NPV. Importantly, in both derivation and external validation cohorts, at least one out five individuals belonged to the rule-out zone, thus avoiding further referral to the liver specialist. Notably, the FNI cut-off of 0.10 had a higher sensitivity for fibrotic NASH as compared to the FIB-4 cut-off of 1.3. Consequently, in the general population with metabolic risk factors, the risk stratification using FNI as opposed to FIB-4 would allow to miss fewer individuals with fibrotic NASH. Importantly, these individuals may require and benefit the most from a prompt intervention in liver clinics due to the presence of an active disease at higher risk of liver-related outcomes. Consistently, we found that, during a median follow-up of 9 years, FNI was more accurate than FIB-4 for predicting incident SLD. However, it is fair to say that FIB-4 has been generated to assess liver fibrosis and the 1.3 cut-off is used to rule out advanced fibrosis rather than progressive NASH.[50]

Conversely, PPV for fibrotic NASH was rather low in the FNI rule-in zone. This is mainly due to the low prevalence of fibrotic NASH in the cohorts used in our study. Indeed, the performance of any disease predictive model is highly dependent on the prevalence of the

disease in the referral population.[50] Indeed, although FNI was generated and validated in individuals at high risk for NAFLD, the prevalence of fibrotic NASH in these individuals was relatively low. However, the performance of the FNI rule-in cut-off is expected to be higher in individuals from secondary/tertiary care centers where the prevalence of advanced fibrosis is higher. Further studies are warranted to assess the performance of FNI in these settings.

Collectively, our data support that FNI may be useful for ruling out rather than diagnosing fibrotic NASH in at-risk individuals in primary healthcare and diabetology/endocrinology clinics. Individuals with indeterminate and positive results would deserve referral to liver clinic for further investigations and follow-up.

The present study has several strengths. First, we used a large and well-characterized derivation cohort with liver biopsy data available. Second, we developed for the first time a predictive model for fibrotic NASH based on routine and widely available laboratory tests which are commonly evaluated in individuals with metabolic risk factors. Third, we validated our findings in three independent and large external validation cohorts. Among them, one included more than 5,000 individuals from the UK Biobank.

Our study has also some limitations. First, FNI has been specifically designed and validated in individuals with dysmetabolism and not in those referred for NAFLD in liver secondary/tertiary care settings. Therefore, its performance should be further verified before being used in this context. Second, we could not compare FNI with other non-invasive blood-based scores for fibrotic NASH, such as MACK-3, because they were not available in most cohorts.

In conclusion, we developed and validated the FNI, an accurate, simple, and affordable non-invasive score for fibrotic NASH based on routine laboratory tests, namely AST, HDL cholesterol, and HbA1c. This score may help clinicians identify at-risk individuals in primary healthcare and diabetology/endocrinology clinics who require a referral to the liver specialist.

SUPPLEMENTARY MATERIAL

UK Biobank

The UK Biobank is a large prospective cohort study recruiting approximately 500,000 participants (age 40-69 years) between 2006-2010 from 22 assessment centers throughout the UK.[63] Clinical information and laboratory data were collected using highly standardized procedures. Medical diagnoses were obtained through linkage of hospital admissions, death register, and cancer register from the National Health Service records (data-fields 41270, 40001, 40002, and 40006).

MRI UK Biobank cohort. To assess the performance of our score for fibrotic non-alcoholic steatohepatitis (NASH), we selected a total of 5,368 European individuals with overweight/obesity and/or type 2 diabetes, without chronic viral hepatitis (International Classification of Diseases 10th edition [ICD-10] B18-B19) from hospital admissions and death register, and with liver magnetic resonance imaging (MRI) proton density fat fraction (PDFF) and iron-corrected T1 (cT1) measurements available. Participants were scanned at the UK Biobank Imaging Centre in Cheadle (UK) using a Siemens 1.5T MAGNETOM Aera as described in detail elsewhere.[65, 66] Briefly, a shortened modified look locker inversion (ShMOLLI) was used to quantify liver T1 and a multi echo-spoiled gradient-echo was used to quantify liver iron and fat. Data were analyzed using LiverMultiScan© Discover 4.0 software.

Prospective UK Biobank cohort. To assess the performance of our score for incident severe liver disease (SLD), we selected a total of 305,745 European individuals with overweight/obesity and/or type 2 diabetes, after excluding those with PDFF and cT1 measurements available. Baseline exclusion criteria were: 1) self-reported history or hospital diagnosis of chronic viral hepatitis, SLD, or other causes of liver disease (ICD-10 B18, B19, C22.0, E83.0, E83.1, I85.0, I85.9, K70.3, K70.4, K70.9, K71, K72.1, K72.9, K74.1, K74.2, K74.3, K74.4, K74.5, K74.6, K75.2, K75.3, K75.4, K75.8, K75.9, K76.6, K76.7, K76.8, K76.9, R18, Z94.4); 2) self-reported history or diagnosis from cancer register of liver cancer (ICD-10 C22); 3) missing data for any score variable. SLD was defined as a composite diagnosis of cirrhosis, decompensated liver disease, hepatocellular carcinoma, and/or liver transplantation from hospital admissions, death register, and cancer register (ICD-10 C22.0, I85.0, I85.9, K70.3, K70.4, K72.1, K72.9, K74.1, K74.2, K74.6, K76.6, K76.7, Z94.4). Follow-up length was calculated from the date of baseline

assessment visit up to the first date of SLD diagnosis, the date of death, or the date of end of follow-up for the assessment center attended (31 January 2018), whichever occurred first. Participants were excluded from the analyses if they received hospital diagnosis of competing liver diseases (ICD-10 B18, B19, E83.0, E83.1, K71, K74.3, K74.4, K74.5, K75.2, K75.3, K75.4, K75.8, K75.9) before the diagnosis of SLD.

CONCLUSION AND FUTURE PERSPECTIVES

NAFLD is a complex and heterogenous entity, deriving from the interplay between genetic, metabolic, and environmental risk factors. Given the high prevalence of the disease and its usually benign nature, it's becoming increasingly important to have simple and accurate non-invasive diagnostic tests to identify in routine clinical practice the minority of individuals evolving to NASH and advanced fibrosis. Indeed, the identification of individuals with advanced liver disease is crucial because these individuals are those who will benefit the most from a prompt referral to liver clinic for further investigation and follow-up. Moreover, these individuals are the ideal candidates for inclusion in clinical trials with novel pharmacotherapies for NASH.

The main objectives of this thesis were 1) to investigate the independent contribution of the most relevant risk factors for NAFLD severity and 2) to generate a simple blood-based score to screen for fibrotic NASH in high-risk individuals in general population settings.

In **Paper I**, we demonstrated that risk factors independently associated with increased risk of incident severe liver disease were abnormal AST, decrease in serum albumin and platelet count, the coexistence of cardiovascular disease and microalbuminuria, and genetic variants in *PNPLA3* and *TM6SF2*.

In **Paper II**, we developed and validated the FNI, a novel, simple, and accurate non-invasive blood-based score to identify fibrotic NASH in high-risk individuals in primary care and diabetology/endocrinology clinics.

These findings may help in clinical care identify individuals at risk for progressive liver disease, in turn leading to personalized risk prediction and prevention strategies. Further longitudinal population-based studies are required to confirm the performance of FNI for detecting at-risk NAFLD, alone or in combination with additional non-invasive NAFLD biomarkers. Finally, histologically characterized cohort studies are required to test the performance of FNI in individuals with NAFLD in secondary/tertiary care settings.

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