Research Article

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Genetic variation in *TNFA* predicts protection from severe bacterial infections in patients with end-stage liver disease awaiting liver transplantation

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Background & Aims: Augmented susceptibility to infections increases mortality in patients with end-stage liver disease (ESLD). We sought to determine the contribution of selected genetic variants involved in inflammatory signalling downstream of the Toll-like receptor 4 (TLR4) to severe bacterial infections (SBIs) in patients with ESLD.

Methods: We retrospectively assessed incidence of SBIs in 336 adult ESLD patients enlisted for orthotopic liver transplantation (OLT) and genotyped them for *TLR4* c.+1196C/T, *CD14* c.−159C/T, *TNFA* c.−238G/A, *TNFA* c.−863C/A, *IL1B* c.−31C/T and *IL1RN* variable number of tandem repeats allelic variants. Principal findings were validated in an independent cohort of 332 ESLD patients.

Results: Thirty-four percent of patients from the identification cohort and 40% of patients from the validation cohort presented with SBI while enlisted for OLT. The presence of the variant allele *TNFA* c.−238A (rs361525) was associated with lower serum levels of TNF-α, and with significantly decreased risk of SBI in both cohorts. Multivariate analysis showed that the relative protection from SBI associated with this allele almost completely negated the increased susceptibility to SBI owed to advanced ESLD. Although not predictive of overall mortality, the presence of the *TNFA* c.−238A allele was associated with a complete prevention of SBI-related pre-transplant deaths.

Conclusions: Our results suggest that genetic variability in inflammatory signalling is associated with the development of SBI in patients with ESLD. Specifically, we identified the importance of the *TNFA* c.−238A allele as a strong predictor of protection from SBI, and as a genetic marker associated with significantly improved pre-transplant survival in patients with SBI.

Keywords: Liver cirrhosis; Inflammatory signalling; Toll-like receptor 4; Innate immune cells; Neutrophil exhaustion.

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Abbreviations: SBI, severe bacterial infections; ESLD, end-stage liver disease; OLT, orthotopic liver transplantation; TLR, toll-like receptors; *TLR4*, toll-like receptor 4; LPS, lipopolysaccharide; SNP, single nucleotide polymorphism; TNF-α, tumour necrosis factor-alpha; IL-1β, interleukin-1 beta; *TNFA*, tumour necrosis factor alpha; *IL1B*, interleukin 1 beta; *IL1RN*, interleukin 1 receptor antagonist; VNTR, variable number of tandem repeats; IL-1RA, interleukin-1 receptor antagonist; SBP, spontaneous bacterial peritonitis; EASL, European Association for the Study of the Liver; CRP, C-reactive protein; PCR, polymerase chain reaction; HWE, Hardy-Weinberg equilibrium; CV, coefficient variability; OR, odds ratio; CI, Confidence Interval; MELD, model for end-stage liver disease.

Introduction

Severe bacterial infections (SBIs) represent the second leading cause of death in patients with end-stage liver disease (ESLD) waitlisted for orthotopic liver transplantation (OLT) [1,2], with mortality approaching 10% in European registries [3] and 23% in the US [4]. The high mortality attributed to SBIs in this patient category is driven by the impaired antimicrobial response associated with ESLD [5,6], and by progression of liver failure that is accelerated by severe infection [1,2]. Cirrhotic patients are prone to develop SBIs because of compromised antimicrobial defence caused by liver synthetic failure, portal hypertension and bacterial translocation from the gut [1,2,5,7,8], in conjunction with altered function of immune cells, including impaired opsonising and neutrophil phagocytic capacity [9,10]. Therefore, early identification of patients with ESLD at risk for SBIs is of paramount importance, but indicators predicting the development of SBIs are missing.

Toll-like receptors (TLRs) play a key role in innate immune responses by recognition of a broad range of microbial components and triggering signals critical for antimicrobial defences [11–13]. Although substantially conserved across species, TLRs

show a genetic variability that modulates their downstream signalling, potentially determining individual susceptibility to infections [14].

It has been shown that the variant p.399Ile of *TLR4* corresponding to the nucleotide substitution *TLR4* c.+1196C/T (rs4986791) changes the ligand-binding site of the receptor [15] and, in one study with limited sample size, predisposed cirrhotic patients to infections [16]. Plasma concentration of CD14 is affected by the promoter polymorphism c.−159C/T (rs2569190) [17], which influences expression of the protein [18] and the risk of death in patients with sepsis [19]. SNPs at the positions c.−863 (rs1800630) and c.−238 (rs361525) of the *TNFA* promoter independently influence transcription of this gene [20–22].

The Interleukin 1 gene cluster on chromosome 2 contains genes *IL1B* and *IL1RN* encoding the pro-inflammatory IL-1β and the anti-inflammatory IL-1 receptor antagonist (IL-1RA) [23]. The variant c.−31T (rs1143627) in the *IL1B* promoter increases the transcriptional activity of this gene [24]. The second intron of *IL1RN* contains a variable number of tandem repeats (VNTR) 86-bp long. Allele 2 (*IL1RN*∗2) increases the concentration of IL-1β *in vitro* [25] and increases mortality in septic patients [26].

The above mentioned studies [16,19,26,27] demonstrated significant associations between genetic variants and susceptibility to bacterial infections. However, these studies were performed mainly on limited numbers of non-cirrhotic patients and were not validated. Therefore, we decided to evaluate the contribution of genetic variants in the TLR4 pathway to the development of SBI in large, well characterised independent cohorts of cirrhotic patients with ESLD enlisted for OLT in two centres.

Patients and methods

Patients and definition of severe bacterial infections

Identification cohort

Three hundred and thirty-six patients with liver cirrhosis with Child-Pugh class B and C were enrolled and retrospectively screened for the occurrence of one or more episodes of extrahepatic SBIs during their time on the waiting list and 270 days before enlistment to include also patients with a recent episode of SBI. These patients were sorted out of 708 adult cirrhotic patients who were enlisted for OLT in Prague between February 1995 and June 2010. Patients with Child-Pugh class A and patients with acute liver failure were excluded. Patients with Caroli disease and primary and secondary sclerosing cholangitis were excluded as well, since intrahepatic bacterial complications are characteristic for the natural course of these diseases.

SBIs were defined as the following bacterial infections requiring hospitalisation and treatment with intravenous antibiotics:

(a) Spontaneous bacterial peritonitis (SBP), diagnosis of which was based on neutrophil cell count exceeding 250/mm^3 and/or positive culture of ascitic fluid if secondary causes of peritonitis were excluded (EASL guidelines [28]).
(b) Urinary tract infections diagnosed on the basis of clinical findings (dysuria, fever), pyuria (leukocytes >10/mm^3) and positive urine culture [29].
(c) Pneumonia, diagnosis of which was determined by clinical symptoms (cough, expectoration, and fever), positive chest X-ray and positive bacteriological finding in sputum [30].
(d) Skin and soft tissue infection, diagnosis of which was established by local cutaneous findings (blush, tumefaction, and pain) and leukocytosis [31].
(e) Bacterial infection of unknown origin defined as a positive blood culture with serum C-reactive protein (CRP) level ⩾70 mg/L,

All clinical data were collected from hospitalisation and outpatient medical records archived at our centre.

Validation cohort

The validation cohort (n = 332 cirrhotic patients enlisted for OLT) was selected from the 522 adult patients evaluated for OLT between September 1995 and April 2011 in Erasmus MC-University Medical Centre, Rotterdam, The Netherlands. The selection process was based on the same criteria as in the identification cohort. In 332 selected patients, SBIs were defined according to the same definitions as used in the identification cohort from Prague.

A higher rate of Child-Pugh B patients was observed in the validation cohort because of the Dutch policy to enlist patients for OLT when they had Child-Pugh score 8 (B) or higher. Another reason to enlist patients with cirrhosis staged as Child-Pugh B was the presence of hepatocellular carcinoma. Due to progression of liver disease on the waiting list, most patients had Child-Pugh score C at the moment they were transplanted.

Genotyping

Patients were genotyped for *TLR4* c.+1196C/T, *CD14* c.−159C/T, *TNFA* c.−238G/A, *TNFA* c.−863C/A, *IL1B* c.−31C/T and *IL1RN* VNTR (UniSTS:156109) allelic variants, as described in [32], using specific primers and PCR conditions shown in Supplementary Table 1. In order to minimise genotyping errors, blank control wells were left on the PCR plates and two operators, unaware of the status of the samples, performed the genotype assignment independently. After testing for Hardy-Weinberg equilibrium (HWE), allele frequencies were checked for consistency with data from the population of European ancestry (Utah Residents with Northern and Western European Ancestry) from the HapMap database [33].

Primary assessment of associations between allelic frequencies and SBIs was performed in the identification cohort from Prague, and positive associations were confirmed in the validation cohort from Rotterdam. The study was approved by the institutional Research Ethics Committee of both participating centres. Written informed consent with DNA sampling was obtained from all patients and the study conformed to the declaration of Helsinki Ethical Guidelines.

Determination of serum levels of TNF-α

Serum levels of TNF-α were determined in blood samples taken from patients at the moment of liver transplantation, i.e., in patients with no physical and laboratory signs of infection. The samples were frozen immediately after serum separation and stored at −80 °C. In the identification cohort, TNF-α was assessed in serum samples of 199 patients, out of which 179 patients were homozygotes for the *TNFA* c.−238G allele and 20 patients were heterozygotes. Additional 36 samples (12 samples of patients carrying the *TNFA* c.−238GA genotype and 24 samples of homozygotes for *TNFA* c.−238G) came from the validation cohort.

Quantitative determination of TNF-α was performed with the Quantikine HS ELISA human TNF-α immunoassay (R&D Systems, Abingdon, UK). All standards, controls and samples were analysed in duplicates and the duplicate readings were averaged. Duplicates with coefficient variability (CV) higher than 50% (5 heterozygotes and 25 homozygotes) were excluded and the remaining 27 heterozygotes were then matched in age, sex, and underlying diseases with 81 of the 188 non-excluded heterozygotes.

Statistical analysis

Data are presented as mean and standard deviation, as median and range, or as frequencies, as appropriate. HWE and differences in genotype frequencies between patients with SBI and controls were analysed using two-sided χ^2 testing. Using standard formulas based on two-by-two tables [34], we calculated basic epidemiology statistics and evaluated the preventable fractions among the population and among the exposed. *t* test or Mann-Whitney tests were used for comparisons of the means. Due to the testing of multiple statistical hypotheses, Bonferroni correction was used in the identification cohort. Cox regression analysis was used to calculate hazard ratio and 95% confidence interval (CI). Significant risk factors from univariate analysis were entered into the multivariate Cox regression analysis, which was performed with a forward stepwise approach. Wald statistics was employed in the regression module to evaluate the relative contribution of significant variables to SBI. Kaplan-Meier analysis with log-rank test was performed to evaluate survival. A p value <0.05 was considered statistically significant throughout the study. Statistical analysis was performed using JMP 9.0.0. and SPSS 13.0 programs.

Results

Demographic, clinical and survival data

Demographic and clinical data of OLT candidates included in the study are shown in Table 1. The median time to develop SBI while on the waiting list, or the median time to OLT in patients who did not develop SBI, was shorter in the identification cohort compared to the validation cohort (337 *vs.* 479 days, p <0.001) and a higher proportion of patients with Child-Pugh B classification were present in the validation cohort (59.9% *vs.* 50.6%, p = 0.015). Both cohorts contained similar spectra of liver diseases (Table 1). The group of cholestatic liver diseases included primary biliary cirrhosis, secondary biliary cirrhosis, PBC/AIH overlap syndrome and benign recurrent intrahepatic cholestasis/Byler's disease. Metabolic liver diseases group comprised Wilson's disease, α_1-antitrypsin deficiency, haemochromatosis, and erythropoietic protoporphyria, and liver cirrhosis of other aetiologies involved cryptogenic cirrhosis, Budd-Chiari syndrome and non-alcoholic steatohepatitis. Significantly more patients with liver cirrhosis due to chronic hepatitis B were present in the validation cohort compared with the identification cohort (15.4% *vs.* 8.6%).

Out of all patients evaluated for OLT, 32.1% patients in the identification cohort and 51.5% in the validation cohort died; 10.7% and 28.6% of patients died pre-transplant at a median of 84 and 206 days after enlistment for OLT, and 21.4% and 22.9% of patients died at a median of 1109 and 969 days after OLT, respectively (Table 1, bottom sections). Approximately 42% pre-transplant and 31% post-transplant deaths were attributable to SBI in the identification cohort. In the validation cohort, SBI were responsible for 38% deaths in the pre-transplant period and for 28% deaths post-transplant.

Severe bacterial infections

Thirty-four percent of patients with advanced liver cirrhosis in the identification cohort and 40% of patients in the validation

Table 1. Demographic, clinical, and survival data of patients listed for OLT.

	Identification cohort	Validation cohort	p value
N	336	332	
Age at OLT (yr, mean ± SD)	53 ± 9.5	52 ± 10.2	n.s.
Male/female (%)	194/142 (57.7/42.3)	216/116 (65.1/34.9)	n.s.
Interval between enlistment and SBI or OLT (days, median-range)	337 (270-1335)	479 (272-2979)	<0.001
Survival after enlistment (days, median-range)	2770 (271-6079)	2994 (274-6834)	n.s.
Child-Pugh B/C (%)	170/166 (50.6/49.4)	199/133 (59.9/40.1)	0.015
MELD (points, median-range)	15.8 (6-40)	15.6 (1-40)	n.s.
Aetiology of cirrhosis	N (%)	N (%)	p value
ALD	119 (35.4)	97 (29.2)	n.s.
HCV	65 (19.3)	70 (21.1)	n.s.
HBV	29 (8.6)	51 (15.4)	0.003
Cholestatic	49 (14.6)	29 (8.7)	n.s.
Metabolic	15 (4.5)	13 (3.9)	n.s.
AIH	23 (6.8)	17 (5.1)	n.s.
Other	36 (10.7)	55 (16.6)	n.s.
Mortality	N (%)	N (%)	p value
No death	228 (67.9)	161 (48.5)	
Death prior to OLT	36 (10.7)	95 (28.6)	<0.001
Death after OLT	72 (21.4)	76 (22.9)	
Pre-transplant deaths			
Survival (median days, IQR)	84 (38-200)	206 (37-477)	0.002
SBI-related deaths (N, %)	15 (41.7)	36 (37.9)	n.s.
SBI-unrelated deaths (N, %)*	21 (58.3)	59 (62.1)	
Post-transplant deaths			
Survival (median days, IQR)	1109 (200-2322)	969 (68-2073)	n.s.
SBI-related deaths (N, %)	22 (30.6)	28 (36.8)	n.s.
SBI-unrelated deaths (N, %)**	50 (69.4)	48 (63.2)	

Child-Pugh and MELD score at the time of enlistment for liver transplantation.
*Causes of SBI-unrelated pre-transplant deaths: Liver failure 9 (42.9%); cardiovascular 7 (33.3%); GIT bleeding related to portal hypertension 3 (14.3%); other 2 (9.5%) in identification cohort and liver failure 24 (40.7%); cardiovascular 4 (6.8%); GIT bleeding related to portal hypertension 6 (10.2%); other and unknown 25 (42.4%) in validation cohort.
**Causes of SBI-unrelated post-transplant deaths: Malignancy 22 (44%); cardiovascular 19 (38%); graft failure 7 (14%); other 2 (4%) in identification cohort and malignancy 10 (20.8%); cardiovascular 15 (31.3%); graft failure 18 (37.5%); other 5 (10.4%) in validation cohort.
ALD, alcoholic liver disease; HCV, hepatitis C virus; HBV, hepatitis B virus; AIH, autoimmune hepatitis; IQR, interquartile range; n.s., not significant.

Table 2. Demographic and clinical data of patients with and without severe bacterial infection.

	Identification cohort			Validation cohort		
	with SBI	without SBI	*p* value	with SBI	without SBI	*p* value
N (%)	115 (34.2)	221 (65.8)		133 (40.1)	199 (59.9)	
Age at OLT (yr mean ± SD)	52 ± 10.6	53 ± 8.9	n.s.	52 ± 10.4	52 ± 10.1	n.s.
Male/female (%)	53/62 (46.1/53.9)	141/80 (63.8/36.2)	0.002	84/49 (63.2/36.8)	132/67 (66.3/33.7)	n.s.
Interval between enlistment and SBI or OLT (days, median-range)	333 (271-1335)	338 (270-709)	n.s.	409 (274-2979)	499 (272-2644)	0.037
Child-Pugh B/C (%)	46/69 (40/60)	124/97 (56.1/43.9)	0.005	60/73 (45.1/54.9)	139/60 (69.8/30.2)	<0.001
MELD (points, median - range)	16 (6-36)	15 (7-40)	0.004	17 (7-40)	14 (1-34)	<0.001
Aetiology of cirrhosis	N (%)	N (%)	*p* value	N (%)	N (%)	*p* value
ALD	41 (35.7)	78 (35.3)	n.s.	46 (34.6)	51 (25.6)	n.s.
HCV	21 (18.3)	44 (19.9)	n.s.	23 (17.3)	47 (23.6)	n.s.
HBV	8 (7.0)	21 (9.5)	n.s.	25 (18.8)	26 (13.1)	n.s.
Cholestatic	16 (13.9)	33 (14.9)	n.s.	10 (7.5)	19 (9.6)	n.s.
Metabolic	5 (4.3)	10 (4.5)	n.s.	6 (4.5)	7 (3.5)	n.s.
AIH	9 (7.8)	14 (6.3)	n.s.	6 (4.5)	11 (5.5)	n.s.
Other	15 (13.0)	21 (9.5)	n.s.	17 (12.8)	38 (19.1)	n.s.

Child-Pugh and MELD score at the time of enlistment for liver transplantation.
ALD, alcoholic liver disease; HCV, hepatitis C virus; HBV, hepatitis B virus; AIH, autoimmune hepatitis; n.s., not significant.

cohort presented with SBI during the observation period (Table 2). In the identification cohort, the risk of SBI was significantly associated with female gender and the degree of liver dysfunction evaluated by MELD score or Child-Pugh score. Both MELD score and Child-Pugh score but not female gender remained significantly associated with SBI in the validation cohort. Patients in the validation cohort suffering from SBI had a significantly shorter observation period than patients without SBI (409 *vs.* 499 days, $p = 0.037$). Age and aetiology of liver cirrhosis were not associated with susceptibility to SBI (Table 2).

A total of 130 episodes of SBI were diagnosed in 115 patients in the identification cohort compared with 170 episodes of SBI diagnosed in 133 patients in the validation cohort. The frequencies of most SBIs were similar in identification and validation cohorts: pneumonia 10/130 *vs.* 20/170, $p = 0.244$, urinary tract infection 21/130 *vs.* 25/170, $p = 0.730$ and infection of skin and soft tissues 8/130 *vs.* 10/170, $p = 0.922$. However, SBP was significantly more frequent in the identification cohort (84/130 *vs.* 90/170, $p = 0.042$) whereas patients with bacterial infections of unknown origin were more prevalent in the validation cohort (7/130 *vs.* 25/170, $p = 0.001$).

Genetic associations with severe bacterial infections

The identification cohort was genotyped for the 6 annotated SNP loci. Genotype frequencies at the individual loci were in HWE except for the *TNFA* c.−863C/A locus, which was excluded from further evaluations. Single locus analysis performed in the identification cohort (n = 336, Table 3 and Fig. 1) revealed a strong association of the *TNFA* c.−238 locus with SBIs. Subjects carrying the minor *TNFA* c.−238A allele showed significantly reduced risk of SBI (OR 0.22, 95% CI 0.07–0.75, $p_{non\text{-}adjusted} = 0.008$, $p_{adjusted\ (Bonferroni)} = 0.042$) compared with homozygotes for the major *TNFA* allele c.−238G. Among the genetic loci under study, only this allele was associated with SBI; neither of the other investigated genes showed any differences in the distribution of genotype frequencies between cases and controls (Table 3 and Fig. 1). Similar to the identification cohort, presence of the *TNFA* c.−238A allele conferred a significantly decreased risk of SBI in the validation cohort (OR 0.37, 95% CI 0.14–1.02, $p = 0.046$, Table 3) and the association was independent of aetiology of SBI in both cohorts (data not shown).

The presence of minor *TNFA* c.−238A allele has been previously shown to decrease the transcriptional activity of *TNFA* resulting in lower production of TNF-α protein [21]. Consistent with this report, we found that 27 carriers of the variant *TNFA c.*−238A allele showed significantly lower levels of serum TNF-α, compared with 81 age-, sex- and diagnosis-matched homozygotes for the major allele *TNFA c.*−238G (2.13 ± 2.11 pg/ml *vs.* 3.11 ± 2.52 pg/ml, $p = 0.022$) (Fig. 2). The difference between the 27 heterozygotes and all 188 homozygotes was even more pronounced (2.13 ± 2.11 pg/ml *vs.* 3.52 ± 3.27 pg/ml, $p = 0.006$).

Determinants of severe bacterial infection and mortality

The potential role of *TNFA* c.−238A as an independent predictor of SBI was evaluated by Cox regression analysis. Due to the low frequency of the *TNFA* c.−238A allele (about 4% in both groups), we pooled both cohorts to achieve a sufficient power to detect moderate and small effect sizes. Calculations of hazard ratios in the univariate mode showed that SBIs were significantly associated with MELD, Child-Pugh class C *vs.* B) and *TNFA* c.−238 status, but not with age and gender. Multivariate analysis confirmed that MELD, Child-Pugh score and the *TNFA* c.−238 genotype represent independent predictors of SBIs (Table 4). Specifically, advanced liver disease (Child-Pugh class C *vs.* B) increased the risk of SBI 1.5-fold and each one-point increment in MELD score increased the risk of SBI by approximately 7%, whereas presence of the variant *TNFA* c.−238A allele decreased risk of SBI by almost 2.5-fold (Table 4, multivariate analysis).

Using Wald statistics to evaluate the relative contributions of these determinants to SBI, we found that the presence of the variant *TNFA c.*−238A allele mitigated the odds of SBI developing as a consequence of Child-Pugh C class liver dysfunction by 76%

Table 3. Genotype distributions in the identification and validation cohort.

Locus	Genotype	Identification cohort			Validation cohort		
		Patients with SBI (n = 115) N (%)	Patients without SBI (n = 221) N (%)	*p* value	Patients with SBI (n = 133) N (%)	Patients without SBI (n = 199) N (%)	*p* value
CD14 c.-159	CC CT TT	32 (27.8) 62 (53.9) 21 (18.3)	62 (28.1) 111 (50.2) 48 (21.7)	n.s.			
TLR4 c.-1196	CC CT TT	101 (87.8) 14 (12.2) 0	196 (88.7) 25 (11.3) 0	n.s.			
TNFA c.-238	GG GA AA	112 (97.4) 3 (2.6) 0	197 (89.1) 24 (10.9) 0	0.008	128 (96.2) 5 (3.8) 0	180 (90.5) 19 (9.5) 0	0.046
TNFA c.-863	CC CA AA	91 (79.1) 18 (15.7) 6 (5.2)	184 (83.3) 26 (11.8) 11 (5.0)	n.a.*			
IL1B c.-31	CC CT TT	13 (11.3) 58 (50.4) 44 (38.3)	33 (14.9) 103 (46.6) 85 (38.5)	n.s.			
IL1RN VNTR	11 12 22 14, 31, 32	47 (40.9) 51 (44.3) 13 (11.3) 4 (3.5)	103 (46.6) 91 (41.2) 14 (6.3) 13 (5.9)	n.s.			

*As *TNFA* c.−863 genotypes were not in HWE, no further calculations were performed.
n.s., not significant; n.a., not available.

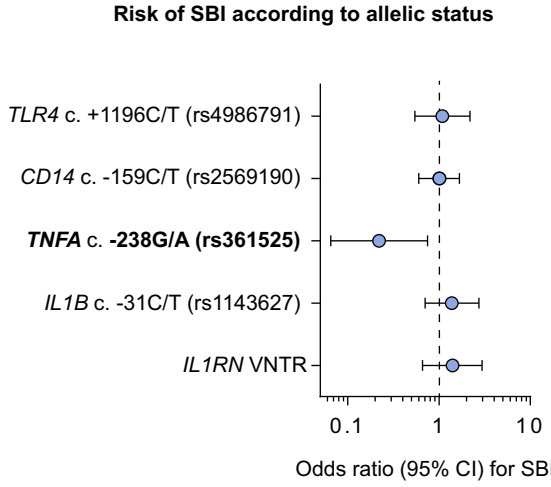

Fig. 1. Association of the investigated variants with SBI in the identification cohort. Bars represent OR with 95% confidence interval. Source data are shown in Table 3.

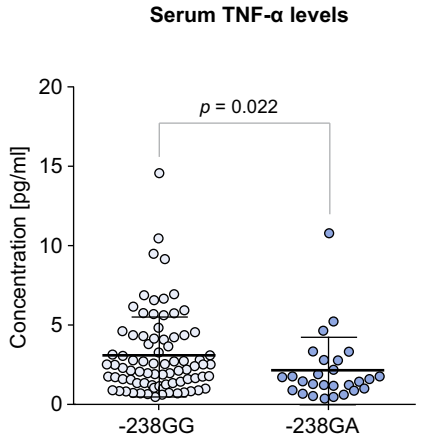

Fig. 2. Decreased serum levels of TNF-α in carriers of the *TNFA* c.−238A variant. The data from 81 homozygotes (c.−238GG) and 27 heterozygotes (c.−238GA) are shown as individual dots. Horizontal bars indicate mean (thick line) and standard deviations (thin lines). Mann-Whitney was used for comparison of the means.

(5.044/6.61) (Table 4, part I.B). Further calculations demonstrated that presence of the minor *TNFA* c.−238A allele prevented 7.2% (95% CI 3.7–8.8%) of SBIs in the whole cohort (preventable fraction among the population), and that among the patients who developed SBIs, 70.6% (95% CI 36.7–86.4%) of these cases would hypothetically be preventable by the presence of the minor *TNFA* c.−238A allele (preventable fraction among the exposed). The presence of *TNFA* c.−238A predicted protection from SBIs in the pooled cohort with a high positive predictive value (84.4%; 95% CI 72.4–98.0%) and specificity (89.8%; 95% CI 86.0–92.5%), whereas sensitivity and negative predictive value were low (9.3% and 38.9%, respectively). Collectively, these findings demonstrate that possession of the variant *TNFA* c.−238A allele provides strong protection against SBI in patients with advanced end-stage liver disease.

Finally, we evaluated *TNFA* c.−238A allele as a predictor of mortality. Multivariate Cox regression analysis identified SBI, age, female gender and MELD score as significant predictors of overall pre-transplant mortality (Table 4, part II and Fig. 3A). In contrast, the *TNFA* c.−238 genotype was associated with all-cause mortality neither in regression modelling (Table 4, part II), nor in Kaplan-Meier analysis (Fig. 3B). However, as our previous data demonstrated significant association of *TNFA* c.−238 genotype with SBI (Table 3, Fig. 1), and SBI was strongly associated with

Table 4. Risk factors of SBI or death.

I. Outcome: severe bacterial infection				
A. Univariate analysis				
Variables	Wald	HR	95% CI	*p* value
MELD per 1 point increment	21.918	1.072	1.041-1.104	<0.001
Child-Pugh class C *vs.* B	6.89	1.550	1.117-2.150	0.009
TNFA c.-238A	4.869	0.427	0.200-0.914	0.028
Sex (female *vs.* male)	2.879	1.293	0.961-1.741	0.09
Cohort (identification *vs.* validation cohort)	0.803	1.149	0.448-1.557	n.s.
Age per 1 year increment	0.580	1.006	0.991-1.020	n.s.
B. Multivariate analysis				
Variables	Wald	HR	95% CI	*p* value
MELD per 1 point increment	19.964	1.067	1.037-1.098	<0.001
Child-Pugh class C *vs.* B	6.61	1.531	1.107-2.119	0.01
TNFA c.-238A	5.044	0.421	0.197-0.896	0.025
II. Outcome: pre-transplant death (all causes)				
A. Univariate analysis				
Variables	Wald	HR	95% CI	*p* value
SBI	32.343	2.36	1.755-3.173	<0.001
Age per 1 year increment	17.609	1.033	1.018-1.049	<0.001
MELD per 1 point increment	11.431	1.056	1.023-1.09	<0.001
Sex (female *vs.* male)	10.647	0.606	0.448-0.819	0.001
Child-Pugh class C *vs.* B	0.972	1.169	0.857-1.595	n.s.
Cohort (identification *vs.* validation cohort)	0.959	1.161	0.861-1.565	n.s.
TNFA c.-238A	0.004	1.017	0.606-1.707	n.s.
B. Multivariate analysis				
Variables	Wald	HR	95% CI	*p* value
SBI	38.58	2.476	1.860-3.296	<0.001
Age per 1 year increment	17.509	1.033	1.017-1.049	<0.001
MELD per 1 point increment	16.259	1.062	1.031-1.093	<0.001
Sex (female *vs.* male)	14.296	0.572	0.428-0.764	<0.001

Analyses were performed in pooled patient cohorts (n = 668) using Cox regression.
HR, hazard ratio; CI, confidence interval; Wald, measurement of influence statistics; n.s., not significant.

mortality (Table 4, part II and Fig. 3A), we hypothesized that *TNFA* c.−238 genotype could be a conditional predictor of mortality based on infection status of the host. Therefore, we performed subgroup analysis and found that patients carrying the *TNFA* c.−238A allele had a significantly decreased risk of succumbing to SBI compared with homozygotes for the major GG genotype (Fig. 3C). Specifically, none of the carriers of the *TNFA* c.−238A allele who developed SBI in the pre-transplant period died, whereas SBI-related mortality in homozygotes for the major allele *TNFA* c.−238G reached 12% (Fig. 3C). As expected, there was no association between *TNFA* c.−238 and pre-transplant death from non-infectious causes (Fig. 3D).

Discussion

Our study provides several lines of evidence suggesting that the *TNFA* c.−238 status is a significant predictor of reduced susceptibility to SBIs. First, the variant allele was associated with SBIs in two independent cohorts of patients with ESLD. Second, its association with SBI was independent of other variables in multivariate Cox regression analysis. Third, the relative significance of the *TNFA* c.−238 allelic status in predicting SBIs was in close succession to MELD score and Child-Pugh C class of liver dysfunction, the strongest determinants of SBI identified in this study.

Given the low prevalence of the *TNFA* c.−238A allele, the potential clinical utility of our finding would be in ruling out the potential risk of SBIs in a minor group of patients with ESLD. Considering that the *TNFA* c.−238A allele is a marker of protection from SBIs, the presence of this allele in any given individual with ESLD will predict significant protection from SBIs (84% positive predictive value for the protection from SBIs in carriers of the variant A allele). Moreover, presence of this allele in patients with SBI awaiting OLT predicts a substantial survival benefit.

The findings are consistent with the relevant contribution of the *TNFA* c.−238 allelic status to the risk of SBI in multivariate Cox regression analysis and indicate that the presence of the *TNFA* c.−238A allele may be truly significant in the clinical scenario. However, although the allelic status aids in ruling out SBI, it has a very low ruling-in characteristic, since the major allele G is abundantly represented in both SBI and non-SBI groups.

Although several allelic variants were investigated in this study, only the *TNFA* c.−238 allele, but not variants in *TLR4*, *CD14*, *IL1B*, and *IL1RN*, were associated with SBIs. This is

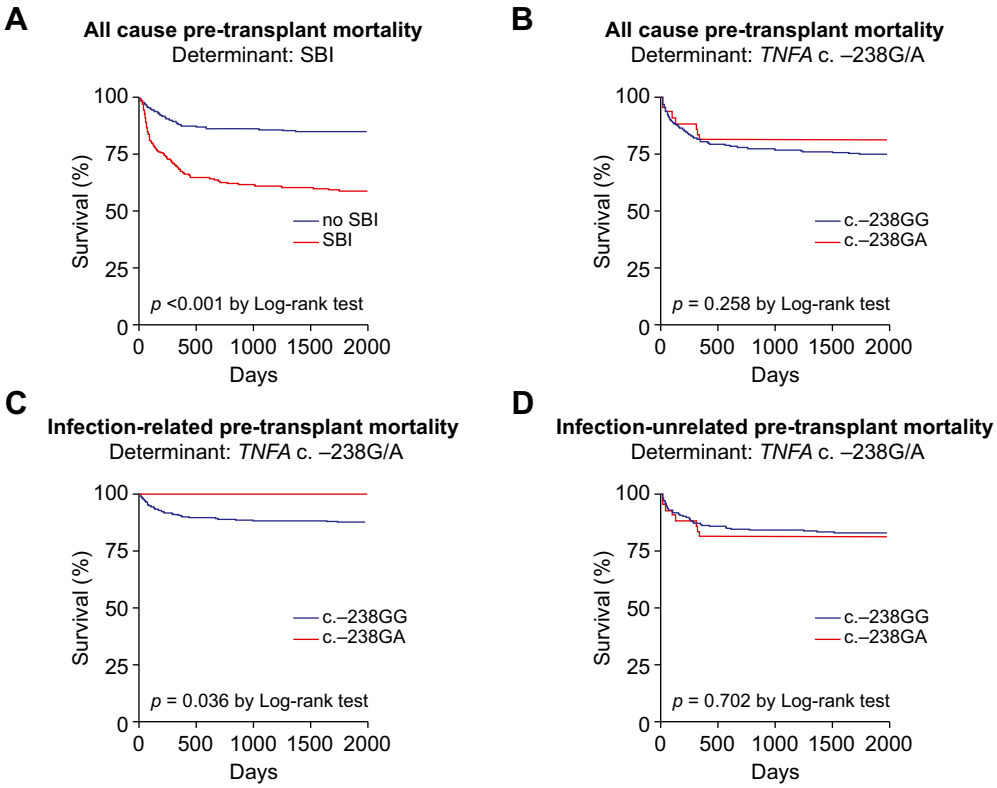

Fig. 3. The presence of the *TNFA* c.−238A variant is associated with significantly decreased risk of SBI-related pre-transplant mortality. The impact of SBI (A) and *TNFA* c.−238GA genotype (B) on overall pre-transplant mortality and the association of *TNFA* c.−238GA genotype with mortality attributable to SBI (C) or non-infectious causes (D) were analyzed in pooled patient cohorts (n = 668). Kaplan-Meier analysis with Log-rank test was used for statistical evaluation.

consistent with the hierarchical pattern of TLR signalling. Although TLR2, 4, 5, and 9 recognize distinct microbial components (peptidoglycan from G^+ bacteria, LPS from G^- bacteria, flagellin from G^+ and G^- bacteria, and unmethylated CpG sequences in bacterial DNA, respectively), they all utilize the common adaptor MyD88 and the nuclear factor kappa-B to induce inflammatory cytokines TNF-α and IL-1β [13]. Therefore, as SBIs are caused by a wide spectrum of pathogens [1,35], it is possible that the major signal for induction of MyD88-dependent inflammatory cytokines in SBIs comes from a source other than LPS, which could explain the lack of association of *TLR4* and *CD14* with SBIs in our study. This notion is further supported by our data that multiple microbial classes determined SBIs in our study.

The lack of association between the *IL1B* gene cluster and SBIs could be attributed to differential roles of TNF-α *vs.* IL-1β in immune responses. Whereas TNF-α is triggered by bacterial ligands and is indispensable for antimicrobial defence, IL-1β serves as an amplifier of inflammatory responses and requires co-activation by host-derived factors released from tissues upon damage [36], which may not always be present in the context of bacterial infection.

The *TNFA* c.−238A allele determines low transcriptional activity of the *TNFA* gene and lower production of TNF-α *in vitro* [21,22] as well as *in vivo* (our study, Fig. 2). This seemingly paradoxical finding is consistent with desensitisation of innate immune cells to bacterial components and significant neutrophilic dysfunction found in patients with liver cirrhosis. Tritto *et al.* [9] reported that severity of liver disease positively correlates with serum levels of TNF-α and negatively correlates with neutrophil phagocytic dysfunction. This suggests that chronic elevation of TNF-α level causes exhaustion of neutrophil opsonising and phagocytic capacity [9]. The hypothesis is further supported by Ono *et al.* [10], who found desensitisation of neutrophils to TNF-α and LPS stimulation resulting in opsonophagocytic dysfunction in patients with liver cirrhosis. Finally, Stadlbauer *et al.* [37] demonstrated the neutrophil phagocytic dysfunction in patients with alcoholic cirrhosis was restored by treatment with probiotics, which resulted in decreased endotoxemia and TNF-α production. Our finding that the low-producer genotype in the *TNFA* c.−238 locus negatively affects the risk of SBI is therefore fully consistent with the aforementioned studies, it furthermore supports the biological concept of immune cell desensitisation by overzealous activation of inflammatory signalling in patients with liver cirrhosis, and implies that the low-producing variant *TNFA* c.−238A allele in patients with ESLD may reset the sensitivity of innate immune cells to microbial ligands. The concept of neutrophil exhaustion is also strongly supported by their lower serum levels of TNF-α.

Low TNF-α production might have unfavourable consequences in cirrhotic patients manifested as higher occurrence of hepatocellular carcinoma as described by Teixeira *et al.* [38]. This observation might be explained by deficient anti-tumour immunity in individuals with decreased levels of TNF-α. Nonetheless, the beneficial effect consisting of lower incidence of SBI and significantly decreased mortality owing to SBIs in patients awaiting OLT predominates over the risk of tumour growth.

The main limitation of our study resides in its retrospective design. For this reason we were not able to validate the published data [21,22] on the decreased transcriptional activity of *TNFA* in heterozygotes for the c.−238A allele. On the other hand, we assessed serum levels of TNF-α in a representative subset of enrolled patients and provided indirect evidence supporting such hypothesis.

In conclusion, we have shown that presence of the *TNFA* c.−238A allele in ESLD patients virtually excludes the probability of developing SBIs or dying of SBI in patients awaiting OLT. The strong effect of this allele supports the biological concept that TNF-mediated desensitisation of innate immune cells drives increased susceptibility to bacterial infections in patients with ESLD. Our findings indicate potential utility of *TNFA* rs361525 genotyping in assessment of individual risk of SBIs in patients waitlisted for OLT and warrant further mechanistic studies.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2013.12.011.

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PLOS ONE

RESEARCH ARTICLE

PNPLA3 rs738409 G allele carriers with genotype 1b HCV cirrhosis have lower viral load but develop liver failure at younger age

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Abstract

Background

PNPLA3 rs738409 minor allele c.444G represents a risk factor for liver steatosis and fibrosis progression also in chronic hepatitis C (HCV). We investigated its impact on the timing of liver transplantation (LT) in patients with genotype 1b HCV cirrhosis.

Methods

We genotyped and evaluated 172 LT candidates with liver cirrhosis owing to chronic HCV infection, genotype 1b. One hundred patients needed LT for chronic liver failure (CLF) and 72 for a small hepatocellular carcinoma (HCC) in the cirrhotic liver without CLF. Population controls (n = 647) were selected from the Czech cross-sectional study MONICA.

Results

The CLF patients were younger (53.5 ± 7.2 vs. 59.6 ± 6.6, $P < 0.001$) with more advanced liver disease than HCC patients (Child-Pugh's score 9.1 ± 1.8 vs. 7.1 ± 1.9, $P < 0.001$, MELD 14.1 ± 3.9 vs. 11.1 ± 3.7, $P < 0.001$). *PNPLA3* G allele increased the risk of LT for CLF in both allelic and recessive models (CG + GG vs. CC: OR, 1.90; 95% CI, 1.017–3.472, $P = 0.045$ and GG vs. CC + CG: OR, 2.94; 95% CI, 1.032–7.513, $P = 0.042$). Multivariate analysis identified younger age ($P < 0.001$) and the G allele ($P < 0.05$) as risk factors for CLF. The genotype frequencies between the CLF group and MONICA study significantly differed in both, allelic and recessive model ($P = 0.004$, OR 1.87, 95% CI 1.222–2.875; $P < 0.001$, OR 3.33, 95% CI 1.824–6.084, respectively). The OR values almost doubled in the recessive model compared with the allelic model suggesting the additive effect of allele G. In contrast, genotype frequencies in the HCC group were similar to the MONICA study in both models.

and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine-aminotransferase; BMI, body mass index; CI, confidence interval; CLF, chronic liver failure; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDL, high density lipoprotein cholesterol; INR, International normalized ratio; LDL, low density lipoprotein cholesterol; LT, liver transplantation; MELD, Model for End-Stage Liver Disease; NASH, non-alcoholic steatohepatitis; OR, odds ratio; PNPLA3, patatin-like phospholipase domain containing 3; SD, standard deviation.

Pretransplant viral load was significantly lower in GG than in CC + CG genotypes (median, IQR; 162,500 (61,550–319,000) IU/ml vs. 570,000 (172,000–1,595,000) IU/ml, $P < 0.0009$).

Conclusions

Our results suggest that *PNPLA3* rs738409 G allele carriage may be associated with a faster progression of HCV cirrhosis to chronic liver failure.

Introduction

Adequate timing of liver transplantation (LT) represents one of the main factors determining favourable posttransplant outcome. Prediction of the patients' prognosis based on the known natural course of a particular liver disease is crucial in the evaluation process [1]. The natural course of liver diseases may be altered by genetic factors. Single nucleotide polymorphism rs738409 c.444C>G (p.Ile148Met) in the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) is nowadays one of the most important genetic factors with an impact on progression of several liver diseases of different etiology [2].

Association between liver fat content as a quantitative trait and *PNPLA3* rs738409 genotype was described in a large genome-wide association mapping study [3] in 2008 and confirmed in a more detailed study [4] by the same group of authors in 2014. More than fifty studies demonstrating that the *PNPLA3* rs738409 G allele is a risk factor for non-alcoholic steatohepatitis (NASH), liver cirrhosis in NASH or alcoholic liver disease have been published in the past decade [5–11]. The same allele was also identified as a risk factor for liver fibrosis and cirrhosis in HCV-monoinfected individuals [12–16] and in those with HCV/HIV coinfection [17–20] and it also turned out to be a predisposing factor of hepatocellular carcinoma (HCC) [21–24]. In a recent study, the increased risk of HCC and *PNPLA3* G allele was found only in alcoholic liver disease, but not in non-alcoholic fatty liver disease or viral hepatitis B and C [25].

Whereas the impact of the G allele on the liver fibrosis progression in chronic hepatitis C seems to be well known, its impact on chronic liver failure (CLF) progression and the need of LT has not been described so far. In this study, we aimed to investigate the impact of *PNPLA3* genotypes on the risk of CLF in a homogenous group of cirrhotic patients infected with HCV genotype 1b.

Patients and methods

Study design and eligibility of patients

We retrospectively evaluated 172 adult patients with HCV-related cirrhosis caused by HCV genotype 1b with Child-Pugh's class A, B and C who underwent LT between January 1995 and August 2018 at our center. One hundred patients were enlisted for LT and transplanted for CLF (CLF group) using standard criteria evaluating liver dysfunction according to the Child-Pugh's and MELD score and 72 patients were transplanted for a small HCC (HCC group). Fifty-two patients fulfilled Milan criteria, remaining 20 complied with San Francisco or up-to-seven criteria based on pre-transplant imaging techniques results [26–28]. The diagnosis of HCC was confirmed in the liver explants using standard histological staining techniques. Neither patients with HBsAg positivity nor those with HBcAb positivity were included. Patients combining HCV infection with excessive alcohol consumption (60 g per day in males and 40 g per day in females) were also excluded. None of HCV-infected patients had obtained antiviral

treatment in the year preceding LT in accordance with our centre anti-HCV treatment policy: very short times in the liver transplant waiting list, 80–90 days, do not allow for a safe entire treatment course before LT, even in the era of direct acting antivirals. The patients were treated after LT according to the period of transplantation, using an interferon-based regimen until 2014 or a direct acting antivirals combination thereafter. Demographic, clinical, laboratory and histological data were collected from the internal hospital and outpatient database (S1 Table).

Genotype frequencies in both CLF and HCC groups were compared with the *PNPLA3* genotype frequencies in 647 subjects 0.566/0.372/0.062 (CC/CG/GG) reported in the Czech cross-sectional population study MONICA [29], genotyping data were taken from Trunecka et al. [30].

HCV viral load and HCV genotype assessment

HCV viral loads (serum HCV RNA levels) were determined in blood samples taken from HCV-infected patients within 24 hours before LT (last value unaffected by immunosuppression or antiviral therapy). In 133 patients, serum HCV RNA level was assessed according to the period of sampling by the Roche COBAS® AmpliPrep/COBAS® TaqMan® HCV Quantitative Test v1.0 or v2.0 (Roche Molecular Systems Inc., South Branchburg, NJ).

In the 39 remaining patients, only an in-house quantitative method was used and therefore those results were not included in the statistical analysis. HCV genotype was assessed using the SIEMENS Versant® HCV Genotype 2.0 Assay (LiPA) (Siemens Healthcare Diagnostics Inc., Tarrytown, NY).

Genotyping

DNA was isolated from the peripheral blood using the Qiagen QIAamp kit (Qiagen, Hilden, Germany). All patients were genotyped for the *PNPLA3* rs738409 c.444C>G polymorphism by the TaqMan predesigned SNP genotyping assay No. C_7241_10 (Thermo Fisher Scientific, Waltham, MA). Genotyping was performed according to the manufacturer's protocol using the Applied Biosystems ABI 7300 Real-Time PCR instrument (Thermo Fischer Scientific). No significant deviation from the Hardy-Weinberg equilibrium was observed in *PNPLA3* genotypes distribution within the CLF and HCC patient groups.

Statistical analysis

Continuous variables are presented as means and standard deviations, whereas categorical variables are expressed as frequencies (%). Categorical data were analyzed using the chi-square test. For continuous data, Student's t-test or the non-parametric Mann-Whitney test were used appropriately. Genotype frequencies were determined and tested for consistency with the Hardy-Weinberg equilibrium using the chi-square test. Testing for genetic associations was performed as described in [31]. Risk factors were examined using multivariate logistic regression analysis. All statistical analyses were two-sided and P value of < 0.05 was considered statistically significant throughout the study. Statistical analysis was performed using the R programming language version 3.2.0 (www.r-project.org).

Ethics statement

This study was approved by the Ethics Committee of the Thomayer Hospital and Institute for Clinical and Experimental Medicine, Prague, Czech Republic, and was carried out in compliance with the Helsinki Declaration. The patients' informed consent was not required by local

law because of the retrospective design of the study and the use of data from which the patients' identification information had been removed. All study participants gave written consent to the storage of blood samples and agreed to using blood for future research including genetic testing. The written consent was obtained before enlistment for LT.

Results

Demographic, clinical data and laboratory data

Demographic, clinical and laboratory data of the CLF and HCC groups are shown in Table 1. Patients transplanted for CLF were younger with a higher proportion of males and suffered from more advanced liver disease according to the Child-Pugh's and MELD score in comparison with the HCC group. Patients in CLF group had lower AFP levels and lower total cholesterol, HDL and serum triglycerides levels.

Pretransplant viral load

Pretransplant viral load was known in 133 of 172 HCV cirrhotic patients. HCV patients with known pretransplant viral load included 66 of 82 patients with the *PNPLA3* rs738409

Table 1. Demographic, clinical and laboratory data of subgroups with CLF and HCC.

Variables	CLF group n = 100	HCC group n = 72	*P* value
Males (n)	68 (68.0%)	38 (52.8%)	0.0428
Age (years)	53.5 ± 7.2	59.6 ± 6.6	< 0.001
BMI (kg/m^2)	26.2 ± 4.2	26.8 ± 3.7	0.175
Type 2 diabetes mellitus	27 (27.0)	25 (34.7)	0.277
Child-Pugh's class			< 0.001
A	6 (6.0)	37 (51.4)	
B	48 (48.0)	27 (37.5)	
C	46 (46.0)	8 (11.1)	
Child-Pugh's score (points)	9.1 ± 1.8	7.1 ± 1.9	< 0.001
MELD score (points)	14.1 ± 3.9	11.1 ± 3.7	< 0.001
Ascites			< 0.001
None	44 (44.0)	53 (73.6)	
Small	32 (32.0)	14 (19.5)	
Large	24 (24.0)	5 (6.9)	
AFP (μg/l)	34.5 ± 50.1	337.1 ± 926.8	< 0.001
Total bilirubin (μmol/l)	51.8 ± 77.3	35.6 ± 46.7	< 0.001
Albumin (g/l)	29.0 ± 6.5	33.5 ± 6.8	< 0.001
ALT (μkat/l)	1.3 ± 0.9	1.5 ± 1.2	0.117
Total cholesterol (mmol/l)	3.4 ± 1.0	3.7 ± 1.0	0.004
HDL cholesterol (mmol/l)	0.9 ± 0.4	1.1 ± 0.4	0.037
LDL cholesterol (mmol/l)	1.9 ± 0.8	2.1 ± 0.7	0.080
Triglycerides (mmol/l)	1.1 ± 0.5	1.3 ± 0.7	0.009
Prothrombin time (INR)	1.4 ± 0.3	1.2 ± 0.2	< 0.001

Data are given as number, number (%), or mean ± SD.

Abbreviations: CLF, chronic liver failure; HCC, hepatocellular carcinoma; BMI, body mass index; MELD, Model for End-Stage Liver Disease; AFP, alpha-fetoprotein; ALT, alanine-aminotransferase; HDL cholesterol, high density lipoprotein cholesterol; LDL cholesterol, low density lipoprotein cholesterol; INR, International normalized ratio.

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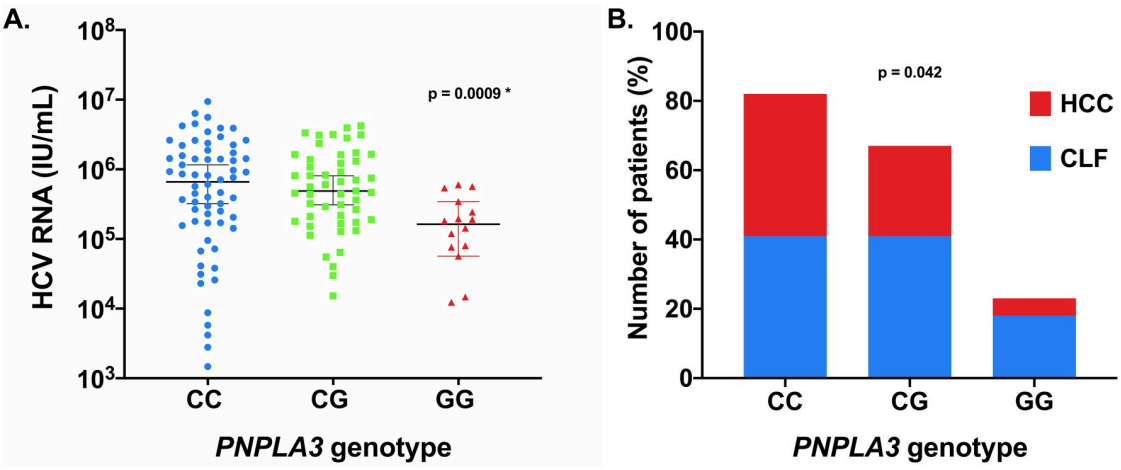

Fig 1. Impact of *PNPLA3* rs738409 genotypes on pre-transplant HCV RNA levels (panel 1*A*) and percentage of patients with CLF (panel 1*B*). Pre-transplant HCV viral load assessed in 133 of 172 patients. Data are given as medians and interquartile ranges. * p value for recessive model.

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CC genotype, 51 of 67 patients with the CG genotype and 16 of 23 patients with the GG genotype.

Similarly, pretransplant viral load was available in 68 of 100 patients with CLF and in 65 of 72 patients with HCC. *PNPLA3* GG homozygotes had a significantly lower pretransplant HCV viral load in comparison with the C allele carriers (median, interquartile range [IQR]; GG 162,500 (61,550–319,000) IU/ml vs. CC+CG 570,000 (172,000–1,595,000) IU/ml, $P < 0.001$, Fig 1*A*). Pre-transplant viral load was significantly lower in patients with CLF in comparison with patients with HCC (median [IQR]; CLF 292,500 (83,725–829,801) IU/ml vs. HCC 806,000 (237,000–1,680,000), $P = 0.008$).

PNPLA3 rs738409 genotype association with CLF

PNPLA3 genotype frequency differences between the CLF and HCC groups were found in both allelic and recessive models (Table 2*A*) ($p < 0.05$). Genotype frequencies between the CLF group and Czech cross-sectional population study MONICA significantly differed with $P = 0.004$ for the allelic model (OR 1.87, 95% CI 1.222–2.875, test power with $\alpha = 0.05$: 0.85) and $P < 0.001$ for the recessive model (OR 3.33, 95% CI 1.824–6.084 (Table 2*B*). The OR values almost doubled in the recessive model compared with the allelic model indicating the additive effect of allele G (Fig 1*B*). By contrast, genotype frequencies in the HCC group were the same as in the MONICA study in both models (Table 2*C*). Importantly, the minor allele frequency in the MONICA study (0.25) did not differ from the frequencies recorded in European population subsets of the GnomAD (0.23) and ExAC (0.23) databases [32].

The proportion of CLF in HCV cirrhotic patients grouped according to their *PNPLA3* rs738409 genotypes is shown in Fig 1*B*.

Risk factors for the need of liver transplantation

In multivariate logistic regression analysis, age and *PNPLA3* rs738409 genotype turned out to be significant determinants of the need of LT. Specifically, presence of the *PNPLA3* G allele increased the risk of LT in CLF 2.4-fold (Fig 2). Other investigated variables such as gender, BMI and type 2 diabetes mellitus did not influence the risk of LT.

Table 2. Genotype frequencies of *PNPLA3* rs738409 C>G polymorphism in the CLF group, HCC group and the MONICA study.

A	Locus	Genotype	CLF group (n = 100)	HCC group (n = 72)	OR	95% CI	*P* value
	PNPLA3 rs738409 c.444C>G	CC	41 (41%)	41 (57%)	1	-	-
		CG	41 (41%)	26 (36%)	1.90	1.017–3.472	0.045[a]
		GG	18 (18%)	5 (7%)	2.94	1.032–7.513	0.042[b]
B	Locus	Genotype	CLF group (n = 100)	MONICA (n = 647)	OR	95% CI	*P* value
	PNPLA3 rs738409 c.444C>G	CC	41 (41%)	366 (57%)	1	-	-
		CG	41 (41%)	241 (37%)	1.87	1.222–2.875	0.004[a]
		GG	18 (18%)	40 (6%)	3.33	1.824–6.084	< 0.001[b]
C	Locus	Genotype	HCC group (n = 72)	MONICA (n = 647)	OR	95% CI	*P* value
	PNPLA3 rs738409 c.444C>G	CC	41 (57%)	366 (57%)	1	-	-
		CG	26 (36%)	241 (37%)	0.98	0.602–1.610	0.951[a]
		GG	5 (7%)	40 (6%)	1.13	0.432–2.968	0.800[b]

[a] Allelic model (*PNPLA3* CG + GG vs. CC),

[b] Recessive model (*PNPLA3* GG vs. CC + CG)

Abbreviations: CLF, chronic liver failure; HCC, hepatocellular carcinoma; MONICA, MONItoring trends and determinants in Cardiovascular disease; OR, odds ratio; CI, confidence interval

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Discussion

The study was prompted by our long-term observation that the liver transplant candidates with HCV genotype 1b decompensated liver cirrhosis (or CLF) had significantly more advanced liver dysfunction and were younger that liver transplant candidates with a small HCC. A similar difference in the degree of liver dysfunction between liver transplant candidates indicated for HCV with or without HCC was reported by others [33–35]. However, the age difference between liver transplant candidates was not significant probably due to the fact

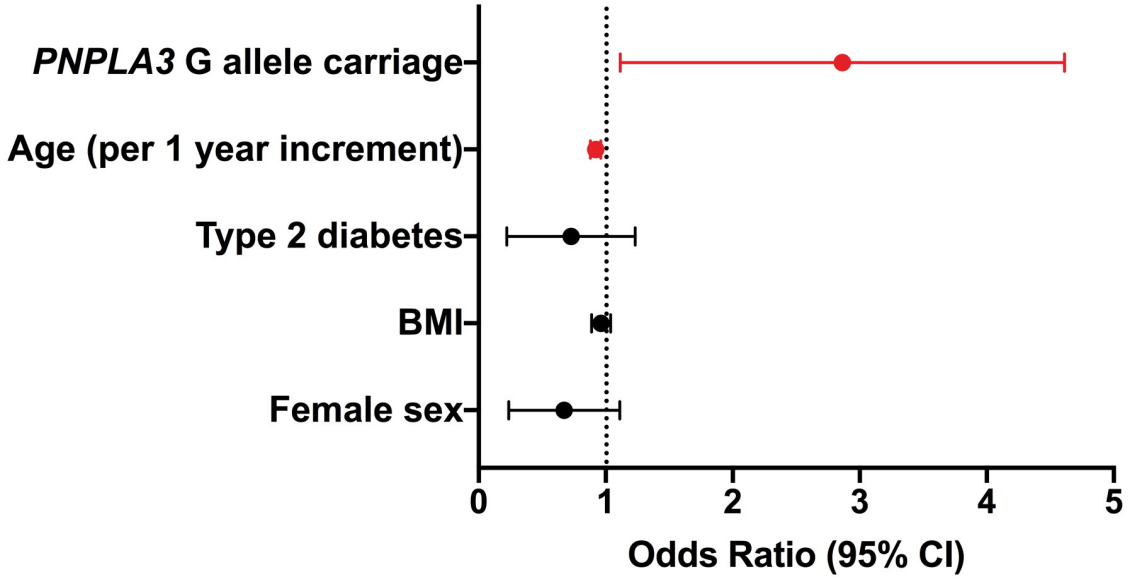

Fig 2. Risk factors for the need of liver transplantation: Multivariate analysis. Bars represent OR with 95% confidence interval.

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that the patients enrolled in these studies were infected with all HCV genotypes and HCV genotype may modify the risk of HCC [36].

To explain the age difference in our cohort, we assumed that the clinical difference between HCV liver transplant candidates with or without HCC might be caused by some genetic factor. A single nucleotide polymorphism *PNPLA3* rs738409 c.444C>G was identified as a risk factor for concurrent liver steatosis and a faster liver fibrosis progression in patients with chronic HCV infection in the past, but its impact on the need and timing of LT has not been evaluated. In our study, we identified further consequences of the carriage of the G allele: accelerated CLF development requiring LT at a younger age and lower pretransplant blood viral load. The CLF patients were younger than HCC patients and had a significantly higher frequency of *PNPLA3* allele G in comparison with HCC patients as well as with population controls.

The earlier need for LT suggests that the G allele carriage is a strong factor contributing to liver fibrosis progression. Consistently with our results, the recently published studies also presented the G allele carriage as a factor accelerating liver fibrosis progression in patients infected with chronic HCV infection. The meta-analysis by Fan and colleagues [12] showed that Caucasians with chronic HCV infection carrying the GG genotype have a more pronounced liver fibrosis and steatosis. In line with these findings, association of the GG and CG genotypes with progression of liver fibrosis was also demonstrated in a large cohort of HCV-infected patients in the HALT-C study [13].

In contrast to studies documenting association of the *PNPLA3* rs738409 genotype with the risk of HCC development in alcoholic liver disease and non-alcoholic fatty liver disease [21–23], no such association was found in HCV-infected subjects [13, 25]. This led us to initial misinterpretation of our data that the *PNPLA3* G allele was protective from HCC. However, since the G allele carriers underwent liver transplant for CLF at younger age, we realized that they were not able to develop HCC later in the course of the disease. Indeed, age is a well-known risk factor of HCC in patients with chronic HCV infection [37, 38].

As mentioned above, *PNPLA3* G allele carriers with chronic HCV infection have also more pronounced liver steatosis. We assume that in these subjects, lipid accumulation in hepatocytes with subsequent steatohepatitis accelerates progression of liver fibrosis caused by the underlying liver disease which is chronic HCV infection. Indeed, coincidence of chronic HCV infection with lipid accumulation and steatohepatitis results in more rapid development of CLF in comparison with HCV-infected individuals without steatohepatitis [12–16]. The hypothesis of two independent synergic processes leading to CLF (HCV infection and steatohepatitis) is further supported by Jimenez-Sousa et al. [15] who demonstrated a dose dependent effect of *PNPLA3* G allele on the progression of liver stiffness in HCV infected individuals. Finally, a dose dependent effect of *PNPLA3* G allele on the serum ALT activity has recently been described in a large study which included patients with chronic liver disease of various aetiologies [39]. When looking at our data, we realized that there is also a notable dose dependent effect of G allele in our cohort: the proportion of patients transplanted for CLF in the subgroups according to *PNPLA3* genotype increased with the number of G alleles (Fig 1B).

A relatively low number of subjects in the HCC group may be considered as the major disadvantage of our study. On the other hand, the comparison with a large number of population controls confirmed the same G allele frequency in the HCC group and population controls.

We also found that G allele carriers had a lower blood HCV viral load. This has been already known but it seems that the impact of the G allele on viral load is different in different HCV genotypes. Rembek et al. [40] reported a significantly lower viral load in GG homozygotes than in CG and CC genotype carriers infected with HCV genotype 2; however, the *PNPLA3* genotype had no impact on the viral load in subjects infected with HCV genotype 3. Contrarily, Eslam et al. [41] found no impact of the *PNPLA3* genotype on the viral load in a large

study group, but the authors included subjects with various HCV genotypes (1–4) and they did not evaluate subjects with different genotypes separately. Our study group was homogenous regarding HCV genotypes: all patients were infected with genotype 1b and this fact allowed us to observe the impact of *PNPLA3* gene variants on the blood viral load. The HCV replication, virus assembly and release is linked to the host cell lipid metabolism. Endoplasmic reticulum–derived membranous web represents the viral RNA replication complex site and lipid droplets serve as virion assembly sites [42, 43]. It has recently been reported that HCV induces complex remodeling of the host cell lipid metabolism in order to enhance both virus replication and virions assembly [44]. The mechanism by which the PNPLA3 variant protein alters lipid turnover in hepatocytes has also been elucidated: the variant protein accumulates on the surface of lipid droplets [45] and binds the cofactor CGI-58 of adipose triglyceride lipase (ATGL or PNPLA2) [46]. Both inactivated ATGL and the barrier of PNPLA3 variant protein on the surface of lipid droplets impede lipolysis of triglycerides and their trafficking in hepatocytes. We assume that changes in lipid metabolism in hepatocytes caused by the PNPLA3 variant protein may affect the HCV life cycle. We consider the lower blood viral load in G allele carriers as a manifestation of the altered lipid trafficking in hepatocytes, but its impact on liver fibrosis progression remains unclear since long-term lowering of viral load by administration of low doses of interferon alpha had no beneficial effect on liver fibrosis progression in the HALT-C study [47].

Conclusions

In conclusion, our results show that the pronounced liver steatosis and fibrosis in *PNPLA3* rs738409 G allele carriers with HCV genotype 1b cirrhosis may have a real impact on the timing and need of liver transplantation. The clinical consequence of G allele carriage could be a faster CLF development and need for liver transplantation at a younger age.

Supporting information

S1 Table. Patients' clinical and laboratory data.
(XLSX)

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ORIGINAL RESEARCH

USP18 downregulation in peripheral blood mononuclear cells predicts nonresponse to interferon-based triple therapy in patients with chronic hepatitis C, genotype 1: a pilot study

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Background and aims: Patients with advanced liver fibrosis owing to chronic hepatitis C virus genotype 1 represent a difficult-to-treat group even if a protease inhibitor is added to pegylated interferon alpha and ribavirin. Therefore, only patients with a high chance of cure should be treated with interferon-based treatment.

Patients and methods: Expression of *IFNG*, *IFNLR1*, and interferon-sensitive genes *CXCL9*, *IFI16*, *IFI27*, *ISG15*, and *USP18* in peripheral blood mononuclear cells was assessed before and during the initial 12 weeks of treatment. The studied group consisted of 26 treatment-experienced patients of average age of 50 years with advanced liver fibrosis compared to seven healthy volunteers. Fourteen patients were treated with pegylated interferon alpha 2b, ribavirin, and boceprevir and 12 patients with telaprevir. The overall sustained virological response (SVR) rate was 69% (18/26).

Results: A significant difference in the initial expression (median, interquartile range [IQR]) of *CXCL9* 2.9×, IQR: 1.7–12.4 vs 1.2×, IQR: 0.5–1.8; (P=0.01) *IFNG* 7.3×, IQR: 1.7–32.6 vs 0.7×, IQR: 0.4–1.3; P=0.002 and *USP18* 3.7×, IQR: 2.1–7.7 vs 1.4×, IQR: 0.9–1.6; (P=0.03) was found between the SVR and non-SVR groups. Expression of all analyzed genes was progressively increasing during the first 12 weeks of therapy, but a significant difference between SVR and non-SVR group was found only in *USP18* expression at week 12 (P=0.001). Initial expression of four genes predicted SVR in univariate analysis (*CXCL9* [OR: 12.00, 95% CI: 1.21–118.89], *IFI27* [OR: 12.00, 95% CI: 1.21–118.89], *IFNG* [OR: 10.50, 95% CI: 1.50–73.67], *USP18* [OR: 21.00, 95% CI: 2.05–215.18]). In multivariate analysis, only the initial expression of *USP18* was identified as a predictor of SVR (P=0.047).

Conclusion: Initial expression of *USP18* and the course of its activation could be a reliable predictor of SVR achievement.

Keywords: chronic hepatitis C, interferon-sensitive gene, *USP18*, protease inhibitor, virological response

Introduction

Hepatitis C virus (HCV) infection represents a leading cause of liver disease.[1] Interferon alpha-based therapies, later co-administered with ribavirin (RBV), have been standard-of-care since the beginning of the 1990s, with their nonspecific immunomodulatory effect inducing the clearance of the virus. In the last 4 years, new direct-acting antivirals (DAAs), inhibiting directly the viral enzymes crucial for its replication, have entered into clinical practice, together with pegylated interferon alpha and RBV (P/R) or in

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different combinations as interferon-free regimens. Nevertheless, interferon-based therapies have remained standard-of-care in many countries and represent a huge proportion of therapies administered worldwide.

The interferon-induced HCV clearance is a complex, immune-mediated process.[2] A detailed clarification of the mechanism of viral elimination is still lacking, nevertheless, previous studies described the induction of many interferon-sensitive genes (ISG) in hepatocytes and other cells following interferon alpha administration.[3] Chen et al[4] first described the differences in ISG activation in the liver in subjects prone to HCV elimination in comparison with nonresponders to therapy. HCV infection modifies the baseline expression profiles of ISG compared with healthy subjects both in hepatocytes and peripheral blood mononuclear cells (PBMC),[4–7] and the course of the ISG expression profiles is most likely unfavorable with persistence of the chronic HCV infection. Clinical data are in accordance with this hypothesis; the capacity to eliminate the virus with interferon alpha and RBV is reduced in the course of the disease. It decreases with the age of the patient and with fibrosis progression, and both these factors are influenced by the duration of the infection.[8] In the livers of nonresponders, long-term exposed to the virus, ISGs are upregulated and are not prone to further stimulation by interferon alpha.[9] Asselah et al[5] proved a significantly higher baseline liver ISG activity in nonresponders in comparison with responders to therapy. Based on the activity of selected ISGs in the liver, they were able to predict sustained virological response (SVR) with 94% probability. Chen et al[10] investigated the ISG baseline expression profile in hepatocytes and liver macrophages in the same individuals, and found a difference. The group of nonresponders was characterized by higher ISG expression in hepatocytes and lower in macrophages when compared with responders to P/R therapy. Furthermore, they confirmed the principal importance of *ISG15* and *USP18* in the specific activation of interferon alpha anti-HCV pathway. Hou et al[6] described a reliable prediction of elimination of the virus based on the expression profiles of 18 ISGs investigated in peripheral blood. On the contrary, Taylor et al[11] investigated relative changes in PBMC ISGs after stimulation with pegylated interferon alpha and did not find any significant changes between responders and nonresponders. Taylor's results describe upregulation of ISGs in PBMC, contrary to MacParland et al, who, when comparing pretreatment ISG expression levels to healthy volunteers, found upregulated as well as downregulated genes in PBMC prior to interferon therapy.[12]

The DAAs inhibit replication of HCV directly by targeting the virus replication cycle, their mechanism of action is not immune-mediated. The first DAAs, first-generation protease inhibitors (boceprevir [BOC] and telaprevir [TVR]), must be administered in combination with P/R. This triple therapy is more effective compared with P/R combination, but its efficacy in the patients with advanced fibrosis and cirrhosis is still unsatisfactory.

The aim of our study was to clarify whether irresponsiveness to interferon-based triple therapy in patients with advanced liver disease depends on the unfavorable pretreatment ISG expression profile and whether we can predict SVR achievement based on the pretreatment expression levels of ISG, *IFNG*, and *IFNLR1* or on the expression variation of these genes during the first 12 weeks of therapy.

With respect to the above-described data, PBMC were used to investigate gene expression because their acquisition was considered easier and safer than the acquisition of liver tissue, especially when analyzing the expression profile in different time points during treatment.

Patients and methods

Study design and eligibility of patients

A total of 26 patients with advanced liver fibrosis (Metavir score ≥ F3) were treated for chronic hepatitis C in two outpatient specialty clinics in Prague, Czech Republic, from December 2011 to April 2014. The cohort consisted of 17 males and 9 females of average age of 50 years (range 30–62). All patients were Caucasians infected with genotype 1 (24 patients with subtype 1b, 2 patients with subtype 1a) and treatment-experienced (all had been treated previously at least once with P/R, 16 were nonresponders and 10 relapsers), distribution of *IL28B* genotypes was as follows: CC 2/26, CT 16/26, and TT 8/26. Pretreatment liver biopsy was performed in all patients, out of whom eleven had fibrosis F3 and 15 had fibrosis F4 according to the Metavir score. All patients had compensated liver disease with no signs of proteosynthetic dysfunction (normal albumin, bilirubin, and prothrombin time values), ascites or encephalopathy. Patients with history of liver disease decompensation, hepatitis B infection or HIV co-infection, and patients receiving any immunosuppressive or immunomodulation therapy at the time of treatment initiation were not included in the study.

Fourteen patients were treated with once weekly subcutaneously administered pegylated interferon alpha 2b together with weight-adjusted RBV 1,000–1,200 mg daily, BOC (total daily dose 2,400 mg) was added at week 4 after a lead-in phase. Twelve patients were treated with once weekly subcutaneously administered pegylated interferon alpha 2a together with weight-adjusted RBV 1,000–1,200 mg daily

and TVR (total daily dose 2,250 mg). The anticipated treatment duration in both groups was 48 weeks in all patients. Virological futility rules were applied at treatment weeks 4, 8, and 12 according to prescribing information of both DAAs. No patient who discontinued therapy owing to an adverse event was included in the final evaluation. HCV RNA was assessed at weeks 4, 8, 12, 24, 36, and 48. SVR was assessed as HCV RNA negativity 24 weeks posttreatment. Blood draws for PBMC isolation were performed at day 0 and treatment weeks 4, 8, and 12 in both groups.

The control group consisted of seven healthy control subjects (two men, five women) of mean age of 42 years. The control subjects did not have any history of liver disease. All had normal activity of alanine aminotransferase, negative serological markers of hepatitis B infection and negative anti-HCV antibodies and HCV RNA.

All study participants provided written consent approved by the Institutional Review Board of the Institute for Clinical and Experimental Medicine and Thomayer's Hospital (Prague, Czech Republic), with prospective personal data collection, PBMC isolation, and DNA and RNA sampling prior to the initiation of therapy, and the study was approved by the Institutional Review Board and conformed to the Declaration of Helsinki Ethical Guidelines.

HCV RNA assessment

HCV RNA was assessed by the Roche AmpliPrep/COBAS® TaqMan® HCV Test v2.0 (Roche Molecular Systems, Branchburg, NJ, USA). Serum HCV RNA levels were determined at baseline, at weeks 4, 12, 24, 36, 48 of treatment and 12 and 24 weeks after the end of therapy.

IL28B genotyping

Patients were genotyped for *IL28B* rs12979860 C/T polymorphism by polymerase chain reaction – restriction fragment length polymorphism assay, as described by Fabris et al.[13]

PBMC and mRNA isolation

Blood was collected in ethylenediaminetetraacetic acid 9 mL tubes, diluted with an equal volume (9 mL) of 1% phosphate-buffer and then layered over a 9 mL Lymphoprep gradient (Axis-Shield, Oslo, Norway). The tubes were centrifuged at 1,400 rpm for 30 minutes at 10°C. The buffy-coat layer was transferred to 15 mL RNAse-free tubes, diluted with phosphate-buffered saline and centrifuged at 1,000 rpm at 10°C. The supernatant was discarded and PBMC were retained and ready for RNA isolation. RNA was extracted from PBMC with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA purity and concentration were assessed by spectrophotometry. mRNA was reverse-transcribed into sscDNA using the RevertAid Premium First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Gene expression analysis

The expression of a set of seven genes was analyzed in PBMC. The set was selected according to the above-cited literature and consisted of *CXCL9*, *IFI16*, *IFI27*, *IFNG*, *IFNLR1*, *ISG15,* and *USP18*. The genes were characterized by the association with immune response to HCV infection. Real-time PCR was performed using TaqMan® Assays in 384-Well Microfluidic Cards (Applied Biosystems, Foster City, CA, USA). Custom cards were designed with the Custom TaqMan array configuration tool. Real-time PCR was performed in a 7900HT Real-Time PCR System (Thermo Fisher Scientific) according to the manufacturer's suggested cycling conditions 50°C 2 minutes and 95°C 20 seconds for the initial cycle and 95°C 1 second and 60°C 20 seconds for the following 40 cycles. All samples were measured in quadruplicates; cDNA was prepared from 400 ng of total RNA and TaqMan Fast Advanced Master Mix (4444963, Thermo Fisher Scientific) were used for each assay. The data were analyzed using the SDS 2.2 software. Each 384-well plate included a set of control reactions quantifying expression of three housekeeping genes *HPRT1*, *ACTB,* and *GAPDH*. Gene expression values (Ct) were normalized to the expression of housekeeping genes present in the card ($2^{-\Delta Ct}$). Relative quantification of gene expression was carried out by the $2^{-\Delta\Delta Ct}$ method. Final results were expressed as folds of expression medians compared with median expression levels in a group of seven healthy controls; *GAPDH* was used as the housekeeping gene in the statistical analysis of the cohort.

Statistical analysis

The data are presented as means and standard deviations, medians and ranges or as frequencies, as appropriate. Mann–Whitney test and chi-square test were used for comparisons of the medians and frequencies, respectively. Paired Mann–Whitney test was used to compare the gene expression changes at different time points. Logistic regression was used to determine significant predictors of SVR. Cutoff points for continuous variables were obtained from Receiver Operating Characteristic analysis. P-value <0.05 was considered statistically significant throughout the study. Statistical analysis was performed using the R programming language version 3.2.0 (www.r-project.org).

Results

Treatment outcomes

The overall SVR rate was 69% (18/26), 64% (9/14) in BOC and 75% (9/12) in TVR group. Of the 26 treated patients, eight did not achieve SVR (four null-responders, two breakthroughs, two relapsers). SVR rates according to *IL28B* genotypes were as follows: CC 1/2 (50%), CT 13/16 (81%), TT 4/8 (50%). In the further analysis, all the patients who did not achieve an SVR were assessed as non-SVR group. The routinely used pretreatment predictors of SVR, ie, age, sex, response to a previous course of therapy, *IL28B* genotype, HCV RNA viral load, stage of liver fibrosis according to Metavir score (F3 vs F4), and the treatment administered (BOC vs TVR) did not differ significantly in patients who had achieved SVR in comparison with non-SVR group – the data are shown in Table 1.

Initial gene expression

The initial expression of *IFNLR1* was decreased in comparison with the group of healthy volunteers whereas expression of the remaining six genes was slightly upregulated compared with healthy controls (Figure 1). A significant difference in the initial expression of *CXCL9*, *IFNG*, and *USP18* was found between the groups of SVR and non-SVR patients (Table 2). The initial expression of *IFI27* 2.2×, interquartile range (IQR): 1.7–3.1 vs 8.3×, IQR: 3.5–43.5; (P=0.02), and *USP18* 1.4×, IQR: 0.9–1.8 vs 6.5×, IQR: 2.4–9.7; (P=0.005), was significantly lower in *IL28B* genotype TT-carriers (8/26) than in CT-carriers (16/26). The initial ISG expression was independent of HCV viral load and patients' age.

Course of ISG activation

Expression of all analyzed genes was progressively increasing during the first 12 weeks of interferon therapy. At weeks 4, 8, and 12, gene expression profiles did not differ between BOC and TVR groups. When comparing their activation between SVR and non-SVR group, a significant difference was found only in *USP18* expression at week 12 (P=0.001), as shown in Figure 2. Its expression rose continuously until week 12 in the SVR group, while in the non-SVR group a decrease at week 12 was observed (Figure S1). The course of gene expressions at different time points did not correlate with HCV RNA levels.

Prediction of SVR

None of the above-described standard clinical pretreatment SVR predictors had predictive value in our group of patients with advanced liver disease (Figure 3A). Out of seven selected genes, the initial expression of four genes was found to be a predictor of SVR achievement in univariate analysis (*USP18*, *IFNG*, *IFI27*, and *CXCL9*), as shows Table 3 and Figure 3B. In multivariate analysis, the initial expression of *USP18* was identified as a predictor of SVR (P=0.047).

Discussion

Our cohort represents a homogenous group of difficult-to-treat individuals of older age with long duration of the disease and with advanced liver fibrosis (stage F3 and F4 according to Metavir score). All had failed in the previous course of P/R therapy. This explains the fact that the expression of most studied genes did not differ between the patient groups (SVR and non-SVR) and did not increase significantly during the retreatment with triple therapy with P/R + DAAs. Nonetheless, we found in this cohort significantly higher initial expression levels of *IFNG*, *CXCL9*, and *USP18* in patients who had achieved SVR than in those in whom the treatment had failed. Another significant difference was observed in *USP18* expression levels at week 12 between the same patient groups. The pretreatment *IFNG* mRNA expression levels in PBMCs are already known to be lower in non-SVR patients treated with P/R without DAAs.[14] Since *CXCL9* is an interferon gamma-sensitive gene, one may expect the same. However, during treatment, expression of both *IFNG* and *CXCL9* did not differ between the SVR and non-SVR patients

Table 1 Patients' characteristics

	SVR (n=18)	Non-SVR (n=8)	*P*-value
Males/females (N)	11/7	6/2	NS
Age (years), median (range)	50 (30–65)	54.5 (45–62)	NS
HCV RNA (IU/mL), median (IQR)	2,210,000 (394,250–4,252,500)	1,415,000 (1,154,000–2,607,500)	NS
HCV genotype (1a/1b)	2/16	0/8	NS
IL28B (CC/non-CC)	1/17	1/7	NS
TVR/BOC (N)	9/9	3/5	NS
Response to previous therapy (REL/NR)	8/10	1/7	NS

Abbreviations: BOC, boceprevir; HCV, hepatitis C virus; REL, relapse; non-SVR, treatment failure (non-response, relapse, breakthrough); NR, non-response; SVR, sustained virological response; TVR, telaprevir; IQR, interquartile range; NS, not significant.

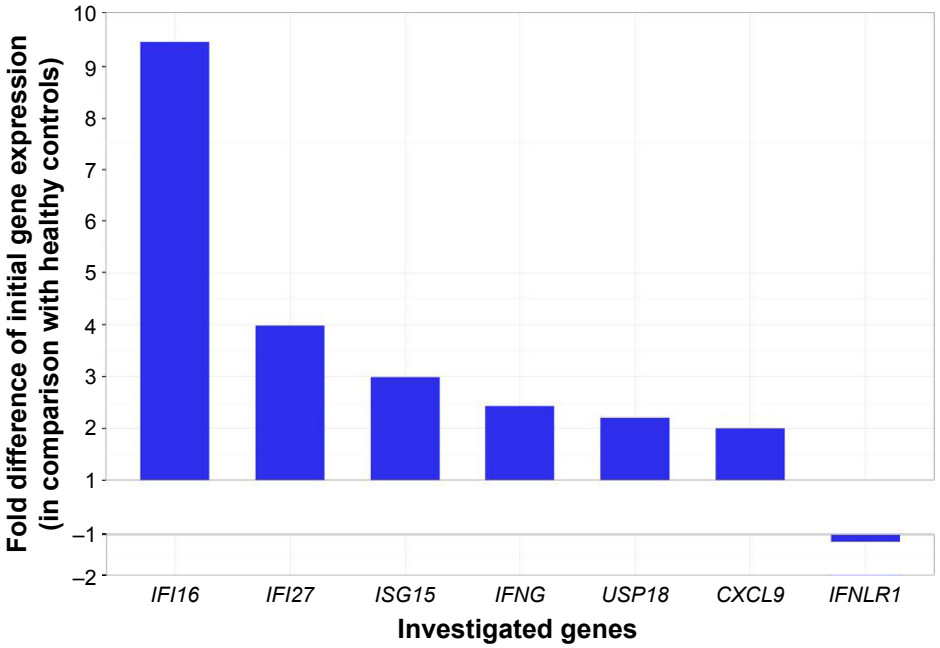

Figure 1 Initial expressions of selected genes (fold difference in comparison with healthy controls).

and it was not upregulated compared with the initial values found in the non-SVR group. We suppose that the observed inability to further upregulate *IFNG* and *CXCL9* and also *USP18* in the non-SVR group may result from the previously reported exhaustion of the immune system owing to longer duration of HCV infection.[15] Exhaustion of the immune system in long-persistent HCV infection has been explained by different mechanisms, one of the mechanisms being the impact of disrupted tetherin signalization on protein kinase R activity.[16] Another mechanism to be considered is the proteolytic degradation of the molecules involved in interferon regulating factor 3 (IRF3) signaling by NS3/4A protease, a nonstructural HCV protein crucial for its replication.[2] Our data show that in patients without response to interferon stimulation treated with P/R in combination even with the NS3/4A protease inhibitor, the interferon-irresponsiveness was not broken through. This indicates that even the pharmacological inhibition of the NS3/4A protease does not lead to full restoration of sensitivity to interferon.

Table 2 Initial expression of selected genes according to response to therapy, fold difference in comparison with healthy controls

Investigated genes	SVR, median (IQR)	Non-SVR, median (IQR)	Non-SVR/ SVR ratio	*P*-value
IFNG	7.3 (1.7–32.6)	0.7 (0.4–1.3)	10.80	**0.002**
CXCL9	2.9 (1.7–12.4)	1.2 (0.5–1.8)	2.20	**0.01**
IFNLR1	1.0 (0.5–1.4)	0.8 (0.6–1.4)	1.10	0.80
IFI16	9.4 (2.8–62.3)	20.2 (6.4–83.9)	0.49	0.70
ISG15	3.0 (0.8–46.4)	2.6 (1.4–4.9)	1.00	1.00
IFI27	6.2 (3.2–18.4)	2.6 (1.7–3.3)	2.00	0.06
USP18	3.7 (2.1–7.7)	1.4 (0.9–1.6)	0.25	**0.03**

Note: Significant *P*-values are shown in bold.
Abbreviations: IQR, interquartile range; non-SVR, treatment failure (non-response, relapse, breakthrough); SVR, sustained virological response.

The *IFNL4* ss469415590 genotype, strongly linked with the *IL28B* rs12979860 variant, is the most reliable genetic predictor of SVR in interferon-based therapies.[17] The *IFNL4* variant ss469415590 ΔG converts the inactive *IFNL4* pseudogene into an active gene producing interferon-lambda 4, which is likely to suppress signaling by other interferons essential for HCV clearance. ISG expression in the liver is strongly associated with *IL28B/IFNL4* genotype.[18,19] The data concerning the association between ISG expression in PBMC and *IL28B/IFNL4* genotype are controversial. In our cohort, we found a significant difference in the initial expression of two genes (*IFI27* and *USP18*) between *IL28B* CT and TT-carriers. Unfortunately, we were not able to evaluate CC group because of a low number of patients (only two in our cohort). The expression of *IFNL4* gene was described only in infected hepatocytes,[20] it has not been studied in PBMC so far. However, the results of other authors support our findings; Bordi et al found an association between favorable *IL28B/IFNL4* genotype and *IFNAR-1* transcription.[21] By this mechanism, *IL28B/IFNL4* genotype would be responsible for different levels of ISG expression in the PBMC. In contrast, Rallon et al did not find an association between ISG expression profile, assessed in PBMC, and *IL28B* genotype in a cohort of HCV–HIV co-infected patients.[22] The absence of an association described by this group could be explained

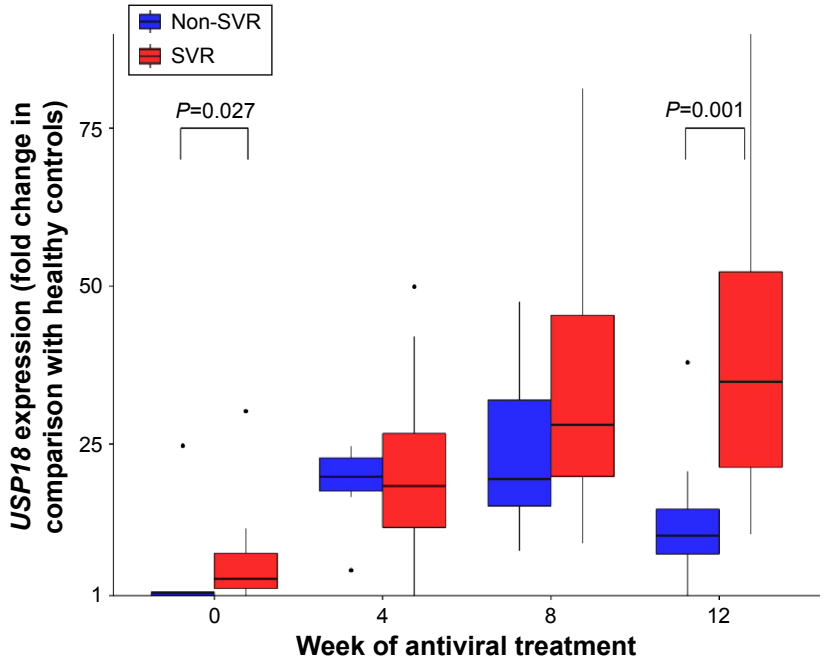

Figure 2 Course of *USP18* activation during the first 12 weeks of therapy (fold difference in comparison with healthy controls).
Abbreviations: SVR, sustained virological response; Non-SVR, treatment failure (non-response, relapse, breakthrough).

by impaired immunoreactivity in HIV/HCV co-infected patients.

We did not find any difference between patients treated with BOC or TVR in the course of ISG activation, although we had anticipated a difference in this point because BOC is added to P/R after a leading-phase of 4 weeks, whereas TVR therapy is initiated at day 1. These data suggest that the addition of a protease inhibitor to the therapy does not influence interferon-induced ISG activation. First-generation protease inhibitors (BOC and TVR) have recently been replaced by simeprevir. The advantage of simeprevir is its better tolerability, but the virological efficacy in patients with advanced liver fibrosis is similar to that of BOC and TVR.[23] Therefore, we assume that our data could be applicable to patients treated with simeprevir, but a validation study of our data is needed before the routine use of this predictor in clinical practice. Simeprevir-containing triple therapy still remains a standard-of-care in many countries, but the patients with genotype 1 and advanced liver fibrosis should rather be candidates for interferon-free regimens. Therefore, in the regions with limited access to the interferon-free treatments, there is a need for the selection of

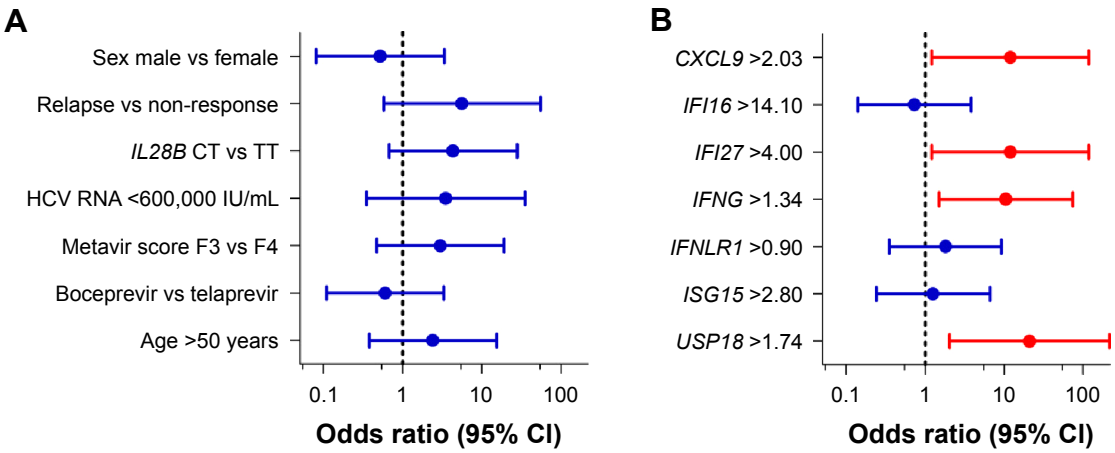

Figure 3 (**A**) Predictive value of demographic pretreatment variables for SVR achievement (univariate analysis). (**B**) Predictive value of initial gene expressions for SVR achievement, fold difference in comparison with healthy controls (univariate analysis).
Abbreviations: HCV, hepatitis C virus; SVR, sustained virological response.

Table 3 Predictive value of pretreatment variables and initial gene expressions (fold difference in comparison with healthy controls) for SVR achievement

	Odds ratio	95% CI
IL28B genotype CT vs TT	4.33	0.68–28.11
HCV RNA <600,000 IU/mL	3.50	0.35–35.37
Age <50 years	2.40	0.38–15.28
Sex (male/female)	0.52	0.08–3.36
DAA (BOC vs TVR)	0.60	0.11–3.30
Metavir score (F3 vs F4)	3.00	0.47–19.04
Previous treatment (REL vs NR)	5.60	0.57–55.43
CXCL9 >2.03	12.00	1.21–118.89
IFI16 >14.10	0.73	0.14–3.82
IFI27 >4.00	12.00	1.21–118.89
IFNG >1.34	10.50	1.50–73.67
IFNLR1 >0.90	1.81	0.35–9.24
ISG15 >2.80	1.25	0.24–6.63
USP18 >1.74	21.00	2.05–215.18

Abbreviations: BOC, boceprevir; DAA, direct-acting antiviral; HCV, hepatitis C virus; NR, non-response; REL, relapse; SVR, sustained virological response; TVR, telaprevir.

patients. Selection according to ISG expression, especially *USP18* in PBMC, may represent a suitable and reliable approach.

USP18, a member of the ubiquitin-specific protease family, has been shown to cleave protein conjugates with *ISG15* (ISGylated proteins) in mice.[24] However, the primary role of *USP18* seems to be different: the protein acts as a negative regulator of type I interferon signaling.[25] Expression of ISG including *USP18* and *ISG15* is increased in hepatocytes in treatment-naïve HCV patients nonresponding to P/R therapy in comparison with responders to the same treatment and expression of these genes increases less in nonresponders than in responders during the therapy. By contrast, higher pretreatment expression levels of *USP18* and *ISG15* have been found in Kupffer cells in responders in comparison with nonresponders.[10]

In our cohort, the initial *USP18* expression in PBMC turned out to be the only factor predicting SVR achievement in multivariate analysis. This finding supports previously published data on the relevance of *USP18* and *ISG15* in induction of antiviral immunity by interferon alpha.[26,27] Furthermore, *USP18* was one of the genes whose initial expression was associated with *IL28B* genotype in our cohort.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

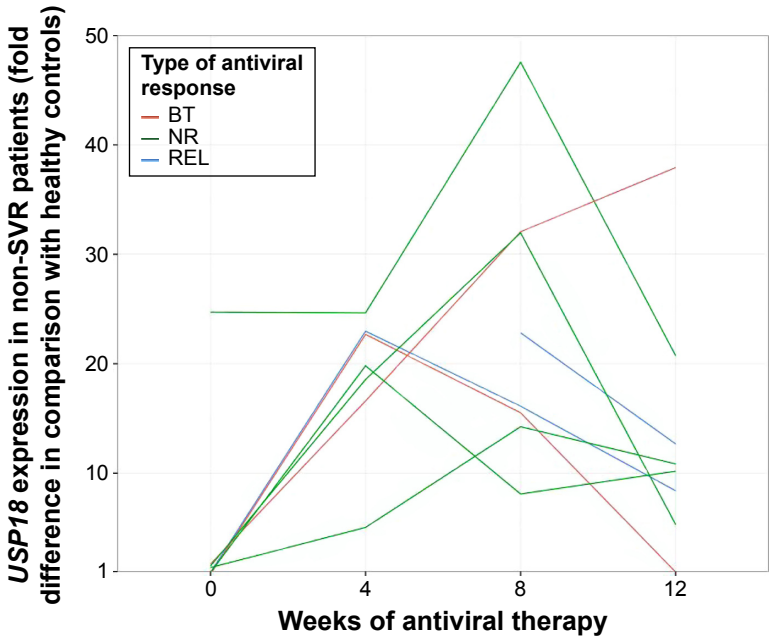

Figure S1 *USP18* expression in non-SVR patients, fold difference in comparison with healthy controls.
Abbreviations: BT, breakthrough; NR, non-response; REL, relapse; non-SVR, treatment failure (non-response, relapse, breakthrough).

PLOS ONE

RESEARCH ARTICLE

Liver stiffness measured by two-dimensional shear-wave elastography predicts hepatic vein pressure gradient at high values in liver transplant candidates with advanced liver cirrhosis

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Abstract

Liver stiffness is a reliable non-invasive predictor of Hepatic Venous Pressure Gradient (HVPG) above 10 mm Hg. However, it failed to predict higher thresholds of HVPG. Our aim was to investigate whether liver stiffness and selected previously published non-invasive blood biomarkers could predict higher HVPG thresholds in liver transplant candidates without ongoing alcohol use. One hundred and nine liver transplant candidates with liver cirrhosis of various aetiologies underwent direct HVPG measurement, liver stiffness measurement by 2D shear-wave elastography (Aixplorer Multiwave, Supersonic Imagine, France) and assessment of blood HVPG biomarkers (osteopontin, VCAM-1, IL-6, TNF-α, IL-1ra/IL-1F3 and ELF score). The correlation between liver stiffness and HVPG was linear up to 30 mm Hg of HVPG ($r = 0.765$, $p < 0.0001$). The regression lines had similar slopes for HVPG values below and above 16 mm Hg ($p > 0.05$) and the correlation in patients with HVPG <16 mm Hg ($r = 0.456$, $p = 0.01$) was similar to patients with HVPG $\geq$ 16 mm Hg ($r = 0.499$, $p < 0.0001$). The correlation was similar in the subgroup patients with alcoholic ($r = 0.718$, $p < 0.0001$), NASH ($r = 0.740$, $p = 0.008$), cryptogenic ($r = 0.648$, $p = 0,0377$), cholestatic and autoimmune ($r = 0.706$, $p < 0.0001$) and viral cirrhosis ($r = 0.756$, $p < 0.0001$). Liver stiffness distinguished patients with HVPG above 16, and 20 mm Hg with AUROCs 0.90243, and 0.86824, sensitivity 0.7656, and 0.7027, and specificity 0.9333, and 0.8750. All studied blood biomarkers correlated better with liver stiffness than with HVPG and their AUROCs did not exceed 0.8 at both HVPG thresholds. Therefore, a composite predictor superior to liver stiffness could not be established. We conclude that liver stiffness is a

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: 2D-SWE, two-dimensional real time shear-wave elastography; AUROCs, areas under the receiver operating characteristic curves; CPS, Child-Pugh score; CSPH, clinically significant portal hypertension; HA, hyaluronic acid; HCC, Hepatocellular carcinoma; HVPG, Hepatic venous pressure gradient; IL-1ra/IL-1F3, Interleukin-1 Receptor Antagonist; IL-6, Interleukin-6; LR, likelihood ratios; LS, Liver stiffness; LSPS, Liver Spleen Platelets Score; MELD, Model for End-Stage Liver Disease; NPV, negative predictive value; NSBB, non-selective β-blockers; PH, Portal hypertension; PIIINP, Amino-Terminal Propeptide of Type III Procollagen; PP, portal pressure; PPV, positive predictive value; TIMP-1, Tissue Inhibitor of Matrix Metalloproteinase 1; TNFα, Tumour Necrosis Factor alpha; VCAM-1, Vascular Cell Adhesion Molecule 1; WHVP, wedge hepatic vein pressure.

clinically reliable predictor of higher HVPG thresholds in non-drinking subjects with advanced liver cirrhosis.

Introduction

Portal hypertension (PH) is the main complication of liver cirrhosis contributing to the development of its life-threatening complications. Hepatic venous pressure gradient (HVPG) represents the reference standard for evaluation of the presence and severity of PH in patients with cirrhosis [1]. HVPG is presumably the most often validated tool for assessing prognosis in patients with liver cirrhosis. HVPG higher than 10 mm Hg is considered clinically significant portal hypertension (CSPH) [2]. Patients with CSPH are at risk of oesophageal varices [3–5], develop ascites and cirrhosis decompensation [6]. HVPG higher than 12 mm Hg is associated with the risk of variceal bleeding, HVPG more than 16 mm Hg with high mortality [7] and HVPG above 20 mm Hg predicts failure to control variceal bleeding [8]. HVPG measurement by hepatic vein catheterization is an invasive procedure and therefore, there is a need for an easy and accurate non-invasive method.

The use of liver stiffness (LS) as a non-invasive predictor of PH has been extensively studied in the last decade. LS measurement by transient elastography showed good predictive value in the diagnosis of both HVPG $\geq$ 10 mm Hg and gastroesophageal varices, and platelet count or spleen diameter were identified as parameters improving the accuracy of the prediction. Platelet count was included in the Baveno VI statement saying that upper gastrointestinal endoscopy could be avoided in patients with compensated cirrhosis and LS $<$ 20 kPa together with platelet count $>$150 x 10^9/L [9–11]. Later on, two-dimensional real time shear-wave elastography (2D-SWE) has been introduced into clinical practice [12, 13] allowing both LS and spleen stiffness measurement even in patients with ascites. Two sequential algorithms based on LS followed by spleen stiffness measurements using 2D-SWE with excellent diagnostic accuracy for CSPH have recently been proposed by Jansen [14, 15] and validated by Elkrief [16, 17]. In the meantime, Buck et al. [18] described a good correlation between blood inflammatory biomarkers and HVPG and proposed a composite diagnostic test based on four biomarkers, which was able to identify 86% of compensated cirrhotic patients with HVPG below 12 mm Hg. Some fibrogenesis biomarkers also showed correlation with HVPG but their predictive value has not been studied [19].

As mentioned above, the researchers focused mainly on the non-invasive prediction of HVPG $\geq$ 10 mm Hg. There are limited data on the non-invasive prediction of HVPG at levels higher than 10 mm Hg; Kim et al. showed a good ability of LS by 2D-SWE to predict HVPG $\geq$ 12 mm [20]. The non-invasive prediction of HVPG $\geq$ 16 mm Hg was described only in the study by Gouya et al. The authors evaluated portal, azygos vein and aortal blood flow by phase contrast MRI and demonstrated that azygos flow was a good predictor of HVPG $\geq$ 16 mm Hg [21]. Loss of correlation or weak correlation [22] between LS and HVPG above 10 mm Hg was the reason impeding prediction of higher levels of PH in most published studies. The weak correlation can be explained by the fact that increase of LS reflects predominantly increased intrahepatic resistance due to liver fibrosis and sinusoidal dysfunction whereas hyperdynamic circulation aggravates portal hypertension only at high pressure values [23]. The mentioned mechanism is supported by Reiberger et al. who demonstrated that treatment with non-selective β-blockers (NSBB) improved the correlation between LS and HVPG in patients with HVPG $>$ 12 mm Hg [24]. NSBB probably ameliorate both hyperdynamic

circulation and sinusoidal dysfunction [25]. Another factor contributing to weaker correlation between LS and HVPG may be liver steatosis since LS becomes overestimated in patients with steatosis whereas HVPG pressure value is not affected [26, 27].

Advanced NASH liver cirrhosis is generally associated with decline of steatosis regardless of high BMI [28, 29]. Contrarily, active alcohol drinkers with advanced liver cirrhosis may have severe steatosis [30]. Therefore, we assume that a different proportion of active alcohol drinkers with various degree of liver steatosis may negatively impact the correlation between LS and HVPG. Liver steatosis is also regularly associated with genotype 3 infection in patients with HCV cirrhosis. This should be considered when explaining the weaker correlation between LS and HVPG above 10 mm Hg in the study by Vizzutti et al. [31].

In this study we focused on non-invasive predictors of HVPG in a group of liver transplant candidates with advanced liver cirrhosis. These candidates represent a unique group of patients with advanced liver cirrhosis characterized by almost complete absence of active alcohol abusers. Since vast majority of such patients have CSPH, these patients need an accurate assessment of potential risk of complications, knowledge of which is the prerequisite for correct treatment during the waiting period. Moreover, despite the fact that NSBB are no more recommended in majority of liver transplant candidates, the rest may still benefit from NSBB administration [32]. Therefore, a non-invasive method for estimation of HVPG in liver transplant candidates capable to evaluate the response to NSBB would be beneficial. Our aim was to assess the predictive power for HVPG of LS and selected blood biomarkers of inflammation and fibrogenesis and their combination(s) in a carefully selected cohort of these steatosis-free patients.

Patients and study design

This prospective, monocentric study included liver transplant candidates with liver cirrhosis admitted between October 2016 and July 2018 to The Department of Hepatogastroenterology at the Institute for Clinical and Experimental Medicine, Prague, Czech Republic. All the patients were admitted to the hospital for evaluation before enrolment into waiting list and followed a protocol-defined work-up, including LS and HVPG measurement. A total number of 119 consecutive patients were eventually prospectively enrolled in the study, ten patients were subsequently excluded from further evaluation. Their demographic data are summarized in Tables 1 and 2. All patients were abstaining from alcohol for at least six months. Exclusion criteria were: complete or partial thrombosis of the portal vein or its right branch diagnosed by abdominal ultrasound or computed tomography, history of transjugular intrahepatic portosystemic shunt (TIPS), hepatorenal syndrome requiring vasoactive drug administration or renal replacement therapy, pulmonary hypertension diagnosed by echocardiography, severe bacterial infection or sepsis, variceal bleeding occurring within 4 weeks prior to hepatic vein catheterisation, and hepatocellular carcinoma outside of the Milan criteria [33]. The patients were screened to assess inclusion and exclusion criteria (clinical examination, routine blood tests, abdominal ultrasound, abdominal CT scan, gastroscopy and LS measurement). The day following LS measurement, they underwent HVPG measurement by liver vein catheterisation and blood sampling for plasma markers of portal hypertension. The study was approved by local Institutional Review Board (IRB of Institute for Clinical and Experimental Medicine and Thomayer's Hospital, Prague). All patients signed the informed consent with the study. The patients were selected for enrolment into the waiting list according to the published medical criteria after the evaluation process [34]. Organ donation was driven by the Czech law No. 285/2002 Coll., On donation, grafts and transplantation of tissues which is compatible with the European Union legislative. The Czech law applies the principle of presumed consent, i.e. the informed consent with organ or tissue donation is not required. People who, during their

Table 1. Baseline clinical and laboratory characteristics of the whole cohort and patient subgroups.

Variable [median, range]	All patients N = 109 (100%)	Child-Pugh A N = 30 (27.5%)	Child-Pugh B/C N = 79 (72.5%)	p
Age [years]	61 (21–84)	66 (38–84)	58 (21–73)	< 0.001
Gender [Male]	73 (67.0%)	23 (76.7%)	50 (63.3%)	N.S.
BMI [kg/m^2]	26.7 (18.4–46.8)	28.6 (20.4–46.8)	25.5 (18.4–38.5)	0.006
Child-Pugh score [points]	8 (5–13)	5 (5–6)	8 (7–13)	< 0.001
Aetiology of liver cirrhosis				< 0.001
Alcohol	38 (34.9%)	10 (33.3%)	28 (35.5%)	
NASH	11 (10.1%)	5 (16.7%)	6 (7.6%)	
Cryptogenic	10 (9.2%)	1 (3.3%)	9 (11.4%)	
Viral	23 (21.1%)	14 (46.7%)	9 (11.4%)	
(HBV/HCV)	(5/18)	(3/11)	(2/7)	
Cholestatic and autoimmune	25 (22.9%)	0 (0%)	25 (31.6%)	
Metabolic—Wilson disease	2 (1.8%)	0 (0%)	2 (2.5%)	
HCC within Milan criteria	38 (34.9%)	20 (66.7%)	18 (22.8%)	< 0.001
MELD score [points]	14 (6–37)	9 (6–21)	15 (7–37)	< 0.001
Overt hepatic encephalopathy	13 (11.9%)	0 (0%)	13 (16.5%)	0.02
Spleen diameter [cm]	15 (9–25)	14 (9–18)	16 (9–25)	0.004
Oesophageal varices (none/small/large)	33/32/44 (30.3/29.4/40.3%)	13/ 7/10 (43.7/23.3/33.3%)	20/25/34 (25.3/31.6/43.1%)	N.S.
History of variceal bleeding	19 (17.5%)	4 (13.3%)	15 (19.0%)	N.S.
Ascites (none/small/large)	55/25/29 (50.4/23.0/26.6%)	27/3/0 (90.0/10.0/0.0%)	28/22/29 (35.4/27.8/36.8%)	< 0.001
Platelets count [x10^9/L]	98 (40–344)	102 (48–286)	98 (40–344)	N.S.
Bilirubin [μmol/L]	34 (5–257)	20 (5–31)	47 (10–257)	< 0.001
Albumin [g/L]	30 (17–49)	39 (29–49)	28 (17–43)	< 0.001

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Table 2. Liver stiffness, HVPG and blood predictors of HVPG and fibrosis.

Variable [median, range]	All patients N = 109 (100%)	Child-Pugh A N = 30 (27.5%)	Child-Pugh B/C N = 79 (72.5%)	p
Liver stiffness median [kPa]	29.7 (9.2–61.6)	19.3 (10.7–39.8)	33.6 (9.2–61.6)	< 0.001
HVPG median [mm Hg]	17 (5–31)	13 (5–20)	18 (6–31)	< 0.001
LSPS [points]	4.4 (0.6–27.9)*	3.0 (0.8–8.8)+	5.4 (0.6–27.9)a	< 0.001
ELF score	12.5 (8.7–16.4)	11.7 (8.7–14.0)	12.7 (10.1–16.4)	< 0.001
Osteopontin [ng/mL]	142 (52–439)**	93 (54–169)	172 (52–439)aa	< 0.001
VCAM-1 [ng/mL]	2718 (579–10268)**	1635 (579–4509)	3348 (1134–10268)	< 0.001
TIMP-1 [ng/mL]	462 (180–1834)	313 (180–562)	515 (226–1834)	< 0.001
PIIINP [ng/mL]	21.8 (6.3–87.7)	16.7 (7.9–50.2)	27.5 (6.3–87.7)	< 0.001
TNF-α [pg/mL]	2.3 (0.1–6.5)***	2.1 (0.1–3.8)++	2.4 (0.3–6.5)aaa	0.02
IL1-Ra/IL-1-F3 [pg/mL]	265 (65–1689) **	331 (171–728)++	254 (65–1689)	N.S.
Hyaluronic acid [ng/mL]	560 (20–20639)	311 (20–1356)	662 (57–20639)	< 0.001

*n = 104,
**n = 107,
***n = 105,
+n = 26,
++n = 29,
an = 78,
aan = 77,
aaan = 76

https://doi.org/10.1371/journal.pone.0244934.t002

lifetime, express a clear opposition to the donation in writing and are registered in the National Registry of people opposed to the post-mortem withdrawal of tissues and organs are excluded from the organ donation. All the donors were referred to our transplant centre from regional hospitals across the Czech Republic via Coordination Centre of Transplantations (https://kst.cz/en/) which is a state organization controlled by the Ministry of Health of the Czech Republic. The organ removal can be performed only after consent of the Coordination Centre of Transplantations that administrates the waiting list and National Registry of people opposed to the post-mortem withdrawal of tissues and organs. The Centre manages organs allocation among the transplant centres.

Blood sampling

The study subjects were in a sitting position for at least 5 min (but not >10 min) before and during sampling. Venous blood was taken between 8 and 10 a. m. The Vacuette system (VACUETTE® TUBE 8 mL Z Serum Separator Clot Activator cat. No. 455071, and VACUETTE® 9 ml K3 EDTA Plasma Separator cat. No. 455036, both from Greiner Bio-One, Kremsmünster, Austria) was used together with 21-gauge needles (Greiner Bio-One). Separation of blood corpuscles was done within 60 min. after sampling at 3000 g for 10 min. (centrifuge Beckman Allegra, Beckman Coulter, Indianapolis, IN). Several serum and plasma aliquots of 500 μL were prepared within 60 min. after centrifugation. CryoKing tubes from Biologix Group Limited, Jinan, China, cat. No. 89–3101, were used to store serum and plasma aliquots at −80˚C until analysis.

Analytical methods

Serum concentrations of hyaluronic acid (HA), Amino-Terminal Propeptide of Type III Procollagen (PIIINP), and Tissue Inhibitor of Matrix Metalloproteinase 1 (TIMP-1) were measured by the ADVIA Centaur® HA assay, lot 25,215,019, the ADVIA Centaur® PIIINP assay, lot 26,290,023, and the ADVIA Centaur® TIMP-1 assay, lot 28,900,016, respectively (Siemens Healthineers, Erlangen, Germany). ADVIA Centaur ELF calibrator was used for calibration of HA, PIIINP, and TIMP-1 assays and ADVIA Centaur ELF quality control materials (three levels) were used as assay controls. Repeatability (within-run CV) assessed as declared by the manufacturer was < 5.6, < 4.2, and < 3.3% for HA, PIIINP and TIMP-1, respectively. Intermediate precisions (between-run CVs) were < 3.2, < 5.1, and < 5.5%. The respective measurement ranges for HA, PIIINP, and TIMP-1 were 1.6–1000, 0.5–150, and 3.5–1300 ng/mL. Traceability was not provided by the manufacturer. Limits of detection of HA, PIIINP, and TIMP-1 were 1.6, 0.5 and 3.5 ng/mL. All measurements were performed in one run during one day by the same laboratory technician using a Centaur CP immunochemistry analyzer (Siemens Healthineers). The Enhanced Liver Fibrosis (ELF) was calculated according to the Centaur CP formula: 0.846 x ln (HA) + 0.735 x ln (PIIINP) + 0.391 x ln (TIMP-1) + 2.494.

Interleukin-6 (IL-6), Vascular Cell Adhesion Molecule 1 (VCAM-1), Interleukin-1 Receptor Antagonist (IL-1ra/IL-1F3), Osteopontin and Tumour Necrosis Factor alpha (TNFα) were assessed in plasma samples obtained from the study subjects according to the manufacturer's instructions using the assays No. HS600B, DVC00, DRA00B, DOST00 and HSTA00E from R&D Systems, Minneapolis, MN. The absorbance was measured on a Synergy™ 2 Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT).

HVPG measurement

Catheterization of the hepatic vein was performed to measure HVPG. Using the transjugular route, an open-end zero-side holes 5F multipurpose angiographic catheter (Cordis, Santa

Clara, CA), was inserted through a 6F sheath (Super Arrow-Flex Percutaneous Sheath Introducer Set, Arrow International brand of Teleflex, Wayne, PA). Iodinated radiological contrast medium was injected into the right or middle hepatic vein to confirm the position of the catheter in a wedged position by fluoroscopy. The pressure was measured five times to demonstrate reproducibility, the mean value was then used for further calculations. HVPG was calculated as the difference between wedged and free hepatic venous pressures. The radiologist performing catheterization was blinded to elastography measurement results.

Liver stiffness measurement

After at least 10 hours of fasting, 2D-SWE was performed using the Aixplorer® ultrasound system (Supersonic Imagine S.A., Aix-en-Provence, France) with an abdominal 3.5 MHz curved array probe (SC6-1) as recommended by three experienced radiologists (more than 50 exams each). 2D-SWE measurements were performed within 7 days before or after HVPG measurement.

The operator was not aware of HVPG results when performing 2D-SWE. LS measurements were performed on the right lobe of the liver through the intercostal spaces with the patient in the supine position and the right arm maximally abducted. All RT-SWE acquisitions were performed using a 3.5 x 2.5 cm box, placed at more than 2 cm under the liver capsule, avoiding large vessels. During the examination the patient was requested to hold breath as needed. After obtaining a stable and homogenous elastographic image inside the box, a region of interest (ROI) was selected using the Q-box tool and placed in the most homogeneous area and the median values of LS within the ROI was displayed and registered. The diameter of the Q-box was set $>$ 15 mm. Three elastographic images from different liver areas were obtained in all patients and the mean value was used for further calculations.

Other known or potential non-invasive predictors of portal hypertension

The MELD score (Model for End-Stage Liver Disease) is a composite predictor of survival in patients with cirrhosis calculated from total serum bilirubin, serum creatinine and the international normalized ratio (INR). MELD score was originally invented to predict short-term survival in cirrhotic patients [35]. Later studies showed that MELD score correlates with MR elastography results, presence of varices and mortality in patients with variceal bleeding [36, 37] and is currently used as a tool for donor livers allocation [35, 38, 39].

LSPS (Liver Spleen Platelets Score) is also a composite predictor combining LS, platelets count and spleen diameter [40]. LSPS was calculated as described previously: [LS (in kiloPascals) x spleen diameter (in centimetres)]/platelet count ratio ($x10^9$/L). LSPS was superior to LS alone for identification of patients with CSPH in a study by Berzigotti [40].

IL-6, IL1-Ra/IL-1-F3, and VCAM-1 are inflammatory biomarkers correlating most significantly with HVPG in the study [18]. TNFα being also an inflammatory biomarker correlated with prehepatic portal hypertension in animal experiments [41, 42]. Osteopontin, acting as a key component of bone matrix and multifunctional cytokine, also correlated well with HVPG in humans [19].

The ELF score was shown to correlate with the stage of liver fibrosis in liver diseases of various aetiologies [43–45]. Therefore, we decided to evaluate also the score and its individual components as potential markers of portal hypertension.

Statistical analysis

The statistical analysis was performed using the SigmaPlot 11.0 (Systat Software Inc., San Jose, CA) or JMP 11.0.0 (2013, SAS Institute Inc., Cary, NC). Quantitative data are presented as

median and range and qualitative data are reported as percentage (%). Shapiro-Wilk test was used to evaluate the normal distribution of data. Spearman's test was used for correlations among continuous variables. Medians were compared using t-test or Mann-Whitney test, as appropriate. The diagnostic performance of each non-invasive parameter was assessed by receiver operating characteristic (ROC) curves analysis. Optimal cut-off values were calculated using a common optimization step that maximized the Youden index. The performance of tested non-invasive parameters to predict various levels of portal hypertension was estimated by calculating the proportion of correctly classified patients—diagnostic accuracy, together with the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratios (LR). The Fischer's exact test and McNemar's test were used in the 2×2 contingency tables for assessing differences in the proportion of misclassified patients with dichotomous cut-offs, as well as for comparing categorical variables. For all calculations, a p value < 0.05 was considered to indicate statistical significance. A pre-study statistical sample size assessment was conducted based on results obtained in patients assessed by the same method for LS and HVPG during one year before the study onset; to achieve a correlation $r = 0.699$ with a power of 0.9, for a level of significance $\alpha = 0.05$, at least 24 patients should have been enrolled.

Results

Patient characteristics

A total number of 119 consecutive patients with advanced liver cirrhosis of various aetiology admitted as liver transplant candidates were prospectively enrolled in the study. Ten patients were subsequently excluded from further evaluation: 7 patients for inability to perform representative LS measurement in 3 ROIs, 2 patients for pulmonary hypertension diagnosed by echocardiography and 1 patient who owned up to excessive alcohol consumption. Interestingly, none of the 7 patients in whom the LS measurement failed had BMI higher than 30. The final number of patients who underwent both LS and HVPG measurement and thus could be evaluated was 109. Eighty-three patients underwent liver transplantation at the time of the manuscript preparation. Liver cirrhosis was proved in all of them and none of them had steatosis in $>$ 5% of hepatocytes in the explanted liver. The median period between study recruitment and liver transplantation was 109 days (range 10–769 days). All the patients received grafts from the heart-beating donors after brain death, age of the donors ranged from 21 to 81 years. None of the organ donors was from a vulnerable population and none of them was registred in the National Registry of people opposed to the post-mortem withdrawal of tissues and organs. For further evaluation, the patients were divided into two groups according to the stage of liver disease based on the Child-Pugh classification: group CPS-A including patients with Child-Pugh class A (30/109, 27.5%) and group CPS-B/C including patients with Child-Pugh class B or C (79/109, 72.5%). The obtained HVPG values ranged from 5 to 31 mm Hg. Only 12/109 (11.0%) patients had HVPG lower than 10 mm Hg; the remaining 97/109 (91%) patients had CSPH. A detailed characterisation of the patient cohort is presented in Tables 1 and 2, the complete patients' characteristics are included in the S1 File.

HPVG and LS values were also compared among the groups classified according to the aetiology of liver cirrhosis. Comparisons were done using the All Pairwise Multiple Comparison Procedures (Holm-Sidak method) at overall significance level 0.05. HVPG was significantly higher in patients with alcoholic liver cirrhosis compared to patients with liver cirrhosis of viral aetiology (19.97±6,58 vs. 12.78±4.24 mm Hg, $p < 0.001$). Similarly, the same groups showed significant difference in LS value; LS was also higher in patients with alcoholic liver

cirrhosis compared to those with liver cirrhosis of viral aetiology (34.93±12.37 vs. 24.49±9.32 kPa, p = 0.005) (Fig 1).

Relations between the studied biomarkers

As a first step of the data analysis, correlations between HVPG, LS and non-invasive blood predictors of portal hypertension were calculated. The obtained Spearman's non-parametric correlation coefficients are presented in Table 3. The strongest correlation with HVPG was achieved for LS. Osteopontin level was the best blood marker for both HVPG and LS and its correlation coefficients with HVPG and LS were closely similar. The remaining blood predictors of portal hypertension correlated better with LS than with HVPG; however, their correlations with both HVPG and LS were weaker than those of osteopontin. Correlations of ELF score and LSPS with HVPG were also calculated for CPS A and CPS B/C patient groups. ELF score did not correlate with HVPG in any of the groups whereas LSPS correlation with HVPG was stronger in the CPS-A group (r = 0.528, p = 0.006 (n = 26), than in the CPS-B/C group (r = 0.308, p = 0.006, n = 78). Importantly, the residual plot and normal distribution of residuals justified the assumed linear relationship between HVPG and LS in the whole range of HVPG values (Fig 2). Moreover, the regression lines had similar β values for patients with HVPG values below and above 16 mm Hg ($p > 0.05$). Aetiology of the cirrhosis had no impact on the relation between LS and HVPG as can be seen from Fig 3; linear regression was calculated separately for each of the five aetiology groups, two subjects representing the metabolic aetiology group were not included in the statistical analysis. The use of linear regression was justified by residual plots and the β values did not differ from the β value calculated for the whole cohort (Table 4).

Non-invasive markers as predictors of HVPG cut-off points

In the next step, areas under the receiver operating characteristic curves (AUROCs) were calculated for all tested non-invasive HVPG predictors at three cut-off points. Whereas the AUROCs of LS to predict 16 and 20 mm Hg of HVPG were all higher than 0.8 (Fig 3), AUROCs of all other tested non-invasive markers to predict the same HVPG cut-offs did not exceeded 0.8 and therefore these markers were not considered suitable for clinical use (Table 5). AUROCs

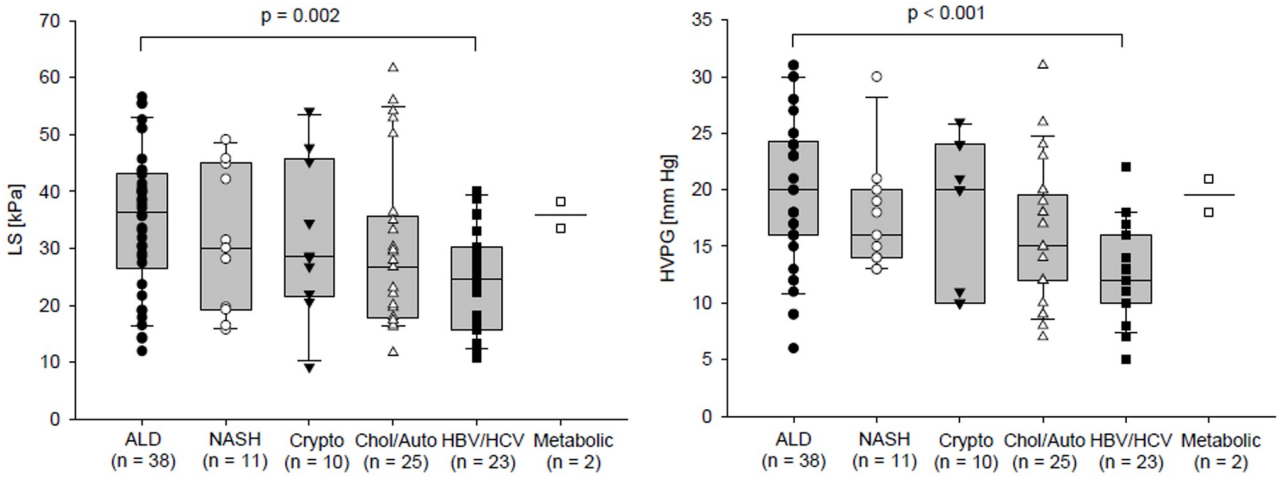

Fig 1. Comparison of LS and HVPG between groups of patients according to the aetiology of the liver cirrhosis. The data of two patients with metabolic liver disease were displayed but not included in the statistical analysis.

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Table 3. Spearman's non-parametric correlation coefficients between HVPG, LS and the evaluated non-invasive markers.

Variable	HVPG		Liver stiffness	
	Spearman's r	p	Spearman's r	p
Liver stiffness [kPa]	0.765	< 0.0001	NA	N.A.
LSPS [points]	0.4488	< 0.0001	0.5046	< 0.0001
MELD score [points]	0.2990	0.0016	0.4254	< 0.0001
Spleen diameter [cm]	0.1100	N.S.	0.0627	N.S.
Platelets [x10^9/L]	–0.0223	N.S.	0.0793	N.S.
Osteopontin [ng/mL]	0.5143	< 0.0001	0.5086	< 0.0001
VCAM-1 [ng/mL]	0.4194	< 0.0001	0.4635	< 0.0001
TIMP-1 [ng/mL]	0.4339	< 0.0001	0.5209	< 0.0001
ELF score [points]	0.3485	0.0002	0.3916	< 0.0001
HA [ng/mL]	0.2880	0.0024	0.348	0.0002
PIIINP [ng/mL]	0.2712	0.0044	0.2940	0.0019
IL-6 [pg/mL]	0.2512	0.015	0.342	0.0008
IL1-ra/IL-1F3 [pg/mL]	–0.1825	N.S.	–0.0457	N.S.
TNF-α [pg/mL]	0.0444	N.S.	0.178	N.S.

N.A. not applicable, N.S. not significant.

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were not calculated for threshold 10 mm Hg since there were only 12/109 patients with HVPG lower that 10 mm Hg.

Diagnostic performance of liver stiffness as the strongest predictor of HVPG cut-off points

The best cut-off values of LS to predict 16 and 20 mm Hg HVPG were selected using the Youden Index (Table 6). The accuracy of LS to predict HVPG above 16 and 20 mm Hg was 83.5 and 81.7%, respectively ($p < 0.05$, McNemar's test). The diagnostic accuracy of LS to predict

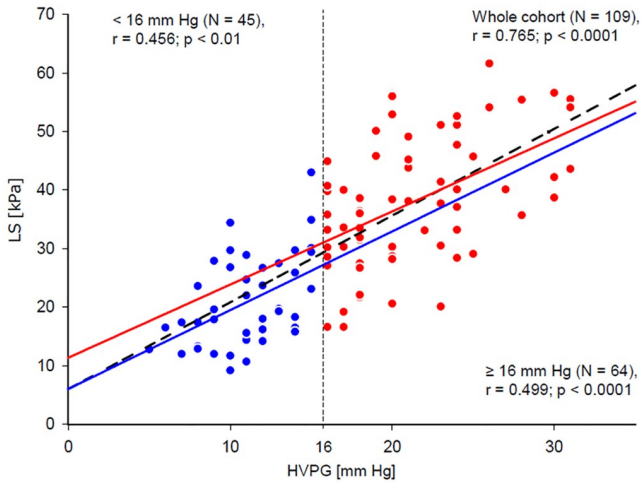

Fig 2. Relation between LS and HVPG. Blue line represents linear fit calculated for patients with HVPG lower than 16 mm Hg (represented by blue dots), red line shows the same for patients having HVPG equal to or higher than 16 mm Hg (represented by red dots). Dashed black line shows linear fit calculated for all patients together. **All patients:** β = 1.52, 95%CI = (1.27–1.77), p <0.001; LS [kPa] = 4.9 + 1.5 x HVPG [mmHg]. **<16 mm Hg:** β = 1.34, 95%CI = (0.59–2.10), p = 0,001; LS [kPa] = 6.0 + 1.3 x HVPG [mmHg]. **≥16 mm Hg:** β = 1.25, 95%CI = (0.73–1.77), p <0.001; LS [kPa] = 11.4 + 1.2 x HVPG [mmHg].

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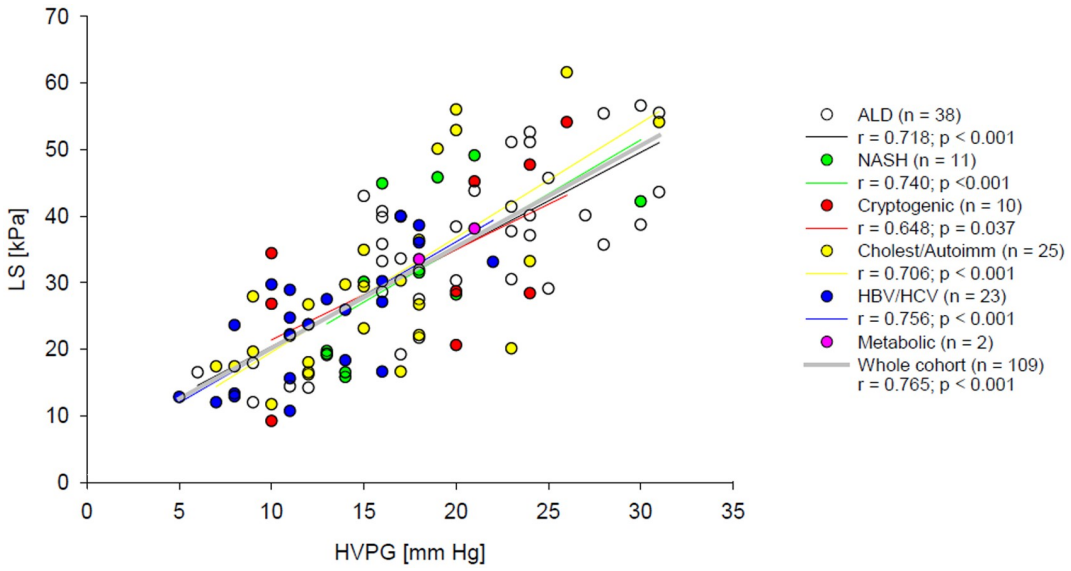

Fig 3. Relation between LS and HVPG. Coloured lines represent linear fits calculated for patients with alcoholic, NASH, cryptogenic, cholestatic/ autoimmune and viral (HBV and HCV) cirrhosis, dotted black line shows linear fit calculated for all patients together.

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16 mm Hg of HVPG in CPS-A or CPS-B/C patients did not differ from the prediction for 20 mm Hg (see Table 6). The comparison of the diagnostic accuracy between CPS-A and CPS-B/C patients could not be calculated at HVPG 20 mm Hg due to the fact that all studied subjects in the CPS-A group had HVPG below 20 mm Hg.

Modelling of a composite predictive factor

HVPG as the dependent variable, and LS together with all other studied non-invasive predictors as independent variables were analysed by the multiple linear regression. The strongest association was found between HVPG and LS ($p < 0.0001$). A weaker association was found between HVPG and IL1-ra/IL-1F3 ($p = 0.01874$) but the stepwise modelling showed minimal increase in r^2 after addition of IL1-ra/IL-1F3 to LS (0.5653 vs. 0.5348). Logistic regression using the same variables at 16 and 20 mm Hg identified LS as significant predictive factor at both cut-off points ($p < 0.0001$ at both 16 and 20 mm Hg (Table 7). None of the tested blood parameters presented with the ability to improve prediction of HVPG by LS. Comparison of correct prediction rates (2D-SWE diagnostic accuracies) among the patients with CPS-A and CPS-B/C at 16 mm Hg HVPG cut-off point revealed 83% (25/30) correctly predicted HVPG

Table 4. LS vs HVPG relationship formulas and β values in groups classified according to the aetiology of liver cirrhosis.

Patient group	LS [kPa] relationship formula	Beta values, 95% CI and p
Alcoholic (N = 38)	5.8 + 1.5 x HVPG [mm Hg]	β = 1.46 (95% CI = 1.07–1.85, p < 0.0001)
NASH (N = 11)	2.7 + 1.6 x HVPG [mm Hg]	β = 1.63 (95% CI = 0.37–2.88, p = 0.0312)
Cryptogenic (N = 10)	7.8 + 1.4 x HVPG [mm Hg]	β = 1.36 (95% CI = 0.26–2.46, p = 0.0419)
Viral (HBV/HCV) (N = 23)	3.9 + 1.6 x HVPG [mm Hg]	β = 1.61 (95%CI = 0.97–2.25, p < 0.0001)
Cholestatic/autoimmune (N = 25)	2.3 + 1.7 x HVPG [mm Hg]	β = 1.72 (95% CI = 1.03–2.42, p < 0.0001)

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Table 5. AUROCs of liver stiffness and other non-invasive markers to predict HVPG 16, and 20 mm Hg.

Cut-off point HVPG 16 mm Hg			
	AUROC	95% CI	p value
Liver stiffness [median, kPa]	**0.9024**	0.8464–0.9585	< 0.0001
LSPS [points]	0.7608	0.6685–0.8531	< 0.0001
ELF score [points]	0.7116	0.6146–0.8086	0.00018
Osteopontin [ng/mL]	0.7765	0.6864–0.8667	< 0.0001
VCAM-1 [ng/mL]	0.7228	0.6228–0.8228	< 0.0001
TIMP-1 [ng/mL]	0.7358	0.6411–0.8304	< 0.0001
PIIINP [ng/mL]	0.6585	0.5572–0.7598	0.0049
TNF-α [pg/mL]	0.512	0.3981–0.6259	N.S.
IL-6 [pg/mL]	0.6326	0.5164–0.7487	0.028
IL1-Ra/IL-1-F3 [pg/mL]	0.4215	0.3122–0.5308	N.S.
Hyaluronic acid [ng/mL]	0.684	0.5810–0.7871	0.00118
Cut-off point HVPG 20 mm Hg			
	AUROC	95% CI	p value
Liver stiffness [median, kPa]	**0.8682**	0.8006–0.9359	< 0.0001
LSPS [points]	0.7151	0.6161–0.8141	0.0002
ELF score [points]	0.6837	0.5757–0.7918	0.002
Osteopontin [ng/mL]	0.7714	0.6828–0.8600	< 0.0001
VCAM-1 [ng/mL]	0.6979	0.5985–0.7972	0.001
TIMP-1 [ng/mL]	0.7241	0.6291–0.8191	0.0001
PIIINP [ng/mL]	0.6552	0.5384–0.7720	0.008
TNF-α [pg/mL]	0.5157	0.4007–0.6307	N.S.
IL-6 [pg/mL]	0.6488	0.5329–0.7648	0.03
IL1-Ra/IL-1-F3 [pg/mL]	0.4036	0.2932–0.5139	N.S.
Hyaluronic acid [ng/mL]	0.6411	0.5335–0.7488	0.016

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pressure values in the CPS-A group and 82% (65/79) correctly predicted HVPG pressure values in the CPS-B/C group.

Discussion

In our carefully selected cohort of patients awaiting liver transplantation due to advanced liver cirrhosis, we obtained a strong correlation between LS and HVPG in a wide range of values ($r = 0.7645$, $p < 0.0001$). The correlation remained linear even in the range from 16 to 30 mg Hg of HVPG, the AUROC values at 16 and 20 mm Hg of HVPG were all above 0.85 and the corresponding cut-off points of LS were 30.2, and 37.1 kPa, respectively. LS of more than 30.2 kPa had diagnostic accuracy 84% and 37.1 kPa 82% for the detection of HVPG higher than 16 and 20 mm Hg, respectively. However, the clinical applicability of these cut-off point should be limited only to the similarly selected group of patients. We did not present the diagnostic accuracy for the prediction of HVPG above 10 mm Hg because there were only 12 of 109 (11.0%) patients with HVPG lower than 10 mm Hg. These data confirm our hypothesis that non-homogeneity of the study group in previous studies regarding alcohol abuse might have been one of the reasons for the weak correlation between LS and HVPG values above 10 mm Hg [12].

Additional support for our hypothesis that active alcohol drinkers with advanced liver cirrhosis have significantly overestimated LS in comparison with abstainers came from the study by Conti at al. [27]. These authors demonstrated that LS in patients with liver cirrhosis is

Table 6. Diagnostic performance of liver stiffness to predict HVPG above 16, and 20 mm Hg*.

HVPG [mm Hg]	16	20
AUROC	**0.90243**	**0.86824**
OR 95% CI	**1.21484 1.1396–1.3174**	**1.157 1.101–1.231**
Cut-off [kPa]	30.2	37.1
Sensitivity	0.7656	0.7027
Specificity	0.9333	0.8750
PPV	94.23%	74.29%
NPV	73.68%	84.00%
+LR	11.47	5.62
–LR	0.25	0.34
Diagnostic accuracy	83.5% (91/109)	81.7% (89/109)
McNemar's test	$p > 0.05$	

PPV, positive predictive values; NPV, negative predictive values; +LR, positive likelihood ratio;–LR, negative likelihood ratio;

* applicable only on a similarly selected group of patients

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proportional to the grade of steatosis. Liver transplant candidates seem to be a unique group of patients for studies of the relation between LS and HVPG in patients with advanced liver cirrhosis due to absence of steatosis.

Another factor contributing to the uniformity of HVPG and LS relationship in our patients is the fact that liver transplant candidates are considered as patients with irreversible end-stage liver disease. There is no detailed definition of the point of no return at which liver cirrhosis becomes irreversible but patients behind this point have uniform clinical presentation and probably also uniform fibrosis and haemodynamic impairment. In contrast to this oversimplified view, Ferrusquía-Acosta et al. proved that aetiology of liver cirrhosis affects liver haemodynamics also in patients with severe portal hypertension [46]. The authors measured wedged hepatic vein pressure (WHVP) and portal pressure (PP) directly before and during TIPS insertion procedure in

Table 7. Composite non-invasive HVPG predictor modelling by linear and logistic regression.

	Stepwise multiple linear regression	Stepwise logistic regression at various cut-off points of HVPG	
Dependent variable	**HVPG**	**16 mm Hg**	**20 mm Hg**
Independent variables	**p value**	**p value**	**p value**
Liver stiffness [kPa]	< **0.0001**	<**0.0001**	< **0.0001**
MELD [points]	0.85768	0.66765	0.6270
Spleen diameter [cm]	0.85706	0.86437	0.8098
Platelets [$x10^9$/L]	0.94557	0.89992	0.2541
Osteopontin (ng/mL)	0.1414	0.04254	0.3603
VCAM-1 [ng/mL]	0.14792	0.6366	0.3268
TIMP-1 [ng/mL]	0.96405	0.63196	0.8637
ELF score [points]	0.47159	0.06959	0.1879
Hyaluronic acid [ng/mL]	0.44322	0.05005	0.6911
PIIINP [ng/mL]	0.53925	0.11706	0.1092
IL-6 (pG/mL) [ng/mL]	0.7389	0.97873	0.5831
TNF-α (pG/mL) [pg/mL]	0.4951	0.29018	0.6445
IL1-ra/IL-1F3 [pg/mL]	**0.01874**	0.2667	0.1257

https://doi.org/10.1371/journal.pone.0244934.t007

patients with NASH, alcohol-related and HCV cirrhosis. They found more often the disagreement between WHVP and PP in NASH than in HCV patients (37.5% vs. 14%) and PP was more frequently underestimated in patients with NASH (32.5% vs. 7.5%). On the other hand, the authors did not provide data on LS values and their correlation with HVPG.

In our study, aetiology of cirrhosis had no impact on the relation between LS and HVPG. Despite the fact that HVPG was significantly higher in the group of patients with alcoholic cirrhosis than in the group of patients with cirrhosis of viral aetiology, the correlation remained unchanged. Higher HVPG and LS in alcoholic liver cirrhosis mirrors the fact that these patients are usually enlisted for liver transplantation with more advanced liver disease than patients with viral aetiology.

Obese patients and patients with cirrhosis owing to NASH were also included in our study group. Severe steatosis should not be present in these patients because it usually fades out in the late stage of NASH cirrhosis, but obesity persists. Obesity itself may increase variability of LS measurement [47]. However, all the 7 patients with unreliable LS measurement excluded from further evaluation had BMI lower than 30 and reliable LS values were obtained in 29 patients with BMI above 30. Neither ascites presented an obstacle for LS measurement as was previously supported by Conti et al [27]. 2D-SWE seems to be the appropriate method for LS measurement in patients with advanced liver cirrhosis: the presence of ascites in most patients with advanced liver disease (more than one half in our cohort of patients) was the crucial reason why we had chosen 2D-SWE as the non-invasive tool for LS assessment in the study. Albeit Fibroscan® advantages include short procedure time and the ability to perform the test at the bedside, it is nearly impossible to obtain the results from patients with ascites [48, 49].

Unfortunately, we were not able to improve the prediction of HVPG using a composite non-invasive predictor consisting of LS combined with one or more blood biomarkers because none of the tested blood biomarkers showed better correlation with HVPG than with LS. Consistently with this statistical concept, stepwise linear regression and logistic regression analysis failed to identify another independent non-invasive predictor being able to improve prediction of HVPG by LS. We did not measure the spleen stiffness because at the beginning of our study, the software upgrade of the 2D-SWE device for spleen stiffness measurement was not available and the measurement of spleen stiffness using liver mode was considered an off-label procedure according to the manufacturer. Furthermore, the results of spleen stiffness measured using the liver mode, which we had evaluated retrospectively before this study, were disappointing since spleen stiffness correlation with HVPG was weak ($r = 0.320$, $p = 0.02$, $n = 32$). Consistently with our experience, spleen stiffness measured by 2D-SWE in the study by Procopet et al. [12] had a lower AUROC for diagnosing CSPH compared with LS. Jansen et al. demonstrated later that 2D-SWE measurement of liver stiffness correlated better with HVPG than 2D-SWE spleen stiffness [14] and proposed a sequential measurement of LS and spleen stiffness for the diagnosis of CSPH. The sequential algorithm was validated by Elkrief et al. [17] 2D-SWE is not considered a suitable method for spleen stiffness measurement [23]; vibration-controlled transient elastography (VCTE, Fibroscan®) combined with B-mode ultrasound localisation of the spleen or point SWE seem to be more promising for CSPH diagnosis [50–52]. However, spleen stiffness measurement seems to be a suitable non-invasive tool for evaluation of response to NSBB. Two recent studies using pSWE [53]and VCTE [54] technique indicate promising results in non-invasive NSBB response evaluation but further data and standardization is needed in this field. Absence of spleen stiffness measurement could be considered a weak point of our study; nonetheless, based on the above-mentioned data, we could have not expected a breaking improvement of HVPG prediction using also spleen stiffness measurement.

Based on the results of study by Bruha and colleagues [19], we initially considered osteopontin as the most promising blood biomarker. However, Bruha et al. [19] achieved a weaker

correlation ($r = 0.25$, $p = 0.0022$, vs. $r = 0.5143$, $p < 0.0001$) between HVPG and plasma osteopontin concentration in their group of 154 patients with liver cirrhosis of various aetiology. The authors declared that none of the patients was an excessive drinker; however, not all were absolutely abstaining. This was probably the reason why the AUROCs did not exceed the value of 0.8 at any point (16 and 20 mm Hg) of HVPG in their study.

LSPS predicted better CSPH than LS alone in the study reported by Berzigotti et al. [40]. LSPS is a composite predictor; it was superior in the prediction of CSPH than LS alone, but the predictive value of LSPS for higher thresholds was not investigated. The study group by Berzigotti [40] included predominantly CPS-A patients, only a few patients of CPS-B but none of CPS-C class. Not surprisingly, the correlation of LSPS with HVPG was weak in our entire group ($r = 0.449$, $p < 0.0001$) consisting predominantly of CPS-B and C patients. When evaluating the correlation between LSPS and HVPG separately in CPS-A and CPS-B/C groups, we noted that the correlation in the CPS-A group was strong despite the low number of patients in this group. In the group of CPS-B/C it became weaker contrasting with the higher number of patients. Since AUROCs for LSPS also did not exceed 0.8 at any of the two HVPG cut-off levels evaluated in our study, we can claim that LSPS was not superior to LS alone for prediction of HVPG at 16 and 20 mm Hg. However, we can neither confirm nor call in question the excellent power of LSPS to predict CSPH in patients with less advanced liver cirrhosis [40].

ELF has originally been constructed as a non-invasive predictor of liver fibrosis stage in non-cirrhotic patients with chronic liver disease [45, 55]. Since ELF represents a reliable tool for distinction between cirrhotic and non-cirrhotic patients, we would have expected a good correlation with HVPG. In reality, both ELF score and its individual components HA, PIIINP and TIMP-1, showed a weak correlation with HVPG in our cohort. Simbrunner et al. [56] published recently a study larger than ours showing better correlation between ELF and HVPG in their cohort of patients of Child-Pugh A, B and C. However, most of the patients (116/201, 58%) were in the Child-Pugh A group and the authors achieved a significant correlation between ELF and HVPG only in Child-Pugh A patients but not in Child-Pugh B and C patients. The authors also demonstrated that the strength of the correlation between ELF and HVPG decreased at higher HVPG levels. Owing to high proportion of Child-Pugh A class patients, they were able to predict CSPH accurately with AUROC 0.833 in the whole cohort. Consistently with the Simbrunner's results, we achieved a weaker correlation in our group with predominance of CPS B/C patients, the correlation between ELF and HVPG in CPS A patients was better than in the entire cohort but weaker than in the Simbrunner's study; in our CPS-B/C group, the correlation was marginally significant. This suggests that ELF score may indeed serve as a non-invasive predictor of HVPG with a better performance in Child-Pugh A patients.

Despite all efforts, our results cannot be extrapolated to general population of patients with advanced liver disease. Nonetheless, the data strongly suggest the feasibility of the non-invasive prediction of HVPG at higher cut-off points by LS.

HVPG is currently the best validated tool to assess prognosis of patients with liver cirrhosis. In this study, we demonstrated the strongest correlation between LS and HVPG in wide range of HVPG values, so far only in a specific group of patients without ongoing alcohol abuse and significant liver steatosis. We speculate that with further technical development in the field of non-invasive assessment of liver fibrosis and steatosis, LS measurement will be able to fully substitute for invasive HVPG measurement to evaluate the risks associated with advanced liver disease.

Supporting information

S1 File. Patients' clinical and laboratory data.
(XLSX)

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Portal Hypertension Is the Main Driver of Liver Stiffness in Advanced Liver Cirrhosis

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Summary

Liver stiffness (LS) is a novel non-invasive parameter widely used in clinical hepatology. LS correlates with liver fibrosis stage in non-cirrhotic patients. In cirrhotic patients it also shows good correlation with Hepatic Venous Pressure Gradient (HVPG). Our aim was to assess the contribution of liver fibrosis and portal hypertension to LS in patients with advanced liver cirrhosis. Eighty-one liver transplant candidates with liver cirrhosis of various aetiologies underwent direct HVPG and LS measurement by 2D shear-wave elastography (Aixplorer Multiwave, Supersonic Imagine, France). Liver collagen content was assessed in the explanted liver as collagen proportionate area (CPA) and hydroxyproline content (HP). The studied cohort included predominantly patients with Child-Pugh class B and C (63/81, 77.8 %), minority of patients were Child-Pugh A (18/81, 22.2 %). LS showed the best correlation with HVPG ($r=0.719$, $p<0.001$), correlation of LS with CPA ($r=0.441$, $p<0.001$) and HP/Amino Acids ($r=0.414$, $p< 0.001$) was weaker. Both variables expressing liver collagen content showed good correlation with each other ($r=0.574$, $p<0.001$). Multiple linear regression identified the strongest association between LS and HVPG ($p<0.0001$) and weaker association of LS with CPA ($p = 0.01883$). Stepwise modelling showed minimal increase in r^2 after addition of CPA to HVPG (0.5073 vs. 0.5513). The derived formula expressing LS value formation is: LS=2.48 + (1.29 x HVPG) + (0.26 x CPA). We conclude that LS is determined predominantly by HVPG in patients with advanced liver cirrhosis whereas contribution of liver collagen content is relatively low.

Key words

Hepatic venous portal gradient • Elastography • Collagen • Hydroxyproline • Osteopontin

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Introduction

Chronic liver disease (CLD) result from the necro-inflammatory process damaging hepatocytes due to

variety of origins and promoting progression of liver fibrosis to cirrhosis. Progression of liver fibrosis is associated with increase of liver stiffness (LS) caused by collagen deposition and distortion of liver architecture. Mechanical changes in liver parenchyma represent the major but not exclusive contributor to increased portal pressure since liver fibrosis is also accompanied by microvascular thrombosis, hepatic sinusoidal endothelial cell dysfunction and hepatic stellate cell activation resulting in increased vascular resistance (McConnell and Iwakiri 2018). Pathophysiology of portal hypertension (PH) development also includes adaptive changes such as splanchnic and systemic arterial vasodilatation, hyperdynamic circulation and formation of portosystemic collaterals. These adaptive changes can further increase the portal pressure (Berzigotti and Bosch 2014).

Hepatic vein pressure gradient (HVPG) represents the gold standard for evaluation of the presence and severity of PH in patients with liver cirrhosis in clinical hepatology (Bosch *et al.* 2009). HVPG is presumably the best validated tool for assessing the risk of severe complications in liver cirrhosis. HVPG higher than 10 mm Hg is considered to be the cut-off value for clinically significant portal hypertension (CSPH) (Lebrec *et al.* 1980). Patients with CSPH are at risk of oesophageal varices, develop ascites and cirrhosis decompensation (Garcia-Tsao *et al.* 1985, Groszmann *et al.* 2005, Ripoll *et al.* 2007). HVPG higher than 12 mm Hg is associated with the risk of variceal bleeding, more than 16 mm Hg with high mortality and HVPG higher than 20 mm Hg predicts failure to control variceal bleeding (Abraldes *et al.* 2008, Silva-Junior *et al.* 2015). HVPG measurement by hepatic vein catheterization is an invasive procedure and therefore, there is a need for an accurate non-invasive method. LS measurement as a non-invasive predictor of portal hypertension has been extensively studied in the last decade. This approach failed to predict HVPG values higher than 10 mm Hg in some studies (Procopet *et al.* 2015); contrarily, other authors proved good correlation between LS and HVPG also for high values of HVPG (Stefanescu *et al.* 2019). The factors influencing correlation between LS and HVPG should be elucidated in the future.

LS can be measured by elastography techniques which can non-invasively estimate liver fibrosis stage. Liver stiffness measurement (LSM) has been widely used in clinical hepatology to characterize the stage of chronic liver disease in the last two decades and almost completely replaced invasive liver biopsy. Apart from non-invasive assessment of liver fibrosis, LSM also offers the possibility of non-invasive evaluation of portal hypertension.

Quantitative elastography is based on the measurement of shear wave propagation through the region of interest in the liver parenchyma (Kennedy *et al.* 2018). Pulse-echo ultrasound acquisition is used to follow the propagation of the shear wave and to measure its velocity; the stiffer the tissue, the faster the shear wave propagates. Transient elastography (TE) is the most commonly used method in clinical hepatology (Sandrin *et al.* 2003). The shear wave is generated by a mechanical vibrator mounted on the axis of the probe. The method is fast and easy, but its use is limited by the presence of ascites. The layer of the liquid represents an obstacle when the mechanical vibration passes across the abdominal wall to the liver. TE showed an excellent diagnostic accuracy for diagnosis of liver cirrhosis in several meta-analyses, with AUROC values > 0.9 (Friedrich-Rust *et al.* 2008, Li *et al.* 2016, Shaheen *et al.* 2007, Stebbing *et al.* 2010, Talwalkar *et al.* 2007, Tsochatzis *et al.* 2011). TE was better at ruling out rather than ruling in liver cirrhosis with negative predictive value > 90 % in these meta-analyses. TE has also been shown to have an excellent performance in predicting CSPH, with two meta-analyses reporting AUROCs ≥ 0.9 (Shi *et al.* 2013, You *et al.* 2017).

The ultrasound-based quantitative elastography systems have recently been implemented in the standard ultrasound systems and therefore they rapidly spread into clinical practice. The shear wave in the ultrasound-based systems is generated by an acoustic radiation force impulse (ARFI). ARFI techniques bring also the benefit of real-time imaging to direct the probe to the region of interest. ARFI is commonly available in two forms, point shear wave elastography (pSWE) and two-dimensional shear wave elastography (2D-SWE). Both ARFI techniques allow LSM also in patients with advanced liver cirrhosis who present with ascites. Furthermore, ARFI methods have demonstrated an excellent diagnostic performance in predicting CSPH and the presence of oesophageal varices (Cassinotto *et al.* 2015, Elkrief *et al.* 2015, Morishita *et al.* 2014, Thiele *et al.* 2020).

Magnetic resonance elastography (MRE) is considered a gold standard in quantitative elastography. This technique allows measurement of tissue stiffness during clinical MRI exams by encoding the propagation of shear waves into the MR phase signal. MRE has shown an excellent ability to detect liver fibrosis and

cirrhosis (Singh *et al.* 2016, Singh *et al.* 2015). Furthermore, some blood biomarkers were identified as non-invasive markers of portal hypertension, but their diagnostic power seems to be less robust in comparison with LSM. On the other hand, the blood biomarkers may serve for the rapid diagnosis and further research in this field is needed (Bruha *et al.* 2016, Simbrunner *et al.* 2020).

The wide clinical experience showed that LS represents a unique parameter reflecting morphological (fibrosis) and circulatory changes associated with the progression of chronic liver disease. It is also obvious that the LS value consists of two components: static (fibrosis) and dynamic (portal hypertension). Liver fibrosis is generally accepted as the driving factor of liver dysfunction and portal hypertension; two human studies (Calvaruso *et al.* 2012, Nielsen *et al.* 2014) showed that in the cirrhotic liver, the correlation between liver collagen content (collagen proportionate area, CPA) and portal hypertension (HVPG) is weaker than the correlation between LS and HVPG in the aforementioned trials. Therefore, it seems likely that in cirrhotic patients, the main component constituting the LS value is portal hypertension, not collagen content. This hypothesis is supported by experiments on an artificial liver model or animal livers (Yang *et al.* 2017, Yarpuzlu *et al.* 2014, Yin *et al.* 2013).

The aim of our study was to assess the contribution of static and dynamic component to LS in patients with advanced liver cirrhosis. To achieve it, we conducted a study in liver transplant recipients. The study design allowed us to assess the collagen content in the explanted liver by two independent laboratory methods. LS and HVPG were assessed in the pre-transplant period. Furthermore, to refine the non-invasive estimation of HVPG, we searched for blood biomarkers specific for portal hypertension independent of liver fibrosis.

Methods

Patients and study design

This prospective study included 81 patients who underwent liver transplantation for liver cirrhosis of various aetiology between October 2016 and July 2018 at our Transplant centre and before liver transplantation had participated in the first part of the clinical study on non-invasive predictors of portal hypertension (Frankova *et al.* 2021). Briefly, 109 liver transplant candidates with liver cirrhosis of various aetiology were included in the clinical study and completed the first part of the study protocol which included liver stiffness measurement, direct HVPG measurement by liver vein catheterisation and blood sampling for biomarkers assessment. All the study participants were evaluated as liver transplant candidates according to the standard criteria (European Association for the Study of the Liver 2016) and 92 of them were enrolled into the waiting list. Eighty-three of them underwent liver transplantation and in 81 of them, the explanted liver was available for the assessment of the collagen content. The aim of the second part of the study was in detail described in the previous section. Patients with portal vein thrombosis, transjugular intrahepatic portosystemic shunt (TIPS), hepatorenal syndrome requiring vasoactive drugs administration or renal replacement therapy, severe bacterial infection or sepsis, pulmonary hypertension, variceal bleeding in the last 4 weeks, hepatocellular carcinoma outside of the Milan criteria (Mazzaferro *et al.* 1996) and ongoing alcohol abuse were not considered for the participation in the study and none of them was included. The study was approved by local Institutional Review Board (IRB of Institute for Clinical and Experimental Medicine and Thomayer's Hospital, Prague). All patients signed the informed consent with the participation in study and the explanted liver assessment.

Blood sampling

The study subjects were in a sitting position for at least 5 min (but not >10 min) before and during sampling. Venous blood was taken between 8 and 10 a.m. The Vacuette system (VACUETTE® TUBE 8 mL Z Serum Separator Clot Activator cat. No. 455071, and VACUETTE® 9 ml K3 EDTA Plasma Separator cat. No. 455036, both from Greiner Bio-One, Kremsmünster, Austria) was used together with 21-gauge needles (Greiner Bio-One). Separation of blood corpuscles was done within 60 min after sampling at 3000 g for 10 min (centrifuge Beckman Allegra, Beckman Coulter, Indianapolis, IN). Several serum and plasma aliquots of 500 µl were prepared within 60 min after centrifugation. CryoKing tubes from Biologix Group Limited, Jinan, China, cat. No. 89-3101, were used to store serum and plasma aliquots at -80 °C until analysis.

Analytical methods

Serum concentrations of hyaluronic acid (HA), Amino-Terminal Propeptide of Type III Procollagen (PIIINP), and Tissue Inhibitor of Matrix

Metalloproteinase 1 (TIMP-1) were measured by the ADVIA Centaur® HA assay, lot 25,215,019, the ADVIA Centaur® PIIINP assay, lot 26,290,023, and the ADVIA Centaur® TIMP-1 assay, lot 28,900,016, respectively (Siemens Healthineers, Erlangen, Germany). ADVIA Centaur ELF calibrator was used for calibration of HA, PIIINP, and TIMP-1 assays and ADVIA Centaur ELF quality control materials (three levels) were used as assay controls. Repeatability (within-run CV) assessed as declared by the manufacturer was < 5.6, < 4.2, and < 3.3 % for HA, PIIINP and TIMP-1, respectively. Intermediate precisions (between-run CVs) were < 3.2, < 5.1, and < 5.5 %. The respective measurement ranges for HA, PIIINP, and TIMP-1 were 1.6–1000, 0.5–150, and 3.5–1300 ng/ml. Traceability was not provided by the manufacturer. Limits of detection of HA, PIIINP, and TIMP-1 were 1.6, 0.5 and 3.5 ng/ml. All measurements were performed in one run during one day by the same laboratory technician using a Centaur CP immunochemistry analyzer (Siemens Healthineers). The Enhanced Liver Fibrosis (ELF) was calculated according to the Centaur CP formula: 0.846 x ln (HA) + 0.735 x ln (PIIINP) + 0.391 x ln (TIMP-1) + 2.494.

Interleukin-6 (IL-6), Vascular Cell Adhesion Molecule 1 (VCAM-1), Interleukin-1 Receptor Antagonist (IL-1ra/IL-1F3), Osteopontin and Tumour Necrosis Factor alpha (TNFα) were assessed in plasma samples obtained from the study subjects according to the manufacturer's instructions using the assays No. HS600B, DVC00, DRA00B, DOST00, and HSTA00E, respectively, all purchased from R&D Systems, Minneapolis, MN. The absorbance was measured on a Synergy™ 2 Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT).

HVPG measurement

The radiologist performing HVPG measurements was blinded to elastography results. HVPG was determined by catheterization of the hepatic veins. An open-end zero-side holes 5F multipurpose angiographic catheter (Cordis, Santa Clara, CA) was inserted through a 6F sheath (Super Arrow-Flex Percutaneous Sheath Introducer Set, Arrow International brand of Teleflex, Wayne, PA) using the transjugular route. Iodinated radiological contrast medium was injected into the right or middle hepatic vein to confirm the position of the catheter in a wedged position by flouroscopy. The pressure was measured five times to demonstrate reproducibility and the mean value was used for further calculations. HVPG was calculated as the difference between wedged and free hepatic venous pressures.

Liver stiffness (LS) measurement

After an overnight fasting, 2D-SWE was performed using the Aixplorer® ultrasound system (Supersonic Imagine S.A., Aix-en-Provence, France) with an abdominal 3.5 MHz curved array probe (SC6-1). The examinations were performed ± 7 days HVPG measurement. The operator was not aware of HVPG results when performing 2D-SWE. All patients were in the supine position and the right arm maximally abducted and LS measurements were performed on the right lobe of the liver through the intercostal spaces. RT-SWE were acquired using a 3.5 x 2.5 cm box, more than 2 cm under the liver capsule and avoiding large vessels. During the examination the patient was requested to hold breath as needed. After obtaining a stable and homogenous elastographic image inside the box, a region of interest (ROI) was selected using the Q-box tool and placed in the most homogeneous area and the median values of LS within the ROI was displayed and registered. The diameter of the Q-box was set > 15 mm. Three elastographic images from different liver areas were obtained in all patients and the mean value was used for further calculations.

Non-invasive predictors of portal hypertension

The MELD score (Model for End-Stage Liver Disease) is a composite predictor of survival in patients with cirrhosis calculated from total serum bilirubin, serum creatinine and the international normalized ratio (INR). MELD score was originally invented to predict short-term survival in cirrhotic patients (Kamath *et al.* 2007). Later studies showed that MELD score correlates with MR elastography results, presence of varices and mortality in patients with variceal bleeding (Conejo *et al.* 2018, Hoffman *et al.* 2020) and is currently used to prioritize liver transplantation (Kamath *et al.* 2007, Wiesner *et al.* 2003, Wiesner *et al.* 2001).

LSPS (Liver Spleen Platelets Score) is also a composite predictor combining LS, platelets count and spleen diameter (Berzigotti 2013). LSPS was calculated as described previously: [LS (in kiloPascals) x spleen diameter (in centimetres)]/platelet count ratio ($x10^9$/l). LSPS was superior to LS alone for identification of patients with CSPH in a study by Berzigotti *et al.* (2013).

VCAM-1 is an inflammatory biomarker

correlating most significantly with HVPG in the study (Buck *et al.* 2014). Osteopontin, acting as a key component of bone matrix and multifunctional cytokine, also correlated well with HVPG in humans (Bruha *et al.* 2016).

The ELF score was shown to correlate with the stage of liver fibrosis in liver diseases of various aetiologies (Miele *et al.* 2017, Parkes *et al.* 2011, Parkes *et al.* 2010). Therefore, we decided to evaluate ELF score and its individual components as potential marker(s) of portal hypertension.

Collagen proportionate area (CPA) evaluation

Four tissue blocks measuring 15 x 15 x 2 mm were prepared from each liver explant and routinely processed for histological evaluation. Two 4 µm-thick sections histochemically stained by Picro-Sirius Red (PSR) technique to visualize collagen were prepared from each paraffin block. Analysis of collagen proportionate area (CPA) was performed by histomorphometry using a 3DHistech Pannoramic Desk II DW digital slide scanner (3DHistech Kft., Budapest, Hungary) and Nikon NIS-Elements AR 3.2 morphometric program (Nikon, Tokyo, Japan). Whole sections were scanned with 60x objective in transmitted light. Total tissue area was determined by thresholding in intensity mode. Subsequently, PSR-stained areas occupied by collagen were separated using Red, Green, and Blue (RGB) thresholding. The CPA percentage was expressed as the proportion of the collagen areas highlighted by PSR to the total tissue section surface.

Hydroxyproline to total Amino Acids ratio (HP/AA)

Colorimetric hydroxyproline assay detecting 3-hydroxy, 4-hydroxy and 5-hydroxyproline species was performed as described (Lunova 2014). Briefly, approximately 1 g of liver tissue sample was deparaffinized and mechanically homogenized to powder. Fifty mg of liver powder was then hydrolyzed in 6 N HCL at 110 °C for 18 hours, filtered to remove the debris and 50 µl of each sample was evaporated by speed vacuum centrifugation. The pellets or standards (trans-4-hydroxy-L-proline, Sigma) were dissolved in 50 µl of distilled water, mixed with 56 mM chloramine – T trihydrate (Sigma, Saint Louis, MO) in acetate - citrate buffer (pH = 6.5) and incubated for 25 min at room temperature. After that, Ehrlich solution (Sigma) was added to form the desired chromophore at 65 °C during 30 min incubation. Absorbance was measured at 570 nm by using the Synergy™ 2 Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT).

To determine total amino acids in liver samples, Ninhydrin reaction was used in pursuance of the manufacturer's recommendations. 50 µl aliquots of the filtered hydrolyzed liver samples (see hydroxyproline assay) were dried by vacuum centrifugation and then dissolved in distilled water. The samples or 40–1400 nM of amino acids (AA-S-18, Sigma) were mixed with Ninhydrin reagent solution (Sigma) in ratio 2:1, respectively, and incubated 10 min at 100 °C. Then the samples and standards were brought to room temperature and further mixed with 95 % ethanol (Sigma) in ratio 3:5, respectively. After that, absorbance was measured at 570 nm using the Synergy™ 2 Multi-Detection Microplate Reader (BioTek).

Statistical analysis

Statistical analyses were performed using the SigmaPlot 11.0 (Systat Software Inc., San Jose, CA) or JMP 11.0.0 software (2013, SAS Institute Inc.). Clinical characteristics were analysed in a descriptive way and reported as mean ± standard deviation, or median and range, as appropriate. Where assumptions of normal distributions were not met, Shapiro-Wilk test was used to evaluate the normal distribution of data. Spearman's test was used for correlations among continuous variables. Medians were compared using t-test or Mann-Whitney test, as appropriate. Similarly, multiple comparisons were done by one way ANOVA or Kruskal-Wallis test. Statistical significance was defined as p value < 0.05 for all calculations.

Results

Patient characteristics

Eighty-one consecutive patients were prospectively enrolled in the study. All patients had advanced liver cirrhosis of various aetiologies and were potential candidates for liver transplantation. All enrolled patients underwent blood draw for biomarkers, LS and HVPG measurement, both examinations were included in the pre-transplant screening protocol. All 81 patients underwent thereafter liver transplantation and CPA and HP/AA were assessed in the explanted liver. The median period between study recruitment and liver transplantation was 105 days (range 10–769 days). All patients received the graft from the heart-beating donors after brain death, age of the donors ranged from 21 to

81 years. None of the organ donors was from a vulnerable population and none of the donors was registered in the National Registry of people opposed to the post-mortem withdrawal of tissues and organs. The aetiology of liver cirrhosis is shown in the Table 1. The patients were divided into two subgroups according to the Child-Pugh classification of liver dysfunction: patients classified as Child-Pugh A (18/81, 22.2 %) and patients with Child-Pugh class B and C (63/81, 77.8 %). The measured HVPG values were from 6 to 31 mm Hg. Only 7 (8.6 %) patients had HVPG lower than 10 mm Hg; the overwhelming majority 74 (91.4 %) had CSPH, i.e. HVPG ≥ 10 mm Hg. There were 34 of 81 patients treated with carvedilol (non-selective β-blocker), all 34 patients had a low daily dose of 6.25 mg (3.125 mg twice daily). Only 3 patients were given atorvastatin at a daily dose of 20 mg, none of the 3 patients on atorvastatin received carvedilol concomitantly. The detailed characteristics of the patients are presented in Tables 1 and 2.

Table 1: Baseline clinical and laboratory characteristics of the whole cohort and patient subgroups

Variable [median, range]	**All patients N = 81 (100 %)**	**Child-Pugh A patients N = 18 (22.2 %)**	**Child-Pugh B and C patients N = 63 (77.8 %)**	**P (test)**
Age [years]	61 (21–74)	68 (53–74)	56 (21–73)	< 0.001 (MW)
Gender [Male]	55 (68.0 %)	13 (72.2 %)	42 (66.7 %)	N.S. (MW)
BMI [kg/m^2]	27.3 (18.4–38.5)	28.7 (21.6–35.4)	25.7 (18.4–38.5)	0.011 (t)
Child-Pugh score [points]	8 (5–13)	6 (5–6)	8 (7–13)	< 0.001 (MW)
Aetiology of liver cirrhosis				
Alcohol	28 (34.6 %)	7 (38.9 %)	21 (33.4 %)	
NASH	9 (11.1 %)	4 (22.2 %)	5 (7.9 %)	
Cryptogenic	7 (8.6 %)	0 (0.0 %)	7 (11.1 %)	
Viral (HBV/HCV)	13 (16.0 %)	7 (38.9 %)	6 (9.5 %)	< 0.001 (F)
Cholestatic and	22 (27.2 %)	0 (0 %)	22 (34.9 %)	
autoimmune	2 (2.5 %)	0 (0 %)	2 (3.2 %)	
Metabolic				
MELD score [points]	15 (6–37)	9 (6–21)	15 (7–37)	< 0.001 (MW)
Overt hepatic	10	0	10	0.026 (F)
encephalopathy	(12.3 %)	(0 %)	(15.9 %)	
Spleen diameter [cm]	16 (9–25)*	15 (9–18)	16 (9–25)*	0.005 (MW)
Oesophageal varices	19/25/37	5/6/7	14/19/30	N.S. (F)
(none/small/large)	(23.4/30.9/45.7 %)	(27.8/33.3/38.9 %)	(22.2/30.1/47.7 %)	
History of variceal	17 (21.0 %)	3 (16.7 %)	14 (22.2 %)	N.S. (F)
bleeding				
Ascites (none/small/large)	40/16/25	16/2/0	24/14/25	< 0.001 (F)
	(49.4/19.7/30.9 %)	(98.9/11.1/0 %)	(38.1/22.2/39.7 %)	
Platelet count [x10^9/l]	93 (40–344)	94 (48-286)	93 (40–344)	N.S. (t)
Bilirubin [μmol/l]	34 (5–257)	19 (5–31)	43 (8–257)	< 0.001 (t)
Albumin [g/l]	30 (17–45)	37 (29–45)	28 (17–40)	< 0.001 (t)

*One value missing. MW – Mann-Whitney rank sum test, F – Fisher exact test, t – t-test

The variables were compared between the subgroups of patients according to the aetiology of liver cirrhosis. There were no significant differences found between the subgroups in LS, CPA, HP/AA, ELF score, osteopontin (Fig. 1A), and MELD and Child-Pugh scores (Fig. 1B). Only HVPG was significantly higher in the subgroup of patients with alcoholic liver disease than in the subgroup of patients with viral aetiology of liver cirrhosis (Fig. 1A).

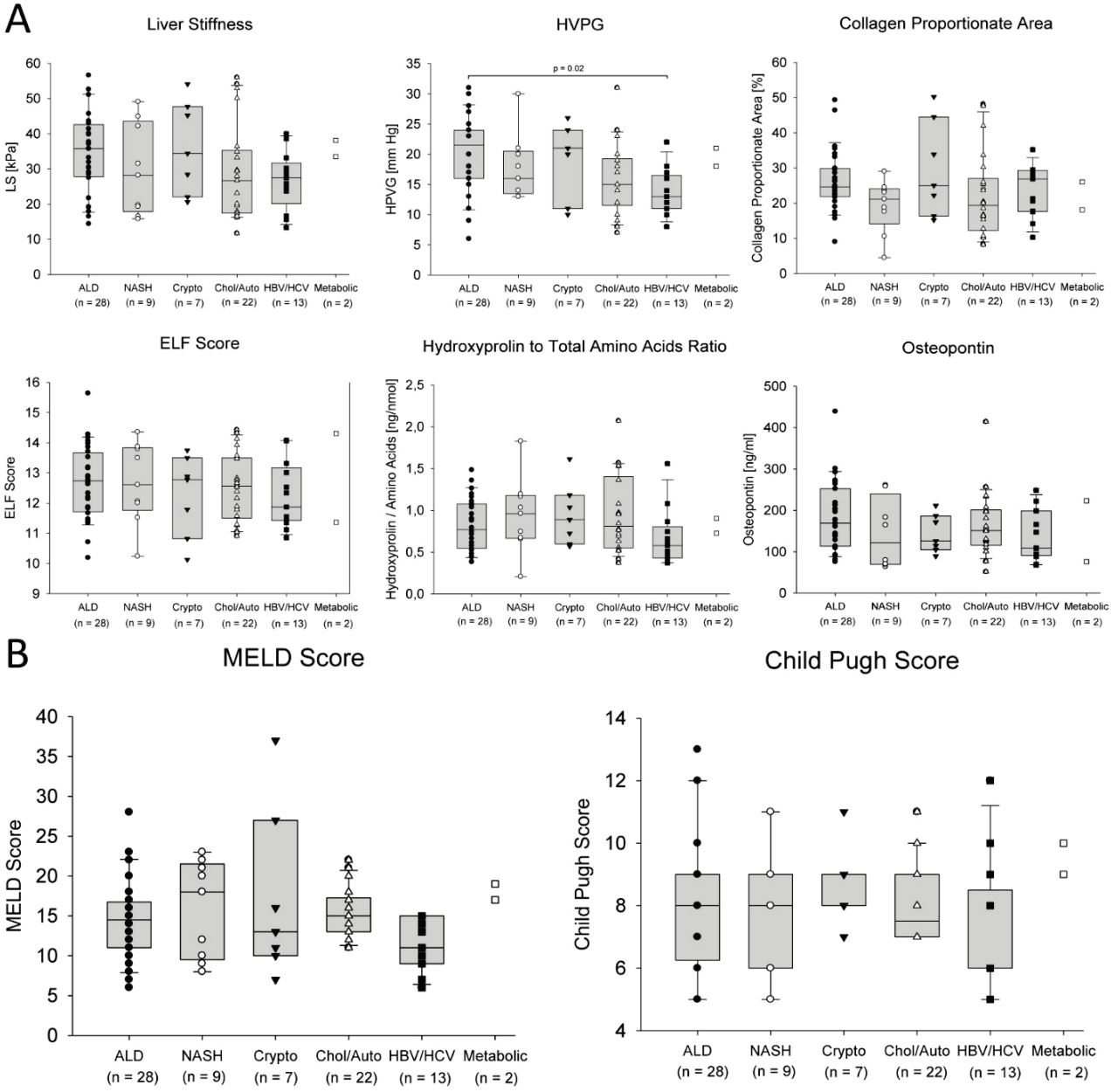

Fig. 1. Comparison of LS, HVPG, collagen content (CPA and HP/AA), ELF and osteopontin (**A**) and liver function scores (**B**) between subgroups of patients according to the aetiology of the liver cirrhosis. The data of two patients with metabolic liver disease were displayed but not included in the statistical analysis.

Correlations of HVPG and collagen content with their non-invasive markers

As the next step, correlations between LS, HVPG, liver collagen content (expressed as CPA or HP/AA) and non-invasive blood biomarkers of liver fibrosis and portal hypertension were calculated. The obtained Spearman's non-parametric correlation coefficients are presented in Table 3. The strongest correlation with LS was achieved for HVPG, the correlations with CPA, HP/AA and osteopontin were weaker. The regression lines for these correlations are displayed in Figure 2. It is evident that the lines have a different slope and HVPG increases well with LS whereas CPA or HP/AA increases more slowly. The correlation of LS with CPA or HP/AA was almost identical since both variables reflect liver collagen content. Accordingly, CPA and HP/AA showed a good correlation between each other ($r = 0.574$; $p < 0.001$). Interestingly, HVPG, LS, LSPS, all associated with portal hypertension rather than collagen content showed better correlation with CPA than with HP/AA. On the other hand, ELF score and its components showed a better

Table 2. Liver stiffness, HVPG, blood predictors of HVPG and fibrosis, and liver collagen content. All comparisons between Child-Pugh A patients and Child-Pugh B and C patients were done by Mann Whitney Rank Sum test.

Variable [median, range]	All patients N = 81 (100 %)	Child-Pugh A patients N = 18 (22.2 %)	Child-Pugh B and C patients N = 63 (77.8 %)	P
Liver stiffness [kPa]	30.0 (11.7–56.6)	19.5 (13.3–39.8)	33.2 (11.7–56.6)	< 0.001
HVPG [mm Hg]	17 (6–31)	14 (8–20)	18 (6–31)	< 0.001
LSPS [points]	4.9 (0.6–27.9)*	3.2 (0.8–8.8)	5.5 (0.6–27.9)*	0.006
ELF score	12.6 (10.1–15.6)	11.8 (10.2–13.0)	12.7 (10.1–15.6)	< 0.001
Osteopontin [ng/ml]	150 (52–439)**	109 (64–169)	177 (52–439)**	< 0.001
VCAM-1 [ng/ml]	2906 (579–10268)*	1833 (579–4509)	3378 (1134–10268)*	< 0.001
TIMP-1 [ng/ml]	475 (219–1834)	316 (219–514)	528 (226–1834)	< 0.001
PIIINP [ng/ml]	21.8 (6.3–71.6)	18.0 (11.3–33.6)	25.5 (6.3–71.6)	0.01
Hyaluronic acid [ng/ml]	564 (43–6872)	350 (43–1269)	700 (57–6872)	0.013
Hydroxyproline/amino acids [ng/nmol]	0.77 (0.21–2.07)	0.53 (0.21–1.11)	0.89 (0.37–2.07)	< 0.001
Collagen area [%]	23.4 (4.5–50.2)	18.4 (4.5–36.2)	24.4 (8.2–50.2)	0.028

*one sample missing, **two samples missing.

Table 3. Correlation between HVPG, LS, collagen content (CPA or HP/AA) and their non-invasive markers.

Variable	HVPG		Liver stiffness		CPA		Hydroxyproline/Amino Acids	
	Spearman's r	p	Spearman's r	p	Spearman's r	p	Spearman's r	p
Liver stiffness [kPa]	0.719	< 0.001	N.A.	N.A.	0.441	< 0.001	0.414	< 0.001
*LSPS [points]**	0.419	< 0.001	0.481	<0.001	0.303	0.006	0.126	N.S.
MELD score [points]	0.216	N.S.	0.413	<0.001	0.073	N.S.	0.234	0.034
Spleen diameter [cm]	0.113	N.S.	0.059	N.S.	0.005	N.S.	0.047	N.S.
Platelets [x10^9/l]	0.008	N.S.	0.140	N.S.	-0.181	N.S.	0.042	N.S.
*Osteopontin [ng/ml]***	0.404	< 0.001	0.390	<0.001	0.071	N.S.	0.188	N.S.
*VCAM-1 [ng/ml]**	0.352	0.002	0.443	<0.001	0.317	0.004	0.236	0.035
TIMP-1 [ng/ml]	0.346	0.002	0.471	< 0.001	0.235	0.004	0.340	0.002
ELF score [points]	0.271	0.015	0.337	0.002	0.347	0.002	0.470	< 0.001
HA [ng/ml]	0.203	N.S.	0.305	0.006	0.321	0.004	0.449	< 0.001
PIIINP [ng/ml]	0.183	N.S.	0.203	N.S.	0.252	0.023	0.407	< 0.001
HP/AA [ng/nmol]	0.327	0.003	0.414	< 0.001	0.574	< 0.001	N.A.	N.A.
CPA [%]	0.324	0.003	0.441	< 0.001	N.A.	N.A.	0.574	< 0.001

*one sample missing, **two samples missing, N.A. not applicable, N.S. not significant

correlation with HP/AA than with CPA. Osteopontin showed a good correlation with LS and HVPG but it did not correlate well with CPA and HP/AA. Therefore, osteopontin represents the most specific blood biomarker

of portal hypertension from the tested set. Similarly, LSPS correlated well with LS and HVPG better than with CPA. Administration of the low dose of carvedilol did not improve the correlation between LS and HVPG (r = 0.6050, CI 95 % 0.3262–0.7870, p = 0.0002 for subgroup on carvedilol; r = 0.7325, CI 95 % 0.5580–0.8450, p < 0.0001 for subgroup without carvedilol). The impact of statin administration could not be assessed because of low number of treated patients.

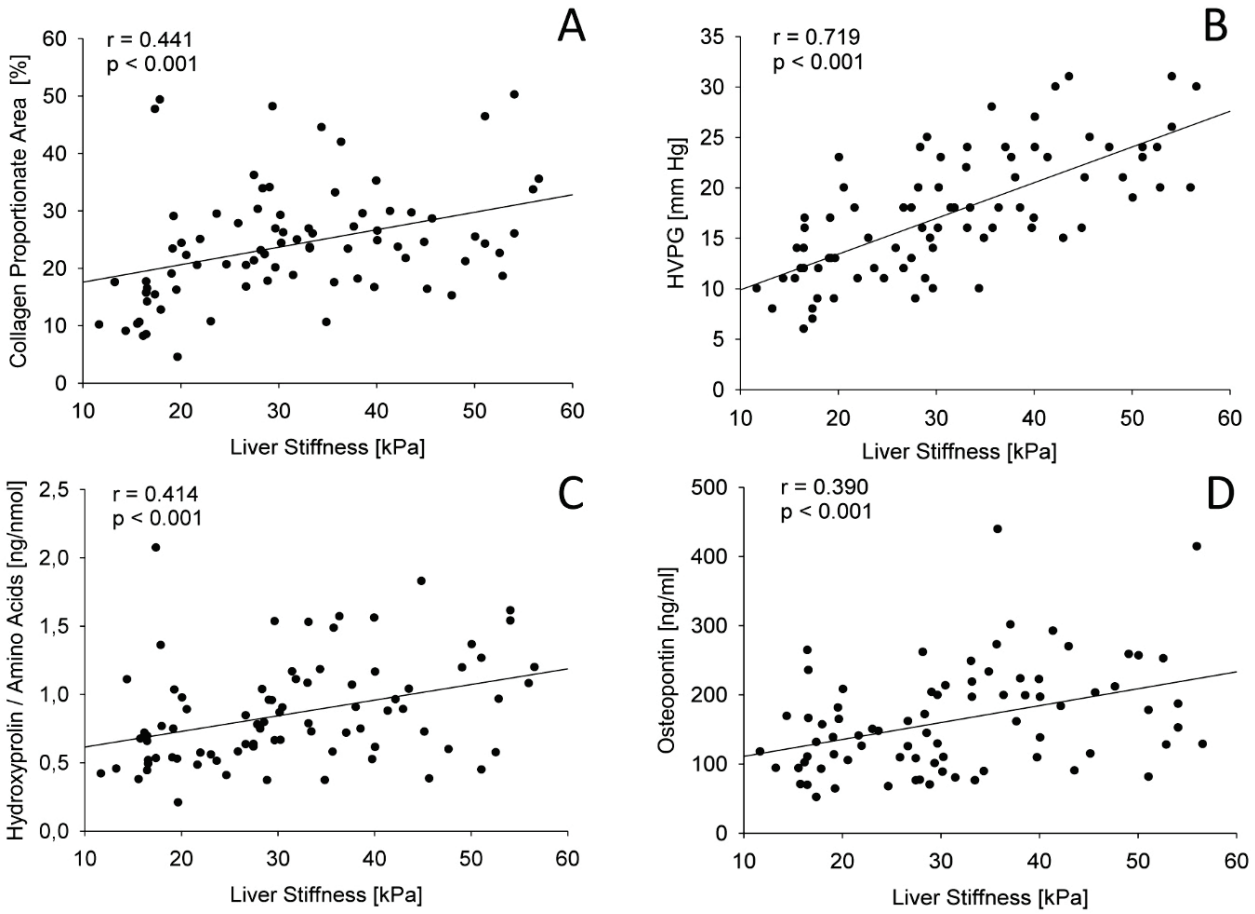

Fig. 2. Regression lines representing relation between LS and CPA (**A**), HVPG (**B**), HP/ AA (**C**) and osteopontin (**D**).

Modelling of a composite predictive factor

LS, considered as a dependent variable, and HVPG, CPA, HP/AA together with other studied non-invasive predictors considered as independent variables, were analysed by the multiple linear regression (Table 4). The strongest association was found between LS and HVPG (p < 0.0001). A weaker association was found between LS and CPA (p = 0.0188) and even weaker association was found between LS and osteopontin (p = 0.0241). The stepwise modelling showed only a minimal increase in r^2 after addition of CPA to HVPG (0.5073 vs. 0.5513) and further addition of osteopontin increased r^2 only to 0.5795. The stepwise modelling showed that major contribution to the LS value formation had HVPG and the contribution of CPA and osteopontin were minimal. The derived formula expressing LS value formation is:

LS = –0.41 + (1,19 x HVPG) + (0,26 x CPA) + (0.03 x osteopontin),

or, after removal of osteopontin, impact of which on LS is low:

LS = 2.48 + (1.29 x HVPG) + (0.26 x CPA).

Discussion

The data obtained in our cohort of patients with advanced liver cirrhosis and 93 % proportion of patients with CSPH strongly suggest that LS is determined predominantly by HVPG whereas contribution of liver collagen content to LS is less variable and relatively low. This conclusion is independently supported by four previously published studies with similar study design –

Table 4. Multiple linear regression analysis.

	Stepwise multiple linear regression
Dependent variable	**LS**
Independent variables	**p value**
HVPG [mm Hg]	**< 0.0001**
CPA [%]	**0.0069**
HP/Aminoacids [ng/nmol]	0.7795
ELF score [points]	0.8659
Hyaluronic acid [ng/ml]	0.9113
PIIINP [ng/ml]	0.4730
TIMP-1 [ng/ml]	0.3691
Osteopontin (ng/ml)	**0.0278**
VCAM-1 [ng/ml]	0.6735

Table 5. Correlation of CPA with HVPG across the published studies.

Publication	Calvaruso *et al.*2009	Isgro *et al.* 2013	Calvaruso *et al.* 2012	Nilesen *et al.* 2014	Current study
Patients population	HCV post LT	HCV post LT	HCV post LT	Mixed aetiology liver cirrhosis	Various aetiology of liver cirrhosis
Liver tissue specimen aquisition	Transjugular biopsy	Transjugular biopsy	Transjugular biopsy	Explanted liver	Explanted liver
Patients total, N	250*	63	62	41	81
Patients with CSPH, N, (%)	21 (8 %)	7 (18 %)	13 (30 %)	31 (76 %)	74 (91 %)
CPA vs. HVPG, Spearman's r, (p)	0.61 ($p < 0.001$)	0.41 ($p = 0.01$)	0.37 ($p = 0.017$)	0.537 ($p < 0.001$)	0.324 ($p = 0.003$)

*250 measurements in 115 patients

HVPG measurement by hepatic vein catheterization and CPA assessment. Three of the studies were conducted on patients after liver transplantation for chronic HCV infection, the fourth study was done on liver transplant candidates with liver cirrhosis of various aetiologies. The proportions of non-cirrhotic and cirrhotic patients and the proportion of cirrhotic patients with and without CSPH were different across the studies. The highest proportion of patients with CSPH was in our cohort which included only cirrhotic patients. This allowed us to compare the relationship between CPA and HVPG across the studies (Table 5). Based on this comparison we realized that correlation between CPA and HVPG becomes weaker with increasing proportion of cirrhotic patients with CSPH in the study cohort.

It is also apparent that correlation between CPA and HVPG in patients after liver transplantation was generally weaker than in the non-transplanted subjects and decreased with the proportion of patients with CSPH in the cohort. The same trend was evident from the comparison of the data presented in (Nielsen *et al.* 2014) with our study.

Another two studies focused on correlation between CPA and LS; the correlation was weaker in the study with higher proportion of patients with cirrhosis. Buzzetti *et al.* (2019) evaluated 76 non-cirrhotic patients with NASH and the Spearman's r was 0.73 whereas in the non-transplanted HCV and HBV subgroups in the study by Calvaruso *et al.* (2012), the Spearman's r was 0.59 and 0.61, respectively, and the proportion of patients with cirrhosis was 18 and 11 %, respectively. The Spearman's r in our cohort of patients with liver cirrhosis was 0.441. Our results thus comply with the concept explaining worsening of portal hypertension by intrahepatic

angiogenesis in patients with advanced liver cirrhosis (Thabut and Shah 2010).

Our conclusion is further supported by the recent animal study indicating that formalin treated porcine liver showed increased stiffness and portal hypertension in the *ex vivo* model (Yang *et al.* 2017). Treatment of the liver with formalin modelled the changes in the liver collagen structure leading to the increase of portal pressure. Another animal study showed that in the perfused bovine liver start both: new collagen fibres production and tissue and vessels remodelling (Yarpuzlu *et al.* 2014).

In addition to CPA, we also assessed the HP/AA ratio as a collagen content marker in the explanted liver as an alternative assuming that HP/AA assessment might be superior for evaluation of liver collagen content. Accordingly, a recent MRI animal study showed good correlation of HP with the extracellular space in the liver (Luetkens *et al.* 2018). The HP/AA ratio showed the same correlation with HVPG as CPA and somewhat weaker correlation with LS. Contrarily, multiple linear regression identified only CPA to be the variable associated with LS. The fact that HP/AA correlated with the ELF score better than CPA is not surprising because ELF score is a composite parameter calculated from blood levels of three biomarkers associated with collagen turn-over. Based on these data, we speculate that CPA reflects not only liver collagen content but also collagen density and remodelling whereas the HP/AA is just an indicator of collagen content.

The design of our study allowed us to assess collagen content in substantially larger samples of the tissue than might have been obtained by transjugular liver biopsy performed together with HVPG measurement. On the other hand, this approach might have been associated with a methodological bias owing to the time period between LS and HVPG measurement and collagen content assessment in the explanted liver. However, in chronic liver diseases, the period between disease onset and advanced liver cirrhosis development takes years or decades whereas the interval between LS and HVPG measurement and LT took only a couple of weeks.

We conclude that our study brought new insight into pathophysiology and relevance of LS in patients with advanced liver cirrhosis. Correct interpretation of the LS value is important for understanding liver pathology and appropriate clinical decisions.

Abbreviations

CSPH, clinically significant portal hypertension; HA, hyaluronic acid; HVPG, Hepatic venous pressure gradient; LR, likelihood ratios; LS, Liver stiffness; LSPS, Liver Spleen Platelets Score; MELD, Model for End-Stage Liver Disease; NPV, negative predictive value, PH, Portal hypertension; PIIINP, Amino-Terminal Propeptide of Type III Procollagen; PPV, positive predictive value; TIMP-1, Tissue Inhibitor of Matrix Metalloproteinase 1; VCAM-1, Vascular Cell Adhesion Molecule 1; 2D-SWE, two-dimensional real time shear-wave elastography.

Conflict of Interest

There is no conflict of interest.

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