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The polymorphic variant of SerpinB3 (SerpinB3-PD) is associated with faster cirrhosis decompensation

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Summary

Background: SerpinB3 is a cysteine protease inhibitor involved in liver disease progression due to its proinflammatory and profibrogenic properties. The polymorphic variant SerpinB3-PD (SB3-PD), presents a substitution in its reactive centre loop, determining the gain of function.

Aims: To disclose the clinical characteristics of a cohort of patients with cirrhosis in relation to the presence of SB3-PD and to assess the effect of this genetic variant on fibrogenic and inflammatory cytokines in vitro.

Methods: We assessed SB3 polymorphism in 90 patients with cirrhosis, prospectively followed up in our referral centre. We used HepG2 and HuH-7 cells transfected to overexpress either wild-type SB3 (SB3-WT) or SB3-PD to assess their endogenous effect, while LX2 and THP-1 cells were treated with exogenous SB3-WT or SB3-PD proteins.

Results: Patients carrying SB3-PD had more severe portal hypertension and higher MELD scores, than patients carrying SB3-WT. In multivariate analysis, SB3-PD was an independent predictor of cirrhosis complications. Patients with SB3-PD polymorphism presented with more severe liver fibrosis and inflammatory features. Hepatoma cells overexpressing SB3-PD showed higher TGF-β1 expression than controls. The addition of recombinant SB3-PD induced an up-regulation of TGF-β1 in LX2 cells and a more prominent inflammatory profile in THP-1 cells, compared to the effect of SB3-WT protein.

Conclusions: The polymorphic variant SB3-PD is highly effective in determining activation of TGF-β1 and inflammation in vitro. Patients with cirrhosis who carry SB3-PD polymorphism may be more prone to develop severe liver disease progression. However, further validation studies are warranted to support the in vivo relevance of this polymorphism.

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1 | INTRODUCTION

Cirrhosis is the end-stage of chronic liver disease. Its natural history can be divided into an asymptomatic phase, termed 'compensated' cirrhosis, characterised by an alteration in the liver tissue and functionality, but still clinically silent, and a 'decompensated' phase, marked by the development of ascites, portal hypertension-related bleeding (PHB), hepatic encephalopathy (HE) and jaundice.[1–3] Transition from a compensated to a decompensated stage is the most common outcome in patients with compensated cirrhosis and occurs at a rate of 5%–7% per year,[4,5] being mainly driven by fibrosis of hepatic tissue and consequently by worsening of portal hypertension (PH).[6–8] The progression of liver disease in the decompensated phase may be accelerated by the development of further episodes of decompensation[2] such as (re)bleeding, renal impairment (refractory ascites, hepatorenal syndrome), hepatopulmonary syndrome and sepsis (spontaneous bacterial peritonitis), and is characterised by poor survival rates.[2] PH is indeed the initial and main consequence of cirrhosis and is responsible for the majority of its complications, with the extent of liver fibrosis representing one of the major determinants of its development.[3,9] Hepatic fibrosis is a dynamic process characterised by the net accumulation of extracellular matrix (ECM), or scar, resulting from chronic liver injury of any aetiology. In this scenario, the activation of hepatic stellate cells (HSCs) into proliferative, fibrogenic myofibroblasts is well established as the central driver of hepatic fibrosis.[3,10,11] Activated HSC are the principal cells able to produce type I collagen in fibrotic liver and they contribute to the development of liver fibrosis through autocrine and paracrine loops of TGF-β1, generally considered as the most potent fibrogenic cytokine stimulating collagen production.[11–13]

Cirrhosis is also characterised by enhanced inflammatory responses, with marked individual variability, irrespective of the grade of liver failure,[8,14,15] and patients who develop acute decompensation and acute on chronic liver failure show an alteration of the inflammatory response and increased levels of different proinflammatory cytokines.[16,17]

Along these lines, SerpinB3 (previously known as squamous cell carcinoma antigen or SCCA1) is a cysteine protease inhibitor that has recently emerged as a mediator involved in chronic liver disease (CLD) progression.[18–20] SerpinB3 is indeed up-regulated in the liver by oxidative stress, iron overload, hypoxia and chronic inflammation.[21–23] Recent findings have characterised SerpinB3 as a novel pro-inflammatory mediator in non-alcoholic steatohepatitis (NASH), as this molecule, produced and released by damaged or stressed hepatocytes, plays an important role among the signals that contribute to promoting the pro-inflammatory phenotype of liver macrophages in NASH.[24] In addition, it has also been demonstrated that SerpinB3 is able to directly activate human HSC, resulting in a strong up-regulation of genes involved in fibrogenesis and angiogenesis.[25,26] Moreover, in vitro studies indicate that TGF-β1 is significantly increased in SerpinB3-transfected cells,[27] with the integrity of the reactive site loop (RSL) of SerpinB3 being crucial to determine the up-regulation of this cytokine, as shown by the results obtained in cells transfected with RSL-deleted mutants.[27] Recently, a new polymorphic variant of SerpinB3 (SCCA-PD, GeneBank Accession No EU852041; rs:3180227G>A), has been characterised,[28,29] and is now termed SerpinB3-PD (SB3-PD), in line with the current nomenclature for SerpinB3.[30] This variant presents the substitution Gly351Ala in the reactive centre loop of the protein and this single amino acid substitution improves the functional activity of the serpin.[29] The aim of the present study was to determine the clinical characteristics and the outcome of a cohort of cirrhotic patients in relation to the presence of SB3-PD and to assess the effect of this genetic variant on TGF-β1 and on inflammatory cytokines using in vitro models.

2 | PATIENTS AND METHODS

2.1 | Patients

The study is based on the analysis of all medical records of eligible outpatients with cirrhosis prospectively and consecutively followed up at the Care Management Program (CMP)[31] of the University Hospital of Padova from April 2007 to December 2013, as previously reported.[32]

The study, which was conducted according to the Declaration of Helsinki, was part of a national Project (FIRB Prot. RBLA03S4SP 005) and was approved by the local Ethics Committee. All patients provided written informed consent. The inclusion criteria were as follows: (a) diagnosis of liver cirrhosis based on histological findings or on clinical, biochemical, ultrasonographic and/or endoscopic findings[33]; (b) age ≥ 18 years. The exclusion criteria were as follows: (a) Patients with hepatocellular carcinoma (HCC) at the baseline, (b) chronic kidney disease (CKD) with serum creatinine ≥2 mg/dL, (c) severe extrahepatic disease (i.e. congestive heart failure New York Heart Association [NYHA] class >2, chronic obstructive pulmonary disease Global Initiative for Chronic Obstructive Lung Disease [GOLD] class >2); (d) extra-hepatic malignancy; (e) previous liver transplant; (f) HIV infection; (g) the lack of availability of medical records. Enrolment flow chart for the study population and numbers of patients included in the study across years are shown in Figures S1 and S2, respectively.

Patients' medical records were reviewed for the following events which defined hepatic decompensation and episodes of further decompensation as defined according to Baveno VII[2]: development of ascites and/or requirement of first paracentesis, admission for grade II-IV hepatic encephalopathy (HE), portal hypertension-related bleeding (PHB), hepatorenal syndrome (HRS-AKI), spontaneous bacterial peritonitis (SBP). These events were recorded if present at baseline and during the first 5 years of follow-up. Data on previous decompensation of cirrhosis were assessed from patients' medical history and the appearance of the

first episode of decompensation or episodes of further decompensation(s) were registered during follow-up. The development of HCC and portal vein thrombosis (PVT) and complications related to HCC and PVT were registered in the medical records, but not considered as clinical complications. During the baseline visit carried out for all patients, a blood sample for the assessment of SerpinB3 polymorphism was taken.

A detailed description of the CMP is reported elsewhere.[31,32] Abdominal ultrasound was performed at least twice a year in all patients and an upper endoscopy was performed yearly in patients with oesophageal varices (EV) and every 2–3 years in patients without EV. Following the classification of Beppu et al,[34,35] the EVs were graded from F0 to F3, with F1 indicating small EVs and F2 and F3 indicating large EVs. All patients received prophylactic treatment for variceal bleeding, when indicated, and etiological treatment of underlying liver disease was administered in all eligible patients. All the charts of emergent admissions to the hospital were carefully reviewed and clinical and laboratory data were recorded. The detection of alcohol consumption during follow-up was also supported by the assessment of urinary ethyl glucuronide (uEtG) and carbohydrate-deficient transferrin (CDT), when available.[36] Patients were followed up until death, liver transplantation, last follow-up visit or for a maximum of 10 years. Cause of death was recorded according to the review of medical records. Clinical complications developed after the diagnosis of HCC were not considered in the analysis, since the presence of the tumour could profoundly affect clinical outcome.

2.2 | Immunohistochemistry, Sirius red staining and histomorphometric analysis

For immunohistochemistry analysis, five patients (three patients carrying SB3-WT and two patients carrying SB3-PD polymorphism) were randomly selected among those transplanted during follow-up. The use of human material conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved for this study by the University of Padova Bioethical Committee.

Paraffin-embedded human liver specimens were immunostained as previously reported.[25] Briefly, paraffin sections (2 μm thick), mounted on poly-L-lysine coated slides, were incubated with the monoclonal antibody against CD68 (Biorad; dilution 1:80) and monoclonal antibody against α-SMA (Sigma Aldrich Spa; dilution 1:400). After blocking endogenous peroxidase activity with 3% hydrogen peroxide and performing microwave antigen retrieval, primary antibodies were labelled by using EnVision, HRP-labelled System (DAKO) and visualised by the substrate 3′-diaminobenzidine. For negative controls, the primary antibodies were replaced by isotype- and concentrations-matched irrelevant antibodies. Collagen deposition was determined by Picro-Sirius Red staining, followed by a rapid immersion of slides in diluted haematoxylin, in order to obtain nuclear counterstaining of liver sections, as previously described.[37]

Quantification of fibrosis (α-SMA positive areas and collagen deposition) as well as of CD68 immuno-positivity were performed by histomorphometric analysis using the PANNORAMIC MIDI (3DHISTECH, Digital Pathology Company) automatic digital slide scanner to collect panoramic images of the entire section which was then analysed using the ImageJ software.

2.3 | Study endpoints

The primary endpoint of the study was to assess the incidence of clinical complications in outpatients with cirrhosis during the first 5 years of follow-up in relation to the presence of SB3-PD polymorphism and of other variables capable of predicting the development of either the primary episode of decompensation and further episodes of decompensations. Moreover, the predictors of the composite endpoint first/further episodes of decompensation or liver-related death were also analysed. Liver-related mortality was defined according to the underlying cause of death registered on the death certificate.

2.4 | Power analysis

The study was designed assuming a cumulative prevalence of SB3-PD of about 30%, an accrual interval of 12 months, and an additional maximum follow-up of 120 months. A median survival time was assumed to be around 72 months, based on previous data from this cohort. If the true hazard ratio (relative risk) of patients carrying SB3-PD relative to subjects carrying SB3-WT is at least 2.5, 63 patients carrying SB3-WT and 25 patients carrying SB3-PD should be included in the analysis to be able to reject the null hypothesis that survival curves of patients carrying SB3-PD and SB3-WT are equal with probability (power) 0.80. Type I error probability associated with this test of this null hypothesis is 0.05. Thus, the overall sample size of the cohort was decided to consist in a total of 90 patients, pending that at least 25 will be SB3-PD carriers.

2.5 | In vitro experiments

2.5.1 | Cell lines

The effect of SB3-WT and SB3-PD on TGF-β1 mRNA and protein expression was evaluated in hepatoma cells (HepG2 and Huh7) transfected with plasmid vectors carrying the wild-type protein SerpinB3 (SB3-WT) or the polymorphic variant SB3-PD, as previously reported,[38] whereas cells transfected with the empty vector (pcDNA3.1D/V5-His-TOPO, Invitrogen Life Technologies) were used as control.

Moreover, the paracrine effect of SerpinB3 was evaluated at different time points (1–48 h) in human stellate cell line (LX2

cells) as well as in the THP-1 cell line, exposed to human recombinant SerpinB3 isoforms, either the wild type (SB3-WT) and the polymorphic variant SB3-PD (200 ng/mL), obtained as previously described.[38]

2.5.2 | Cell culture conditions

Human monocytic leukaemia cell line (THP-1, American Type Culture Collection, ATCC) and human LX2 cells (kindly provided by Prof. Scott L. Friedman, Icahn School of Medicine) were cultured in Dulbecco's modified Eagle's medium (Sigma Aldrich Spa), supplemented with 10% fetal calf serum and 100 U/mL penicillin, 100 μg/mL streptomycin and 25 μg/mL amphotericin-B (Sigma Aldrich Spa). Both cell lines were treated with human recombinant SB3-WT or SB3-PD, at the final concentration of 200 ng/mL for the indicated time points. Before incubation with the different SerpinB3 isoforms, THP-1 cells were seeded 7×10^5 in 35 mm petri dishes, differentiated for 48 h with phorbol 12-myristate 13-acetate (PMA, 50 nM) and cultured for 24 h in fresh medium.

2.5.3 | mRNA quantification

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. After determination of the purity and the integrity of total RNA, complementary DNA synthesis and quantitative real-time PCR reactions (RT-PCR) were carried out as previously described[39] using the CFX96 Real-Time instrument (Bio-Rad Laboratories Inc).

The relative gene expression was generated for each sample by calculating $2^{-\Delta\Delta Ct}$.[40] The list of primers used for the experiments is listed in Table S1. Samples were run in triplicate and mRNA expression was generated for each sample. Specificity of the amplified PCR products was determined by melting curve analysis.

2.5.4 | Pro-inflammatory cytokines release quantification

The presence of biologically active TGF-β1 protein in the supernatant of transfected cells was determined using the TGF-β1 Emax ImmunoAssay system, following the manufacturer's instructions (Promega Corporation). Biological activity was quantified using standard curves, with a linear range between 15.6 and 1000 pg/mL, as previously reported.[27]

Moreover, collected medium from THP-1 differentiated macrophages untreated or treated with SB3-WT or SB3-PD at a concentration of 200 ng/mL were processed in order to evaluate levels of the cytokines primarily involved in the development and in worsening of PH (TGF-β and VEGF)[3,8] and of the most important cytokines driving the inflammatory process (TNF-α, IL-6, IL-8 and MCP-1), favouring acute decompensation and further episodes of decompensation,[3,16]

by employing human ELISA kit (Invitrogen-Thermo Fisher Scientific, EHOSM), according to the manufacturer's instructions.

2.5.5 | SB3-PD polymorphism

Genomic DNA was extracted from frozen whole blood, obtained from the patients with cirrhosis and stored at −80°C, with EuroGold Blood DNA Mini Kit Plus (Euroclone) according to the manufacturer's instructions. Nucleic acid was eluted in 50 μL elution buffer and quantified with Nanodrop 2000c spectrophotometer (ThermoScientific).

High-resolution melting (HRM) analysis of Genomic DNA was performed using SsoFast EvaGreen Supermix (Bio-Rad). Short amplicon covering the mutation region of the SerpinB3 gene was amplified using the CFX96 real-time PCR (BioRad) and results were analysed using the CFX Manager™ and Precision Melt Analysis™ software (BioRad). The PCR reaction was performed in a 20 μL final reaction volume containing 200 nmol of each primer and SsoFastTM EVA Green 5X SuperMix (BioRad). The system amplification protocol was: 95°C for 3 min; 50 cycles of 95°C for 10 s; 27 cycles of 60°C for 30 s. Subsequently, a melt curve was generated by heating from 65 to 95°C with 0.2°C increment. Precision Melt Analysis software was then used to identify areas of stable pre- and post-melt fluorescence from the HRM curve and automatically determined a cluster of each genotype. Some positive (SNP) and negative (wild type) controls were examined in the same PCR and melt reaction to verify the precision of melt analysis.

The genetic results were confirmed randomly by direct sequencing, using an ABI 310 automated DNA sequencer (Apply Biosystems), according to the manufacturer's instructions.

2.6 | Statistical analysis

Normally distributed continuous variables were reported as means with standard deviation and compared with the Student's t test. Non-normally distributed continuous variables were reported as median, interquartile range (IQR) and compared with the Mann–Whitney U test. Categorical variables were reported as proportions and compared with the chi-square test with continuity correction and/or Fisher's exact test when appropriate. The incidence of complications was estimated by the Kaplan–Meier method and comparisons between groups were made with the log-rank test. Patients who died or who received a transplant before developing a complication of cirrhosis (ascites, HE, GHB and SBP) were censored at the time of death or transplantation. Variables found to have a $p < 0.05$ in the univariate analysis were included in a multivariate Cox proportional hazards model, with backward elimination. The hazard ratios (HR) and their 95% confidence intervals (CIs) were calculated. When scores for liver disease were included in the multivariate analysis, their components were excluded to avoid multicollinearity. Platelet count and not spleen diameter were included in the multivariate analysis to avoid

multicollinearity. Non-normally distributed continuous variables were log-transformed to be included in the multivariate models. Fine-Grey competing risk model was calculated to estimate the cumulative incidence of first/further decompensation or liver-related death, using liver transplantation as a competing risk factor. A nomogram for Complications Free Survival (CFS) time, defined as the time (months) from the enrolment (first visit of the patient at our outpatient clinic) and the development of the first decompensated event or further decompensated events, has been derived from the multivariable model and calculated with Rms-package. To assess the net improvement attributable to SB3-PD, the Net Incremental Reclassification index has been computed.[41] Multiple testing was not accounted for. All tests were two-tailed and $p < 0.05$ were considered significant. The statistical analysis was performed with the use of the SPSS statistical package, version 29.0 and the R-System ver 4.1.

3 | RESULTS

3.1 | In vivo results

3.1.1 | Clinical features at presentation in relation to SB3-PD polymorphism

Baseline characteristics of the study population and the comparison between patients carrying SB3-PD polymorphism or SB3-WT are reported in Table 1. Twenty-seven patients (30%) carried SB3-PD polymorphism (only 1 patient carried homozygous SB3-PD mutation). No differences were found between the two populations in terms of age, sex, aetiology of liver disease (alcohol use and hepatitis C virus [HCV] infection were the most common aetiologies of cirrhosis), duration of follow-up (72 months, 31.7–118) and presence of a previous decompensation. Even if there were no patients registered with NASH/MAFLD aetiology, a significant prevalence of our patients had metabolic features of metabolic syndrome and MAFLD aetiology (diabetes, dyslipidaemia and arterial hypertension), but none of these three comorbidities were significantly different in relation to the presence of SerpinB3-PD polymorphism. Percentage of patients taking therapy with beta-blockers was also comparable. Despite these similar demographic characteristics, patients carrying SB3-PD polymorphism had more frequently large EV (41.7% vs. 18.6%; $p = 0.029$), a larger spleen diameter (16.5 vs. 15.1 cm; $p = 0.036$) and accordingly, a more severe thrombocytopenia (73 vs. 101×10^9/L; $p = 0.007$) and higher values of fibrosis score (Apri test and FIB-4 score, $p = 0.005$ and $p = 0.014$, respectively). It is worth to note that at the first episode of decompensation, a trend to younger age was observed in patients carrying SB3-PD, compared to patients carrying SB3-WT polymorphic variant (46.4 ± 9.8 vs. 52.7 ± 10 years, $p = 0.078$). The percentage of patients with ascites at the baseline was similar between the two groups (25.9% vs. 22.6%; $p = 0.733$), but patients carrying SB3-PD and ascites were significantly younger than patient carrying SB3-WT and ascites (48.3 vs. 60.5 years, $p = 0.01$; Figure S3A). Similar data were also observed for patients

with features of severe liver disease, since among patients with MELD > 15, those carrying SB3-PD polymorphism were significantly younger than patients carrying SB3-WT (49 vs. 56 years; $p < 0.05$; Figure S3B).

In line with these results, patients carrying SB3-PD polymorphism had a significantly higher Child-Pugh score at baseline (7.3 vs. 6.5; $p < 0.05$), were more frequently in Child-Pugh class C (22% vs. 4.8%; $p = 0.033$) and had a higher MELD score (14 vs. 10.9; $p = 0.001$) at the baseline. Among the clinical parameters of Child-Pugh and MELD score, INR (International Normalised Ratio) was significantly higher in patients carrying SB3-PD polymorphism (1.49 vs. 1.28; $p = 0.004$). Table S4 summarises the allele frequency for SB3-PD polymorphism in patients included in the study, all of which were in the range of previously reported frequencies.[28]

It should be noted that four out of 41 patients (9%) with alcoholic cirrhosis and HCV plus alcoholic cirrhosis did not maintain abstinence during follow-up and none of them carried SB3-PD polymorphism.

3.1.2 | SB3-PD polymorphism and incidence of clinical complications

During 5 years of follow-up, patients carrying SB3-PD developed more frequent clinical complications (57.7% vs. 23.8%; $p = 0.002$). The type of complications was similar between the two groups of patients and ascites and HE were the most common types of complications of cirrhosis, accounting for three-quarters of overall complications. Twenty patients were transplanted and 30 patients died during follow-up.

The risk of development clinical complications during the first 5 years of follow-up was significantly higher in patients carrying SB3-PD than in those carrying SB3-WT ($p = 0.003$) (Figure S4), even considering separately the onset of first decompensation ($p = 0.035$) or further episodes of decompensation ($p = 0.006$) (Figure 1A,B). Moreover, the probability of transplant-free survival, estimated by Kaplan–Meier method, was significantly lower in patients carrying SB3-PD polymorphism than in those carrying SB3-WT ($p = 0.007$) (Figure 1C). In Figure 1D, the composite endpoint first/further episodes of decompensation or liver related death was analysed with fine-grey model with liver transplantation as competing risk, and patients carrying SB3-PD polymorphism resulted at higher risk of decompensation and death.

3.1.3 | Predictors of clinical complications

The development of clinical complications at 60-month was assessed in patients carrying SB3-PD or SB3-WT by univariate analysis. As reported in Table S3, no differences in terms of age, gender, aetiology and frequency of beta-blockers therapy were detected. Patients who developed clinical complications suffered more frequently a previous decompensation and had more frequently large EV and, as expected, patients who developed clinical complications had more severe signs

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TABLE 1 Baseline characteristics of patients included in the study and comparison of patients with and without polymorphic variant SerpinB3-PD.

Variable	Study population (n = 90)	SB3-PD (n = 27)	SB3-WT (n = 63)	p
Age (years)	54.7 (±9.1)	53.5 (±8.7)	55.1 (±9.2)	0.505
Gender (male – %)	69 (76.7)	23 (85.2)	46 (73)	0.211
Follow-up, months	72.5 (31.7–118)	73 (13–118)	66 (36–119)	0.449
Etiology				
HCV, n (%)[a]	37 (41.1)	12 (44.4)	25 (39.7)	0.618
HBV, n (%)	20 (22.2)	7 (25.9)	13 (20.6)	
Alcohol, n (%)	30 (33.3)	8 (29.6)	22 (34.9)	
Others	3 (3.3)	0 (0)	3 (4.8)	
Type 2 diabetes, n (%)	18 (20)	5 (18.5)	13 (20.6)	0.818
Dyslipidemia, n (%)	11 (12.2)	6 (22.2)	5 (7.9)	0.058
Arterial Hypertension, n (%)	26 (28.9)	5 (18.5)	21 (33.3)	0.155
Beta-Blockers treatment, n (%)	58 (64.4)	19 (70.3)	39 (61.9)	0.408
ALT (U/L)	45 (23–88)	52 (23–93)	39 (29–86)	0.843
AST (U/L)	64 (39–105)	66 (37–107)	62 (39–102)	0.827
Haemoglobin (g/dL)	13.2 (±2)	13.3 (±2)	13.2 (±2)	0.904
Apri test	2.85 (±2.6)	4.04 (±3.7)	2.34 (±1.8)	0.005
FIB-4	2.53 (1–7.4)	3.9 (0.8–14)	2.4 (1.1–6.3)	0.014
Large EV, n (%)	21 (23.3)	10 (37)	11 (17.4)	0.029
Ascites, n (%)	21 (23.3)	7 (25.9)	14 (22.2)	0.733
Spleen (cm)	15.6 (±2.9)	16.5 (±2.9)	15.1 (±2.7)	0.036
Platelet count (10^9/L)	88 (53–122)	73 (41–98)	101 (64–123.5)	0.007
Child-Pugh score	6.7 (±1.7)	7.3 (±2.2)	6.5 (±1.5)	0.046
Child-Pugh class n (%)				
A	53 (59)	15 (55)	38 (60)	0.033
B	28 (31)	6 (22)	22 (35)	
C	9 (10)	6 (22)	3 (5)	
MELD	11.8 (±4)	14 (±5)	10.9 (±3)	0.001
Bilirubin (μmol/L)	22.1 (15.1–32.2)	24.7 (13.1–39.1)	22 (16.4–31.3)	0.186
Albumin (g/L)	37.8 (±6)	37.1 (±6)	38.1 (±6)	0.478
INR	1.34 (±0.27)	1.49 (±0.3)	1.28 (±0.2)	0.004
Creatinine (μmol/L)	78 (±16)	77 (±17.3)	78 (±15.7)	0.738
HCC development n (%)	28 (31.1)	7 (25.9)	21 (33.3)	0.487
PVT development (%)	11 (12.2)	5 (18.5)	6 (9.5)	0.233
Previous decompensation, n (%)	46 (51.1)	12 (44.4)	34 (54)	0.408
Age of first decompensation (years)	51 (±10.2)	46.4 (±9.8)	52.7 (±10)	0.078
New onset of complications, n (%)	30 (33.3)	15 (55.5)	15 (23.8)	0.002
Type of complication				
Ascites, n (%)	16 (17.7)	9 (33.3)	7 (11.1)	0.533
SBP, n (%)	2 (2.2)	1 (3.7)	1 (1.5)	
HE, n (%)	7 (7.7)	4 (1.4)	3 (4.7)	
PHB, n (%)	5 (5.5)	1 (3.7)	4 (6.3)	

Note: Values are number (%), mean (±SD) or Median (interquartile range).

Abbreviations: ALT, alanine transaminase; APRI, AST to Platelet Ratio Index; AST, aspartate transaminase; EV, oesophageal varices; FIB-4, Fibrosis-4; HCC, hepatocellular carcinoma; HE, hepatic encephalopathy; INR, International Normalised Ratio; MELD, model of end-stage liver disease; PHB, portal hypertension-related bleeding; PVT, portal vein thrombosis; SB3-WT, SerpinB3 wild-type; SBP, spontaneous bacterial peritonitis; SD, standard deviation.
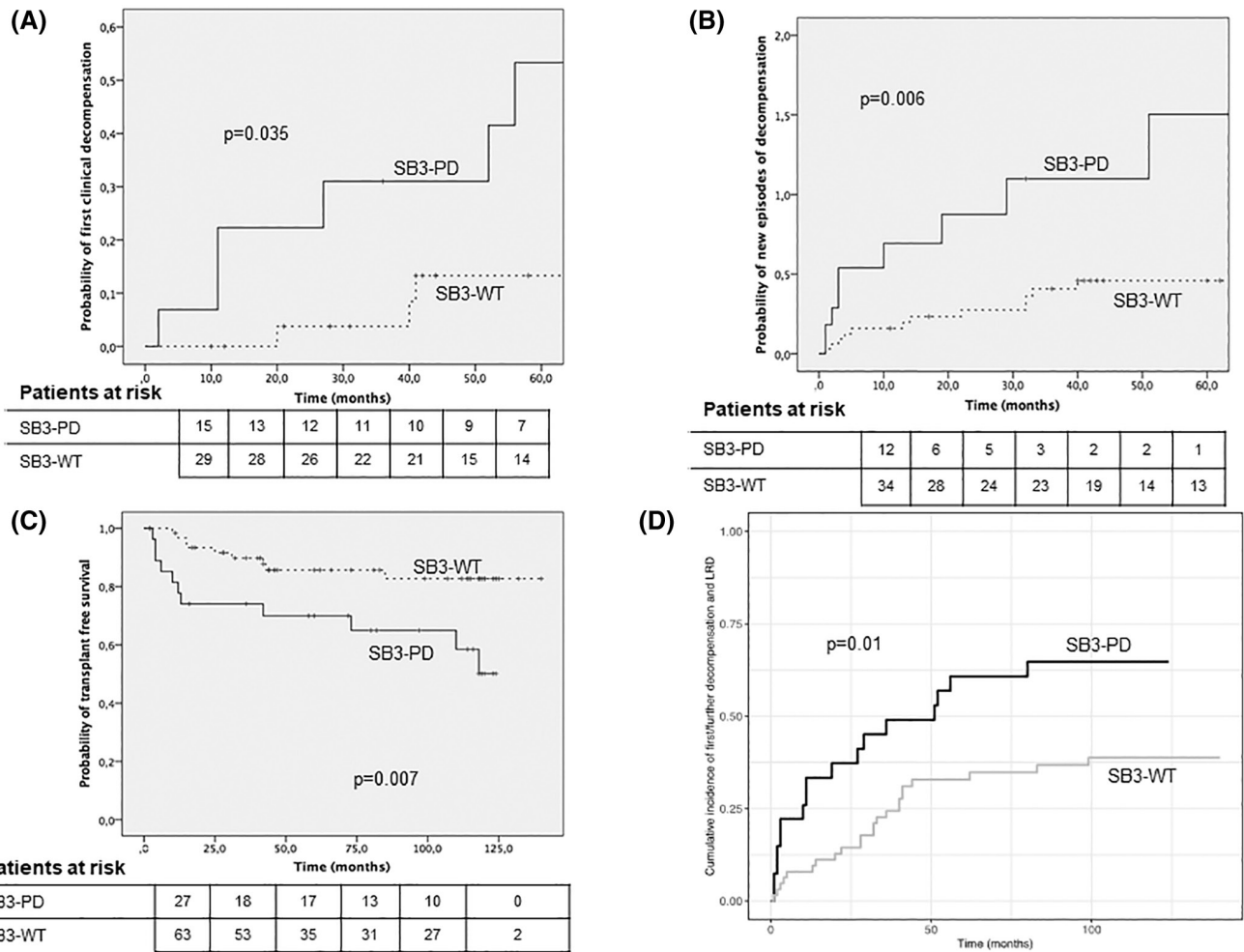
[a]11 patients had a concomitant alcoholic aetiology.

FIGURE 1 Patients with SB3-PD polymorphism have more severe liver disease. (A, B) Cumulative incidence of risk of first decompensation (A), and new onset of clinical complications (B), in cirrhotic patients with or without SB3-PD polymorphism during 60 months of follow-up was estimated by the Kaplan–Meier method and comparisons between groups were made with the log-rank test ($p = 0.035$ and $p = 0.006$, respectively). (C) The probability of transplant-free survival according to the presence of SB3-PD polymorphism was estimated by the Kaplan–Meier method and comparisons between groups were made with the log-rank test ($p = 0.007$). (D) Fine-Grey competing risk model to estimate the cumulative incidence of first/further decompensation or liver-related death, using liver transplantation as a competing risk factor. LRD, liver-related death.

of PH, as significantly higher frequency of ascites (42.9% vs. 13%; $p = 0.003$), lower platelet count (64 vs. 109; $p = 0.022$), larger spleen diameter (16.4 vs. 14.9 cm; $p = 0.023$), higher MELD score (13.5 vs. 10.8; $p = 0.003$), more severe class of Child-Pugh ($p < 0.0001$) and lower levels of haemoglobin (12.5 vs. 13.8 g/dL; $p = 0.005$).

In the multivariable analysis, MELD score (HR: 1.18; CI 95%: 1.04–1.35; $p = 0.008$), SB3-PD polymorphism (HR: 3.16; CI 95%: 1.31–7.6; $p = 0.010$), albumin (HR: 0.89; CI 95%: 0.82–0.98; $p = 0.019$), were found to be independent predictors of new onset of complications at 60 months (Table 2). The information about SerpinB3 polymorphism improved significantly the accuracy in model prediction (NRI: 0.99, 95% CI: 0.18–1.48) as compared with the model including only albumin and MELD score, and these data are in line with the results shown in Figure S5. MELD score, albumin and the presence of SB3-PD polymorphism, were graphically reported into a nomogram, stratifying complication-free survival (CFS) probability at 12,

36 and 72 months after the inclusion in the study (Figure S5). It is remarkable to observe that the presence of the polymorphism per se, increases the probability of decompensated events of more than 30 points. The composite endpoint first/further decompensation or liver-related death was also evaluated at univariate Cox regression analysis (Table S4) and multivariate analysis (Table 3). SerpinB3-PD polymorphism resulted independent predictor of first/further decompensation or liver-related death 3, together with MELD, albumin and haemoglobin. Then, we analysed the presence of fibrosis and inflammation at the histological level, in a subgroup of patients of similar age (SB3-WT patients were 51.3 ± 3 vs. SB3-PD patients were 47 ± 4; $p = 0.50$) who underwent liver transplantation (Figure 2). Patients carrying SB3-PD polymorphism had a more severe liver fibrosis, measured as collagen deposition ($p < 0.0001$), and significantly higher levels of alpha SMA ($p = 0.0002$). Moreover, CD68, a well-known marker of macrophage activation, was significantly more

TABLE 2 Multivariate analysis of predictors of onsets of first/further decompensation.

	aHR	(CI 95%)	p
MELD	1.18	1.04–1.35	0.008
SB3-PD	3.16	1.31–7.61	0.010
Albumin (g/L)	0.89	0.82–0.98	0.019

Note: Variables included in the model: large EV (0.079), male gender (0.053), haemoglobin (0.052), ascites ($p=0.255$), platelet count ($p=0.620$), age at inclusion ($p=0.869$) and previous decompensation ($p=0.579$).

Abbreviations: aHR, adjusted hazard ratio; CI, confidence interval; EV, oesophageal varices; MELD, model of end-stage liver disease; SB3, SerpinB3.

TABLE 3 Multivariate analysis of predictors of onset of first/further decompensation events or liver-related death.

	aHR	(CI 95%)	p
MELD	1.20	1.09–1.32	<0.0001
SB3-PD	2.14	1.10–4.18	0.024
Albumin (g/L)	0.911	0.85–0.96	0.002
Haemoglobin (g/dl)	0.66	0.54–0.83	0.005

Note: Variables included in the model: haemoglobin (0.052), EV (0.088), male sex (0.650), age at inclusion (0.387), platelet count (0.545) and previous decompensation (0.488).

Abbreviations: aHR, adjusted hazard ratio; CI, confidence interval; EV, oesophageal varices; MELD, model of end-stage liver disease; SB3, SerpinB3.

expressed in patients carrying SB3-PD polymorphism ($p<0.05$) (Figure 2).

3.2 | In vitro results

3.2.1 | SB3-PD polymorphism induces high levels of profibrotic and proinflammatory cytokines

As a first step, we decided to evaluate whether the SB3-PD polymorphism may affect TGF-β1 synthesis and release, as compared with the SB3-WT variant. For this purpose, liver cancer cells, (HepG2 and Huh-7) were transfected with plasmid vectors carrying the wild-type SerpinB3 (SB3-WT) or the polymorphic variant SerpinB3 (SB3-PD) sequence. We observed a significant increase of TGF-β1 mRNA expression in HepG2 and in Huh-7 cells transfected to overexpress SB3-WT, compared to controls, but transfection with the plasmid vector carrying the SB3-PD sequence induced a further significant increase of the levels of this cytokine in both cell lines (Figure 3A,B).

In parallel, by performing ELISA assay on the supernatant of HepG2 and HuH-7 transfected cells (Emax ImmunoAssay test), we have evaluated the biologically active TGF-β1 protein production. As reported in Figure 3C,D, the cytokine production was increased in the supernatant of cells transfected with the plasmid carrying the SB3-WT sequence, compared with the control, and, even more significantly, in cells transfected with the plasmid vector carrying the SB3-PD.

We next decided to investigate the paracrine effect of SerpinB3 on human stellate cells (LX2 cells) and macrophages (THP-1 cell line). LX2 cells were exposed to human recombinant SerpinB3 at different time points. The results documented that, also in these cells, the recombinant form of the polymorphic variant SB3-PD was able to induce higher levels of TGF- β1 than those obtained with the recombinant SB3-WT (Figure 3E). Along these lines, the treatment of THP-1 differentiated macrophages with recombinant SB3-WT or SB3-PD resulted in the induction of an intermediate M1/M2 phenotype. In fact, analyses of M1 (TNF-α, IL-6) or M2 (TGF-β, vascular endothelial growth factor or VEGF, IL-8) pro-inflammatory cytokines and of the Monocyte chemoattractant protein-1 (MCP-1 or CCL2) showed an up-regulation of their gene expression (by qPCR, Figure 4) as well as protein release in culture medium (by ELISA assay, Figure 5) in THP-1

cells treated with SB3-WT or SB3-PD. Of relevance, the treatment with SB3-PD resulted in a significantly higher and more prolonged effect in THP-1, compared with that determined by SB3-WT, suggesting a potential role of this polymorphic variant in more severe progression of chronic liver disease.

4 | DISCUSSION

Several studies indicate that the protease inhibitor SerpinB3 plays a relevant role in the main steps of liver disease progression, including fibrosis, angiogenesis and inflammation. It has been previously reported that in patients with chronic liver disease SerpinB3 up-regulation correlates with TGF-β1 expression and the extent of hepatic fibrosis.[27] Moreover, SerpinB3 can exert a pro-fibrogenic role in the progression of experimental chronic liver disease by directly acting on activated HSC, forcing these cells to upregulate the expression of major genes involved in fibrogenesis and angiogenesis.[24,25] In addition, in vitro experiments have shown that for the induction of TGF-β1, the integrity of the reactive site loop of the protein is required.[27] In the present study the polymorphic variant SB3-PD, which determines a gain of anti-protease activity,[29] was found even more effective in determining increased levels of fibrogenic and inflammatory cytokines, supporting its role in faster progression of cirrhosis evolution. In a cohort study of outpatients with advanced chronic liver disease we have indeed observed that at presentation patients carrying the polymorphic variant SB3-PD had signs of more severe portal hypertension (large EV, splenomegaly and thrombocytopenia) and accordingly, they had a higher incidence of clinical complications of liver cirrhosis. These complications included either the first episode of decompensation or further episodes of complications. In line with these results, the polymorphic variant SB3-PD resulted, together to MELD score and albumin levels, an independent predictor of clinical complications. Moreover, patients carrying the polymorphic variant SB3-PD had a lower transplant free survival and among patients with ascites at baseline, the SB3-PD polymorphism was characterised by a significantly younger age.

The increased hepatic vascular resistance and the modulation of portal blood flow are the two main determinants of the establishment and worsening of portal hypertension.[3] The relevant increase of intra-hepatic vascular resistance is mainly caused by pro-fibrotic
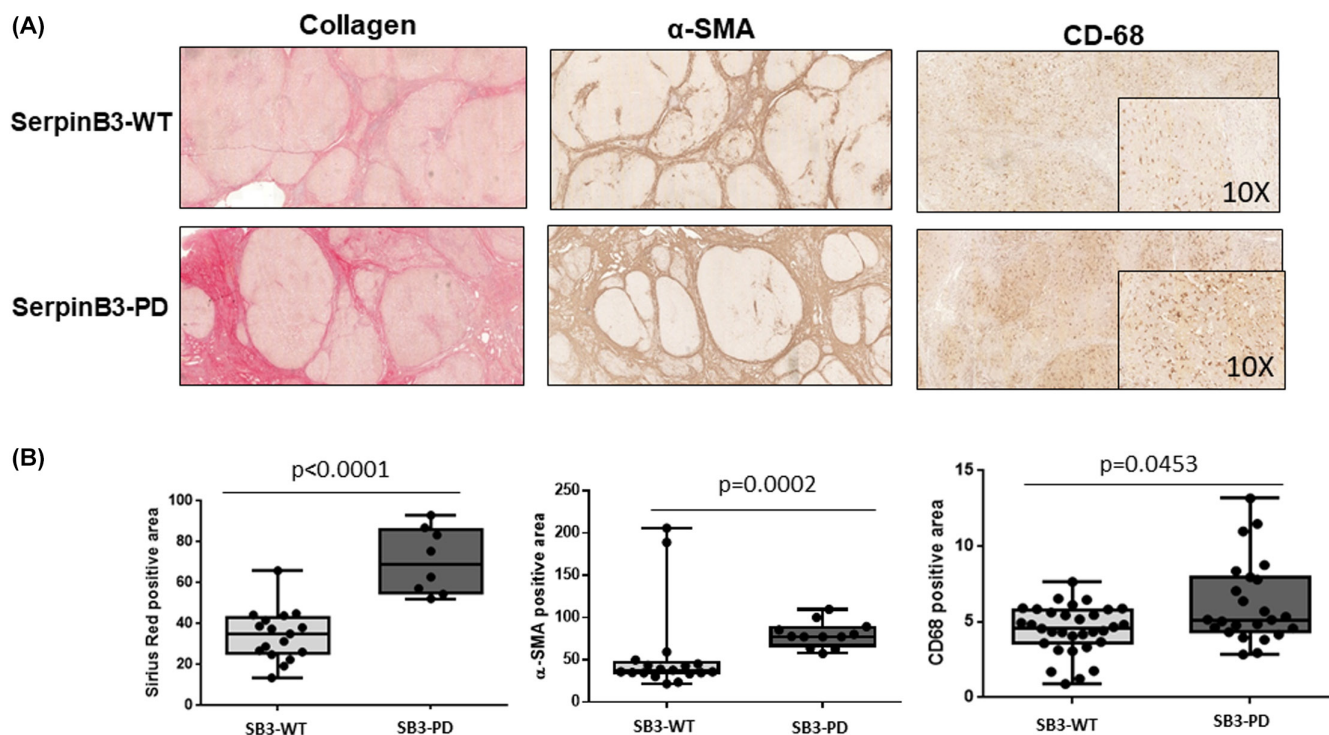
FIGURE 2 Inflammation and liver fibrosis quantification in patients with or without SB3-PD polymorphism. (A) Examples of fibrosis and inflammation markers in the liver of a patient with SerpinB3 wild type (WT) and in one with SerpinB3-PD polymorphism. Liver fibrosis was morphologically evaluated by Sirius red staining or immunohistochemistry for α-SMA. Inflammation was performed by immunohistochemistry for the macrophage marker CD68. Original magnification 2×. The inner image, as indicated. (B) Quantification of fibrosis, stellate cell activation and inflammation markers. ImageJ software analysis was performed for Sirius red staining, α-SMA and CD-68 immunohistochemistry results. Analysis was performed in five human liver specimens (three specimens with SB3-WT and two specimens with SB3-PD polymorphism) randomly selected among patients of our cohort who underwent liver transplantation. Quantification of positivity for each parameter is referred to 5–15 microscopy field (dots on the graphs) per patient. Statistical differences were assessed by the Student's t test (Sirius Red) or the Mann–Whitney test for nonparametric values (α-SMA and CD-68).

architectural rearrangement of the liver[3,8] and, in this setting, TGF-β1 is extensively considered a critical mediator in the activation of HSC into proliferative and fibrogenic myofibroblasts.[11,42] On the other hand, in the splanchnic vasodilation the contribution of angiogenesis, throw the activation of VEGF, has been widely demonstrated.[3,8]

Our in vitro experiments have shown that the polymorphic variant SB3-PD, when expressed by cultured cell line, is able to determine significantly higher levels of TGF-β1 than the SB3-WT isoform. In addition, the exogenous form of SB3-PD was able to induce paracrine effects resulting in higher synthesis and release of TGF-β1 in stellate cells and of VEGF in THP-1 cells that were characterised by a more prominent mixed M1/M2 profile, compared with the effects induced by the wild-type form. These findings were supported by the results observed in histological liver specimens of our cohort of patients with cirrhosis who underwent liver transplantation, where a more remarkable extent of fibrosis and of inflammatory cell infiltrate was observed in patients carrying the SB3-PD polymorphism. These in vitro and in vivo data are consistent with the crucial role of systemic inflammation in liver disease progression, favouring the development of acute decompensation already described in the literature.[3,8,43]

Therefore, our results suggest that SB3-PD released by damaged/hypoxic hepatocytes in the cirrhotic liver could contribute more efficiently to the microenvironment modifications implicated in architectural rearrangement and fibrosis progression than its wild type isoform.

The precise mechanism responsible for the in vitro and clinical differences observed in presence of the SerpinB3 polymorphism have not been addressed yet. We are currently exploring the pathway involved in this effect and we have recently identified a membrane receptor whose activation, involved in inflammation and TGF-β1 synthesis, is highly dependent on the anti-protease activity of SerpinB3 (manuscript in preparation). Preliminary results indicate a lack of its activation in presence of experimental models carrying SerpinB3 knock out in the reactive center loop sequence. In addition, steric inhibition of this cellular receptor protects from inflammation and fibrosis response in animal models of steatohepatitis carrying different extent of expression of SerpinB3.

The clinical significance of different single nucleotide polymorphisms (PNPLA3, TM6SF2, MBOAT7 and Pi*ZZ genotype), as risk factor for the progression of chronic liver disease has been reported in literature.[44–49] Recently, a polymorphic variant of the MHC class I-related chain A (MICA) gene has been related to liver fibrosis progression in patients with chronic hepatitis C, where association with alterations in TGF-β1 and HSC pathways were described.[50] Previously, one study has linked the presence of a single nucleotide polymorphism (PNPLA3-gene, rs738409 and G/G genotype) as a risk
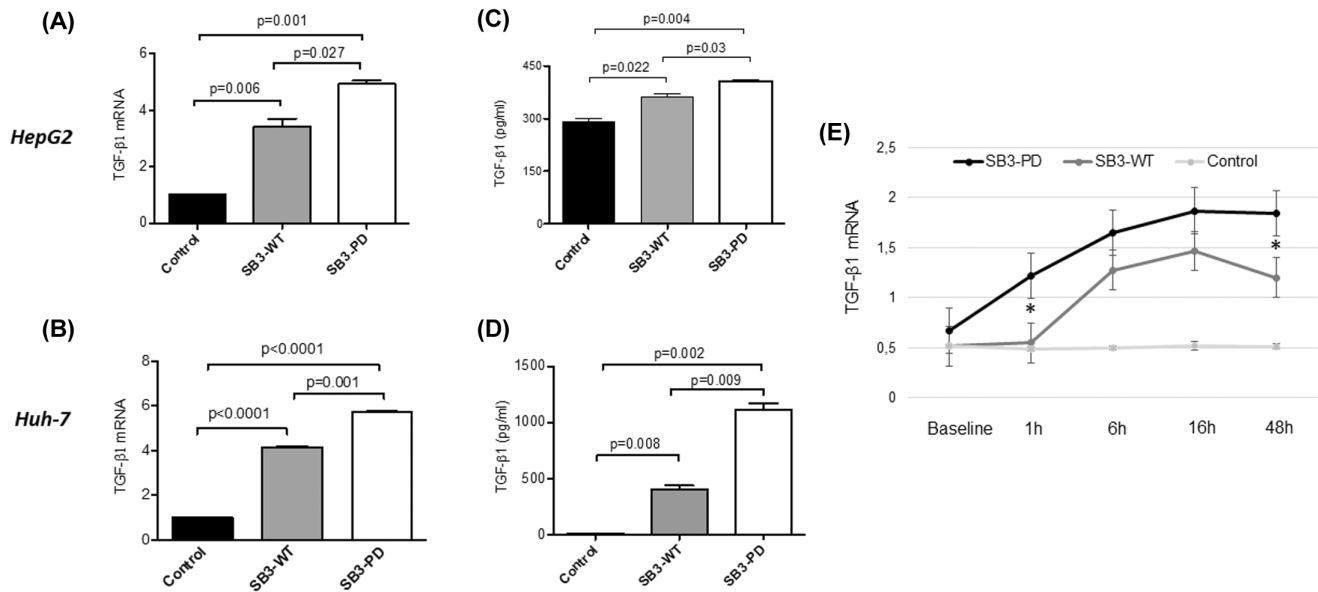
FIGURE 3 TGF-β1 mRNA and protein expression are increased by SB3-PD in Hepatoma cell lines. Fold increase of TGF-β1 mRNA and protein expression in HepG2 (A, C) and HuH-7 cell line (B, D) transfected with SB3-WT and with the polymorphic variant SB3-PD. TGF-β1 mRNA was evaluated with RT-PCR and data are expressed as ($2^{-\Delta\Delta Ct}$); TGF-β1 protein expression was evaluated with ELISA. Data are mean±SD (unpaired t test). (E) Time course increase of TGF-β1 mRNA after treatment of LX2 cell line with recombinant protein SB3-WT (grey line) and with recombinant protein SB3-PD (black line) compared to LX2 cell line treated with medium alone (light grey line) ($p<0.01$). Data are mean±SD (unpaired t-test) and data are expressed as ($2^{-\Delta\Delta Ct}$), *$p<0.01$.
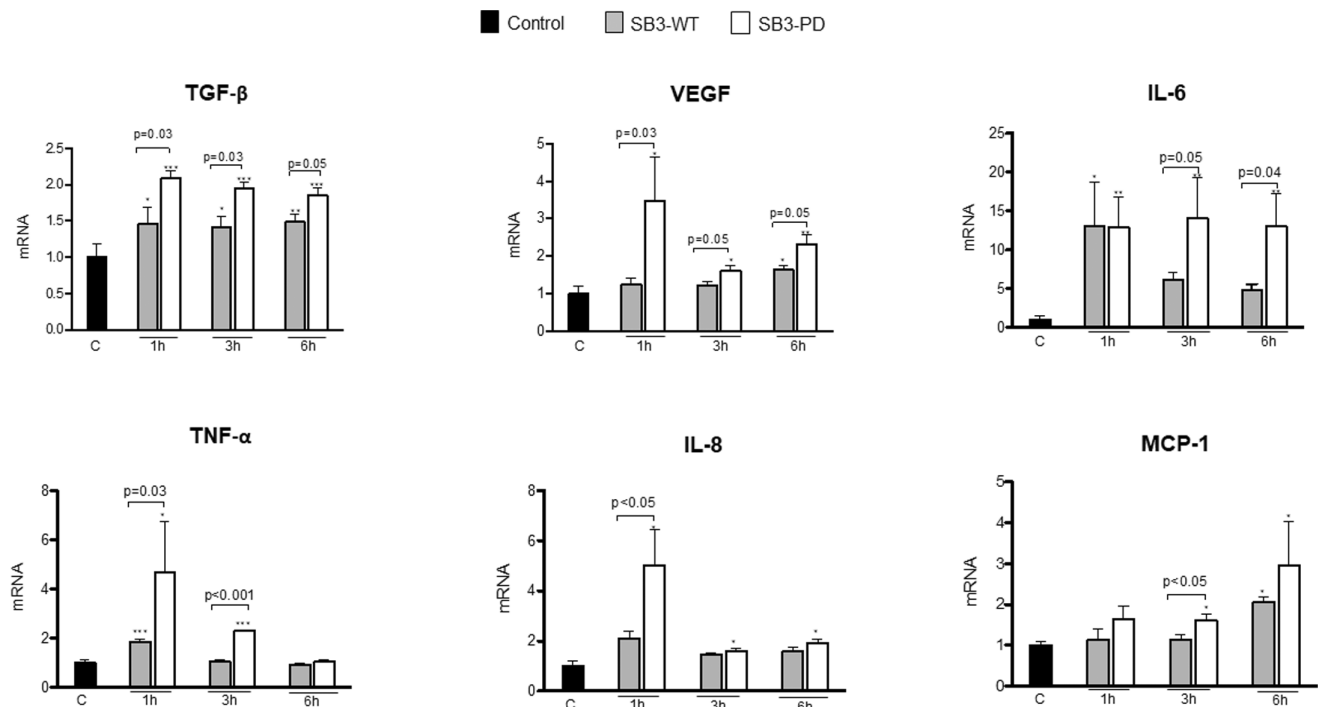

FIGURE 4 Pro-inflammatory cytokine expression is increased by SB3-PD in THP1 cell line. qPCR analysis of human pro-inflammatory cytokines (TGF-β, VEGF, IL-6, TNF-α and IL-8) and MCP-1 expression in THP-1 cells treated with SerpinB3-WT (SB3-WT) or SerpinB3-PD (SB3-PD) at the concentration of 200 ng/mL. Data in graphs are expressed as means±SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control condition, whereas p value of SB3-WT vs. SB3-PD is indicated).
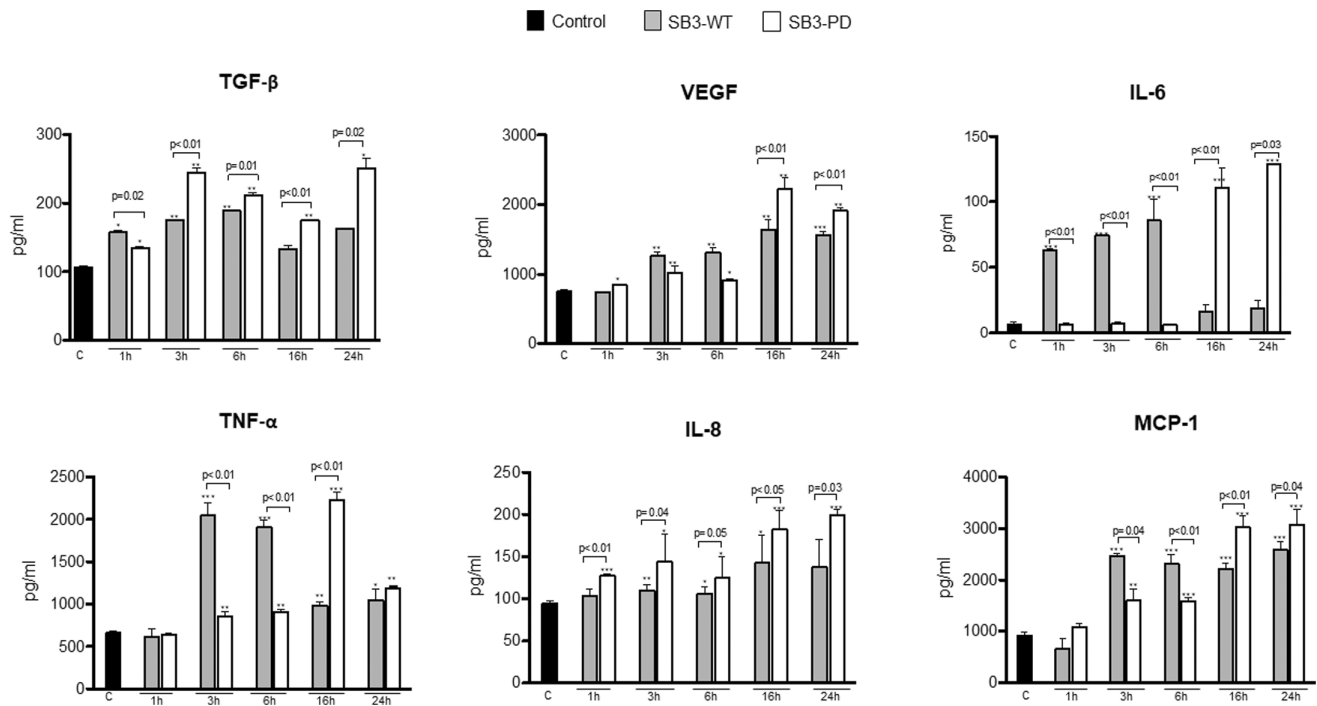
FIGURE 5 Increased expression of pro-inflammatory cytokines by SB3-PD is reflected in their increased release from THP-1 differentiated macrophages. THP-1 cells were differentiated in macrophages by PMA and then exposed to SB3-WT or SB3-PD at a concentration of 200 ng/mL. Cytokines release were quantified by ELISA assay. Data are expressed as mean ± SD in three independent experiments run in duplicate (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control condition, whereas p value of SB3-WT vs. SB3-PD are indicated).

factor for hepatic decompensation in patients with both alcoholic and non-alcoholic fatty liver disease, but not in patients with chronic hepatitis C liver disease.[51] The genetic variant of SERPINA1 (Pi*Z variant) is a strong genetic modifier of metabolic liver disease and a genetic risk factor for hepatic decompensation.[52] Like other human serpins, both SERPINA1 and SERPINB3 share a highly conserved region comprising the serpin-enzyme complex binding domain described by Joslin et al[53] which binds the low-density lipoprotein receptor-related protein-1 on the cell surface, allowing the internalisation of the serpin-protease complex.[54] This interaction determines the activation of pro-survival signalling pathways, including Wnt/β-catenin upregulation.[55,56] Beside this reactive loop-independent common mechanism of action, the specific anti-protease activity of the serpins provides peculiar effects to the different serpins, likely due to their binding to different proteases. This, for example, could explain the fact that among the SERPINB3/4 isoforms, SERPINB3 has been more closely associated with cancer progression. Indeed, despite these two polymorphic variants share a 92% identity at amino acid sequence, their degree of homology drops to 54% at the catalytic site, accounting for the different targeted proteases.[57]

Concerning the Pi*Z variant of SERPINA1, one of the main mechanisms determining liver disease progression in patients carrying this genetic mutation is the misfolding and polymerisation of the protein in liver hepatocytes. This occurrence enhances ER stress,[52] increasing inflammation and oxidative stress.[58] It is worth to note that these events are recognised factors that promote SerpinB3 expression.[59] On the basis of these considerations, it would be interesting to

explore the relationship of these two serpins in future studies, also in view of novel therapeutic strategies.

The clinical part of our study has some methodological limitations, such as: (a) its retrospective design, (b) the lack of a validation cohort, (c) the absence of a direct measurement of PH (HVPG) in the two groups of patients with cirrhosis. Nevertheless, the strengths of this study encompass: (a) patients were prospectively studied and they were well characterised at the time of assessment of polymorphism,[31,32] (b) the quantification of fibrosis and inflammation in a subgroup of transplanted patients with available histological data (c) the use of widely accepted criteria to detect the severity of PH in patients with advanced chronic liver disease,[60,61] (d) the long and thorough follow-up.

In conclusion, our data provide evidence that the gain of function of SB3-PD polymorphism may determine increased fibrosis through higher TGF-β1 and VEGF induction and greater inflammatory profile, supporting the finding that patients with cirrhosis carrying this polymorphism could be more prone to develop a severe liver disease progression. However, future multicentric and prospective studies with larger sample size are needed to support the clinical relevance of the SB3-PD polymorphisms in patients with different extents of liver disease, including patients with Acute on Chronic Liver Failure. An increased sample size and the inclusion of validation cohorts may also help to allow for providing aetiology-specific information. If our results will be confirmed, the inclusion of SB3-PD polymorphism in tailoring patient management could allow us to better define the timing of clinical follow-up and the choice of the most suitable therapeutic approaches.

AUTHOR CONTRIBUTIONS

Andrea Martini: Data curation; formal analysis; investigation; writing – original draft. **Cristian Turato:** Data curation; methodology. **Stefania Cannito:** Formal analysis; investigation. **Santina Quarta:** Formal analysis; investigation. **Alessandra Biasiolo:** Formal analysis; methodology. **Mariagrazia Ruvoletto:** Investigation; methodology. **Erica Novo:** Formal analysis; investigation. **Filippo Marafatto:** Data curation; investigation. **Pietro Guerra:** Data curation; investigation. **Marta Tonon:** Data curation; investigation. **Nausicaa Clemente:** Formal analysis; investigation. **Claudia Bocca:** Data curation; validation. **Salvatore Piano:** Data curation; validation. **Maria Guido:** Investigation; validation. **Dario Gregori:** Data curation; methodology; software; supervision. **Maurizio Parola:** Supervision; writing – review and editing. **Paolo Angeli:** Supervision; writing – review and editing. **Patrizia Pontisso:** Conceptualization; funding acquisition; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

Nothing to report.

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SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section.