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ORIGINAL ARTICLE

Non-invasive candidate protein signature predicts hepatic venous pressure gradient reduction in cirrhotic patients after sustained virologic response

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Abstract

Background and Aims: A reduction in hepatic venous pressure gradient (HVPG) is the most accurate marker for assessing the severity of portal hypertension and the effectiveness of intervention treatments. This study aimed to evaluate the prognostic potential of blood-based proteomic biomarkers in predicting HVPG response amongst cirrhotic patients with portal hypertension due to Hepatitis C virus (HCV) and had achieved sustained virologic response (SVR).

Abbreviations: AUROC, area under the receiver operating characteristic curve; BL, baseline; BMI, body mass index; CSPH, clinically significant portal hypertension; DA, differential abundance; DAA, direct-acting antiviral; ELF, enhanced liver fibrosis score; EOS, end of study; EOT, end of therapy; FHVP, free hepatic vein pressure; fM, femtomole; HA, hyaluronic acid; HCV, hepatitis C virus; HVPG, hepatic venous pressure gradient; ICC, Intra-class correlation coefficients; LOD, limits of detection; LOOCV, leave-one-out cross-validation; LOQ, limits of quantification; LSM, liver stiffness measurement; LT, liver transplantation; ML, machine Learning; NASH, non-alcoholic steatohepatitis; NIR, no information rate; NPV, negative predictive value; NSBBs, non-selective beta-blockers; PIIINP, procollagen III N-terminal propeptide; PLT, platelet count; PPV, positive predictive value; PH, portal hypertension; SOC, standard of care; SOMAmers®, Slow Off-rate Modified Aptamers; SVR, sustained virologic response; TE, transient elastography; TIMP-1, tissue inhibitor of metalloproteinase; WHVP,, wedged hepatic venous pressure.

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Methods: The study comprised 59 patients from two cohorts. Patients underwent paired HVPG (pretreatment and after SVR), liver stiffness (LSM), and enhanced liver fibrosis scores (ELF) measurements, as well as proteomics-based profiling on serum samples using SomaScan® at baseline (BL) and after SVR (EOS). Machine learning with feature selection (*Caret*, *Random Forest and RPART*) methods were performed to determine the proteins capable of classifying HVPG responders. Model performance was evaluated using AUROC (*pROC R package*).

Results: Patients were stratified by a change in HVPG (EOS vs. BL) into responders (greater than 20% decline in HVPG from BL, or <10 mmHg at EOS with >10 mmHg at BL) and non-responders. LSM and ELF decreased markedly after SVR but did not correlate with HVPG response. SomaScan (SomaLogic, Inc., Boulder, CO) analysis revealed a substantial shift in the peripheral proteome composition, reflected by 82 significantly differentially abundant proteins. Twelve proteins accurately distinguished responders from non-responders, with an AUROC of .86, sensitivity of 83%, specificity of 83%, accuracy of 83%, PPV of 83%, and NPV of 83%.

Conclusions: A combined non-invasive soluble protein signature was identified, capable of accurately predicting HVPG response in HCV liver cirrhosis patients after achieving SVR.

KEYWORDS

hepatic venous pressure gradient, machine learning, predictive analysis, proteomics, sustained virologic response

1 | INTRODUCTION

Cirrhosis, the last stage of the fibrogenic process in chronic hepatic injury leads to over one million annual deaths worldwide from hepatic dysfunction and hepatocellular cancer.[1,2] Portal hypertension (PH) represents a main driver of decompensation, and hence mortality in patients with cirrhosis.[3] HVPG is a clinically significant measure of PH that has shown to be an independent prognostic indicator for predicting clinical outcomes in patients with cirrhosis. HVPG-response, described as a reduction of 10%–20% or <12 mmHg from baseline HVPG, reduces the risk of clinical complications[3] and was established a surrogate marker for evaluating effectiveness of non-selective beta-blockers (NSBBs). Improvement in fibrosis has been shown in patients who have lower pretreatment HVPGs when obtained before the initiation of medical management. HVPG <10 mmHg accurately predicted the possibility of regression of cirrhosis in hepatitis C virus-infected liver transplantation (LT) recipients who achieved SVR.[4] Valuable insights have been gained from chronic hepatitis C patients concerning the relationship between changes in HVPG resulting in liver fibrosis reversal and the improvement of clinical outcomes.[5,6] More recently, Mandorfer et al.[7], evaluated change in HVPG after SVR as a tool to assess the prognosis of patients with pretreated CSPH. HVPG measurements, however, are limited by the invasiveness of the procedure and availability being restricted to specialized facilities. The clinical need for non-invasive measures capable of diagnosing progression and regression in chronic liver diseases is therefore paramount.

Key points

Our study identified novel, non-invasive candidate biomarkers from serum capable of accurately distinguishing HVPG responders from non-responders (AUROC .86, sensitivity 83% and specificity 83%). These findings would be valuable in assessing the effectiveness of treatments in liver disease patients without having to endure invasive procedures.

Recently, several non-invasive measurements have been proposed as surrogates for invasive HVPG determination. One transient elastography (TE) study[8,9] suggested that a post-treatment LSM/PLT criteria (LSM < 12 kPa & PLT > 150 G/L) could exclude clinically significant portal hypertension (CSPH) ([HVPG] ≥ 10 mmHg) (sensitivity: 99.2%) in HCV patients achieving SVR.[10] Despite ongoing efforts to identify non-invasive biomarkers that correlate with HVPG for diagnostic purposes, efforts aimed at identification of biomarkers that correlate with or are capable of diagnosing a change in HVPG response after treatment remain to be reported. We hypothesize that a soluble biomarker approach utilizing the SomaScan® proteomic platform can identify protein changes that correlate with similar changes in HVPG. SomaScan involves a unique protein measurement procedure that utilizes Slow Off-Rate Modified Aptamers (SOMAmers) that bind to proteins with high affinity and specificity[11] and can be used to identify diagnostic

signatures of several other diseases.[12–14] In particular, SomaScan and machine learning methods were used to identify novel proteins to classify the different stages of fibrosis.[15]

This study focused on patients with HCV-related cirrhosis and PH who achieved SVR after standard of care (SOC) antiviral therapy. Paired HVPG, LSM and serum samples were collected from patients at BL and EOS. The aim of our study was to identify which non-invasive biomarkers are capable of classifying patients with an HVPG response after eradication of Hepatitis C virus. In this population, we evaluated: (a) the changes of non-invasive biomarkers and their relationship with HVPG response, (b) the prognostic potential of a multi-marker protein panel to classify HVPG responders in HCV cirrhosis patients after SVR.

2 | METHODS

2.1 | Patients

This analysis used data from two non-interventional studies in patients with compensated cirrhosis due to HCV infection (Figure 1).

2.1.1 | CBASICHR0006 (Cohort 1)

In this retrospective cohort, consenting patients were selected from local databases of individuals with CSPH (HVPG ≥10 mmHg),[16–19] who were referred to the hospital clinic of Barcelona between 2015 and 2017. This study included patients with compensated cirrhosis who were treated with direct-acting anti-viral (DAA) agents (treatment durations ranged from 12 to 24 weeks) and who attained SVR, defined as the continued absence of HCV 6 months post-therapy. Compensated cirrhosis was defined clinically as the absence of or no history of ascites, variceal haemorrhage, hepatic encephalopathy. In addition to HVPG, all patients underwent a clinical examination, liver assessment, including LSM and blood chemistry. All patients underwent a clinical follow-up of at least 1 year without addition or dose change or stoppage of non-selective beta-blocker (NSBB). In addition, serum was banked at the same time points. In total, 40 patients enrolled in the study with available HVPG measurements, liver stiffness and other biomarkers and endpoints were considered in the study.

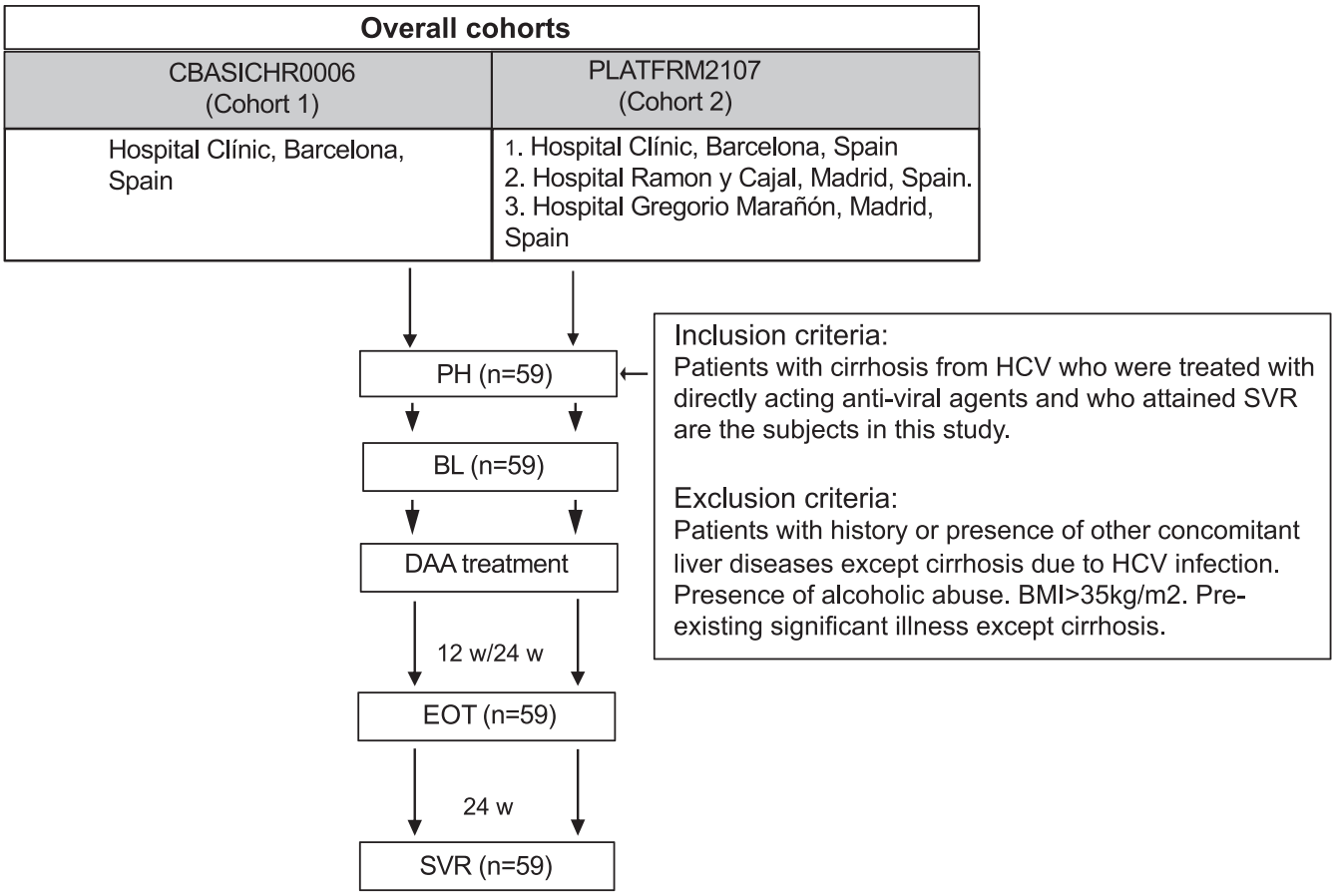

FIGURE 1 Patient flowchart. The study resulted in 59 patients with confirmed PH. All the patients underwent antiviral treatment and all the patients achieved SVR. BL, baseline; DAA, direct-acting antiviral; EOS, end of study; EOT, end of therapy; PH, portal hypertension; SVR, sustained virologic response.

2.1.2 | PLATFRM2107 (Cohort 2)

This was a prospective, multicentre, exploratory biomarker study focusing on chronically infected hepatitis C virus patients with compensated cirrhosis. The multicentre study consisted of three European centres (Hospital Clinic, Barcelona; Hospital Universitario Ramon y Cajal, Madrid; Hospital General Universitario Gregorio Marañon, Madrid) with extensive experience in hepatic hemodynamic procedures and HVPG measurements.

This cohort comprised a mixed demographic of male and female patients aged between 18 and 70 years with compensated cirrhosis from HCV infection. Patients with a diagnosis of compensated cirrhosis had PH as confirmed by HVPG. Nineteen patients provided informed consent and those with 7 ≤ HVPG ≤ 20 mmHg were considered eligible to continue in the study and return to the clinic within 10 days for HCV therapy. For inclusion as a compensated cirrhotic patient in this study, a documented history of a prior liver biopsy or imaging (such as ultrasonograms, computed tomographic scans, magnetic resonance imaging) with findings consistent with cirrhosis. Otherwise, the patients required a liver stiffness measurement of ≥16 kPa as determined by transient elastography. Furthermore, patients with evidence or history of hepatic decompensation, defined as ascites, variceal haemorrhage or hepatic encephalopathy were excluded from the study. Patients were excluded upon initiation or dose change of a NSBB or nitrate within 3 months prior to the study and until EOS. A total of 19 patients received local DAA anti-viral therapy for 12- or 24-weeks agents and who attained SVR (Continued absence of HCV 6 months post-therapy). This part of the study consisted of screening and baseline visits to establish eligibility, two study visits and a follow-up visit approximately 24 weeks after completion of therapy.

2.2 | HVPG measurement, liver stiffness, and serum markers

HVPG measurements were performed according to a standardized protocol at baseline and 24 weeks after completion of antiviral treatment. Measurements of wedged (occluded) hepatic venous pressure (WHVP) and free hepatic venous pressure (FHVP) were made in triplicate. Permanent tracings for each measurement were obtained, and the mean value was recorded for that visit. HVPG was calculated as the difference between the mean WHVP and mean FHVP. All tracings were evaluated centrally by a single reader; the intra-class correlation coefficients (ICCs) at each time point were .97 or greater.

Measurement of liver stiffness with Fibroscan was performed according to a standardized protocol at baseline and 24 weeks after completion of antiviral treatment by the clinic and followed with standard protocol. A commercially available soluble biomarker panel, ELF, as well as its components, tissue inhibitor of metalloproteinase (TIMP-1), hyaluronic acid (HA) and procollagen III N-terminal propeptide (PIIINP) were obtained at the time of HVPG measurement.

In this study, a clinically relevant HVPG response was achieved after SVR and defined as: (1) Achieving less than 10 mmHg at EOS in patients with greater than 10 mmHg at BL or (2) A decrease in HVPG >20%.

2.3 | Serum acquisition and storage

Fasting plasma samples or serum samples were collected at the same time as baseline and 24 weeks after end of the treatment for measurement using the multiplex assay. Blood was collected in EDTA-treated tubes for plasma or serum separating tubes for serum, centrifuged at 1.9 *g* for 15 min within 120 min of collection, and the supernatant aliquoted and frozen at −80°C.

2.4 | SomaScan proteomic profiling

The SomaScan proteomic profiling platform utilizes SOMAmers® (Slow Off-rate Modified Aptamers) that binds to target proteins with high affinity and specificity.[20] The assay version used in this study includes 5034 SOMAmers, of which 4783 measure human proteins from 4137 distinct human genes with femtomole (fM) limits of detection over a wide range of protein levels in plasma or serum (>8 logs of concentration). The platform exhibits median limits of detection and quantification (LOD/LOQ) of 40 and 100 fM, respectively and ~5% coefficients of variation for median intra- and inter-assay variability.[21] A hybridization array to capture SOMAmers, quantitatively determines the proteins present by converting the assay signal (relative fluorescence units) into the relative abundance of an analyte.[22] Assays were performed by SomaLogic in collaboration with Novartis according to the protocol described by our group and others.[23,24]

2.5 | Statistical analysis and predictive modelling

Statistical tests were performed according to the distribution of the data. Clinical characteristics and patient numbers were expressed as counts. Percentages were used for categorical variables, means (SDs) were calculated for normally distributed continuous variables and medians were calculated for variables with skewed distributions. Spearman rank-order correlation was used to determine the correlations between HVPG versus LSM versus ELF variables. *T*-tests' were used for continuous data and the fisher exact test for categorical data.

Differential abundance (DA) of SOMAmers based on the pairwise comparison between BL and EOS were determined using Limma, an R package previously applied to microarray data from R/Bioconductor framework. DA SOMAmers were identified using empirical Bayes (eBayes) moderation to determine precision weights for

each observation.[25] Upregulated SOMAmers were defined as those with a $\log_2$ (fold change) greater than +.2 (20%), and down-regulated SOMAmers as those with a $\log_2$ (fold change) less than −.2 (20%). Significance was defined using an adjusted *p*-value of <.05 defined by the Benjamini-Hochberg procedure.

Machine learning (ML) methods provided through the R/Bioconductor framework R version 4.4.3[26] were used to identify proteins capable of classifying patients with clinically significant reductions in HVPG specified in this study. Propensity score matching was completed using *MatchIt* (package version 4.4.0), a recognized method of controlling selection bias due to confounding variables.[27] The model included all variables with potential clinical relevance and comparator group (responder and non-responder). To construct a balanced matrix of HVPG responder ($n=22$) and non-responder ($n=22$) groups for biomarker identification, the nearest neighbour matching without replacement (1:1) on the estimated propensity score method was applied.

The balanced dataset was randomly split and stratified into a train and test set with a ratio of 70:30, using the "createDataPartition" method within the *CARET* package. The classification model was trained to differentiate between HVPG responders and non-responders. A leave-one-out cross-validation was performed on the training set and the optimal hyperparameters were alpha=.5 and lambda=.1.

The identification of features, as candidate biomarkers, involved a two-step process. (1) The full list of proteins was used as input and feature selection performed. (2) The features from step 1 were combined with the DA SOMAmers attained from a union from the pairwise comparison from the responders. The union of SOMAmers were used as input and the most important features were extracted from the model (feature selection). Feature selection methods were performed using *Caret (ElasticNet)*, *Random Forest*, and *RPART*, to reduce the number of input variables/proteins to those most important for the model.

The performance of the protein panel for predicting HVPG response was determined using logistic regression models and the performance evaluated using the areas under receiver operating characteristic curves (AUROCs). Specifically, AUROC using *pROC* (package version 1.17.0.1) was used to illustrate/calculate the models' ability to classify the responder and non-responder groups.

Accuracy, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and no information rate (NIR) of the model was determined using standard definitions.

3 | RESULTS

3.1 | BL characteristics

A total of 59 patients diagnosed with HCV-related cirrhosis were included in the study. Cohort 1 comprised 40 patients from Hospital Clinic, Barcelona and with SOC therapy. Cohort 2 comprised 19 patients with SOC therapy, 10 patients were from Hospital Ramon y Cajal, 8 patients were from Hospital Clinic and 1 patient from Hospital General Universitario Gregorio Marañón. The BL characteristics of these patients are found in Table 1. Briefly, the patients' mean age was 63.2 years old, ranging from 43 to 81 years, and 61.0% ($n=36$) were male. The mean HVPG at baseline was 14.1 ± 3.3 mm Hg, all the patients had PH (HVPG >7 mmHg), 37 (62.7%) patients had CSPH (HVPG >=10 mmHg, but <16 mmHg) and 17 (28.8%) patients had high-risk PH (HVPG >=16 mmHg) (Table S1). Nine patients (16.7%) were on stable NSBB treatment during the period described in the methods (Table S3).

TABLE 1 Baseline clinical characteristics of patients in cohort 1 and cohort 2. Demographic characteristic average and range values for participating subjects.

Mean (SD)	Overall ($n=59$)	Cohort 1 ($n=40$)	Cohort 2 ($n=19$)
Demographics			
Age (y)	63.2 (7.1)	66.6 (8.0)	56.2 (6.2)
Male sex, *n* (%)	36 (61.0%)	17 (42.5%)	19 (100.0%)
Weight (kg)	73.2 (12.1)	70.1 (11.7)	77.1 (12.5)
Height (cm)	166.6 (9.3)	162.7 (10.6)	170.5 (7.9)
BMI (kg/m^2)	26.6 (3.1)	26.4 (2.7)	26.5 (3.4)
HVPG (mmHg)	14.1 (3.3)	14.4 (3.1)	13.6 (3.9)

Note: *n* = number of participants in the full study.
Abbreviations: BMI, body mass index; HVPG, hepatic venous pressure gradient.

3.2 | HVPG, liver stiffness and ELF measurement after SVR

Overall, 59 patients completed the study with an EOS visit. The EOS HVPG measurement was performed 6.54 (±.83) months after the end of IFN-free therapy, .54 (±.83) months after of patients was achieved SVR. Serum samples for ELF and proteomic measurements were attained simultaneously at the time of HVPG measurement. The EOS liver stiffness measurement was performed 6.08 (±1.67) months after the end of IFN-free therapy, .28 (±1.22) months of achieving SVR. SVR resulted in a statistically significant decrease in HVPG from baseline of 14.1 ± 3.3 mmHg to 11.9 ± 3.9 mmHg ($p<.001$; Figure S1 and Table 2). In addition, SVR ameliorated portal hypertension across all HVPG strata at baseline (Table S1). While measures of free hepatic vein pressure (FHVP) were not altered after SVR (7.1 to 7.5 mmHg, $p=.2799$), wedged hepatic vein pressure values (WHVP) decreased from 20.9 to 19.4 mmHg ($p<.001$) (Table S5).

Overall, the change of LSM, ELF score and individual markers of ELF are listed in Table 2. A highly significant reduction ($p<.001$) in mean LSM from BL (28.6 ± 13.9 kPa) to EOS (19.4 ± 9.2 kPa) was reported after SVR. A decrease in mean ELF was also observed from BL to EOS (11.4 ± 1.1 kPa to 10.2 ± 1.0 kPa, $p<.001$). Amongst the individual components of ELF score, TIMP-1, PIIINP and HA were significantly reduced in comparison to baseline.

TABLE 2 Changes in clinical characteristic measured at BL and at 24 weeks after DAA therapy (EOS).

Variable	BL ($n=59$) Mean (SD) (min–max)	EOS ($n=59$) Mean (SD) (min–max)	p-Value
HVPG (mmHg)	14.1 (3.3) (7.5–20.0)	11.9 (3.9) (5.0–20.0)	<.001
LSM (Kpa)	28.6 (13.9) (10.4–70.6)	19.4 (9.2) (7.6–46.4)	<.001
ELF	11.4 (1.1) (9.2–13.7)	10.2 (1.0) (8.5–12.7)	<.001
TIMP-1 (ng/mL)	423.5 (159.8) (192.2–1300.0)	277.7 (79.0) (101.3–516.9)	<.001
PIIINP (ng/mL)	17.5 (8.3) (5.5–46.5)	10.7 (5.2) (3.9–26.7)	.004
HA (ng/mL)	429.3 (426.3) (53.4–2064.5)	166.8 (177.1) (20.6–867.4)	<.001

Note: n = number of participants in the full analysis set. p-Values are for the comparison between BL and EOS.

Abbreviations: BL, baseline; ELF, enhanced liver fibrosis score; EOS, end of study; HA, hyaluronic acid; HVPG, hepatic venous pressure gradient; LSM, liver stiffness measurement; PIIINP, procollagen III N-terminal propeptide; TIMP-1, tissue inhibitor of metalloproteinase.

TABLE 3 Clinical characteristics of patients with HVPG responders and non-responders within total cohort.

Variable mean(SD) (min–max)	Overall					
	HVPG responders ($n=28$)			HVPG non-responders ($n=31$)		
	BL	EOS	p-Value	BL	EOS	p-Value
HVPG (mmHg)	13.7 (3.6) (7.5–20)	9.6 (2.9) (5–16)	<.0001	14.4 (3.1) (7.5–20)	14.0 (3.5) (6.5–20)	.1459
LSM (Kpa)	26.3 (11.6) (10.4–70.6)	16.8 (7.4) (7.6–35.8)	.0003	30.7 (15.6) (10.4–69.1)	21.8 (10.0) (8.6–46.4)	<.0001
ELF	11.3 (1.0) (9.7–13.4)	9.9 (.8) (8.5–11.9)	<.0001	11.5 (1.2) (9.2–13.7)	10.5 (1.0) (8.5–12.7)	<.0001
TIMP-1 (ng/mL)	406.7 (102.1) (235.2–610.9)	253.6 (48.7) (165.2–307.7)	<.0001	434.2 (200.0) (192.2–1300.0)	299.9 (94.3) (101.3–516.9)	<.0001
PIIINP (ng/mL)	17.3 (9.6) (6.4–46.5)	9.3 (4.1) (3.9–25.1)	<.0001	17.6 (7.2) (5.5–32.2)	12.1 (5.7) (4.3–26.7)	<.0001
HA (ng/mL)	364.5 (405.2) (59.9–1682.0)	134.3 (163.5) (28.6–867.4)	.0003	485.5 (442.9) (53.45–2064.5)	200.0 (187.1) (20.6–807.3)	.0002

Note: n = number of participants in the full analysis set. Clinical characteristic average and range values for participating subjects. p-Values generated from HVPG responder and HVPG non-responder comparisons.

Abbreviations: BL, baseline; ELF, enhanced liver fibrosis score; EOS, end of study; HA, hyaluronic acid; HVPG, hepatic venous pressure gradient; LSM, liver stiffness measurement; PIIINP, procollagen III N-terminal propeptide; TIMP-1, tissue inhibitor of metalloproteinase.

The characteristics of HVPG, LSM, ELF and each of the components of ELF in HVPG responders and non-responders are summarized in Table 3. Patients classified as HVPG responders or and non-response had similar HVPG values at BL. HVPG responders had a HVPG value of 13.7 ± 3.6 mmHg at BL with a mean decrease to 9.6 ± 2.9 mmHg ($p=1.6e{-}.5$). The patients classified as non-responders had a minimal HVPG change from 14.4 ± 3.1 mmHg in BL to 14.0 ± 3.5 mmHg at EOS. On the contrary, the value of LSM, ELF and each component of ELF decreased in both HVPG responders and non-responders after SVR. There was no correlation observed between a reduction of LSM or ELF and a change of HVPG either in the responder or the non-responder groups (Figure S2). Five patients were lacking paired LSM, ELF or SomaScan results at BL and EOS were consequently excluded from further analysis.

3.3 | The discovery of serum proteins arising from SomaScan

The 54 patients with paired serum samples were SomaScan profiled to identify 82 circulating proteins (from 86 SOMAmer measurements) that were significantly different between BL and EOS in both cohorts ($p < .05$ and fold change >.2; Figure 2; Table S4).

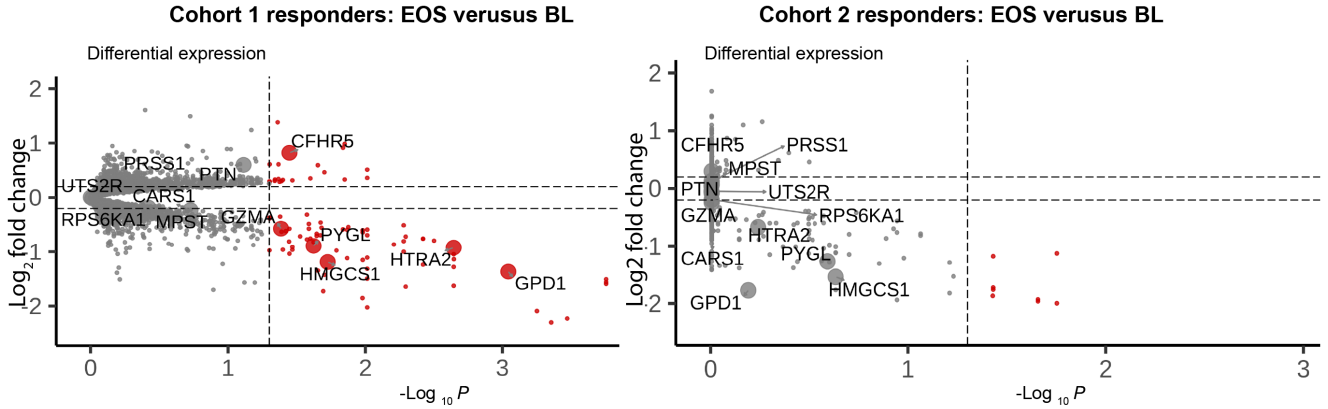

FIGURE 2 'Volcano plot' of EOS versus BL of HVPG responders: The x-axis is the negative logarithm (base 2) of the Benjamini–Hochberg adjusted p values and the y-axis is the fold change (FC, logarithm base 2). The vertical and horizontal lines reflect the filtering criteria 2 (FC = ±0.2 and p value = 1.3, -log10(0.05)). The dots represent the proteins detected by the somamers.

TABLE 4 HVPG prediction (responder vs. non-responder classification) performance using ElasticNet (Caret).

	Sensitivity	Specificity	Accuracy	PPV (%)	NPV (%)	*p*-Value	NIR	95% CI	AUC
Protein panel	.83	.83	.83	83	83	.0008	.5	.63–.95	.86
Clinical features	0	.67	.33	0	40	.97	.5	.16–.55	.5
Protein panel + Clinical features	1	.5	.74	67	100	.0113	.5	.53–.9	.94
ELF	.64	.76	.65	67	64	.006	.51	.54–.75	.63
LSM	.41	.59	.5	50	50	.54	.50	.39–.61	.50

Note: Multivariate modelling was used to classify HVPG response based on the 12 protein-panel. The model's performance was evaluated using AUROC with 95% confidence intervals, sensitivity, specificity, PPV and NPV. Clinical features included age, gender, height, weight, and BMI. ELF and LSM were also incorporated separately into the model.

Abbreviations: AUROC, the area under the receiver operating characteristic curve; BMI, body weight index; HVPG, hepatic venous pressure gradient; NIR, no information rate; NPV, negative predictive value; PPV, positive predictive value; SPMS, SOMAmer-pull down mass spectrometry.

Thirty-seven percent of the up-and down-regulated proteins observed within the HVPG responder sub-group were liver-specific proteins. Among these proteins, the majority (50%) were linked to lipid metabolism, oxidoreductases (17%), haemostasis (17%), amino acid metabolism (3%), whilst 13% were non-specific. Our findings also highlighted differential regulation of proteins that contribute to innate and adaptive immune response, metabolic pathways, extracellular matrix remodelling, antimicrobial activity, muscle contraction and angiogenesis.

3.4 | Candidate novel soluble/non-invasive biomarkers identified capable of diagnosing HVPG response and their predictive performance evaluated

Based on the definition of HVPG response in this study, various supervised machine learning algorithms were used to generate classification models. The machine learning methods used were ElasticNet, RPART and RF, and all samples were cross-validated using a leave-one-out method due to the limited number of samples.

Feature selection or the importance of each feature for the classification of responders and non-responders, was performed by the ML algorithms, and their union resulted in a 12-protein panel: GPD1, PYGL, RPS6KA1, MPST, HMGCS1, UTS2R, CARS1, PTN, PRSS1, HTRA2, GZMA and CFHR5 (Tables 4 and 5).

We then evaluated the performance of the prediction model (12-protein panel) using AUROC on the test cohort. The model performed well, with an AUROC .86 (95% CI .63–.95).

Two patients with subclinical PH (<10 mmHg) at baseline were noted in the training set. To determine the robustness of the model, these two patients were removed from the train set and the performance of the model was re-evaluated. Again, the model performed well, with an AUROC .91 (95% CI .63–.95) (Table S2).

3.5 | Association of selected novel soluble/non-invasive candidate biomarkers with clinical variables

Baseline clinical features (age, gender and Body Mass Index [BMI] (height, weight)) were evaluated as potential confounders of HVPG response. There were no differences in BMI or the prevalence of overweight/obesity between patients with HVPG response and

TABLE 5 Biological functional class and major tissue expression for proteins included in HVPG response prediction panel.

Gene symbol	Protein name	Protein function	Tissue expression
PYGL	Glycogen phosphorylase	Lipid metabolism	Liver
HMGCS1	Hydroxymethylglutaryl-coa synthase	Lipid metabolism	Liver
MPST	3-mercaptopyruvate sulfurtransferase	Lipid metabolism	Liver
CFHR5	Complement factor h-related protein 5	Liver haemostasis	Liver
GPD1	Glycerol-3-phosphate dehydrogenase	Glycerophospholipid metabolism	Adipose tissue, skeletal muscle
UTS2R	Urotensin-2 receptor	Peptide ligand-binding receptors	Multiple
CARS1	Cysteinyl-TRNA Synthetase 1	tRNA Aminoacylation	Multiple
PTN	Pleiotrophin	Neuronal signalling	Brain, parathyroid gland
HTRA2	Serine protease HTRA2	Apoptosis	Multiple
GZMA	Granzyme A	Immune response	Lymphoid tissue
RPS6KA1	Ribosomal protein S6 kinase alpha-1	Immune response	Macrophage
PRSS1	Trypsin-1	Digestion	Pancreas

non-response at BL (Table S3). Moreover, the change in weight and BMI from BL to EOS HVPG did not differ between these subgroups. Whilst LSM, ELF score and each component of ELF at BL were comparable between both groups, patients achieving a HVPG response had a lower LSM (16.8 [7.37] kPa vs.21.8 [10.04] kPa; $p = .03450$) and ELF (9.9 [.80] vs. 10.5 [1.05]; $p = .0270$). However, there is no significant absolute (ΔLSM, $p = .8407$; ΔELF $p = .1047$) and relative (ΔLSM, $p = .1614$; ΔELF $p = .0534$) decreases in patients with a HVPG response. PLT followed the same pattern with no difference at BL and EOS with absolute and relative values of decreases after antiviral therapy in patients. Please see Table S3. Matching was performed to control or exclude confounding variables which might otherwise influence the results. Multivariate modelling of the clinical features alone resulted in an AUROC of .5 (95% CI .16–.55) LSM alone with AUROC of .5 (.39–.61) and ELF alone with .6 (.54–.75), which demonstrates minimal impact on the prognostic potential of HVPG response. The 12-protein panel associated with the HVPG response was further analysed for correlation with the clinically relevant features. Combining the clinical features with the protein panel did not significantly improve the performance of the protein panels' prediction results. And despite not outperforming ΔHVPG (AUROC = 1), which was used to define the responder and non-responder groups, the 12-protein-only model was capable of classifying HVPG with high accuracy (83%, $p = .0008$) (Figure 3, Table 4).

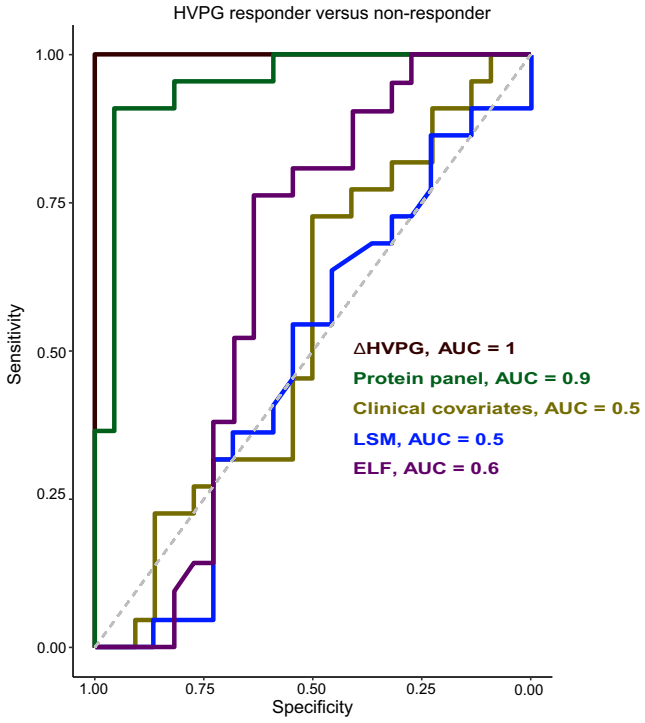

FIGURE 3 ROC curves generated to evaluate the model's ability to classify HVPG responder and non-responder groups, based on a 70%:30% train:test set split. The protein panel alone (green) resulted in an AUC = 0.9 (95% CI 0.63-0.95, P = 0.0008), LSM alone (blue) resulted in an AUC of 0.5 (95% CI 0.39-0.61), ELF alone (purple) resulted in an AUC of 0.6 (95% CI 0.54 - 0.75) and the clinical variables alone (brown) with an AUC of 0.5 (95% CI 0.16-0.55, P = 0.97). ROC: receiver operating characteristic; AUC: area under the receiver operating characteristic curve; LSM: liver stiffness measurement; ELF: enhanced liver fibrosis score. 95% confidence intervals based on a final leave-one-out-cross-validation (LOOCV) for GLM-LR.

4 | DISCUSSION

Regression of liver fibrosis after achieving SVR through interferon-based regimens or DAA's has been well established.[28–31] The reversibility of liver disease is an excellent model to study the dynamic changes in HVPG after the cure of the primary etiologic factor. Data from patients with HCV and HBV have shown that with long-term follow-up, decreases in HVPG mirror hemodynamic and fibrotic changes that occur after treatment.[32,33] Furthermore, reductions in HVPG observed at earlier time points could translate into clinically meaningful benefits in patients with CSPH by reducing the risk of complications,[5,7,34] as well as decreasing hepatic necro-inflammatory activity.[6] This demonstrates that portal hypertension and chronic liver disease can be

reversed, reflected by the reduction in HVPG after the removal of the injurious etiological agent or through pharmacological interventions. It is likely, however, that outcomes of cirrhosis vary amongst individuals. For example, minimal changes in HVPG are observed in a high proportion of cirrhosis patients, consequently leaving the risk for further decompensation.[35–37] Monitoring the change in HVPG is therefore paramount in gauging treatment response and guiding further management. Consistent with previous studies from HCV-related or other aetiologies, we observed 47.4% (28/59) of patients achieved HVPG response with SVR. Moreover, our study provides valuable insights into the prognostic value of HVPG response by evaluating LSM, ELF and most importantly, proteomic signatures. These findings may contribute to a better understanding of the underlying mechanisms involved in HVPG response and inform the development of novel diagnostic strategies for cirrhotic patients with portal hypertension. In this study, LSM and ELF were considered as promising markers for evaluating fibrotic content in chronic liver diseases, was elevated to levels consistent with the diagnosis of cirrhosis in both cohorts.[38–40] We further evaluated the reduction of LSM and ELF in patients after DAA treatment, to confirm correlations with changes in HVPG. With therapy, LSM, ELF score and individual components of ELF, HA, TIMP-1 and PIIINP declined after SVR which suggested continued remodelling of the fibrotic matrix after successful viral eradication, reflecting the intrinsic regenerative properties of the liver. Despite evidence of spontaneous regression after removal of the etiologic cause, we observed that the reduction in LSM, ELF and its components, did not correlate with the reduction of HVPG. LSM and ELF have been commonly accepted to support the assessment of liver fibrosis. LSM shows a high accuracy for diagnosing CSPH.[40] However, in our study, these are likely inappropriate for evaluating dynamic HVPG response and correlated clinical outcome, especially for patients where HVPG does not regress or even progress. This result is consistent with the findings of Mandorfer[7] who showed LSM, platelet count, VWF, and VITRO, as well as their relative/absolute changes during treatment for diagnosing an HVPG reduction greater than 10% was inadequate for clinical use.

The serum biomarker panel from the robust proteomic profiling allowed for the relative quantification of proteins linked to the biological plausibility of HVPG response. Protein-based platform analysis can provide incremental insights into certain proteins involvement in the pathogenesis of portal hypertension and enable the prediction of HVPG response. Using multiplex proteomic profiling platform (SomaScan), with 5034 SOMAmers, we selected 12 proteins linked to biological processes related to lipid metabolism, immune response, haemostasis, oxidoreductase, and others which correlated with metabolic disorders, tissue injury and fibrosis. Our study revealed a subset of proteins that were significantly decreased in HVPG responders compared with non-responders. This subset of proteins (ADAMTSL2, ACY1, ADH4, ALDOC, ASL, ENPP7, FBP1 and FTCD) were also reported to correlate with fibrosis in a recent study.[15] Particularly, ADAMTSL2 was strongly classified as a non-invasive biomarker of significant and advanced fibrosis in non-alcoholic steatohepatitis (NASH) patients. These results indicate the association of these proteins with advanced fibrosis.

SomaScan profiling combined with machine learning algorithms identified a candidate circulating protein biomarker panel associated with clinically significant decreases in HVPG. This yields an AUROC of .86, thus supporting the utility of this proteomic-based panel for predictive purposes. Furthermore, the performance of the protein panel for classifying HVPG responders was not influenced by the clinical variables which were included in our study. Among the selected proteins within the panel, UTS2R, a ligand-binding receptor, has been reported to correlate with liver cirrhosis and portal hypertension. Notably, a report showed the antagonist of UTS2R could reduce hepatic resistance and portal hypertension.[41] Consistent with 82 proteins identified by SomaScan, the major representation of the proteins (PYGL, HMGCS1, MPST and GPD1) were involved with lipid metabolism or glycerophospholipid metabolism. The second group of proteins (GZMA, CFHR5 and RPS6KA1) were related to immune response. Another approach to test whether the non-invasive soluble biomarkers recognize changes in HVPG is to examine associations between the metabolites and HVPG response to propranolol. The combination of metabolites helps identify acute HVPG response to propranolol.[42]

Our study has several strengths, including the measurement of HVPG at specialist centres, expert evaluation of liver stiffness and measurement of serum biomarkers at a dedicated central laboratory before and after HCV elimination. Using the proteomics platform and machine learning offers an innovative tool to discover novel disease biomarkers and an understanding of the different pathophysiological processes involved in disease development and progression. However, several limitations of the study warrant discussion. First, the number of patients was small; hence, further validation in an additional, independent study is necessary. Second, the study was restricted to patients with compensated cirrhosis due to HCV infection. Validation of the protein panel in patients with cirrhosis of alternate aetiologies is necessary to confirm generalizability of our findings. To ensure that the results are not confounded by the effects of other drugs on HCV clearance, we excluded patients who experienced change in their initial dosages or discontinuation of NSBBs closely related to HVPG. We acknowledge that data on concomitant medication use, including statins or angiotensin-converting enzyme inhibitors were not collected. However, our study had a longer follow-up period of 24-week post-treatment, which may have minimized the potential interference of temporary changes in statin use on our study outcomes. Nonetheless, we recognize that the impact of concomitant medication use in our findings cannot be fully determined without comprehensive data on medication changes during the study.

5 | CONCLUSIONS

In conclusion, our results indicate a proteomic signature of 12 proteins capable of diagnosing clinically significant reductions in HVPG among patients with HCV cirrhosis following curative therapy. Such factors may have potential utility as non-invasive biomarkers in predicting the improvement of portal hypertension. In turn, to identify

the clinical outcome in cirrhosis patients with PH and guiding appropriate disease management in chronic fibrotic liver disease.

AUTHOR CONTRIBUTIONS

Juan Carlos Garcia-Pagán, Agustin Albillos, Rafael Bañares and Chinweike Ukomadu designed the study and interpreted the data. Shola M. Richards, Fang Guo, Anna Baiges, Sabela Lens, Luis Tellez, Javier Martinez-González, Fanny Turon, José Ferrusquía-Acosta, Valeria Perez-Campuzano, Marta Magaz, Xavier Forns and Virginia Hernández-Gea collected and assembled the data. Shola M. Richards, Fang Guo, Florian Nigsch, Alok Pachori, Heng Zou, Yiming Zhang, Rebecca Pitts, Nancy Finkel, Joseph Loureiro, Dale Mongeon, Shenglin Ma, Mollie Watkins, Florine Polus, Michael Badman and Andreas W. Sailer performed data analysis and/or interpretation. All authors reviewed and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

SMR, FG, FN, HZ, YZ, RP, NF, JL, DM, SM, MW, FP, MB, and AWS are employees and stockholders of Novartis. All other authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data are available on request due to privacy and ethical restrictions. SomaScan data are available through a data use agreement with Novartis Institutes for BioMedical Research and are available upon reasonable request (lori.jennings@novartis.com).

ETHICS APPROVAL AND PATIENT CONSENT STATEMENT

Both studies were conducted in accordance with the Declaration of Helsinki and approved by the local ethics committees, CEIm Hospital Clinic de Barcelona, CEIm Hospital Universitario Ramón y Cajal and CEIm Hospital General Universitario Gregorio Marañón. Eligible patients were included in the study after providing written, IEC approved informed consent. Informed consent was obtained before conducting any study-specific procedures and documented in the patient's source documents.

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SUPPORTING INFORMATION

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