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Pharmacogene expression during progression of metabolic dysfunction-associated steatotic liver disease: Studies on mRNA and protein levels and their relevance to drug treatment

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ABSTRACT

Metabolic dysfunction-associated steatotic liver disease (MASLD) is common worldwide. Genes and proteins contributing to drug disposition may show altered expression as MASLD progresses. To assess this further, we undertook transcriptomic and proteomic analysis of 137 pharmacogenes in liver biopsies from a large MASLD cohort.

We performed sequencing on RNA from 216 liver biopsies (206 MASLD and 10 controls). Untargeted mass spectrometry proteomics was performed on a 103 biopsy subgroup. Selected RNA sequencing signals were replicated with an additional 187 biopsies.

Comparison of advanced MASLD (fibrosis score 3/4) with milder disease (fibrosis score 0–2) by RNA sequencing showed significant alterations in expression of certain phase I, phase II and ABC transporters. For cytochromes P450, CYP2C19 showed the most significant decreased expression (30 % of that in mild disease) but significant decreased expression of other CYPs (including CYP2C8 and CYP2E1) also occurred. CYP2C19 also showed a significant decrease comparing the inflammatory form of MASLD (MASH) with non-MASH biopsies. Findings for CYP2C19 were confirmed in the replication cohort. Proteomics on the original discovery cohort confirmed decreased levels of several CYPs as MASLD advanced but this decrease was greatest for CYP2C19 where levels fell to 40 % control. This decrease may result in decreased CYP2C19 activity that could be problematic for prescription of drugs activated or metabolized by CYP2C19 as MASLD advances. More limited decreases for other P450s suggest fewer issues with non-CYP2C19 drug substrates. Negative correlations at RNA level between CYP2C19 and several cytokine genes provided initial insights into the mechanism underlying decreased expression.

1. Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD), previously referred to as non-alcoholic fatty liver disease or NAFLD, is an increasingly important public health problem worldwide with approx. 25 % of individuals in many countries estimated to have this disease [1].

Abbreviations: CI, confidence interval; MASLD, metabolic dysfunction-associated steatotic liver disease; MASH, metabolic dysfunction-associated steatohepatitis; MASL, metabolic dysfunction-associated steatosis; RNA-seq, RNA sequencing; ADME, absorption, distribution, metabolism, excretion; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; S9, fraction; 9000g, supernatant of tissue homogenate.

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Despite its high prevalence and associated morbidity, as of January 2024, there are no regulatory approved drugs to treat MASLD. Multiple compounds addressing a range of relevant pathophysiological targets have been trialled, with many failing to demonstrate efficacy but a few still currently under investigation [2]. As MASLD progresses from steatotic fatty liver (MASL) towards steatohepatitis (MASH) and cirrhosis with increasing inflammation and fibrosis, the liver shows considerable changes in gene expression [3]. The possibility that expression of genes relevant to drug metabolism and transport (absorption, distribution, metabolism, and excretion, ie ADME) are among those altered is of considerable importance and interest in light of their potential to impact target engagement/drug efficacy, or adversely impact existing comorbidities requiring drug treatment, including for cardiovascular disease [4,5]. Several studies have reported significant changes in gene expression and protein levels in MASLD, particularly genes and proteins relevant to ADME as well as pharmacogenes in general [6–11]. One limitation is that most studies to date have involved measurement of enzyme activity and protein in liver microsomes, or other similar fractions such as the 9000 g supernatant (S9), isolated from donor livers. These donors included individuals with MASLD, with some changes in ADME proteins especially for cytochromes P450 reported. The use of donor livers have the advantage of relatively large amounts of tissue being available for analysis but this approach does not necessarily allow systematic study of all MASLD stages. A recent transcriptomics study has focussed on a set of pharmacogenes in MASLD liver biopsies and reports specific changes in expression of certain genes [11]. Though the cohort was well characterised in terms of MASLD phenotype, overall numbers of livers (n = 93) were relatively small, the extent of replication of findings was limited to use of data from other published transcriptomics analysis and no information on protein levels was provided.

We have recently performed a transcriptomic study on liver biopsies from MASLD patients using RNA sequencing and involving over 200 cases of MASLD covering all disease stages with detailed histological analysis [3]. The strongest altered expression detected mainly involved genes relevant to extracellular matrix and oxidative stress but overall, we were able to confirm a large number of changes in gene expression, especially when advanced disease was compared with early stages involving steatosis only (MASL).

We have undertaken a further targeted study of pharmacogenes in this cohort and now describe some changes as MASLD progresses, though levels of other transcripts encoding key pharmacogenes remain unchanged. For a subgroup of livers, we also compare levels of certain proteins by mass spectrometry-based proteomics.

2. Materials and methods

2.1. Liver biopsy selection and characterisation

A total of 436 liver biopsies (from 414 MASLD cases and 22 controls without liver disease) from European Caucasian patients were included in this study. Cases were derived from the European NAFLD Registry (NCT04442334) metacohort [12]. All liver biopsies were scored by expert pathologists, as described previously in detail for these samples [3,12]. This was according to the semiquantitative NASH-Clinical Research Network NAS and the FLIP SAF scoring system [13,14] with fibrosis staged during the pathological scoring from F0 to F4. F0 corresponds to no liver fibrosis and F4 to liver cirrhosis. Patients with alternate diagnoses and etiologies, including excessive alcohol intake (30 g per day for males and 20 g per day for females), viral hepatitis, autoimmune liver diseases, and steatogenic medication use, were excluded. As described previously [12], collection and use of samples and clinical data for this study were approved by the relevant local and/or national Ethical Review Committee covering each participating centre, with all patients providing informed consent for participation. All participant recruitment and informed consent processes at recruitment centres were conducted in compliance with nationally accepted practice in the respective territory and in accordance with the World Medical Association Declaration of Helsinki 2018. For transcriptomic analysis by RNA sequencing, the discovery cohort comprised 216 snap-frozen biopsy samples from 206 patients diagnosed with MASLD and 10 healthy obese control cases without any biochemical or histological evidence of MASLD from patients undergoing bariatric surgery. A replication cohort, which was analysed for selected signals only using nanoString mRNA measurement, consisted of biopsies from 175 patients with MASLD and 12 healthy obese control cases. Additional proteomics analysis was undertaken on protein extracts from 5 control biopsies and 98 MASLD biopsies from the RNA sequencing discovery cohort.

2.2. Sample preparation for transcriptomic and proteomic analysis

Frozen tissue samples were lysed using Trizol (Sigma-Aldrich, Poole, UK) and mRNA was extracted with the Allprep DNA/RNA Micro kit (Qiagen, Manchester, UK), following the manufacturer's instructions. To obtain protein for proteomics analysis, the combined trizol-chloroform phase plus interphase following trizol extraction after removal of aqueous phase for RNA extraction was used, with storage at −80 °C prior to processing. After thawing, 100 μL of trizol extract was mixed with 20 % (w/v) SDS to a final concentration of 5 % (w/v) SDS to fully unfold proteins. Proteins were reduced with 120 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) (Pierce,Thermo Fisher, Loughborough, UK) added to a final concentration of 12 mM and incubated for 15 min at 55°. Disulphides were alkylated by adding 500 mM iodoacetamide (Sigma-Aldrich, Poole, UK) to a final concentration of 30 mM and incubating for 30 min in the dark. Each sample was then mixed with S-trap binding buffer (100 mM tetraethylammonium bromide (TEAB) (Sigma-Aldrich, Poole, UK) in 90 % methanol) and loaded on an S-Trap cartridge (Protifi, Fairport NY, USA) by centrifugation at 10,000g. Proteins were trapped on the column and the flow-through discarded. The protein-containing columns were then washed with 3 rounds of 150 μL 100 mM TEAB in 90 % methanol. Trypsin (Worthington-Biochem, Lakewood, New Jersey, USA) (5 ug) was added to each sample and incubated at 47 °C for 2 h. Peptides were eluted with sequential washes of 50 μL 50 mM TEAB, 0.1 % formic acid and 50 % acetonitrile. The 3x eluates were pooled and dried by centrifugal evaporation. The peptide pellet was dissolved in 0.5 % formic acid and loaded onto C18 Evotips (Evosep, Odense, Denmark) for LC-MS/MS analysis.

The replication cohort of 187 samples consisted of both frozen and formalin-fixed paraffin-embedded (FFPE) biopsy samples. For frozen samples, the protocol described above was used for mRNA isolation. For FFPE samples, mRNA was isolated using the High Pure FFPET RNA Isolation Kit (Roche Life Science, Welwyn Garden City, UK).

2.3. Transcriptomics analysis

For RNAseq, samples were processed with the TruSeq RNA Library Prep Kit v2 and sequenced on the NextSeq 550 System (Illumina, Cambridge, UK) as described previously [3]. Sequencing quality and alignment to the reference genome GRCh38 (Ensembl release 76) was implemented using Fastqc (v0.11.5) and MultiQC (v1.2dev), and count tables were produced using HT-Seq. Counts were normalised using the trimmed mean of M values method and transformed using limma's voom methodology.

For the replication cohort, mRNA was analysed using nanoString® assay panels for selected genes on the nanoString® nCounter system by the manufacturer's recommended protocol (NanoString, Seattle, USA). Quality control metrics were performed using the internal positive and negative control, and normalisation to housekeeping genes was done using the nSolver 3.0 software (nanoString®).

2.4. Proteomics analysis by LC-MS/MS

Sample-loaded Evotips (section 2.2) were subjected to liquid

chromatography using an Evosep One system (Evosep), with a manufacturer-standardised 200 samples per day method: Solvent A was 0.1 % formic acid (FA) in HPLC grade water, solvent B was 0.1 % formic acid in acetonitrile. The gradient ran from 0-30 % solvent B in a total of 5.6 min gradient, with wash at 85 % solvent B. Total run time was 7.2 min. Column equilibration, gradient flow, and column washing was performed at 4, 2, and 4 µl/min) with an EV1107 Endurance column (Evosep). This was used in line with a timsToF HT mass spectrometer (Bruker Daltonics, Billerica, Massachusetts, USA), with the instrument operated in DIA-PASEF mode, acquiring mass and ion mobility ranges of 450–950 *m/z* and 0.6–1.4 1/K0, with a total of 8 IM-*m/z* windows with two *m/z* ranges each. Analysis of raw (bruker.d) files was performed using DIA-NN version 1.8.25 (beta 27) [15], with using the built-in predicted spectral library function and the *H. sapiens* proteome (Uniprot ID UP000005640, reviewed proteins only with isoforms, FASTA file downloaded 01/03/2023) and a contaminant database [16]. Further processing was performed using R-studio, where contaminants and proteins identified/quantified using < 2 peptides excluded from further analysis.

2.5. Statistical analysis and protein interaction analysis

Kolmogorov-Smirnov or the Shapiro-Wilk normality test, unpaired Student's *t*-test or Mann-Whitney *U* test, one way ANOVA or Kruskal-Wallis test with respectively Tukey's or Dunn's post hoc multiple comparison test were performed using IBM SPSS statistics 26 and GraphPad Prism 9.5.0. To correct for multiple testing assessing targets in the RNA sequencing data, the Bonferroni Correction was implemented. Pearson correlations, simple linear regression and graphical plots were done using RStudio 2023.06.2 or GraphPad Prism 9.5.0. A p-value < 0.05 was considered significant.

Functional protein–protein interaction network analysis was done using the online String application (https://string-db.org/).

3. Results

3.1. Pharmacogene selection

Data on 137 pharmacogenes (Table 1) from a larger set of RNA sequencing data was selected for detailed analysis. These genes were selected based on known key role of the gene product in drug disposition (66 phase I metabolism, 47 phase II metabolism, 17 transporter) or as a regulator of genes relevant to drug disposition (7), using individual expertise (AKD, OG, QMA), known hepatic expression and review of the DMET^{TM} list of 231 genes developed by Affymetrix (Santa Clara, California, USA) [17]. A number of the DMET^{TM} list members were not included due to limited relevance to hepatic metabolism or drug targets, especially certain transporters and "other genes"..

3.2. Studies on pharmacogene expression by RNA sequencing

Three different analyses of gene expression were performed: (i) MASLD versus non-MASLD controls, (ii) Mild MASLD versus advanced MASLD and (iii) MASH versus no MASH (MASL). The results obtained are summarised in Fig. 1 and Table 2. A total of 54 genes showed either increased or decreased expression in one or more of these comparisons (Fig. 1a).

For all MASLD cases against controls, 14 genes showed significantly increased expression and 9 decreased expression, after correction for multiple testing. The strongest effect seen was a 38.9 fold increase in expression of AKR1B10, in line with a number of previous reports [3,18,19], but smaller changes were seen for a range of other genes. The pharmacogenes showing significant changes encode a range of different enzymes including P450s, transporters and transcriptional regulators. When profiles for advanced MASLD on the basis of fibrosis score (F3 and F4) were compared with milder disease (<F3), we saw significantly increased expression for 8 target genes but decreased expression for 26

Table 1
Genes selected for study.

Cytochromes P450	*Oxidoreductases*	*Transcriptional regulators*	*Glutathione S-transferases*	*UDP-glucuronosyltransferases*	*Drug transporters*
CYP1A1	ADH1A	AHR	GSTA1	UGT1A1	ABCB1
CYP1A2	ADH1B	NR1I2	GSTA2	UGT1A10	ABCB7
CYP1B1	ADH1C	NR1I3	GSTA3	UGT1A3	ABCB8
CYP2A13	ADH4	PPARA	GSTA4	UGT1A4	ABCB9
CYP2A6	ADH5	*Oxidases*	GSTA5	UGT1A5	ABCC1
CYP2A7	AKR1A1	AOX1	GSTK1	UGT1A6	ABCC2
CYP2B6	AKR1B1	NQO1	GSTM1	UGT1A7	ABCC3
CYP2C19	AKR1B10	*Carboxyesterases*	GSTM2	UGT1A9	ABCC4
CYP2C8	AKR1B15	CES1	GSTM3	UGT2A1	ABCC5
CYP2C9	AKR1C1	CES2	GSTM4	UGT2A3	ABCC6
CYP2D6	AKR1C2	PON1	GSTM5	UGT2B10	ABCC8
CYP2E1	AKR1C3	*Epoxide hydrolases*	GSTO1	UGT2B11	ABCC9
CYP2J2	AKR1C4	EPHX1	GSTO2	UGT2B15	ABCG1
CYP2R1	AKR7A3	EPHX2	GSTP1	UGT2B17	ABCG2
CYP2S1	ALDH1A1	*Flavin-linked monooxygenases*	GSTT2	UGT2B28	SLC22A1
CYP2U1	ALDH1A2	FMO3	GSTZ1	UGT2B4	SLCO1B1
CYP2W1	ALDH1A3		*N-acetyltransferases*	UGT2B7	SLCO1B3
CYP3A4	ALDH1B1		NAT1		*Selected drug targets of particular relevance*
CYP3A43	ALDH2		NAT2		VKORC1
CYP3A5	ALDH3A1		*Sulfotransferases*		HMGCR
CYP3A7	ALDH3A2		SULT1A1		
CYP4A11	ALDH3B1		SULT1A2		
CYP4B1	ALDH4A1		SULT1A3		
CYP4F11	ALDH5A1		SULT1B1		
CYP4F12	ALDH6A1		SULT1C2		
CYP4F2	ALDH7A1		SULT1C4		
CYP4F22	ALDH8A1		SULT1E1		
CYP4F3	ALDH9A1		SULT2A1		
P450-related genes	DPYD		SULT2B1		
POR	PTGR1		SULT4A1		
			SULT6B1		
			Methyltransferases		
			TPMT		

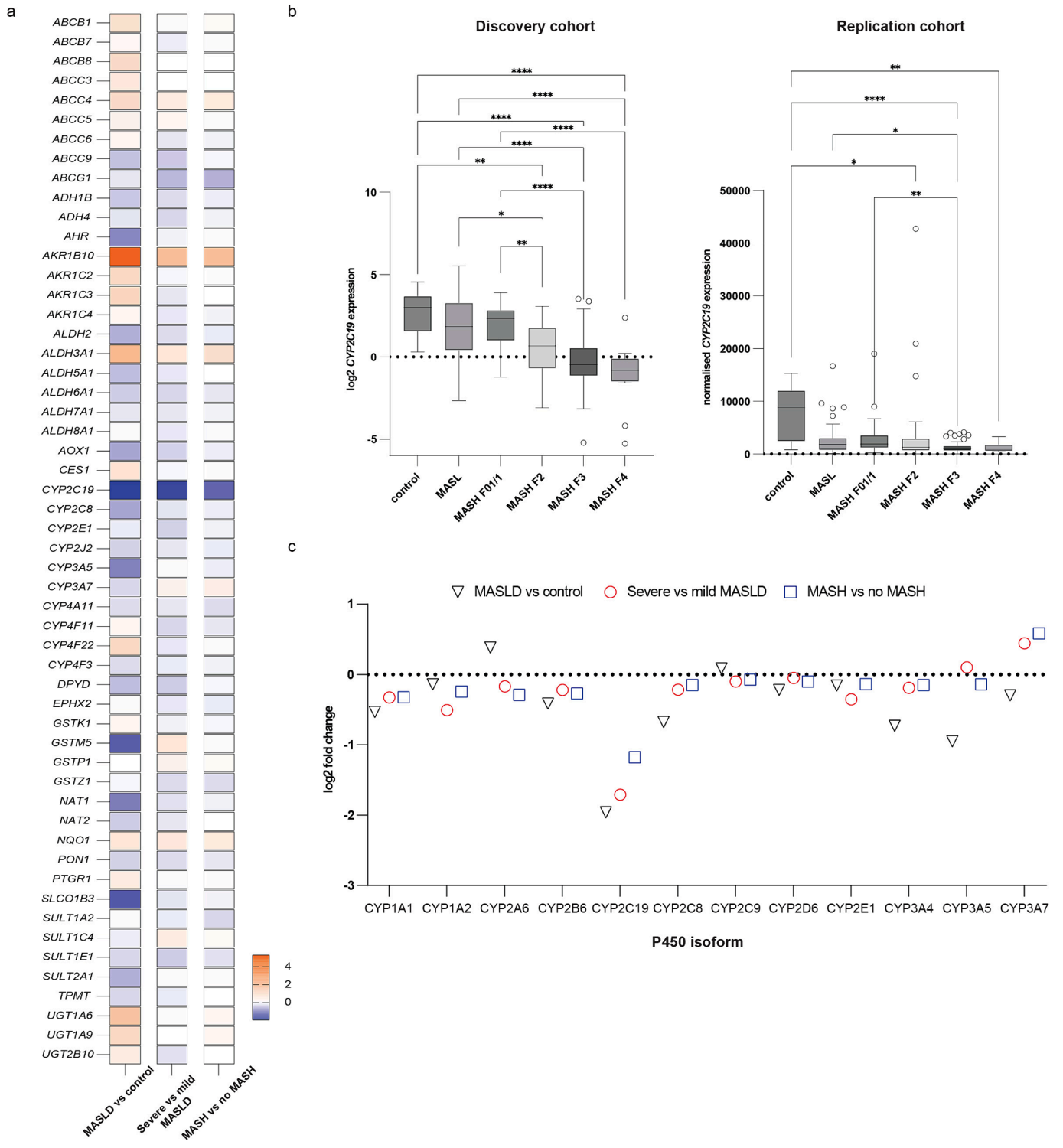

Fig. 1. Changes in pharmacogenes at the transcriptomic level. (a). Heat map for 54 genes showing alterations in expression in MASLD assessed by three different parameters. (b) CYP2C19 expression with increasing fibrosis severity for the discovery cohort (RNA sequencing) and the replication cohort (Nanostring). (c) Changes in expression of 12 P450 isoforms most relevant to drug metabolism as MASLD progresses.

genes. As expected, increased expression of AKR1B10 occurred but we also saw increased other phase I expression (ALDH3A1 and NQO1), increased phase II (GSTP1, GSTM5 and SULT1C4) and increased ABC transporter levels (ABCC4 and C5). The pharmacogene showing the largest and most significant decrease in expression was CYP2C19, which fell to 0.3 of the level detected in milder disease but smaller decreases in other CYPs (CYP2C8, CYP2E1, CYP2J2, CYP4F3 and CYP4F11) were also detected. A range of additional phase I, II and transporter genes also showed decreased expression. Finally, we focussed specifically on the presence of MASH since the inflammatory changes in this condition might be expected to affect expression of certain pharmacogenes. The number of changes was less compared with the effect of fibrosis with 5 genes showing increased expression and 7 decreased. Four of the 5 genes showing increased expression in MASH were those also associated with

Table 2
Pharmacogene expression levels determined by RNA sequencing.

	MASLD vs controls			Severe vs mild MASLD			MASH vs no MASH		
Gene ID	*Fold change*	*p value*	*corrected p value*	*Fold change*	*p value*	*corrected p value*	*Fold change*	*p value*	*corrected p value*
ABCB1	1.91	6.07E-05	**8.31E-03**	1.08	2.17E-01	1.00E + 00	1.15	3.57E-02	1.00E + 00
ABCB7	1.20	3.00E-03	4.11E-01	0.90	3.00E-04	**4.11E-02**	0.97	2.63E-01	1.00E + 00
ABCB8	2.12	5.39E-06	**7.38E-04**	1.02	6.58E-01	1.00E + 00	1.02	4.42E-01	1.00E + 00
ABCC3	1.64	5.52E-06	**7.57E-04**	1.01	8.82E-01	1.00E + 00	0.99	5.41E-01	1.00E + 00
ABCC4	2.09	9.00E-04	1.23E-01	1.53	5.85E-08	**8.01E-06**	1.62	1.50E-07	**2.05E-05**
ABCC5	1.32	1.11E-01	1.00E + 00	1.22	1.00E-04	**1.37E-02**	1.07	1.71E-01	1.00E + 00
ABCC6	1.26	2.14E-02	1.00E + 00	0.88	8.83E-06	**1.21E-03**	0.93	7.80E-03	1.00E + 00
ABCC9	0.73	2.88E-02	1.00E + 00	0.76	5.60E-06	**7.67E-04**	0.95	3.77E-01	1.00E + 00
ABCG1	0.87	3.96E-01	1.00E + 00	0.69	6.15E-06	**8.43E-04**	0.67	9.58E-06	**1.31E-03**
ADH1B	0.75	4.00E-03	5.48E-01	0.83	5.66E-05	**7.76E-03**	0.90	1.05E-02	1.00E + 00
ADH4	0.86	8.26E-02	1.00E + 00	0.81	8.28E-07	**1.13E-04**	0.92	5.14E-02	1.00E + 00
AHR	0.53	1.48E-05	**2.03E-03**	0.92	8.41E-02	1.00E + 00	1.05	5.52E-01	1.00E + 00
AKR1B10	38.92	1.87E-06	**2.57E-04**	4.25	2.56E-09	**3.50E-07**	4.12	1.68E-07	**2.31E-05**
AKR1C2	2.27	3.71E-06	**5.09E-04**	0.94	2.09E-01	1.00E + 00	1.04	5.12E-01	1.00E + 00
AKR1C3	2.38	2.91E-07	**3.98E-05**	0.87	4.10E-03	5.62E-01	1.01	8.13E-01	1.00E + 00
AKR1C4	1.22	1.69E-02	1.00E + 00	0.88	2.00E-04	**2.74E-02**	0.94	4.17E-02	1.00E + 00
ALDH2	0.66	1.60E-06	**2.20E-04**	0.83	2.11E-07	**2.89E-05**	0.89	3.00E-04	**4.11E-02**
ALDH3A1	4.73	5.13E-06	**7.03E-04**	1.75	8.60E-05	**1.18E-02**	2.01	1.51E-05	**2.07E-03**
ALDH5A1	0.71	4.16E-05	**5.70E-03**	0.88	1.80E-03	2.47E-01	1.00	8.78E-01	1.00E + 00
ALDH6A1	0.77	2.06E-02	1.00E + 00	0.81	2.00E-04	**2.74E-02**	0.87	2.30E-03	3.15E-01
ALDH7A1	0.87	1.84E-02	1.00E + 00	0.88	2.39E-06	**3.27E-04**	0.92	4.70E-03	6.44E-01
ALDH8A1	0.97	8.12E-01	1.00E + 00	0.88	1.89E-05	**2.59E-03**	0.96	1.70E-01	1.00E + 00
AOX1	0.63	6.92E-05	**9.48E-03**	0.80	1.00E-04	**1.37E-02**	0.90	3.25E-02	1.00E + 00
CES1	1.84	7.22E-05	**9.90E-03**	0.95	4.19E-01	1.00E + 00	1.04	2.15E-01	1.00E + 00
CYP2C19	0.26	1.20E-03	1.64E-01	0.31	2.48E-10	**3.40E-08**	0.44	1.00E-04	**1.37E-02**
CYP2C8	0.63	8.56E-06	**1.17E-03**	0.86	1.00E-04	**1.37E-02**	0.90	4.20E-03	5.75E-01
CYP2E1	0.90	3.70E-01	1.00E + 00	0.78	3.68E-08	**5.05E-06**	0.91	1.28E-02	1.00E + 00
CYP2J2	0.79	5.00E-04	6.85E-02	0.87	1.21E-06	**1.66E-04**	0.89	4.00E-04	5.48E-02
CYP3A5	0.52	2.00E-04	**2.74E-02**	1.07	1.68E-01	1.00E + 00	0.91	1.74E-01	1.00E + 00
CYP3A7	0.81	6.29E-02	1.00E + 00	1.36	1.00E-03	1.37E-01	1.50	8.82E-05	**1.21E-02**
CYP4A11	0.84	7.14E-02	1.00E + 00	0.87	1.10E-03	1.51E-01	0.84	2.00E-04	**2.74E-02**
CYP4F11	1.21	1.22E-02	1.00E + 00	0.81	1.24E-08	**1.70E-06**	0.88	9.00E-04	1.23E-01
CYP4F22	2.29	1.96E-05	**2.69E-03**	0.88	9.64E-02	1.00E + 00	1.11	1.16E-01	1.00E + 00
CYP4F3	0.82	7.90E-03	1.00E + 00	0.89	8.08E-05	**1.11E-02**	0.92	7.40E-03	1.00E + 00
DPYD	0.71	3.80E-03	5.21E-01	0.77	4.86E-06	**6.66E-04**	0.96	2.34E-01	1.00E + 00
EPHX2	1.08	5.44E-01	1.00E + 00	0.88	2.27E-06	**3.12E-04**	0.89	7.89E-05	**1.08E-02**
GSTK1	1.28	6.77E-05	**9.27E-03**	0.93	6.00E-04	8.22E-02	0.94	2.20E-03	3.01E-01
GSTM5	0.41	9.20E-03	1.00E + 00	1.77	3.00E-04	**4.11E-02**	1.11	4.95E-01	1.00E + 00
GSTP1	0.99	7.88E-01	1.00E + 00	1.33	1.61E-07	**2.21E-05**	1.12	6.26E-02	1.00E + 00
GSTZ1	0.94	6.51E-02	1.00E + 00	0.83	1.00E-04	**1.37E-02**	0.83	5.08E-05	**6.95E-03**
NAT1	0.51	5.74E-07	**7.86E-05**	0.85	2.61E-05	**3.57E-03**	0.91	1.20E-02	1.00E + 00
NAT2	0.76	2.03E-02	1.00E + 00	0.87	1.00E-04	**1.37E-02**	1.00	7.80E-01	1.00E + 00
NQO1	1.73	2.14E-02	1.00E + 00	1.64	8.93E-07	**1.22E-04**	1.61	3.47E-05	**4.75E-03**
PON1	0.78	5.20E-03	7.12E-01	0.82	1.07E-05	**1.47E-03**	0.88	6.10E-03	8.36E-01
PTGR1	1.58	3.00E-04	**4.11E-02**	0.97	7.35E-01	1.00E + 00	1.03	1.83E-01	1.00E + 00
SLCO1B3	0.40	5.20E-05	**7.12E-03**	0.87	7.89E-02	1.00E + 00	0.93	5.01E-01	1.00E + 00
SULT1A2	0.98	7.40E-01	1.00E + 00	0.89	5.56E-02	1.00E + 00	0.80	3.00E-04	**4.11E-02**
SULT1C4	0.90	1.86E-01	1.00E + 00	1.55	2.57E-07	**3.52E-05**	1.12	1.51E-01	1.00E + 00
SULT1E1	0.80	1.92E-01	1.00E + 00	0.76	3.17E-05	**4.35E-03**	0.86	2.73E-02	1.00E + 00
SULT2A1	0.66	2.00E-04	**2.74E-02**	1.08	2.73E-01	1.00E + 00	1.06	1.26E-01	1.00E + 00
TPMT	0.80	1.15E-02	1.00E + 00	0.89	2.00E-04	**2.74E-02**	1.00	9.97E-01	1.00E + 00
UGT1A6	3.74	8.12E-07	**1.11E-04**	1.03	3.53E-01	1.00E + 00	1.21	3.90E-03	5.34E-01
UGT1A9	2.35	3.00E-04	**4.11E-02**	1.00	7.98E-01	1.00E + 00	1.24	2.30E-03	3.15E-01
UGT2B10	1.58	2.20E-05	**3.02E-03**	0.86	8.70E-03	1.00E + 00	0.99	8.99E-01	1.00E + 00

Expression levels are shown in units of overall counts. Corrected p values were calculated to correct for 137 genes being studied.

more severe fibrosis in the disease severity analysis but CYP3A7 showed a significant increase in the MASH group. Similar to the disease severity analysis, CYP2C19 showed the largest and most significant decrease in the MASH comparison with a fall to 0.44 of non-MASH with the other 6 genes showing significance also the same as those decreasing as fibrosis progressed. Data showing in detail the fall in CYP2C19 expression as NAFLD progresses to cirrhosis (MASH F4) is shown in Fig. 1(b).

3.3. Replication of selected RNA sequencing findings by nanostring analysis

To replicate the findings for MASLD progression for CYP2C19, AKR1B10, ALDH3A1 and ABCC4, RNA levels in 175 additional MASLD cases and 12 controls were measured by Nanostring gene expression

Table 3
Analysis of expression of selected genes by Nanostring in an independent cohort.

	MASLD (n = 175) vs control (n = 12)		Mild MASLD (n = 94) vs advanced (n = 81)		MASH (n = 136) vs no MASH (n = 39)	
Gene	*Fold change*	*p value*	*Fold change*	*p value*	*Fold change*	*p value*
CYP2C19	0.15	p < 0.0001	0.79	p = 0.25	0.59	p < 0.0001
ABCC4	1.21	p = 0.07	0.98	p = 0.37	1.11	p = 0.03
AKR1B10	39.84	p < 0.0001	2.94	p < 0.0001	2.53	p < 0.0001
ALDH3A1	3.01	p = 0.0002	3.15	p < 0.0001	2.27	p = 0.0008

analysis. The data obtained are summarised in Table 3 which shows the fold changes obtained comparing: (i) MASLD versus controls (ii) Mild MASLD versus advanced MASLD and (iii) MASH versus no MASH (MASL). Decreased expression for CYP2C19 was confirmed by analyses (i) and (iii) which showed statistically significant ($P < 0.0001$) decreases to 0.15 and to 0.59 of comparator respectively (Fig. 1b). A decrease was also seen for MASH versus non-MASH cases but this was not statistically significant. In the case of ABCC4, while increases in expression were seen for all three comparisons, only analysis (ii) for disease severity showed borderline significance. Significant increases for both AKR1B10 and ALDH3A1 expression were seen in all 3 comparisons (Table 3).

3.4. Cytochrome P450 expression in the discovery cohort

In view of the key role for certain P450 isoforms in metabolism of commonly prescribed drugs, we focussed more specifically on 12 specific isoforms with well-defined roles in drug metabolism. As summarised in Fig. 1c and Table 4, the most significant changes at the level of transcription were seen for CYP2C19 and CYP2C8. For CYP2C19, significant changes with respect to both severity of fibrosis and presence of MASH were seen (Fig. 1c). For CYP2C8, there was a significant decrease for MASLD generally compared with controls together with a decrease as fibrosis became more severe. Levels of CYP2E1 expression decreased in severe MASLD, with CYP3A7 expression increased in MASH. Though levels of CYP3A5 were lower in MASLD cases compared with the control group, no effect was seen for disease severity for this isoform.

Table 4
Key cytochrome P450 mRNA expression levels in MASLD from RNA sequencing.

MASLD versus controls				
Isoform	Controls	MASLD	Fold change	Corrected p-value
CYP1A1	4.54	4.01	0.69	>1
CYP1A2	8.09	7.95	0.90	>1
CYP2A6	9.63	10.01	1.30	>1
CYP2B6	7.87	7.46	0.75	>1
CYP2C8	11.13	10.46	0.63	**0.001**
CYP2C9	10.25	10.34	1.06	>1
CYP2C19	2.67	0.72	0.26	0.16
CYP2D6	7.34	7.12	0.86	>1
CYP2E1	12.59	12.43	0.90	>1
CYP3A4	11.34	10.62	0.60	>1
CYP3A5	10.21	9.26	0.52	**0.03**
CYP3A7	5.76	5.46	0.81	>1
Mild versus severe MASLD on the basis of fibrosis score				
Isoform	Mild MASLD	Advanced MASLD	Fold change	Corrected p-value
CYP1A1	4.12	3.79	0.8	>1
CYP1A2	8.12	7.61	0.71	0.14
CYP2A6	10.06	9.90	0.89	>1
CYP2B6	7.53	7.32	0.86	>1
CYP2C8	10.53	10.31	0.86	**0.01**
CYP2C9	10.37	10.27	0.93	>1
CYP2C19	1.28	−0.43	0.3	**3.40E-08**
CYP2D6	7.14	7.09	0.97	>1
CYP2E1	12.55	12.19	0.78	**5.05E-06**
CYP3A4	10.68	10.49	0.88	>1
CYP3A5	9.22	9.33	1.07	>1
CYP3A7	5.31	5.76	1.36	0.14
MASH versus no MASH				
Isoform	No MASH	MASH	Fold change	Corrected p-value
CYP1A1	4.25	3.93	0.8	>1
CYP1A2	8.13	7.89	0.84	>1
CYP2A6	10.22	9.94	0.82	0.07
CYP2B6	7.66	7.39	0.83	>1
CYP2C8	10.57	10.42	0.9	0.58
CYP2C9	10.39	10.32	0.95	>1
CYP2C19	1.59	0.41	0.44	**0.01**
CYP2D6	7.19	7.09	0.93	>1
CYP2E1	12.53	12.40	0.91	>1
CYP3A4	10.73	10.58	0.9	>1
CYP3A5	9.36	9.22	0.91	>1
CYP3A7	5.03	5.61	1.49	**0.01**

RNA seq data (overall counts) from a set of 206 MASLD liver biopsies for 12 P450 isoforms of particular relevance to drug disposition are shown. Corrected p values are based on the entire 137 drug disposition gene set studied. Mild MASLD is defined as fibrosis F0 to F2 and advanced as F3 and F4.

3.5. Proteomics analysis

To extend the data obtained by transcriptomic analysis, we performed proteomics analysis by untargeted LC-MS/MS using extracts from a subgroup of the same biopsy samples analysed by RNA sequencing where available. A total of 102 samples (5 control biopsies and 98 MASLD biopsies) provided adequate data for analysis of the signals of interest for selected P450s, together with POR. Attempts to detect certain other proteins of interest, including ABCC4 (MRP4) were not successful. The results obtained are summarised in Fig. 2. The heat map (Fig. 2a) shows selected protein levels at different MASLD stages compared with controls and indicates a decrease in CYP2C19 protein levels to approx. 0.4 fold of control in MASH cases, regardless of fibrosis grade, and an increase in AKR1B10 levels over control from 3.5 fold to 12 fold as MASLD progresses from MASL to cirrhosis (MASH F4). When protein levels were compared for all MASLD against controls, MASH versus MASL only and advanced disease against mild disease, the only proteins showing statistical significance after correction for multiple testing were CYP2C19, where levels in the entire MASLD cohort compared with controls (Fig. 2b) and the MASH only cohort compared with non-MASH cases were significantly decreased, and AKR1B10 where levels in the advanced MASLD cohort compared with milder MASLD were increased significantly.

3.6. Gene expression of CYP2C19 regulators during MASLD progression

The possibility that the altered expression of CYP2C19 at the mRNA level correlated with altered expression of inflammatory regulators considered to be relevant to its expression based on previous reports was investigated by use of the RNA sequencing data described in Section 3.1. To identify possible regulators, we performed a correlation of the entire transcriptome in the cohort of 216 individuals (206 MASLD and 10 controls) with CYP2C19 expression. The top 50 most significant genes showing negative correlations with CYP2C19 expression were analysed for functional protein–protein interaction networks using String. As shown in Fig. 3a, we observed a large number of genes relevant in Extracellular Matrix remodelling (such as ADAMTSL2, COL1A1, LOXL1, LOXL4 and ITGBL1), as well as a large number of pro-inflammatory cytokines (such as LTBP2, PDGFA and IL32) (Fig. 3a). The strongest correlations were seen for genes LTBP2 (a member of the TGF-β family), IL-32 and PDGFA (Fig. 3b). We also assessed possible correlations for CYP2C19 levels with IL-6, CRP and TNFA, plus a related gene TRAF2 in view of previous reports suggesting these decrease CYP2C19 expression [20,21]. As summarised in Table 5, significant negative correlations were seen with LTBP2, IL32, PDGFA, TNFA and TRAF2 mRNA levels for CYP2C19 mRNA. There were no significant associations detected with IL-6 and CRP.

4. Discussion

MASLD is a very common disease worldwide affecting up to 30 % of adults [1]. A subgroup of those affected may advance to a serious inflammatory condition (MASH) with increasing formation of fibrotic tissue which may advance to liver cirrhosis. Several recent studies have demonstrated that there are specific changes in gene expression as MASH and severe fibrosis develop [3,18,19] but less is known about changes at the protein level. The changes in gene expression include alterations in expression of extracellular matrix, inflammatory mediator and cellular stress genes [3] but there is also evidence for altered

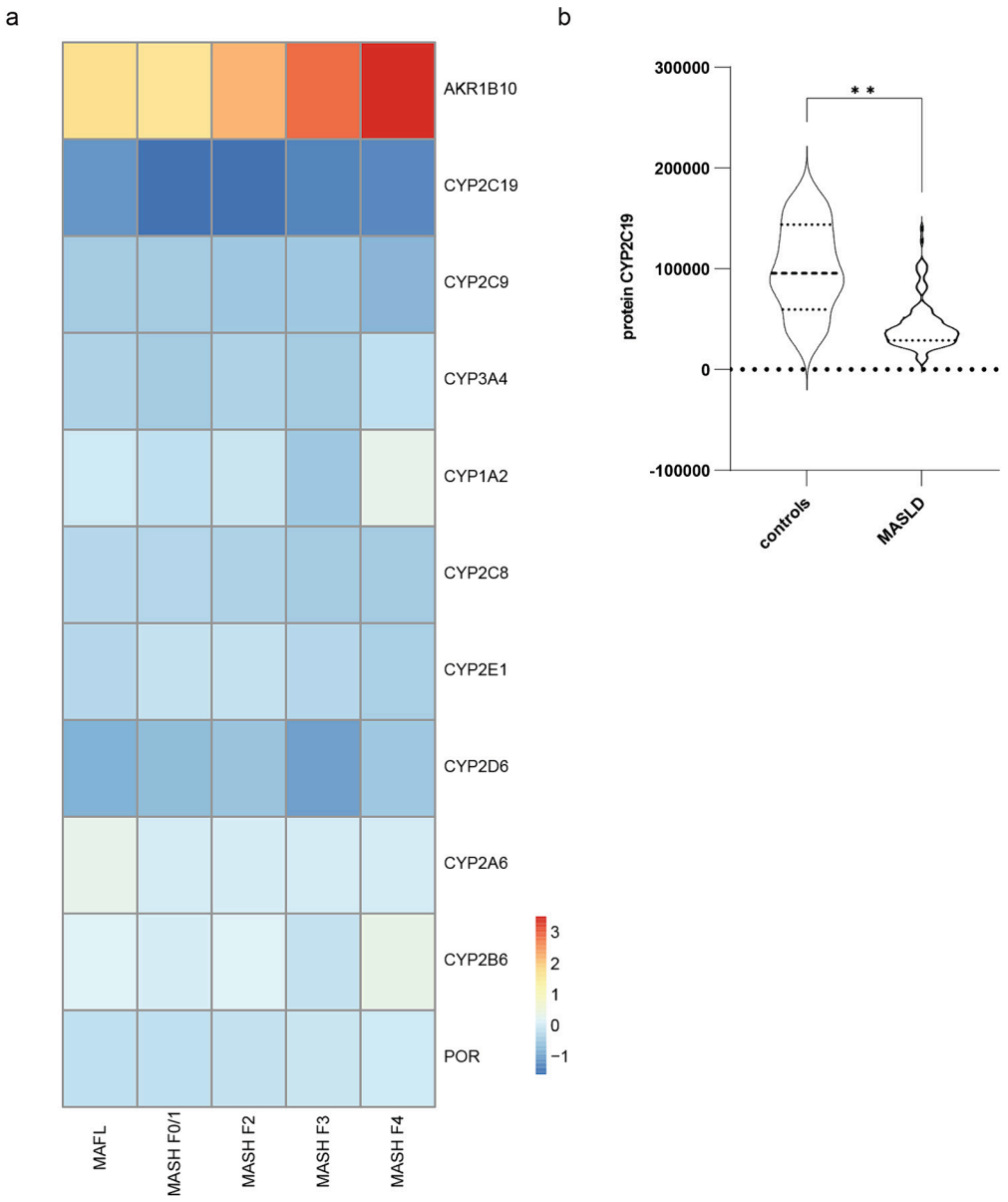

Fig. 2. Analysis of P450 and related proteins in MASLD by proteomics. (a) Heat map comparing log_2fold change at the protein level for the different MASLD stages compared with control. (b) CYP2C19 levels in MASLD compared with controls with data shown in overall counts, **p < 0.01. AKR1B10 was included as a positive control.

expression of genes relevant to normal hepatic functions including drug disposition [11]. The current study aimed to define changes in pharmacogenes during MASLD more precisely by detailed analysis of the different stages of disease using both RNA sequencing and proteomic analysis of liver biopsies. Pharmacogenes are generally unlikely to be the drivers of MASLD progression but a detailed understanding of expression changes is important in relation to prescription of drugs to MASLD patients who often suffer from a range of other conditions including cardiovascular disease and type II diabetes. In addition, new drug treatments for MASLD specifically are being developed [2] and in this context, a clear understanding of drug disposition within livers affected by MASLD is vital.

Using a well phenotyped set of liver biopsies, we have performed a detailed analysis on pharmacogene mRNA and protein levels as MASLD advances. The key finding in terms of relevance to drug disposition is the significant decrease in CYP2C19 levels determined by both mRNA and protein analysis as MASLD progresses. In addition, we find some changes for other pharmacogenes but also demonstrate that for most P450 genes, levels of mRNA and protein are maintained at close to the levels seen in controls, even with advanced disease. The decreased levels of CYP2C19 mRNA as MASLD progresses have been reported elsewhere [7,11] but the current study is the first to determine both RNA and protein levels in the same patient samples.

CYP2C19 has a key role in metabolism of several drugs that may be prescribed to patients with MASLD, particularly the anti-platelet drug clopidogrel but also omeprazole and certain antidepressants. While the decrease in protein levels in advanced disease is to the region of 40 % of normal activity, this would be comparable to the decrease in activity seen in those heterozygous for loss of function alleles in this gene, particularly *CYP2C19*2*. CPIC guidelines for clopidogrel prescription recommend use of alternative anti-platelet drugs in those with either heterozygous (intermediate metabolizer) or homozygous variant (poor metabolizer) genotypes [22] so our current data combined with the previous report [11] does raise some concerns about choice of anti-platelet therapy as MASLD progresses. On the other hand, for many other prescribed drugs, CYP3A4, CYP2D6 and CYP2C9 will be of more

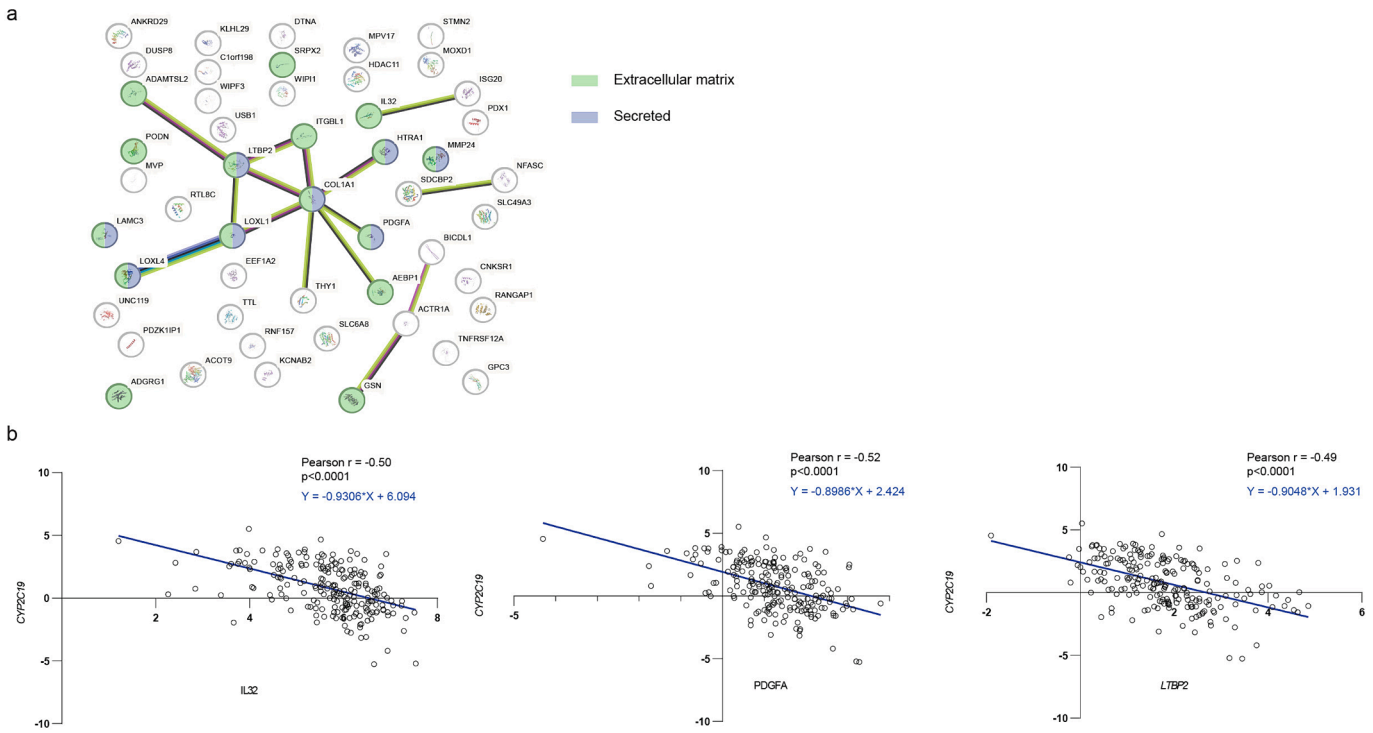

Fig. 3. Transcriptomic analysis of possible CYP2C19 regulators. (a) String analysis of relationships between 50 genes showing strongest negative correlations to CYP2C19 expression in the MASLD cohort based on RNA sequencing. (b) Negative correlations between CYP2C19 expression and LTBP2/PDGFA/IL32 expression.

Table 5
Putative CYP2C19 regulators showing significant negative correlations with CYP2C19 expression at the mRNA level.

Gene name	*Pearson R*	*p value*
LTBP2	−0.52	4.4E-16
IL32	−0.50	5.4E-15
PDGFA	−0.49	1.8E-14
TRAF2	−0.36	3.4E-08
TNFA	−0.16	1.6E-02
IL6	−0.11	9.5E-02
CRP	−0.11	1.0E-01

importance in their metabolism and the current data suggests fewer concerns with changes in these enzymes in MASLD, though the altered architecture of the liver during progression to cirrhosis will also be relevant to drug pharmacokinetics. A previous report involving use of probe drugs in patients with liver disease who were mainly suffering from cirrhosis found CYP2C19-mediated metabolism of mephenytoin showed impairment in what was described as “mild” disease but would be equivalent to F3 fibrosis in the current patient cohort [23]. In that study, all patients with cirrhosis showed decreased clearance of probe drugs for other P450s but impairment of CYP2C19-mediated metabolism occurred in earlier disease and is in line with what we have found in the current study. It is likely that our findings on P450 levels as MASLD progresses will be relevant to several of the novel MASLD drug treatments currently under development [2].

The RNA sequencing studies showed very pronounced increases in levels of AKR1B10 and ALDH3A1 as MASLD progresses. Though these can be regarded as pharmacogenes, neither is likely to be important in the metabolism of commonly prescribed drugs and the increases observed may well be due to the increased oxidative and other cellular stresses associated with MASLD progression. Both genes are already known to increase in response to a variety of cellular stresses [24,25]. The ABC transporter ABCC4 also shows increased expression as the disease progresses, as well as in MASH generally, and this could be of more relevance to disposition of prescribed drugs in view of the well established role for ABCC4 in outward transport of conjugated drugs across the sinusoidal membrane [26]. While we could not confirm our findings for ABCC4 by either mRNA measurement by NanoString in the independent cohort or by proteomic analysis of the samples used in the original sequencing study, which is likely due to the low abundance of ABCC4 mRNA and protein in hepatocytes, there are independent reports of a similar increased ABCC4 expression from two other RNA sequencing studies on MASLD [11,27]. We also saw significantly increased expression of CYP3A7 (fold change 1.5) in MASH compared with MASL. This increase in a fetal P450 is in line with increases reported for this isoform in a number of different disease states [28].

When we compared mild and advanced MASLD mRNA levels in our primary analysis, a number of other pharmacogenes in addition to CYP2C19 show decreased expression as the disease advances though the fold changes seen are less than that for CYP2C19. For example, DPYD, CYP2E1 and AOX1 all showed decreases to approx. 75 % of levels seen in mild disease. The overall relevance of these relatively small changes at the pharmacokinetic level is still unclear but in view of the general importance of these gene products in drug metabolism, further investigations would be useful.

In addition to assessing levels of key drug metabolising P450s in detail, we analysed P450 oxidoreductase (POR) expression. We did not detect any significant changes at the mRNA level as MASLD progressed but, in a recent study involving analysis of circulating proteins with the SomaScan aptamer-based system [29], we found a significantly increased level of circulating POR as MASLD progressed with maximal levels seen for patients with F4 disease. In view of this finding and the key role of the POR gene product in P450-mediated metabolism, we assessed POR levels in the current cohort by mass spectrometry-based proteomics. No significant change in expression in the liver extracts was seen so the basis for the finding of increased POR in serum and plasma remains unclear. Coverage of P450s in the SomaScan system used in the previous study was limited [29] and we did not find any significant changes for any CYPs included.

The underlying mechanism for the early decrease in CYP2C19

expression as MASLD progresses remains unclear. However, there has been some recent interest in an apparent decrease in clearance of the CYP2C19 substrate voriconazole in patients suffering inflammation who require anti-fungal therapy, mainly in an intensive care setting [30]. This decrease has been suggested to involve interactions of CYP2C19 with raised CRP, IL-6, TNF-α and other inflammatory cytokines. Studies on patients undergoing surgery and involving phenotyping with probe drugs also suggest that post-surgery cytokine increases result in decreased CYP2C19 levels [21,31]. Though there is also some evidence from *in vitro* studies for cytokine effects on CYP2C8 and CYP2C9 genes which are adjacent to CYP2C19 on chromosome 10 with all three CYP2C genes often considered to be subject to similar regulation [20], our current findings and those from another recent study [11] are more consistent with a CYP2C19-specific effect as MASLD progresses to MASH. We attempted to assess the relevance of cytokine expression by interrogation of our publicly available RNA sequencing data. While we did not see any significant associations with CRP and IL-6 levels, we saw significant negative correlations with mRNAs relevant to the TNF-α pathway, with LTBP2, which encodes members of the TGF-β family, and with IL32 and PDGFA. The TNF-α finding is in line with a report on CYP2C19 phenotype in patients with raised TNF-α levels due to congestive heart failure [32]. Decreased CYP2C19 expression has been reported *in vitro* following exposure of human hepatocytes to TGF-β [20].

Our study suffers from a number of limitations, especially with only a limited number of mRNAs covered in the Nanostring replication study, and also a relatively low overall coverage of proteins in our untargeted proteomic analysis. We were also unable to determine enzyme activities and assess pharmacokinetic parameters as MASLD progresses. In addition, the availability of liver biopsies from individuals without MASLD was very limited and our control groups are therefore small. The important limitations of current animal and cellular models for studying MASLD progression [33,34] means that it is not immediatly possible to follow up our findings on decreased CYP2C19 in patient samples to fully understand the underlying events that occur at the transcriptional level. However, our relatively large MASLD discovery cohort and the expert pathology assessment of MASLD stage are strengths of the study.

In summary, we have shown that levels of both CYP2C19 mRNA and protein fall to approx. 40 % of normal as MASLD progresses with some evidence that this occurs early in MASH. However, we did not detect similar changes for other P450s that contribute to metabolism of commonly prescribed drugs. Some other alterations seen in pharmacogene expression such as the increased expression of ABCC4 may also be of clinical relevance.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ann Daly and Quentin Anstee reports financial support was provided by European Union. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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