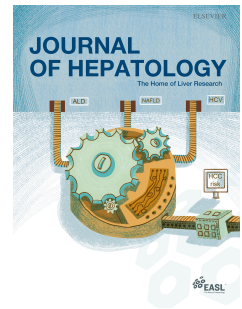


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Genome-wide association study of non-alcoholic fatty liver and steatohepatitis in a histologically-characterised cohort

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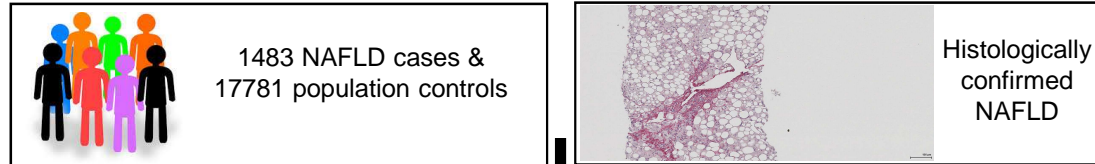
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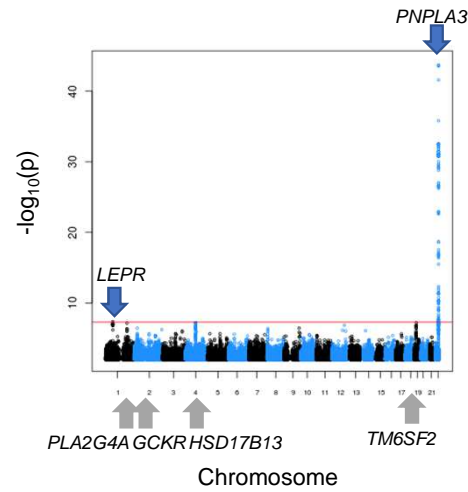
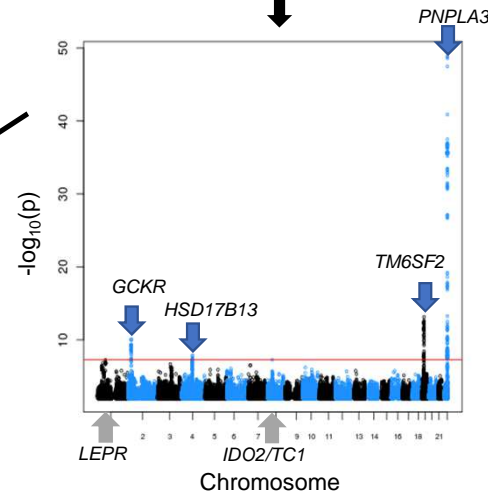
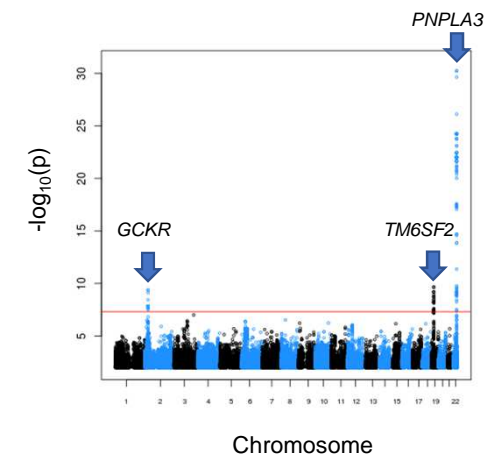
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Genome-wide
association study

NASH only

Advanced fibrosis (F3/F4) only

Replication cohort of 559 NAFLD cases
and 945 controls genotyped for top
SNPs

Genome-wide association study of non-alcoholic fatty liver and steatohepatitis in a histologically-characterised cohort

Running title: GWAS on NAFLD/NASH

Quentin M. Anstee^{1,2}, Rebecca Darlay³, Simon Cockell⁴, Marica Meroni⁵, Olivier Govaere¹, Dina Tiniakos^{1,6}, Alastair D. Burt^{1,7}, Pierre Bedossa¹, Jeremy Palmer¹, Yang-Lin Liu¹, Guruprasad P. Aithal⁸, Michael Allison⁹, Hannele Yki-Järvinen¹⁰, Michele Vacca^{9,11}, Jean-Francois Dufour¹², Pietro Invernizzi^{13,21}, Daniele Prati⁵, Mattias Ekstedt¹⁴, Stergios Kechagias¹⁴, Sven Francque¹⁵, Salvatore Petta¹⁶, Elisabetta Bugianesi¹⁷, Karine Clement¹⁸, Vlad Ratziu¹⁹, Jörn M. Schattenberg²⁰, Luca Valenti⁵, Christopher P. Day¹, Heather J. Cordell³, Ann K. Daly¹ *on behalf of the EPoS Consortium Investigators*[#]

¹ Translational & Clinical Research Institute, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom.

² Newcastle NIHR Biomedical Research Centre, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, United Kingdom.

³ Population & Health Sciences Institute, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom.

⁴ Bioinformatics Support Unit, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom.

⁵ Department of Pathophysiology and Transplantation, University of Milan, Translational Medicine - Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy.

⁶ Dept of Pathology, Aretaieio Hospital, National & Kapodistrian University of Athens, Greece.

⁷ Faculty of Health and Medical Sciences, The University of Adelaide, Adelaide, Australia

⁸ NIHR Nottingham Biomedical Research Centre, Nottingham University Hospitals NHS Trust and University of Nottingham, Nottingham, UK.

⁹ Liver Unit, Department of Medicine, Cambridge Biomedical Research Centre, Cambridge University NHS Foundation Trust, United Kingdom.

¹⁰ Department of Medicine, University of Helsinki, Helsinki, Finland & Helsinki University Hospital, Helsinki, Finland

¹¹ Department of Biochemistry and Wellcome Trust/MRC Institute of Metabolic Science, MRC Metabolic Diseases Unit, Metabolic Research Laboratories, University of Cambridge, UK

¹² University Clinic for Visceral Surgery and Medicine, University of Berne, Freiburgstrasse, Berne 3010, Switzerland

- 13 Division of Gastroenterology and Center for Autoimmune Liver Diseases, Department of Medicine and Surgery, University of Milano - Bicocca, Monza, Italy.
- 14 Division of Gastroenterology and Hepatology, Department of Medicine and Health Sciences, Linköping University, Linköping, Sweden.
- 15 Department of Gastroenterology and Hepatology, Antwerp University Hospital, Antwerp, Belgium.
- 16 Sezione di Gastroenterologia, Dipartimento Promozione della Salute, Materno-Infantile, di Medicina Interna e Specialistica di Eccellenza "G. D'Alessandro", Università di Palermo, Palermo, Italy.
- 17 Department of Medical Sciences, Division of Gastro-Hepatology, A.O. Città della Salute e della Scienza di Torino, University of Turin, Turin, Italy.
- 18 Sorbonne University, Inserm, Nutrition and obesity: Systemic approaches, Nutrition department, Pitié-Salpêtrière hospital, Assistance Publique-Hôpitaux de Paris, 75013 Paris, France.
- 19 Sorbonne Université, Assistance Publique-Hôpitaux de Paris, Hôpital Pitié Salpêtrière, Institute of Cardiometabolism and Nutrition (ICAN), Paris, France.
- 20 NAFLD Research Center, Department of Medicine, University Medical Center of the Johannes Gutenberg University, Mainz, Germany.
- 21 European Reference Network on Hepatological Diseases (ERN RARE-LIVER), San Gerardo Hospital, Monza, Italy

Corresponding Authors:**Prof Ann K. Daly PhD**

Professor of Pharmacogenetics,
Translational & Clinical Research Institute,
The Medical School, Newcastle University,
4th Floor, William Leech Building,
Framlington Place,
Newcastle upon Tyne, NE2 4HH,
United Kingdom.

Telephone: + 44 (0) 191 208 7031

Email: a.k.daly@ncl.ac.uk

Prof Quentin M. Anstee PhD, FRCP

Professor of Experimental Hepatology & Honorary Consultant Hepatologist,
Translational & Clinical Research Institute,
The Medical School, Newcastle University,
4th Floor, William Leech Building,
Framlington Place,
Newcastle upon Tyne, NE2 4HH,
United Kingdom.

Telephone: + 44 (0) 191 208 7012

Email: quentin.anstee@ncl.ac.uk

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Authors contributions:

Study concept and design: QMA, CPD, AKD; acquisition of data: QMA, CPD, LV, MM, DT, ADB, PB, OG, JP, YL-L, GPA, MA, HY-J, MV, J-FD, PI, DP, ME, SK, SF, SP, EB, KC, VR, JMS; analysis and interpretation of data: HJC, RD, QMA, SC, AKD; drafting of the manuscript: AKD, RD, HJC, QMA; critical revision of the manuscript for important intellectual content: all; statistical analysis: RD, HJC; obtained funding: QMA, CPD, AKD; administrative, technical, or material support: OG, JP, YLL; study supervision: QMA, HJC, CPD, LV, AKD.

The EPoS Consortium Investigators

NEWCASTLE UNIVERSITY, UK

Quentin M. Anstee

Simon Cockell

Heather J. Cordell

Ann K. Daly

Rebecca Darlay

Christopher P. Day

Olivier Govaere

Katherine Johnson

Yang-Lin Liu

Fiona Oakley

Jeremy Palmer

Helen Reeves

Dina Tiniakos

Kristy Wonders

ICAN, FRANCE

Karine Clement

Raluca Pais

Vlad Ratziu

Timothy Schwartz

UNIVERSITY OF MAINZ, GERMANY

Jörn M Schattenberg

Detlef Schuppan

*CONSIGLIO NAZIONALE DELLE
RICERCHE, ITALY*

Amalia Gastaldelli

UNIVERSITY OF TURIN, ITALY

Elisabetta Bugianesi

Fabio Marra

Maurizio Parola

Chiara Rosso

Ramy Younes

UNIVERSITY OF OREBRO, SWEDEN

Tuulia Hyötyläinen

Matej Orešič

UNIVERSITY OF HELSINKI, FINLAND

Hannele Yki-Järvinen

Panu K. Luukkonen

UNIVERSITY OF CAMBRIDGE, UK

Michael Allison

Sergio Rodriguez Cuenca

Vanessa Pellegrinelli

Michele Vacca

Antonio Vidal-Puig

NORDIC BIOSCIENCES, DENMARK

Morten Karsdal

Diana Leeming

Mette Juul Nielsen

IXSCIENT, UK

Dave Wenn

UNIVERSITY OF ZURICH

F. Stickel

Abstract

Background and Aims: Genetic factors associated with non-alcoholic fatty liver disease (NAFLD) remain incompletely understood. To date, most GWAS studies have adopted radiologically assessed hepatic triglyceride content as reference phenotype and so cannot address steatohepatitis or fibrosis. We describe a genome-wide association study (GWAS) encompassing the full spectrum of histologically characterized NAFLD.

Methods: The GWAS involved 1483 European NAFLD cases and 17781 genetically-matched population controls. A replication cohort of 559 NAFLD cases and 945 controls was genotyped to confirm signals showing genome-wide or close to genome-wide significance.

Results: Case-control analysis identified signals showing p-values $\leq 5 \times 10^{-8}$ at four locations (chromosome (chr) 2 *GCKR/C2ORF16*; chr4 *HSD17B13*; chr19 *TM6SF2*; chr22 *PNPLA3*) together with two other signals with $p < 1 \times 10^{-7}$ (chr1 near *LEPR* and chr8 near *IDO2/TC1*). Case-only analysis of quantitative traits steatosis, disease activity score, NAS and fibrosis showed that the *PNPLA3* signal (rs738409) was genome-wide significantly associated with steatosis, fibrosis and NAS score and identified a new signal (*PYGO1* rs62021874) with close to genome-wide significance for steatosis ($p = 8.2 \times 10^{-8}$). Subgroup case-control analysis for NASH confirmed the *PNPLA3* signal. The chr1 *LEPR* SNP also showed genome-wide significance for this phenotype. Considering the subgroup with advanced fibrosis ($\geq F3$), the signals on chromosomes 2, 19 and 22 remained genome-wide significant. With the exception of *GCKR/C2ORF16*, the genome-wide significant signals replicated.

Conclusions: This study confirms *PNPLA3* as a risk factor for the full histological spectrum of NAFLD at genome-wide significance levels, with important contributions from *TM6SF2* and *HSD17B13*. *PYGO1* is a novel steatosis modifier, suggesting relevance of Wnt signalling pathways in NAFLD pathogenesis.

Lay summary

Non-alcoholic fatty liver disease (NAFLD) is a common disease where excessive fat accumulates in the liver and may result in cirrhosis. To understand better who is at risk of developing this disease and suffering liver damage, we undertook a genetic study whereby we compared genetic profiles of people suffering from fatty liver disease with genetic profiles seen in the general population. We found that particular sequences in four different areas of the human genome were seen at different frequencies in the fatty liver disease cases. Knowledge of people's genotype for these sequences may help predict individual risk of developing advanced disease. Some genes where these sequences are located may also be good targets for future drug treatments.

Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of progressive liver disease characterised by increased hepatic triglyceride content (HTGC) in the absence of excess alcohol consumption.[1] NAFLD encompasses steatosis (non-alcoholic fatty liver, NAFL), steatohepatitis (non-alcoholic steatohepatitis, NASH), fibrosis and ultimately cirrhosis. It is strongly associated with features of the metabolic syndrome (obesity, type 2 diabetes mellitus [T2DM] and dyslipidaemia).[1] Although common, affecting approximately 25% of the global adult population, only a minority of NAFL patients develop NASH, progress to significant fibrosis or experience associated morbidity.[1, 2] NAFLD is best considered a complex trait where disease phenotype results from environmental exposures acting on a susceptible polygenic background comprising multiple independent modifiers.[3]

Genome-wide association studies (GWAS) have contributed greatly to our understanding of the genetic contribution to NAFLD pathogenesis and variability of prognosis.[3] Amongst the loci identified, the non-synonymous single nucleotide polymorphism (SNP) in *PNPLA3* (phospholipase domain-containing 3) (rs738409),[4, 5] and more recently, a non-synonymous SNP in *TM6SF2* (transmembrane 6 superfamily member 2) (rs58542926), originally ascribed to the neighbouring *NCAN* gene,[5] have been associated with ¹H-MRS quantified HTGC.[6] Both genetic associations have been replicated in further studies where they have been associated not only with steatosis, but also with clinically relevant factors including grade of steatohepatitis and stage of hepatic fibrosis/cirrhosis[7, 8] and, in the case of *PNPLA3*, with the development of NAFLD-related HCC.[9, 10] A number of other associations, with *LYPLAL1*, *GCKR*, and *PPP1R3B*, have been reported by GWAS comprising relatively few histologically characterised cases and are currently less robustly replicated[3, 5]. A recent study using exome sequencing[11] confirmed a previously reported association of raised alanine transaminase (ALT) with a *HSD17B13* SNP (rs6834314)[12] in a general patient population

and then demonstrated an association of this polymorphism with NAFLD. Two further studies genotyped for *HSD17B13* only and broadly confirmed this association.[13, 14]

To date, most adequately powered GWAS studies relevant to NAFLD have addressed either radiologically determined HTGC[4, 6, 12] or clinical biochemistry parameters such as ALT.[12, 15] They have therefore been unable to address the more clinically relevant phenotypes of steatohepatitis grade or fibrosis stage (reviewed[3]). One GWAS has assessed a large number of histologically characterised patients, reporting associations with both *PNPLA3* and with chromosome 19 close to *TM6SF2*. [16] These patients, however, were recruited from bariatric surgery programs with dietary restrictions prior to surgery and wedge biopsy collection which may affect liver histology; in addition such patients tend to be younger and with a higher average BMI than NAFLD cases more generally.[17] The current study aims to seek genetic modifiers of steatohepatitis and fibrosis attaining genome-wide levels of statistical significance using a large internationally-derived cohort of patients with histologically characterised NAFLD with all stages of the disease well represented. We now report the largest histology-based NAFLD GWAS to date in a cohort of 1483 European patients exhibiting the full spectrum of biopsy-proven NAFLD.

Materials and Methods

NAFLD CASES

For the main GWAS study, patients were recruited from clinics at a number of leading European tertiary liver centres (see Supplementary Methods). Additional cases for replication were recruited at Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy. The study had the necessary ethical approvals from the relevant national/institutional review boards (see Supplementary Methods) and all participants provided informed consent. All cases were unrelated patients that had undergone a liver biopsy as part of the routine diagnostic workup for presumed NAFLD having originally been identified due to abnormal biochemical tests (ALT and/or GGT) and/or an ultrasonographically detected bright liver, associated with features of the metabolic syndrome; or having abnormal biochemical tests (ALT and/or GGT) and macroscopic appearances of a steatotic liver at the time of bariatric surgery. Full details of inclusion/exclusion criteria are provided in the Supplementary Methods.

CONTROLS

We used general population samples with existing genome-wide genotype data as study controls. For the GWAS, we selected European ancestry controls (n=17,781) from multiple sources as described in the Supplementary Methods. To replicate GWAS associations, we used an Italian control cohort (n=945) consisting of controls described previously[18] with some newly collected individuals. Any that were found to match the Hypergenes controls already used in our discovery GWAS were excluded.

HISTOLOGY

Liver biopsy specimens (at least 1.6 cm length and ~1 mm diameter) were formalin-fixed and paraffin-embedded. Tissue sections (5 µm-thick) were

routinely stained with haematoxylin and eosin and trichrome stain for visualizing collagen. All cases were recruited at tertiary centres where liver biopsies were routinely assessed according to accepted criteria by experienced liver pathologists and scored using the well validated NIDDK NASH-CRN system.[19] To ensure optimum data quality, biopsies were retrieved from archival storage where possible (78% of cases) and scored centrally by an expert liver pathologist from the FLIP/EPoS central pathology team (DT, ADB, PB), as described in detail previously.[20] Where archival samples were unavailable for central reading, the local liver pathologist's scores were used. To maximise insights into the specific pathophysiological processes that occur as NAFLD progresses, six phenotypes of interest were studied: degree of Steatosis (S0-3); degree of Ballooning (B0-2); degree of Lobular Inflammation (I0-3); severity of NASH activity (calculated as 'Disease Activity' = Hepatocyte Ballooning (B0-2) + Lobular Inflammation (I0-3) and also an overall 'NAS' combining all three parameters (NAS0-8)); and stage of Fibrosis (F0-4).

GENOTYPING

DNA was prepared from blood samples collected with EDTA as anticoagulant as described previously.[21] For GWAS genotyping, genotyping of cases was carried out in two phases. For phase I, genotyping was performed initially using the Illumina OmniExpress BeadChip by Edinburgh Clinical Research Centre. To obtain data for additional exomic SNPs, further genotyping of these samples was performed using the Illumina HumanCoreExome BeadChip (Aros, Denmark). Genome-wide genotyping of the phase II cases was performed using the Illumina OmniExpressExome BeadChip by the Edinburgh Clinical Research Centre. A total of 721078 markers shared across the batches passed quality control (QC) (see Supplementary Methods). SNP imputation was performed as described in detail in the Supplementary Methods.

Top associated SNPs were further confirmed in replication cases using TaqMan[®] SNP genotyping assays (ThermoFisher Scientific, Waltham, MA) in accordance with the manufacturer's recommendations. If an assay could not be designed for the SNP showing the strongest signal for the region, a suitable proxy SNP was chosen (<https://ldlink.nci.nih.gov/?tab=home>).

RNA SEQUENCING AND IN VITRO STUDIES

RNA sequencing (RNAseq)

RNAseq data on samples from 206 liver biopsies from NAFLD patients as described elsewhere (Govaere et al., submitted) was used to further investigate functional significance of *HSD17B13* variants.

Bioluminescent retinol dehydrogenase assays for HSD17B13

Retinol (75 μ M; Sigma-Aldrich, St. Louis, Missouri, USA) was incubated with recombinant HSD17B13 (TP313132; Origene, Maryland, USA) for 1h at room temperature in the presence of 0.5 mM NAD in 200 mM Tris-HCl, pH7.5. As a control, the known HSD17B13 substrate β -estradiol (75 μ M) was incubated in parallel assays. NADH production was measured by Bioluminescent NAD/NADH-Glo[™] Assay (Promega, Wisconsin, USA) according to manufacturer's guidelines.

STATISTICAL ANALYSIS

We used principal component (PC) analysis of the genome-wide genotype data to investigate the ancestry of the cases and controls; this showed the expected north/south variation commonly seen across Europe[22] but, importantly, suggested adequate matching between cases and controls (Fig S1a and Fig S1b). Case/control analysis and quantitative trait analysis (QTA) of GWAS data was performed as described in detail in the Supplementary Methods, using a linear mixed modelling approach with the incorporation of

the top 5 PCs as covariates to adjust for any population stratification. Examination of the resulting genome-wide QQ plots and genomic control inflation factors (λ)[23] (see Results) indicated that this adjustment adequately corrected for any population differences.

Significance of findings in the replication cohort was assessed by calculation of odds ratios, 95% confidence intervals and p-values by univariate analysis and multiple logistic regression using PLINK.[24]

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Results

CLINICAL CHARACTERISTICS OF THE CASES

Clinical details of the NAFLD cases included in the main GWAS are summarized in Table 1. The replication cohort details are shown in Table S1. All cases in both cohorts were of white European ethnicity. The percentage with advanced fibrosis (stage F3 or F4) was similar in both cohorts ($p > 0.05$) but other parameters including age, BMI, T2DM, sex and incidence of NASH were different.

OVERALL NAFLD CASE-CONTROL ANALYSIS

The overall NAFLD case-control analysis is presented as a Manhattan plot (Fig 1). PCA scattergrams for cases and controls are shown in Fig S1 and the QQ plot of the association results in Fig S2. As summarised in Table 2, 4 different regions (on chromosomes 2, 4, 19 and 22) passed conventional genome-wide significance ($p < 5 \times 10^{-8}$) with two other regions (on chromosomes 1 and 8) showing p-values $< 1 \times 10^{-7}$ (for LocusZoom plots see Fig S3). Data presented in Fig. 1 were obtained from imputation analysis. Primary case-control analysis without imputation showed similar signals in chromosomes 2, 4, 19 and 22 only but no additional signals at $p < 1 \times 10^{-7}$ (Fig. S4 and Table S2). Correction of the imputed data for sex in addition to the first 5 principal components used in the main analyses did not result in large changes in p-value (Table S3). Together, these results point to *PNPLA3*, *TM6SF2*, *HSD17B13* and the *GCKR/C2ORF16* region being the major risk factors for disease susceptibility with borderline signals for chromosome 1 near *LEPR* and for chromosome 8 adjacent to *IDO2* and *TC1(C8orf4)*. In view of the well-established strong association of *PNPLA3* rs738409 with NAFLD, additional analysis using a model conditioning on this SNP was performed. This analysis gave broadly similar findings to those summarised in Table 2 with no new signals (data not shown).

QUANTITATIVE TRAIT ANALYSIS ON NAFLD PHENOTYPES

Case-only analyses assessing relevance of genotype to grade of steatosis, grade of steatohepatitis (assessed as predefined 'Disease Activity' and 'NAS') and stage of fibrosis were also performed using the imputed data. Results of these analyses are shown in Fig. 2 with the most significant signals summarised in Table 3 (for QQ and LocusZoom plots see Figs. S5 and S6). The primary data without imputation are summarised in Fig. S7 and Table S4. For steatosis, NAS and fibrosis as quantitative traits, signals with $p < 10^{-10}$ were detected for *PNPLA3* rs738409 and other SNPs in this region of chromosome 22. For steatosis, a signal with $p = 8.2 \times 10^{-8}$ on chromosome 15 (rs62021874 in *PYGO1*) was also detected (Table 3). This variant is in complete linkage disequilibrium with a missense variant rs11858624 which also showed a signal close to significance ($p = 1.7 \times 10^{-7}$). No signals reached conventional genome-wide significance ($P < 5 \times 10^{-8}$) for disease activity score alone or when ballooning or inflammation were considered as individual traits (Fig. S8). The effect of correction of the imputed data for clinical covariates was also assessed for each trait (Table S5), giving results very similar to those obtained originally.

To further assess relevance of genotype to particular NAFLD phenotypes, the contribution to NAFLD progression of the four major genome-wide significant genetic risk factors identified in the case-control GWAS was assessed by calculating a combined genetic risk score based on summing the allele count (with no weighting by effect size) for *PNPLA3* rs738409, *TM6SF2* rs58542926, *GCKR* rs1260326 and *HSD17B13* rs9992651 and relating the resulting score to grade of steatosis, NAFLD activity score (NAS score) and fibrosis stage (Fig. S9). Trend tests by linear regression showed that there was a statistically significant relationship between the value of the semi-quantitative steatosis/NAS/fibrosis scores and the value of the genetic risk score for all three phenotypes, with the most significant relationship ($p = 4.68 \times$

10^{-13}) detected for fibrosis stage (Fig S9). Those with a risk score of 2 (n=216) had a mean fibrosis score of 1.27 (se 0.08) compared with 1.94 (se 0.09) for a risk score of 5 (n=260).

ADDITIONAL SUBGROUP CASE-CONTROL ANALYSIS

Since both steatohepatitis and advanced fibrosis are clinically important phenotypes in NAFLD,[25] additional case-control analyses were undertaken including cases with NASH only (n=836) and fibrosis stage F3 and F4 only (n=386). The findings for both phenotypes are summarised in Fig. 3 and Table 4 (for QQ and LocusZoom plots see Fig. S10 and S11). For NASH, signals showing p-values of $<5 \times 10^{-8}$ were detected for chromosome 1 (*LEPR*) and chromosome 22 (*PNPLA3*) (Table 4). For *LEPR* rs12077210, the p-value of 4.4×10^{-9} was lower for NASH than for NAFLD overall (Table 2). A second novel chromosome 1 signal with $p=7.1 \times 10^{-8}$ located in an intergenic region downstream of phospholipase A2 group IVA (*PLA2G4A*) was also detected. The SNPs in chromosomes 2, 4 and 19 that were significant in the main case-control analysis showed p values in the region of 2×10^{-7} so came close to significance for NASH. For fibrosis stages F3 and F4, chromosome 2, 19 and 22 signals showing p-values of $<5 \times 10^{-8}$ were detected but the signals from the main case-control analysis detected previously for chromosomes 1, 8 and 4 showed p values $>1 \times 10^{-7}$. For *HSD17B13* rs9992651 (chromosome 4), the p value was 1.16×10^{-5} .

REPLICATION OF GWAS SIGNALS AND INVESTIGATION OF ADDITIONAL POSSIBLE NAFLD RISK FACTORS

A replication cohort of 559 Italian NAFLD cases was assembled from a different centre to the discovery cohort. Allele frequencies for selected SNPs in these cases were compared with those for Italian controls. Findings for 8 separate loci giving signals with $p < 1 \times 10^{-7}$ in either the main GWAS or the quantitative trait studies are summarised in Table 5. The *PNPLA3*, *TM6SF2* and *HSD17B13* signals seen in the main GWAS replicated ($p < 0.05$) but we

found only borderline effects or no significance for 4 other loci. However, the *PYGO1* signal, which was associated with steatosis by quantitative trait analysis, showed a significant association in the analysis in the same protective direction as observed for steatosis. The *GCKR/C2Orf16* signal did not replicate either in the main replication cohort (Table 5) or in a subgroup of replication cases (n=134) with fibrosis stage 3 or 4. Due to the relatively low number of NASH cases in the replication cohort, we did not seek to replicate the novel rs80084600 signal seen for this phenotype. Multiple logistic regression analysis with adjustment for *PNPLA3* rs738409 and *TM6SF2* rs58542926 (Table 5) generated similar findings to the univariate analysis, apart from small decreases in p-values for the *HSD17B13* and *PYGO1* signals.

Results for selected variants reported recently by others as risk factors for NAFLD but which had not shown p-values of $p < 1 \times 10^{-7}$ in the current GWAS were also extracted from the main case-control analysis. Only rs2642438 in mitochondrial amidoxime reducing component 1 (*MARC1*) and rs28929474 in alpha1-antitrypsin (*AAT*) showed p-values < 0.05 (Table S6). For rs2642438, the p-value was 6×10^{-6} with a protective odds ratio of 0.816, in line with that reported previously.[26]

EQTL ANALYSIS AND STUDIES ON EXPRESSION OF GWAS SIGNALS IN LIVER BIOPSIES FROM DIFFERENT NAFLD STAGES

While the signals seen for NAFLD relating to *PNPLA3*, *TM6SF2* and *GCKR* are already well-established risk factors for this disease from population studies[4-6, 27] and studies on functional significance,[28-30] evidence for functional significance for the other signals is limited. The relationship of rs9992651 and rs72613567 in *HSD17B13* with gene expression was evaluated by sequencing RNA samples from liver biopsies. Three different *HSD17B13* transcripts were detected (Fig. S12), including a full length transcript with all 7 exons, a variant with exon 2 deleted and a variant without

exon 6. Based on genotype for rs9992651 from the RNAseq data, the variant missing exon 6 was generally not detectable in homozygotes for the reference G allele but was expressed at a higher level in homozygotes for the minor A allele and also heterozygotes. The ability of recombinant HSD17B13 to oxidise retinol[13] was also confirmed (Fig. S13).

Other loci showing associations in the case-control studies including rs12077210 in *LEPR* (intronic), rs139648192 on chromosome 8 and rs80084600 on chromosome 1 could not be investigated by RNA sequencing due to their locations. The borderline significant rs11858624 in *PYGO1* (Table 3) is a missense variant (P299H). Analysis with data obtained from GTEx (<https://gtexportal.org/home/>) indicated no difference in RNA expression between rs11858624 homozygous wild-types and heterozygotes in liver tissue (Fig. S14).

Discussion

This study is the largest GWAS to date on histologically characterised NAFLD enrolled in a hepatology setting that addresses the full disease spectrum from steatosis to cirrhosis. This contrasts with the only previous GWAS involving more than 1000 histologically characterised cases, which was in a predominantly female bariatric cohort with extreme obesity but relatively mild NAFLD.[16] Furthermore, that study only considered grade of steatosis, not the more clinically relevant phenotypes of steatohepatitis or fibrosis.[16] The current study confirms the well-established signals in *PNPLA3*, *TM6SF2* and *GCKR*, together with the more recently reported *HSD17B13* signal.[11] The findings for *GCKR* are in line with several candidate gene studies on NAFLD however, this is the first GWAS study reporting this four gene combination as NAFLD risk modifiers.

HSD17B13 has been reported to be relevant to NAFLD with several variants associated with decreased risk.[11, 13] The current study found a protective effect against NAFLD generally, with the strongest effect related to the SNPs rs9992651 and rs13118664. These SNPs are in non-coding regions of *HSD17B13* but are in strong linkage disequilibrium with rs72613567, which is associated with a single base-pair insertion that has been suggested to be of functional significance in relation to RNA splicing.[11] The current study confirms that an *HSD17B13* isoform lacking exon 6 is associated with rs9992651 and a protective effect against NAFLD; consistent with a report showing a similar splicing pattern with the SNPs rs6834314 and rs72613567[13] but differing from that described in the original report.[11] Consistent with that recent study,[13] we also show the *HSD17B13* gene product possesses retinol dehydrogenase activity. Retinol metabolism is a complex multistep process involving a number of different enzymes.[31] While it remains unclear whether loss of *HSD17B13* retinol dehydrogenase activity can explain the protective effect of the variant, it is likely that enzyme activity in the reverse direction involving retinal reduction to retinol could also be impaired since these enzymes operate in both oxidising and reducing

directions.[31] Thus, increased levels of retinal and the biologically active retinoic acid isomers could occur in those carrying *HSD17B13* variants. This effect might protect against NAFLD development, in line with recent evidence that 13-*cis* and all-*trans* retinoic acid are found at significantly decreased levels in human livers with NAFLD.[32] A clear trend towards a protective effect against advanced hepatic fibrosis was observed, although this did not reach genome-wide significance levels (p-value approx. 10^{-5}). Given that the strength of association with NASH was stronger (p-values approx. 2×10^{-7}), it may be that the protective effect of *HSD17B13* is more relevant to development of steatohepatitis than progression of fibrosis.

The *GCKR* signal in both the main GWAS and advanced fibrosis-only analysis identified rs1260326 as the most significant SNP within this region, with T-variant carriage increasing NAFLD risk. This common missense variant has been studied widely both as a risk factor for T2DM and for NAFLD. An upstream SNP, rs780094, in strong linkage disequilibrium with rs1260326, has also been shown to be a NAFLD risk factor in candidate gene studies.[33] The relationship between both SNPs and susceptibility to NAFLD and T2DM is complex. Rs1260326 is well established to have a protective effect against T2DM, probably due to the *GCKR* variant showing weaker interaction with glucokinase compared with the wild-type.[34] This promotes hepatic glucose metabolism, decreasing plasma glucose levels, and is associated with an increased risk of NAFLD.[33] The underlying mechanism is unclear but rs1260326 is associated with higher levels of circulating lactate,[35] presumably due to increased glucose metabolism via glycolysis. The inability to replicate the *GCKR* association was slightly surprising but may reflect the overall lower severity of NAFLD in the replication cohort. There are a relatively large number of reports of a significant increased risk for *GCKR* variants in NAFLD generally, especially for paediatric cases.[27, 36, 37]

A further interesting finding relates to a signal on chromosome 15 (rs11858624) that was close to genome-wide significance for steatosis and was validated in the replication study. The gene involved is *PYGO1*, which

encodes a transcription factor that contributes to the Wnt signalling pathway.[38] The exact impact of *PYGO1* in Wnt signalling remains unclear, though a homologue *PYGO2* appears to contribute to several physiological pathways including increased adiposity and impaired glucose tolerance in mice lacking this protein.[39]

Signals on chromosomes 1 and 8 were detected in the case-control analysis however these just failed to meet genome-wide significance and did not replicate. The chromosome 1 SNP was genome-wide significant in the NASH-only case-control analysis and lies in the region encoding *LEPROT* and *LEPR*; both genes share the same promoter and first two exons but encode separate proteins. This association is notable given that *db/db* mice, carrying a spontaneous loss of function mutation in the OB-Rb leptin receptor, have been widely used to model NAFLD [40]. There are also some previous reports from candidate gene studies that *LEPR* variants are risk factors for NAFLD but the current variant lies considerably upstream of these previously studied variants [41, 42]. The signal on chromosome 8 relates to an area between *IDO2* and *TC1*. Of potential relevance to NAFLD, both genes have roles in modulating inflammation with *IDO2* inducible by lipopolysaccharide and contributing to immune function[43] while *TC1* modulates NF κ B signalling. Further investigation of these variants is needed. The subgroup analysis on NASH grade showed a second novel chromosome 1 signal separate from *LEPR*. The p-value for NASH, though not genome-wide significant at 7×10^{-8} , was considerably lower than that seen for this variant in the main case-control study (0.0049). The variant is in an intergenic region but is downstream of *PLA2G4A*, which shows elevated expression in adipose tissue in obesity and may contribute to T2DM susceptibility.[44]

The most significant associations in this study were obtained for NAFLD in the binary case-control design. The quantitative trait analyses has shown a clear association for *PNPLA3* rs738409 with steatosis, NAS score and fibrosis, which is generally in line with previous reports in NAFLD and ALD.[45] However, there were no significant associations of any genotype with disease

activity when considered separately from steatosis. The failure to see more specific associations for *TM6SF2* and *HSD17B13* with other histological traits similar to those reported previously in candidate gene studies may reflect the complex nature of the histological disease phenotype[8, 11] and also limited statistical power. In contrast to quantification of HTGC by imaging techniques, which provides a highly reproducible quantitative measure of a single biochemical entity, the histological scoring systems used to evaluate steatohepatitis and fibrosis provide only non-linear, semiquantitative or categorical assessments of disease and are subject to intra- and inter-observer variation. Indeed, clear diagnostic consensus regarding the presence or absence of steatohepatitis among pathologists is not always feasible.[19, 20, 46] Thus, the conduct of a histologically-based GWAS, whilst addressing the most clinically relevant phenotypic characteristics, is technically more challenging. We have addressed this challenge by having expert liver pathologists providing histological diagnosis and scoring. The reduced statistical power due to limited number of cases in particular histological categories, may limit the number of variants that attain the genome-wide significance threshold to only the most strongly associated such as the *PNPLA3* variant. Despite these limitations, disease severity was correlated with genetic risk score based on the most significant case-control GWAS signals, statistically significant relationships for association of the risk score with increasing degree of steatosis, grade of steatohepatitis and fibrosis stage were found, which suggests that a risk score approach may be of value prognostically although further studies on this are needed.

Despite a fairly extensive supporting literature, we and others[47] have not found *MBOAT7* to be a risk factor for NAFLD. Notably, no NAFLD focussed GWAS to date has reported a significant association with *MBOAT7*. Other signals for NAFLD reported by others previously including in *PPP1R3B*,[5] alpha1 antitrypsin[48] and interferon lambda 4[49] also failed to show genome-wide significance in the case-control analysis. This is not surprising in the case of alpha-1-antitrypsin as patients known to have this condition were specifically excluded from the cohort, limiting the MAF substantially.

However, the gene *MARC1*, where a nonsynonymous variant has been reported to protect against both "all cause" cirrhosis and fatty liver disease,[26] showed a similar protective effect against NAFLD with a low p value, though this did not attain genome-wide significance. This gene encodes the mitochondrial amidoxime-reducing component enzyme which can reduce trimethylamine N-oxide (TMAO) generated by oxidation of trimethylamine. Elevated plasma TMAO has been suggested to be a risk factor for cardiovascular disease and type 2 diabetes so could also be relevant to risk of NAFLD.[50]

There are several limitations to our study. NAFLD is a common phenotype in the general population, affecting up to 25% of individuals in Europe.[51] Our population controls cannot therefore be considered to be entirely free of NAFLD and there is no way of investigating this further. Our use of large numbers of controls with genetic matching helps mitigate the risk that this will lead to underestimate of genuine genetic risk factors but does not eliminate it entirely. We undertook some "case only" studies, which included a small group of patients with biochemical evidence of NAFLD but liver biopsies showing steatosis below the normal disease definition, to further mitigate this. It is generally accepted that histological interpretation of liver biopsies is subject to some inter-observer variation, even amongst experienced hepatopathologists.[19, 52] This is therefore inherent to a histopathological phenotype. However, all data used in the analysis were generated by highly experienced liver pathologists based in tertiary centres and, to further mitigate against this issue, the majority of liver biopsies were scored by a member of the project's central pathology team. Finally, our replication cohort was not perfectly matched with our discovery cohort in terms of disease severity and factors such as sex, T2DM and BMI. This is due, at least in part, to this being from a single centre from Southern Europe where NAFLD risk factors such as diet may be different to those further north in the continent, resulting in lower obesity rates within the NAFLD population.[53] We were unfortunately not able to identify another suitable European replication cohort involving patients who had undergone liver biopsy following referral to a hepatology clinic.

In conclusion, this relatively large GWAS of histologically characterised NAFLD cases has confirmed previously reported associations and provided evidence for four novel signals. Much larger meta analyses may be helpful in investigating the relevance of these novel signals.

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Table 1**Characteristics of the cohort (n=1483)****Patient demographic and clinical characteristics**

Age (years) (mean±SD)	50.1 ± 13.0
Sex (% female)	47.3 %
BMI median kg/m ² (IQR)	35.19 (29.1-39.7)
T2DM n (%)	593(40.0)*

Histologic characteristics

Steatosis n (%)	
0	53 (3.6)
1	483 (32.6)
2	541 (36.5)
3	390 (26.3)
Missing	16 (1.1)
NAS score n (%),	
0	19 (1.3)
1	138 (9.3)
2	225 (15.2)
3	258 (17.4)
4	271 (18.3)
5	283 (19.1)
6	178 (12.0)
7	80 (5.4)
8	15 (1.0)
Missing	16 (1.1)
Disease activity score n (%)	
0	255 (17.2)
1	285 (19.2)
2	418 (28.2)
3	308 (20.8)
4	166 (11.2)
5	35 (2.4)
Missing	16 (1.1)
NASH n (%)	
Yes	836 (56.4)
No	631 (42.5)
Missing	16 (1.1)
Fibrosis n (%)	
0	432 (29.1)
1	350 (23.6)
2	312 (21.0)
3	240 (16.2)
4	147 (9.9)
Missing	2 (0.13)

*For T2DM, 5 (0.33%) missing

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Table 2**Summary of top findings in the NAFLD case-control analysis**

SNP	Chr	A1	Gene	P	OR (95% CI)
rs12077210*	1	T	LEPR	5.62E-08	1.484 (1.287-1.711)
rs1260326*	2	T	GCKR	1.06E-10	1.278 (1.186-1.377)
rs1919127*	2	C	C2orf16	5.61E-10	1.290 (1.190-1.398)
rs2068834	2	C	ZNF512	8.49E-11	1.302 (1.202-1.410)
rs9992651	4	A	HSD17B13	2.78E-08	0.744 (0.671-0.826)
rs13118664	4	T	HSD17B13	1.41E-08	0.740 (0.667-0.821)
rs139648192	8	T	-	5.20E-08	1.538 (1.317-1.796)
rs58542926*	19	T	TM6SF2	2.05E-11	1.609 (1.400-1.849)
rs8107974	19	T	SUGP1	2.58E-12	1.632 (1.423-1.872)
rs17216588	19	T	-	7.25E-14	1.612 (1.423-1.827)
rs10500212	19	T	PBX4	3.40E-12	1.549 (1.369-1.752)
rs738409*	22	G	PNPLA3	1.45E-49	1.827 (1.687-1.979)

7412561 imputed SNPs included, Total number of cases and controls=19264

*Denotes validated SNP following imputation

The first 5 principal components were included as covariates

Table 3**Summary of top findings in quantitative trait analysis**

SNP	Chr	A1	Gene	Phenotype	n	P (no clinical covariates)	Beta (95% CI)
rs738409*	22	G	PNPLA3	Steatosis	1469	2.37E-09	0.183 (0.123-0.243)
rs62021874	15	T	PYGO1	Steatosis	1469	8.16E-08	-0.303 (-0.414- -0.192)
rs11858624*	15	T	PYGO1	Steatosis	1469	1.64E-07	-0.295 (-0.406- -0.185)
rs738409*	22	G	PNPLA3	Fibrosis	1481	7.58E-11	0.318 (0.222-0.414)
rs738409*	22	G	PNPLA3	NAS	1467	8.78E-09	0.364 (0.240-0.488)

Results for 7900223 imputed SNPs. First 5 principal components were included as covariates

*validated directly by genotyping

Table 4

Summary of top findings from case-control analysis for NAFLD cases with NASH or with fibrosis scores F3 and F4 only

NASH

SNP	Chr	Gene	P-value (no clinical covariates)	OR (95% CI)
rs12077210	1	LEPR	4.42E-09	1.671 (1.390-2.008)
rs80084600	1	-	7.08E-08	1.977 (1.543-2.533)
rs1260326	2	GCKR	3.78E-07	1.302 (1.176-1.442)
rs9992651	4	HSD17B13	2.92E-07	0.718 (0.633-0.815)
rs13118664	4	HSD17B13	2.37E-07	0.716 (0.631-0.813)
rs58542926	19	TM6SF2	1.90E-07	1.606 (1.344-1.919)
rs8107974	19	SUGP1	1.36E-07	1.609 (1.348-1.920)
rs738409	22	PNPLA3	2.58E-44	2.053 (1.856-2.271)

N = 18617 (Cases = 836, Controls = 17781), covariate model includes first 5 principal components

Fibrosis F3/F4

SNP	Chr	Gene	P-value (no clinical covariates)	OR (95% CI)
rs1260326	2	GCKR	4.07E-10	1.678 (1.427-1.974)
rs56255430	19	-	2.11E-10	1.863 (1.538-2.257)
rs738409	22	PNPLA3	5.66E-31	2.374 (2.051-2.748)

N=18167 (Cases=386, Controls=17781), covariate model includes first 5 principal components.

Table 5

Genotype frequencies in replication cohort

Gene	SNP	Case frequency	Control frequency	Univariate analysis		Multiple logistic regression adjusting for <i>PNPLA3</i> rs738409 and <i>TM6SF2</i> rs58542926	
				Odds ratio	P value	Odds ratio	P value
<i>LEPR</i>	rs12077210	0.05877	0.05983	0.98 (0.71-1.35)	0.91	0.96 (0.69-1.34)	0.81
<i>GCKR</i>	rs1260326	0.5407	0.5305	1.04 (0.90-1.21)	0.59	1.08 (0.92-1.27)	0.36
<i>C2ORF16</i>	rs1919127	0.382	0.3566	1.12 (0.96-1.30)	0.16	1.1 (0.94-1.29)	0.25
<i>HSD17B13</i>	rs72613567	0.2101	0.2462	0.81 (0.68-0.97)	0.025	0.78 (0.64-0.95)	0.013
<i>IDO2</i> <i>/TC1(C8orf4)</i>	rs79137099	0.03789	0.03891	0.97 (0.66-1.44)	0.89	1.05 (0.6-1.59)	0.83
<i>PYGO1</i>	rs11852624	0.05144	0.0709	0.71 (0.52-0.98)	0.035	0.67 (0.48-0.96)	0.027
<i>TM6SF2</i>	rs58542926	0.08813	0.05027	1.83 (1.36-2.45)	4.63E-05	NA	NA
<i>PNPLA3</i>	rs738409	0.4436	0.2754	2.10 (1.80-2.45)	6.60E-21	NA	NA

Figure legends

Figure 1. Manhattan plot from imputed GWAS case-control analysis.

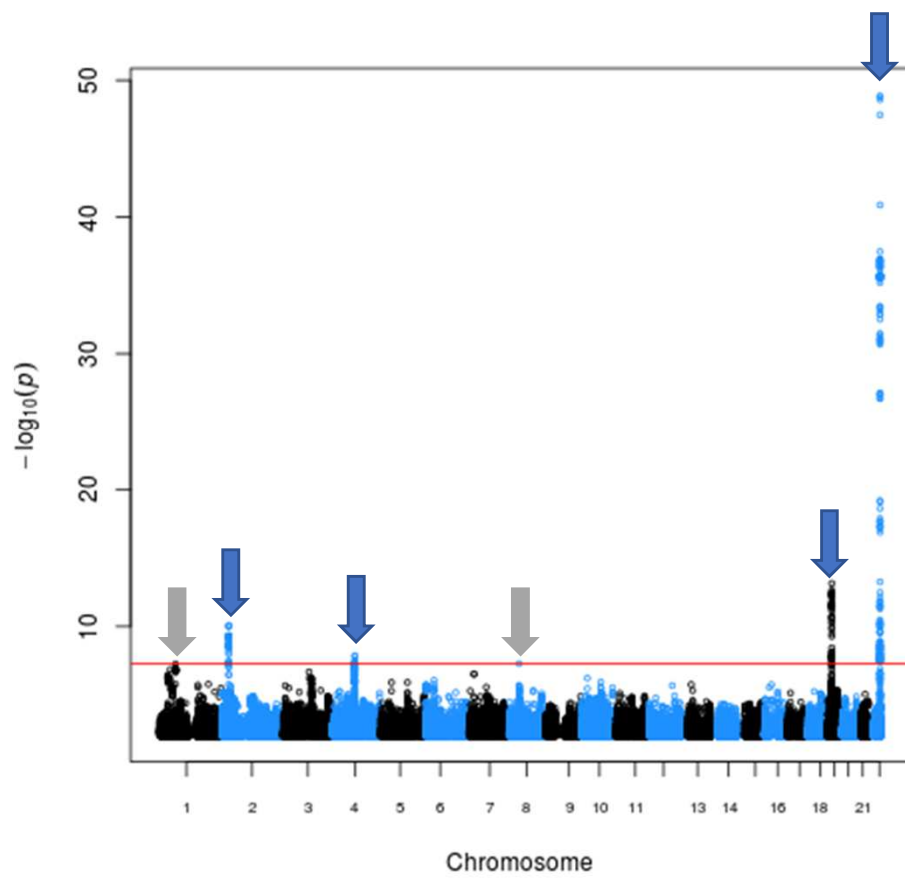
Included 1483 NAFLD cases and 17781 controls. Threshold for genome-wide significance was taken to be 5×10^{-8} . The first 5 principal components were included as covariates. Genome-wide significant signals are indicated by blue arrows with those showing p in the range 1×10^{-7} to 5×10^{-8} shown by grey arrows.

Figure 2. Manhattan plots from imputed GWAS analysis on the basis of quantitative traits.

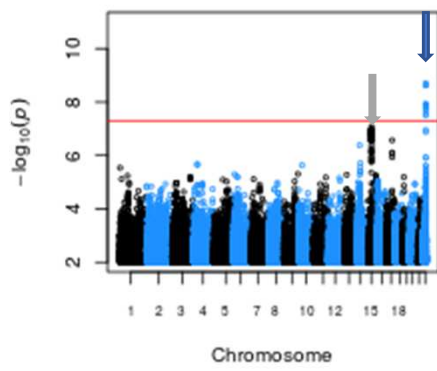
Included 1483 NAFLD cases. Threshold for genome-wide significance was taken to be 5×10^{-8} but signals showing $p < 1 \times 10^{-7}$ are also indicated. Panel A shows data for steatosis, B for fibrosis, C for disease activity score and D for NAS score. The first 5 principal components were included as covariates. Genome-wide significant signals are indicated by blue arrows with those showing p in the range 1×10^{-7} to 5×10^{-8} shown by grey arrows.

Figure 3. Manhattan plots from imputed GWAS case-control analysis of NASH and severe fibrosis (F3/F4).

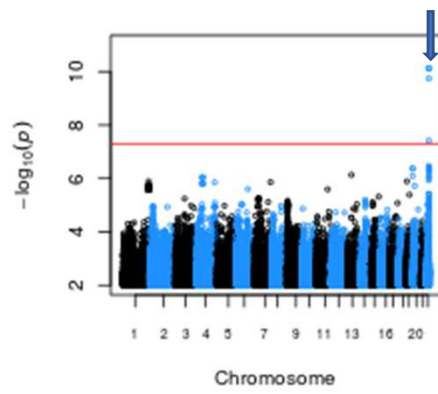
Threshold for genome-wide significance was taken to be 5×10^{-8} . The first 5 principal components were included as covariates. Panel A. NASH analysis. 836 cases and 17781 controls. Panel B. F3/F4 analysis. 386 cases and 17781 controls. Genome-wide significant signals are indicated by blue arrows with those showing p in the range 1×10^{-7} to 5×10^{-8} shown by grey arrows.



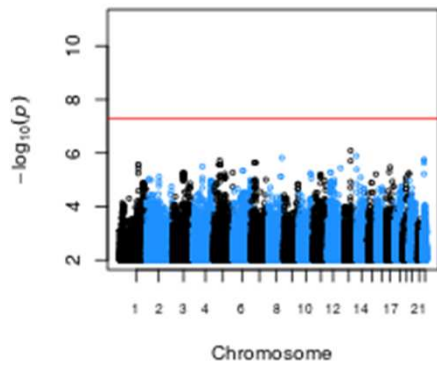
A.



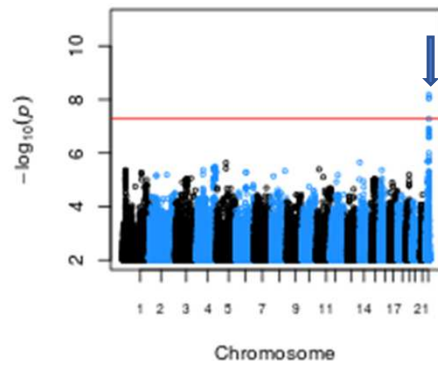
B.



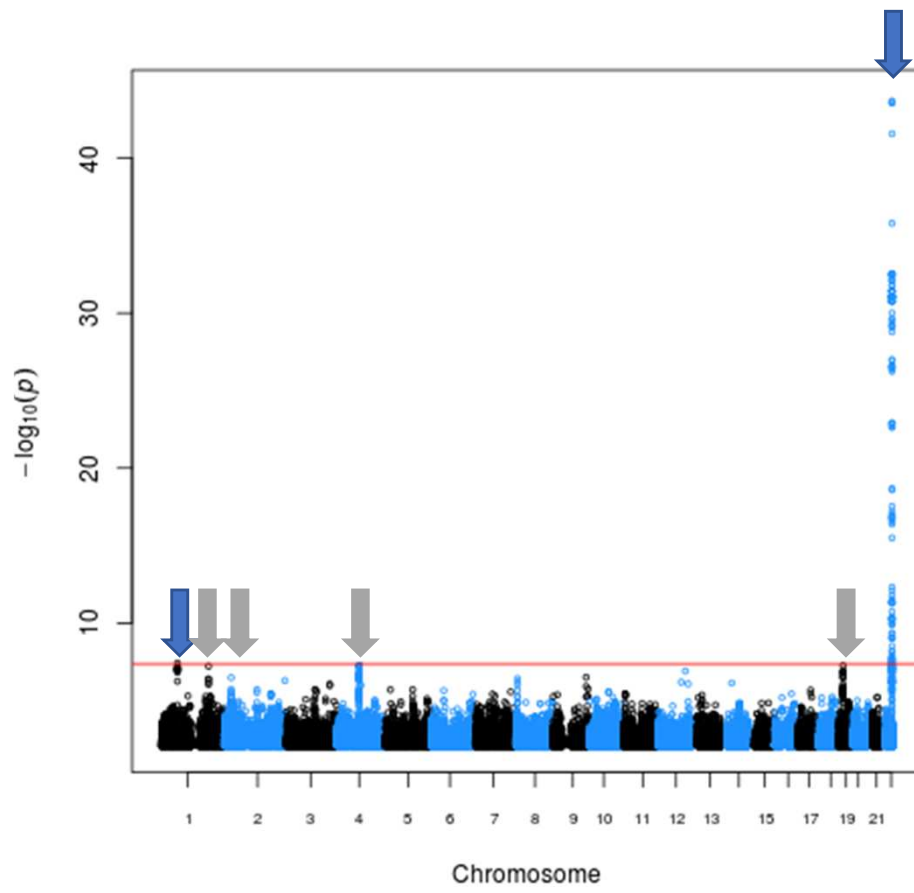
C.



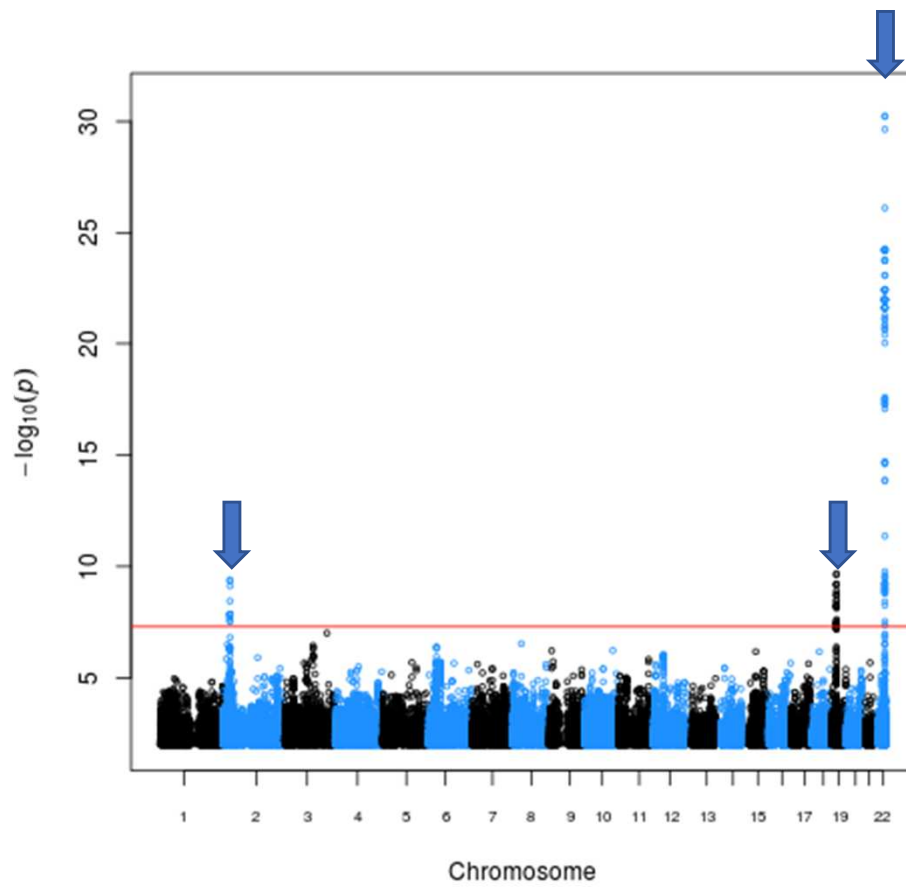
D.



A.



B.



Highlights for JHEPAT-D-19-01900

- Genome-wide association study involved 1483 biopsied NAFLD cases and 17781 controls
- Main analysis shows genome-wide significance for *PNPLA3*, *TM6SF2*, *HSD17B13* & *GCKR*
- Subanalyses show significance near *LEPR* for NASH and near *PYGO1* for steatosis
- The genome-wide significant signals replicated except for *GCKR*

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