**ORIGINAL ARTICLE**

**PDCD1** and **IFNL4** genetic variants and risk of developing hepatitis C virus-related diseases

Valli De Re1 | Maria Lina Tornesello2 | Mariangela De Zorzi1 | Laura Caggiari1  
Francesca Pezzuto2 | Patrizia Leone3 | Vito Racanelli3  
Gianfranco Lauletta3 | Stefania Zanussi1 | Ombretta Repetto1  
Laura Gragnani4 | Francesca Maria Rossi5 | Riccardo Dolcetti6  
Franco M. Buonaguro2 | Agostino Steffan1  

1Immunopathology and Cancer Biomarkers/Bioproteomic facility, Department of Translational Research, Centro di Riferimento Oncologico (CRO) IRCCS, Cancer Institute, Aviano, Italy  
2Molecular biology, viral oncology Istituto Nazionale Tumori IRCCS “Fondazione G. Pascale”, Napoli, Italy  
3Biomedical Sciences and Human Oncology, University of Bari Medical School, Bari, Italy  
4Center for Systemic Manifestations of Hepatitis Viruses (MaSVE), Internal Medicine and Liver Unit, Department of Experimental and Clinical Medicine, Careggi University Hospital, Florence, Italy, Florence, Italy  
5Clinical and Experimental Oncohematology Unit, Centro di Riferimento Oncologico (CRO) IRCCS, Aviano (PN), Italy  
6The University of Queensland Diamantina Institute, Translational Research Institute, Brisbane, Australia

**Correspondence**  
Valli De Re, Immunopathologia e Biomarcatori Oncologici/Bio-proteomics Facility, Department of Research and Advanced Tumor Diagnostics, Centro di Riferimento Oncologico di Aviano (CRO), Via F. Gallini 2, I-33081 Aviano (PN), Italy.  
Email: vdere@cro.it

**Funding information**  
FIRE/AISF ONLUS, Grant/Award Number: FIRE/AISF ONLUS (Fondazione Italiana per la Ricerca; SX1000), Grant/Award Number: SX1000_2010_Mds

**Abstract**

**Background:** Genetic variants of **IFNL4** and **PDCD1** genes have been shown to influence the spontaneous clearance of hepatitis C virus (HCV) infection. We investigated the **IFNL4** rs12979860 and the **PDCD1** polymorphisms in 734 HCV-positive patients, including 461 cases with liver disease of varying severity and 273 patients with lymphoproliferative disorders to determine the association of these genes with patient's outcome.

**Methods:** Expression levels of **PDCD1** mRNA encoded by haplotypes were investigated by quantitative PCR in hepatocellular carcinoma (HCC) tissue and peripheral blood mononuclear cells. Flow cytometry was used to detect PD-1 and its ligand PD-L1.

**Results:** The frequency of **IFNL4** rs12979860 C/T or T/T genotypes was significantly higher in patients with HCV-related diseases than blood donors (P < .0001). Patients expressing the IFNλ4 variant with one amino acid change that reduces IFNλ4 secretion was found increased in frequency in HCV-related diseases compared to HCC. **PDCD1** mRNA levels in HCC tissue were significantly higher in cases carrying the PD-1.3 A or the PD-1.7 G allele (P = .0025 and P = .0167). Linkage disequilibrium (LD) between PD-1.3 and IFNL4 was found in patients with mixed cryoglobulinaemia (MC) only (LD = 0 in HCC; LD = 72 in MC). PBMCs of MC patients expressed low levels of PD-L1 in CD19+IgM+ B cells and of PD-1 in CD4+ T cells suggesting the involvement of regulatory B cell-T cell interaction to the pathogenesis of MC.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2020 The Authors. Liver International published by John Wiley & Sons Ltd
Introduction: Collectively, our data indicate an important contribution of IFNλ4 expression to the development of HCV-related HCC and an epistatic contribution of IFNL4 and PDCD1 in MC.

Lay summary: Studies of IFNL4 and PDCD1 genes are helpful to better understand the role of host genetic factors and immune antigens influencing the outcome of HCV-related diseases. Our data support an association between the expression of IFNλ4, which prevents the expression of IFNλ3, with all the different HCV-related diseases studied, and besides, evidence that a higher IFNλ4 expression is associated with hepatocellular at a younger age. The expression pattern of low PD-L1 on B cells and high PD-1 on CD4+ T-cells in patients with HCV-positive cryoglobulinaemia suggests a critical role of the PD-1/PD-L1 signaling in modulating B cell-T cell interaction in this lymphoproliferative disease.

Keywords: cryoglobulinaemia, hepatitis virus C, hepatocellular carcinoma, IFNλ4, PD-1

1 | Introduction

Individuals with a hepatitis C virus (HCV) infection develop chronic hepatitis C (CHC) or remain asymptomatic. Recently, the incidence of hepatocellular carcinoma (HCC) has increased in association with the increase in HCV infection. Chronic viral infection is characterized by dysfunctional B cells, which render some patients susceptible to the development of B lymphocyte proliferative diseases including mixed cryoglobulinaemia (MC) and B-cell non-Hodgkin lymphoma (B-NHL). Interferon-lambda 4 (IFNL4) and programmed cell death protein-1 (PD-1) expression levels have emerged as important modulators of altered host response to HCV infection and as cause of a variety of autoimmune diseases. The IFNL4 gene encodes the IFNλ4 protein that increases the expression of IFN-stimulated genes (ISGs) in the liver. The receptors for IFNλ4 are expressed on selective cell types like hepatocytes and some immune cells. IFNλ4 is expressed only in individuals carrying the IFNL4 rs368234815 ΔG allele, which creates an open reading frame. In contrast, individuals with the rs368234815 T/T allele do not express the protein because of a premature stop codon. Other IFNL4 polymorphisms include a common variant (rs117648444) resulting in a P70S amino acid change and two others (L79F and K154E), which lead to lower IFNλ4 secretion and antiviral activity. High IFNλ4 expression is strongly associated with spontaneous HCV clearance and a lower response to IFN-based treatment. The IFNL4 rs12979860[T] allele variant may be used as an alternative to IFNL4 rs368234815 ΔG variant to test for IFNL4 expression since their almost complete linkage is well established. IFNλ4 was recently found to have selective pressure on the HCV genome. Genome-wide association studies of patients with hepatitis infection identified polymorphisms in IFNL4 and PDCD1 genes conferring a higher risk of chronic infection, HCV-related diseases and treatment response. Data from patients with HCV-related lymphoproliferative diseases are limited, and there is a clear need to verify the role of IFNλ4 in the Caucasian population. The rs11568821 (PD-1.3) was associated with the development of autoimmune diseases in Europeans and Mexicans but not in African Americans. In addition, the incidence of HCC is higher among Asian Americans than White Americans, while a high incidence of HCV-related MC was present in the Mediterranean basin, suggesting that genetic polymorphisms and environmental risk factors may influence the risk of these diseases. Investigation of IFNL4 and PDCD1 immunogenetic profiles in patients with different HCV-related diseases could help to elucidate these pathologies. We therefore studied IFNL4 and PDCD1 genetic variants distribution and their relative expression in Italian patients diagnosed with different HCV-related diseases. Results could underlie the complex immune interactions leading to different clinical outcomes in chronic HCV-positive patients.

2 | Patients and Methods

This study investigated data and biological samples obtained from 734 HCV-positive patients with different liver or lymphoproliferative diseases and 94 non-HCV-infected blood donors. Liver diseases taken into consideration were CHC (n = 148), cirrhosis (n = 113) and HCC (n = 200), while the studied lymphoproliferative diseases were MC (n = 138) and NHL (n = 135). Patients were in treatment at one of the three participating Italian hospitals where they had been diagnosed and had undergone HCV molecular analysis. For each patient,
we obtained from clinical records the following data: age at diagnosis, sex, HCV positivity, HCV viral load and genotype, and specific diagnosis. Data on blood donors’ age and sex were collected.

In agreement with the Centre for Disease Controls and Prevention (CDC) guidelines, we defined as CHC a patient with long-lasting HCV infection who had persistent viraemia for more than 6 months after initial exposure diagnosed by blood test. Such test is based on the detection of serum anti-HCV antibodies performed by an enzyme immunoassay (III-generation EIA) against antigens from the HCV-core and from the HCV-nonstructural regions and is confirmed by the qualitative analysis that detects the presence of HCV RNA (Cobas Amplicor HCV assay). All patients we have included in the CHC group were positive to both these tests. The viral load was determined in 192 patients (Table 1) to monitor their response to treatment. Data reported in Table 1 were referred to tests performed before treatment. The viral load (branched DNA, Chiron, Emeryville, USA) was measured only in a subgroup of patients with HCV infection because in most cases a positive or a negative result was sufficient for clinical determinations. Sustained virological response (SVR) is defined as a

| TABLE 1 | Baseline characteristics of HCV patients and blood donors |
|---------|----------------------------------------------------------|
| **Characteristics** | **BD** | **CHC** | **Cirrhosis** | **HCC** | **MC** | **NHL** |
| Age (years) | | | | | | |
| BD | 42 ± 10 | | | | | |
| CHC | 57 ± 14 | | | | | |
| Cirrhosis | 64 ± 11 | | | | | |
| HCC | 67 ± 9 | | | | | |
| MC | 66 ± 10 | | | | | |
| NHL | 68 ± 15 | | | | | |
| Sex male | | | | | | |
| BD | 90.1% | | | | | |
| CHC | 48.3% | | | | | |
| Cirrhosis | 65.4% | | | | | |
| HCC | 71.2% | | | | | |
| MC | 29.3% | | | | | |
| NHL | 47.7% | | | | | |
| HCV positive viraemia | | | | | | |
| BD | 0% | | | | | |
| CHC | 100% | | | | | |
| Cirrhosis | 100% | | | | | |
| HCC | 100% | | | | | |
| MC | 100% | | | | | |
| NHL | 100% | | | | | |
| HCV viral load | | | | | | |
| CHC | 2.5 ± 0.3 | | | | | |
| Cirrhosis | 2.2 ± 0.4 | | | | | |
| HCC | 1.9 ± 0.2 | | | | | |
| MC | 3.1 ± 0.2 | | | | | |
| NHL | 3.0 ± 0.4 | | | | | |
| HCV genotype | | | | | | |
| CHC | genotype 1 | 60% | genotype 2 | 26% | genotype 3 | 13% | genotype 4 | 2% |
| Cirrhosis | 63% | | 21% | | 10% | | 6% |
| HCC | 58% | | 28% | | 9% | | 4% |
| MC | 63% | | 26% | | 7% | | 4% |
| NHL | 60% | | 32% | | 4% | | 4% |
| CHC with | | | | | | |
| Mild-moderate fibrosis | 60 | 40.5% | | | | |
| Severe fibrosis | 59 | 39.9% | | | | |
| Data not available | 29 | 19.6% | | | | |
| MC with | | | | | | |
| Mild-moderate fibrosis | 27 | 19.6% | | | | |
| Severe fibrosis | 24 | 17.4% | | | | |
| Data not available | 87 | 75.0% | | | | |

Note: Continuous variables reported as mean ± standard deviation. Categorical variables reported as %.

BD, blood donors, CHC, patients with a chronic HCV infection for more than 6 months; HCC, patients with hepatocellular carcinoma; MC, patients with mixed cryoglobulinaemia; NHL, patients with a non-Hodgkin lymphoma.

\(^a\)HCV infection for more than 6 months confirmed at both serum anti-HCV antibodies and for the presence of HCV RNA.

\(^b\)Data available for 192 cases;

\(^c\)Data available for 323 cases.
negative response to serum HCV RNA viral load performed at least 12 weeks after the end of HCV treatment.

In addition, fibrosis stage assessment by liver biopsy (Metavir score) or fibroscan measure in patients with CHC and MC were retrieved when available (n = 119 CHC; n = 51 MC, Table 1). A good correlation was established between fibroscan and Metavir score as follow: F0-F1, 7.1 kPa; F2, 7.1-9.4 kPa; F3, 9.5-12.5 kPa; and F4, 12.5 kPa. We categorized patients with F0-F2 stage as a ‘mild-moderate fibrosis’ and those with a F3-F4 stage as ‘advanced fibrosis’.

HCV genotypes have been determined using the Versant HCV Genotype 2.0 assay (Siemens Healthcare Diagnostics). A diagnosis of HCC was based on the criteria of the European Association for the Study of the Liver using non-invasive criteria, namely two imaging techniques both demonstrating a focal lesion >2 cm in diameter with features of arterial hypervascularization. The diagnosis of cyroglobulinemia was based on the detection of cryoglobulins, performed according to guidelines of the Associazione Italiana per la Lotta alle Crioglobulinemie. The diagnosis of NHL in the course of cyroglobulinemia was based on the detection of cryoglobulins, performed according to guidelines of the Associazione Italiana per la Lotta alle Crioglobulinemie. The diagnosis of NHL in the course of HCV infection has been histopathologically confirmed based on the WHO classification.26

The study protocol was approved by the Comitato Etico Indipendente of the Azienda Ospedaliero-Universitaria Consorziale Policlinico di Bari, the Scientific Board and Ethics Committee of Fondazione G. Pascale Istituto Nazionale Tumori, the institutional review board code SPE 14.084 AOU, Comitato Etico Area Vasta Centro AOU Careggi, Firenze and Comitato Etico Bio-banca CRO. The study was done in accordance with the Declaration of Helsinki. All research subjects provided written informed consent for the collection, storage and analysis of their samples and data. The analyses were carried out at the Centro di Riferimento Oncologico (Aviano, Italy) and Fondazione G. Pascale (Naples, Italy) in the period 2017-2019.

2.2 | Selection of known IFNL4 and PDCD1 polymorphisms and IFNL4 mutational analysis

For IFNL4, we chose to genotype rs12979860 because of its known linkage disequilibrium with rs368234815 which is required for IFNL4 expression4 and because we had validated a method for rs12979860 genotyping in a previous study.17

For PDCD1, we selected single nucleotide polymorphisms (SNPs) known to be associated with susceptibility to cancer or autoimmune diseases28–45 or to influence the spontaneous resolution of HCV infection or the response to antiviral treatment46–49 (Table S1). From this preliminary list of six SNPs, we selected four SNPs (ie PD-1.3, PD-1.5, PD-1.6 and PD-1.7) for subsequent analysis because they had a minor allele frequency (MAF) ≥0.05 in the Italian population according to the Ensemble website database (http://www.ensembl.org/Homo_sapiens; Table S1).

To identify other genetic variations potentially affecting IFNL4 activity, we sequenced the IFNL4 gene (intron 1 to exon 5) in genomic DNA from PBMCs of 36 cases homozygous at rs12979860 (16 C/C and 20 T/T). Sequencing was done as previously described4 but with a modified sequencing primer (IFNL4 int IV1, Table S2). The amino acid changes G58R and P70S in the IFNL4 protein flank residue N61, whose glycosylation is required for secretion of active IFNL4 protein.50 Consequently, these variants lower the antiviral activity of the reference IFNL4.51

2.3 | Genotyping and sequencing of IFNL4 and PDCD1

Genotyping of IFNL4 rs12979860 and rs117648444 (C>T, P70S) was performed using custom TaqMan SNP genotyping assays (Applied Biosystems) on a 7900HT Fast Real-Time PCR system (Applied Biosystems).

To sequence IFNL4 (from intron 1 to exon 5), we first amplified the gene from genomic DNA by PCR in a reaction volume of 25 μl (200 ng dNTPs and 0.5 U GoTaq DNA Polymerase, Promega) using primers as reported in Table S2 and cycling conditions as described in reference 4. Sequencing was performed as previously reported.52

To genotype PDCD1 SNPs, genomic DNA (30–300 ng) was amplified in a 50 μl reaction mixture containing 10 pmol of each primer (Table S2), 1.25 U Hot Master Taq DNA Polymerase (5 Prime) and 25 μl PreMixJ (MasterAmp PCR, Epicentre, Madison, USA) on a Sure Cycler 8800 thermal cycler (Agilent Technologies). Amplification started with an initial denaturation at 94°C for 3 min, followed by 30 amplification cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, elongation at 72°C for 1 min, and a 10 min final elongation at 72°C. PCR products were subjected to Sanger automated sequencing analysis.
2.4 | LD analysis

Linkage disequilibrium (LD) was estimated among PD-1, that is, PD-1.3, PD-1.5, PD-1.7 and IFNL4 rs12979860 SNP. Distribution of the Lewontin's coefficient D' and correlation coefficient r² was calculated as the measures of LD using the SHEsis online tool (http://analysis.bio-x.cn). LD within each genomic region was explored and the extent of statistical significance of each pairwise association was represented by a scale of colour intensity.

2.5 | Analysis of PDCD1 mRNA expression

The expression levels of haplotypes within PDCD1 gene were evaluated by real-time quantitative PCR using total RNA from PBMCs of selected cases based on their PDCD1 haplotype (n = 12) and from selected HCC tissues (n = 12). Briefly, total RNA (500 ng) was reverse transcribed in a 20 µL volume with Superscript II RNase H and an oligo d(T) primer (Life Technologies). The obtained cDNA (2 µL) was then subjected to qPCR using forward (5'-GAGGGACAAATAGGAGCCAGG-3') and reverse (5'-TCTTCTCTGCCACTGGAAA-3') primers targeting PDCD1 exons 4 and 5, respectively, to exclude the amplification of cross-contaminating DNA. Each reaction contained 12.5 µL of 1x iQ SYBR Green supermix (Bio-Rad Laboratories), 10 pmol of each primer, 2 µL of CDNA and nuclease-free water in a final volume of 25 µL. Amplifications were performed in triplicate using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). Gene expression was analysed using the comparative Ct (2^-ΔCt) method of quantification and SRSF4 as reference sample. The ΔCt values for each transcript were calculated by subtracting the respective Ct (cycle threshold) value from the corresponding SRSF4 Ct (ΔCt = Ctⱼ - CtSRSF4). Ct values were corrected for the efficiency of primer pairs.53

2.6 | PD-1 and PD-L1 flow cytometry analysis

Cryopreserved peripheral blood mononuclear cells (PBMCs) from two HCV-negative patients with HCC, two patients with HCV-related HCC and two patients with HCV-related MC were used for PD-1 and PD-L1 flow cytometry analysis. Before the analysis, the dimethyl sulfoxide (DMSO) cryoprotectant was removed by washing and centrifugation. PBMCs were re-suspended in phosphate-buffered saline (PBS). Sample were stained with combination of vital dye 7-aminoactinomycin (7-AAD), CD4 PE (cl. SK3), PD-1 FITC (cl. MIH4), or CD19 APC (cl.HIB19), PD-L1 PE (cl.MIH1), all from Becton Dickinson, San Jose, CA) and IgM FITC (Dako). All samples were acquired on a FACSCantoll flow cytometer and DiVa software (Becton Dickinson). At least 1,000 T or B cells were acquired per tube. Media of percent positive cells from vital lymphocytes were calculated between HCC HCV-negative, HCC HCV-positive or MC-HCV-positive samples.

2.7 | Statistical analysis

Fisher’s exact test and ANOVA analysis of variance were used to compare allele and genotype frequency of gene polymorphisms between patient groups with different pathologies and control subjects.

Multivariate logistic regression analysis was performed with diagnosis as the dependent variable and genotype as independent variable; age and sex were valuated as covariables. Odds ratios and 95% confidence intervals were calculated. Genotypes of each polymorphism were assessed according to dominant (0 wild-type homozygote; 1 heterozygote and variant homozygote), recessive (0 wild-type homozygote and heterozygote; 1 variant homozygote) and additive genetic models (ie overdominant and log-additive).

The chi-squared test for trends was used to assess associations with genotype for liver diseases of increasing severity (chronic HCV infection, cirrhosis and HCC).

Statistical analyses were performed using MedCalc software (version 17.2), SNPStats (https://www.snpstats.net/start.htm) and SHEsis online tool (http://analysis.bio-x.cn). A P < 0.5 was taken to indicate statistical significance. Bonferroni for dependent SNPs and Sidak corrections for independent SNPs were utilized to conduct multiple comparisons test.

3 | RESULTS

This study examined the immunogenetic profiles of 734 HCV-infected patients, 461 with a liver disease (148 CHC, 113 cirrhosis, 200 HCC) and 273 with a lymphoproliferative disease (138 MC, 113 NHL) and 98 non-HCV-infected blood donors, all living in Italy (Table 1). Among the patients with liver disease, there were 148 with chronic HCV infection, 113 patients with cirrhosis and 200 patients with HCC. Among those with a lymphoproliferative disease, there were 138 with MC and 135 with B-NHL. Male sex predominated among cirrhosis and HCC patients, while female sex predominated among MC patients. Blood donors (BD) were mostly male. Fibrosis data were available for 119 patients included in the CHC group and for 51 patients included in the MC group (Table 1). Among the CHC group, 60 patients (40.5%) were diagnosed with mild-moderate liver fibrosis (fibrosis F1-F2) and 59 (39.9%) with severe fibrosis (F3-F4); in the MC group, 27 patients (19.6%) had mild-moderate liver fibrosis and 24 (17.4%) a severe fibrosis (F3-F4). Response data to anti-HCV treatment were available for 319 patients: 101 with CHC, 82 with cirrhosis, 34 with HCC, 66 with MC and 36 with NHL.

3.1 | IFNL4 and PDCD1 genotype frequencies

Patients and controls were genotyped for five SNPs having possible immune-modulating effects, including rs12979860 in IFNL4 and four
## Table 2: Frequencies of PD-1 and IFNL4 genotypes in patients with HCV-related diseases and in blood donors

|                | PD-1.3 (%) | PD-1.5 (%) | PD-1.6 (%) | PD-1.7 (%) | IFNL4 (%) |
|----------------|------------|------------|------------|------------|-----------|
|                | rs11568821 | rs2227981  | rs10204525 | rs7421861  | rs12979860 |
| **BD (n = 98)**| G/G 82 (84%) | C/C 36 (37%) | C/C 79 (81%) | A/A 42 (43%) | C/C 44 (45%) |
|                | G/A 16 (16%) | C/T 44 (45%) | C/T 19 (19%) | A/G 47 (48%) | C/T 48 (49%) |
|                | T/T 18 (18%) | T/T 0     | G/G 9 (9%)   | G/G 9 (9%)   | T/T 6 (6%)   |
| **CHC (n = 148)**| G/G 114 (77%) | C/C 39 (26%) | C/C 125 (84%) | A/A 78 (53%) | C/C 39 (26%) |
|                | G/A 34 (23%) | C/T 85 (57%) | C/T 20 (14%) | A/G 56 (38%) | C/T 87 (59%) |
|                | T/T 24 (16%) | T/T 3 (2%)  | G/G 14 (9%)  | G/G 14 (9%)  | T/T 22 (15%) |

A) Compared to BD

|                | PD-1.3 (%) | PD-1.5 (%) | PD-1.6 (%) | PD-1.7 (%) | IFNL4 (%) |
|----------------|------------|------------|------------|------------|-----------|
|                |            |            |            |            |           |
| **CHC (n = 148)**| G/G 114 (77%) | C/C 39 (26%) | C/C 125 (84%) | A/A 78 (53%) | C/C 39 (26%) |
|                | G/A 34 (23%) | C/T 85 (57%) | C/T 20 (14%) | A/G 56 (38%) | C/T 87 (59%) |
|                | T/T 24 (16%) | T/T 3 (2%)  | G/G 14 (9%)  | G/G 14 (9%)  | T/T 22 (15%) |
| **Cirrhosis (n = 113)**| G/G 92 (81%) | C/C 34 (30%) | C/C 125 (82%) | A/A 53 (47%) | C/C 39 (26%) |
|                | G/A 45 (22%) | C/T 58 (51%) | C/T 19 (19%) | A/G 44 (39%) | C/T 87 (59%) |
|                | A/A 4 (2%)  | T/T 21 (19%) | T/T 0       | G/G 9 (9%)   | T/T 6 (6%)   |
| **HCC (n = 200)**| G/G 151 (75%) | C/C 74 (37%) | C/C 159 (80%) | A/A 75 (38%) | C/C 51 (26%) |
|                | G/A 45 (23%) | C/T 94 (47%) | C/T 39 (20%) | A/G 114 (57%) | C/T 103 (52%) |
|                | A/A 4 (2%)  | T/T 32 (16%) | T/T 2 (1%)  | G/G 11 (5%)  | T/T 46 (23%) |
| **MC (n = 138)**| G/G 99 (72%) | C/C 34 (30%) | C/C 93 (82%) | A/A 53 (47%) | C/C 51 (26%) |
|                | A/A 4 (2%)  | C/T 58 (51%) | C/T 19 (17%) | A/G 44 (39%) | C/T 87 (59%) |
|                | T/T 24 (16%) | T/T 3 (2%)  | G/G 14 (9%)  | G/G 14 (9%)  | T/T 22 (15%) |
| **NHL (n = 135)**| G/G 113 (84%) | C/C 41 (30%) | C/C 106 (79%) | A/A 69 (51%) | C/C 45 (33%) |
|                | G/A 22 (16%) | C/T 60 (45%) | C/T 27 (20%) | A/G 54 (40%) | C/T 66 (49%) |
|                | T/T 34 (25%) | T/T 2 (2%)  | G/G 12 (9%)  | G/G 12 (9%)  | T/T 24 (18%) |
| **Total liver diseases (n = 461)**| G/G 357 (77%) | C/C 147 (32%) | C/C 377 (82%) | A/A 206 (45%) | C/C 116 (25%) |
|                | G/A 100 (22%) | C/T 237 (51%) | C/T 78 (17%) | A/G 214 (46%) | C/T 256 (56%) |
|                | A/A 4 (1%)  | T/T 77 (17%) | T/T 6 (1%)  | G/G 41 (9%)  | T/T 89 (19%) |
| **Lymphoproliferative diseases (n = 273)**| G/G 212 (78%) | C/C 94 (34%) | C/C 215 (79%) | A/A 124 (45%) | C/C 103 (38%) |
|                | G/A 61 (22%) | C/T 122 (45%) | C/T 55 (20%) | A/G 117 (43%) | C/T 127 (47%) |
|                | T/T 57 (21%) | T/T 3 (1%)  | G/G 32 (12%) | G/G 32 (12%) | T/T 43 (16%) |
| **All HCV-positive patients (n = 734)**| G/G 569 (78%) | C/C 241 (33%) | C/C 592 (81%) | A/A 330 (45%) | C/C 219 (30%) |
|                | G/A 161 (22%) | C/T 359 (49%) | C/T 133 (18%) | A/G 331 (45%) | C/T 383 (52%) |
|                | A/A 4 (1%)  | T/T 134 (18%) | T/T 9 (1%)  | G/G 73 (10%) | T/T 132 (18%) |

B) Compared to CHC

|                | PD-1.3 (%) | PD-1.5 (%) | PD-1.6 (%) | PD-1.7 (%) | IFNL4 (%) |
|----------------|------------|------------|------------|------------|-----------|
|                |            |            |            |            |           |
| **Cirrhosis (n = 113)**| G/G 92 (81%) | C/C 34 (30%) | C/C 93 (82%) | A/A 53 (47%) | C/C 26 (23%) |
|                | G/A 21 (19%) | C/T 58 (51%) | C/T 14 (12%) | A/G 44 (39%) | C/T 66 (58%) |
|                | T/T 21 (19%) | T/T 6 (1%)  | G/G 16 (14%) | G/G 16 (14%) | T/T 21 (19%) |
| **HCC (n = 200)**| G/G 151 (75%) | C/C 74 (37%) | C/C 159 (80%) | A/A 75 (38%) | C/C 51 (26%) |
|                | G/A 45 (22%) | C/T 94 (47%) | C/T 39 (19%) | A/G 114 (57%) | C/T 103 (52%) |
|                | A/A 4 (2%)  | T/T 32 (16%) | T/T 2 (1%)  | G/G 11 (5%)  | T/T 46 (23%) |

(Continues)
### 3.2 | IFNL4 mutational analysis

The IFNL4 gene was sequenced from intron 1 to exon 5 in 36 cases homozygous at IFNL4 rs12979860 with either a C/C (n = 16) or T/T (n = 20) genotype. This analysis identified eight polymorphisms in 24 patients (Figure 1). Already known non-synonymous variants rs117648444 (P70S, five cases) and rs746231316 (G58R, one case) were identified in exon 2. The amino acids changed by these SNPs flank residue N61, which glycosylation is required for functional IFN-4.50

---

**Table 2** (Continued)

| Allele Distribution | Liver Diseases | MC (n = 273) | Lymphoproliferative Diseases Excluded CHC (n = 273) |
|---------------------|----------------|---------------|-------------------------------------------------|
| C/C                 | 53 (38%)       | 33 (35%)      | 39 (37%)                                        |
| T/T                 | 12 (9%)        | 13 (14%)      | 11 (11%)                                        |
| G/G                 | 108 (84%)      | 99 (106%)     | 95 (90%)                                        |
| A/A                 | 12 (9%)        | 10 (11%)      | 10 (10%)                                        |

Abbreviations: BD, blood donors; CHC, chronic HCV infection; HCC, hepatocellular carcinoma; MC, autoimmune lymphoproliferative mixed cryoglobulinaemia; NHL, non-Hodgkin lymphoma.

| Allele Distribution | Liver Diseases | MC (n = 273) | Lymphoproliferative Diseases Excluded CHC (n = 273) |
|---------------------|----------------|---------------|-------------------------------------------------|
| C/C                 | 53 (38%)       | 33 (35%)      | 39 (37%)                                        |
| T/T                 | 12 (9%)        | 13 (14%)      | 11 (11%)                                        |
| G/G                 | 108 (84%)      | 99 (106%)     | 95 (90%)                                        |
| A/A                 | 12 (9%)        | 10 (11%)      | 10 (10%)                                        |

Abbreviations: BD, blood donors; CHC, chronic HCV infection; HCC, hepatocellular carcinoma; MC, autoimmune lymphoproliferative mixed cryoglobulinaemia; NHL, non-Hodgkin lymphoma.

The most significant model for association with diagnosis (OR (95% CI) in comparison with BD is reported only when

P < 0.05). A Bonferroni's correction for multiple SNPs results in a

P-value threshold of 0.0125 and of Sidak's correction of 0.0127. Significant SNPs after corrections are in bold text.
Consequently, these variants reduce the protein antiviral activity.\textsuperscript{51} Our results showed that these two variants associate with the rs12979860 T/T genotype (six of 20 cases, 30\%) only. Finally, a new synonymous mutation was identified in exon 3 (one case), and the known synonymous variant rs12971396 was found in exon 5 (16 cases). This latter SNP associated with the rs12979860 T/T genotype (15 of 20, 75\%). Although based on a small number of patients, these data suggest an association between HCC and secretion of fully active IFN\textsubscript{λ}4 (patients homozygous for rs12979860[T] without a G58R or P70S variant in IFNL4; Table 3). Indeed, the rs12979860[T] polymorphism necessary for IFN\textsubscript{λ}4 production is associated with HCC (Table 2) and the frequency of the genotype necessary for fully active IFN\textsubscript{λ}4 expression is higher in HCC (no G58R or P70S variant in 15 of 28, 54\%) than in all other HCV-positive patients (17 of 48, 35\%) (Table 3). HCC patients with a fully active IFNL4 expression were diagnosed with tumour at younger age than those with reduced expression (Figure 2). These data suggest that IFN\textsubscript{λ}4 levels are a risk factor for HCC development.

### 3.3 | PDCD1 polymorphisms and mRNA expression

We next examined whether PDCD1 polymorphisms affected mRNA expression in tumour biopsies and PBMCs from HCC patients (Figure 3A). We found higher PDCD1 mRNA expression in HCC tissues carrying the PD-1.3 A/G genotype than the G/A genotype ($P = .0025$, Mann-Whitney test; Figure 3B and C). Similarly, expression was higher in HCC tissues with the PD-1.7 G polymorphism (A/G and G/G genotypes) than with the A/A genotype ($P = .0167$, Mann-Whitney test; Figure 3B and C). Conversely, no significant difference in PDCD1 mRNA expression was observed among PBMCs from HCC patients with different PD-1 genotypes (Figure 3C). Considering the important role of the PD-1–PD-L1 axis in regulating T-cell function, these results suggest that a high expression of PD-1 in the HCC microenvironment contributes to hepatocarcinogenesis by locally impairing antitumour immunity.
3.4 Epistatic interactions between IFNL4 and PDCD1 variants

Haplotype data for the PDCD1 SNPs PD-1.3, PD-1.5 and PD-1.7 and for rs12979860 of IFNL4 were analysed using SHEsis software, yielding eight common haplotypes with a frequency >5% (Table 4). In addition, a comparison between cirrhosis and HCC has been performed (Table S7). These results suggested that specific haplotypes may influence the susceptibility to or progression of different HCV-related diseases (Table 4). In addition, linkage disequilibrium analysis (LD) evidenced a linkage that did not occur by chance (LD >50) between PDCD1 SNPs and rs12979860 of IFNL4 in BD and in patients with CHC, MC and B-NHL but not in patients with cirrhosis or HCC. An association between LD-1.3 and IFNL4 was found in MC (LD = 72) but not in HCC (LD = 0) (Figure 4). In addition, real-time polymerase chain reaction (PCR) indicated higher PDCD1 expression in HCC tissue samples carrying a PD-1.3 G/A allele and a PD-1.7 G/A or G/G allele than in those with A/A genotypes, respectively, but not in PBMCs of the same patients (Figure 3).

Overall these results underline the importance of IFNL4 expression (IFNL4 rs12979860[T] variant) and low hepatic expression of PD-1, found in the GCAT haplotype, in predicting HCV-related HCC (OR = 2.101; 95% CI, 1.265-3.491, Table 4). Conversely, expression of PDCD1 (ACGC haplotype) in addition to the more potent anti-HCV IFNL3 expression (IFNL4 rs12979860[C] carrier) was in large part associated with MC (OR = 4.237; 95% CI, 1.923-9.334). Moreover, when IFNL4 was expressed (IFNL4 rs12979860[T] carrier), its antiviral effect in MC patients was frequently reduced by the presence of a G58R or P70S variant in IFN-λ4, by lowering the level of IFN-λ4 secretion (present in 69% of patients carrying the IFNL4 rs12979860[T] allele in our series). When IFNL4 is produced in MC, PDCD1 expression seems less important (GCAT haplotype; OR = 1.895; 95% CI, 1.097-3.275). Overall, these data suggest that the PD-1 SNPs, coupled with a low antiviral effect as a result of a reduced IFNL4 expression, have an important role in MC while expression of a fully active IFNL4 (IFNL4 rs12979860[T] allele without G58R or P70S mutation) is more important in determining hepatic outcomes.

3.5 PD-1 expression on T cells and PD-L1 expression on B lymphocytes of MC patients

A new B-cell subpopulation has recently been described, namely CD19+ PD-L1+ regulatory B cell, which requires PD-L1 expression to regulate CD4+ PD-1+ T follicular helper (Tfh) cell expansion and differentiation and to suppress autoimmune diseases.54 Also in peripheral tissues, and particularly during chronic pathological immune situations, CD4+ Tfh helper T cells belonging to follicular helper lineage have an important role in stimulating B-cell responses, including plasma cell differentiation and production of high-affinity antibodies.55,56 Given that PD-1hi B cells limit both memory B-cell development and plasma cell differentiation (pathognomonic signs of MC) by interacting with PD-1hi T cells,56,57 we analysed the expression of PD-1hi in CD4+ T cells and of PD-L1hi in IgM+ CD19+ B lymphocytes from patients with HCC without HCV infection (n = 2), with HCV-related HCC (n = 2) and with HCV-related MC (n = 2). We observed >10% levels of PD-1hi expression on CD4+ T cells and ≥1% levels of

---

**TABLE 3** Frequency of IFNL4 genetic variants associated with IFN-λ4 in patients and blood donors

| IFN-λ4 expression | Absent | Present |
|-------------------|--------|---------|
| patients with rs12979860-TT genotype tested for G58R or P70S IFNL4 variants |        |         |
| BD (n = 5) | 3 (60%) | 2 (40%) |
| Patients with liver disease (n = 47) |        |         |
| CHC (n = 11) | 5 (45%) | 6 (55%) |
| Cirrhosis (n = 8) | 2 (25%) | 6 (75%) |
| HCC (n = 28) | 15 (54%) | 13 (46%) |
| Patients with lymphoproliferative disease (n = 29) |        |         |
| MC (n = 16) | 5 (31%) | 11 (69%) |
| NHL (n = 13) | 5 (38%) | 8 (62%) |
| 17 (35%) | 31 (65%) |
| All HCV-positive patients excluded HCC (n = 48) |        |         |

---

**FIGURE 2** Boxplot describing the relationship of IFNL4 genotype related to a fully active IFNL4 secretion with a younger median age of HCC patients. HCC patients carrying a fully active (rs12979860-T/T combined without P70S mutation, rs117648444 C/C) were diagnosed with tumour at younger age compared to those with a reduced expression (rs12979860-T/T combined with P70S mutation, rs117648444 C/T or T/T), median age 61y and 72y, respectively. P = .02 ANOVA test. Boxes range from the 25th to the 75th percentile with a horizontal black line at the median and vertical lines extending to the 10th and 90th percentiles. These data suggest that IFNL4 levels may represent a risk factor for HCC development.
(A) PD-1 mRNA expression

2^-ΔCT

| Sample | LC-297 | LC-342 | LC-374 | LC-379 | LC-386 | LC-340 | LC-366 | LC-416 | LC-421 | LC-424 | LC-432 | LC-433 |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| PD-1.3 | G>A    | G>G    | G>G    | G>G    | A>G    | A>G    | A>G    | A>G    | A>G    | A>G    | A>G    | A>G    |
| rs11568821 | TT     | TT     | CC     | CT     | N/A    | CC     | CT     | CC     | CT     | CT     | CT     | CT     |
| PD-1.4 | T>C    | C>T    | C>T    | C>T    | C>T    | C>T    | C>T    | C>T    | C>T    | C>T    | C>T    |
| rs10204525 | CT     | CC     | CC     | CT     | CT     | CC     | CC     | CC     | CC     | CT     | CC     |
| PD-1.7 | A>G,T  | AA     | AA     | AA     | AG     | AG     | GG     | AG     | AG     | AG     | AG     | AG     |
| rs7421861 | AA     | AA     | AA     | AG     | AG     | GG     | AG     | AG     | AG     | AG     | AG     | AG     |

(B) PD-1 mRNA expression in HCC tissues

(1) PD-1 POLYMORPHISM (HCC Tissues) COMPARISON OF EXPRESSION LEVELS (Mann-Whitney test) P Value

| PD-1.3 (rs11568821) G>A |
|-------------------------|
| AA (n = 5)              |
| AG (n = 7)              |
| GG (n = 5)              |

P = 0.0025

| PD-1.5 (rs2227981) C>T |
|------------------------|
| CC (n = 3)              |
| TT (n = 2)              |
| CT (n = 6)              |

P = 0.4600

| PD-1.6 (rs10204525) C>T |
|-------------------------|
| TT (n = 6)              |
| CT (n = 4)              |
| CC (n = 6)              |

P = 0.2302

| PD-1.7 (rs7421861) A>G |
|------------------------|
| AA (n = 3)              |
| AG (n = 4)              |
| GG (n = 1)              |

P = 0.0102

(C) PD-1 POLYMORPHISM (PBMCs) COMPARISON OF EXPRESSION LEVELS (Mann-Whitney test) P Value

| PD-1.3 (rs11568821) G>A |
|-------------------------|
| AA (n = 5)              |
| AG (n = 5)              |
| GG (n = 3)              |

P = 0.2500

| PD-1.5 (rs2227981) C>T |
|------------------------|
| CC (n = 2)              |
| TT (n = 2)              |
| CT (n = 8)              |

P = 0.1010

| PD-1.6 (rs10204525) C>T |
|-------------------------|
| TT (n = 6)              |
| CT (n = 3)              |
| CC (n = 6)              |

P = 0.2510

| PD-1.7 (rs7421861) A>G |
|------------------------|
| AA (n = 3)              |
| AG (n = 3)              |
| GG (n = 1)              |

P = 0.4285
PD-L1<sup>hi</sup> on IgM<sup>+</sup> CD19<sup>+</sup> B cells in HCC patients without HCV infection (Figure 5A), but lower than 10% levels of PD-1<sup>hi</sup> expression on CD4<sup>+</sup> T cells and absence of PD-L1<sup>hi</sup> in patients with HCV-related MC (Figure 5B).

4 | DISCUSSION

This study confirms the significant associations between the IFNL4 rs12979860[T] variant and both persistence of HCV infection and risk of HCC. The IFNL4 rs12979860[T] variant is in high LD with the rs3682348[G] polymorphism necessary for producing the antiviral cytokine IFN<sub>λ</sub> while preventing the production of IFN<sub>λ</sub>.<sup>3,5,19</sup> which has a stronger antiviral activity than IFN<sub>λ</sub>.<sup>4</sup> We identified in our series four SNPs in LD with the rs12979860[T] variant: two functional G58R and P70S variants whose minor allele decreased IFN<sub>λ</sub> secretion,<sup>15</sup> the intronic (IV3) rs111531283[G] variant of unknown role but present in all cases, and the synonymous rs12971396[C] variant in exon 5.<sup>15</sup> The frequencies of polymorphic alleles encoding a fully active IFN<sub>λ</sub> and a reduced secretion of IFN<sub>λ</sub> (ie G58R and P70S) were not significantly different between HCC patients and BD. Nonetheless, patients with variants leading to a higher IFNL4 production showed a delay in HCC development (median age, 72 vs 60 years, P = .02). A possible explanatory hypothesis is that fully active IFN<sub>λ</sub>-4 protein, associated with HCC in our series. Moreover, the ACGC haplotype, exhibiting higher IFNL4 mRNA and a lower IFN<sub>λ</sub>-4 protein expression, discriminates cirrhosis from HCC. Furthermore, LD analysis indicated that PD-1.3 and PD-1.7 are in linkage (LD = 72) in HCC and that IFNL4 is an independent gene (LD = 0). The expression of PDCD1 in HCC cells is not well characterized. A recent study reported that PD-1 participates in the mTOR pathway in PD-1-positive tumour cells.<sup>62</sup> However, the specific biological role and target implications of PD-1-positive cells in HCC biopsies require further investigation.

The association of IFNL4[TT] genotype in patients with HCV-related lymphoproliferative disorders was less evident than in patients with liver diseases. Furthermore, the association with HCV-related lymphoproliferative disorders was lower when considering the less active form of IFN<sub>λ</sub>-4 (IFNL4 rs12979860[T] coupled to P70 mutation), which causes an increase in viral replication.<sup>60</sup> We hypothesize that HCV replication intensifies the antigen-driven immune stimulation that in turn sustains B-cell proliferation.<sup>63</sup>

The LD analysis showed an epistatic contribution between IFNL4 and PD-1.3 polymorphisms in MC patients (LD = 72). The study of haplotypes indicated that PD-1[ACG]-IFNL4[C] was positively associated with MC (OR = 4.237) and that the opposite haplotype [GTA] [T] (OR = 0.458) is negatively associated with MC. These results suggest that PD-1.3 G allele may produce a PD-1 molecule that counterbalances the absence of IFN<sub>λ</sub>-4 protein (rs12979860 = CC) in the risk of MC and vice versa.

Previous studies have demonstrated that high expression of PD-1 in hepatic lymphocytes, especially exhausted T cells and Tregs, is associated with a dysfunctional immune response in chronic HBV infection and HCC.<sup>64-67</sup> and that PD-1 had influence on the viral profile.<sup>68</sup> Studies on CHC<sup>69,70</sup> showed that overexpression of PD-1 on HCV-specific CD8<sup>+</sup>T cells was associated with a reduced efficiency of T cell-mediated cytolysis compared to non-HCV-specific T cells, and correlated with the maintenance of an exhausted phenotype of CD8<sup>+</sup>T cells.<sup>71</sup> Notably, blockade of PD-1-PD-L1 interactions restored the activity of HCV-specific T cells,<sup>72</sup> and controlled HCV replication in a chimpanzee model of CHC.<sup>73</sup>

Lymphoid follicles with a germinal centre architecture were commonly observed in the livers of patients with HCV infection.<sup>74</sup>

Since it is widely accepted that mutations have different effects in combination than individually, we analysed the relationship between the PDCD1 haplotype and IFNL4 in HCC and compared it to that in patients with CHC. The GCAT haplotype, exhibiting lower PDCD1 mRNA expression in tumour biopsies and a fully active IFN<sub>λ</sub>-4 protein, associated with HCC in our series. Moreover, the ACGC haplotype, exhibiting higher PDCD1 mRNA and a lower IFN<sub>λ</sub>-4 protein expression levels than those with wild-type homozygote (PD-1.3 genotype G/G, P = .0025 and PD-1.7 genotype A/A, P = .0182, Mann-Witney test respectively).
### TABLE 4  Epistatic interaction defined by PD-1 and IFNL4 polymorphisms and their associations with HCV-related diseases compared to patients with a chronic HCV infection

| Haplotype | Frequency | X² | P value | Odds ratio [95%CI] | Frequency | X² | P value | Odds ratio [95%CI] |
|-----------|-----------|----|---------|-------------------|-----------|----|---------|-------------------|
| PD        |           |    |         |                   |           |    |         |                   |
| 1.3       | 1.5       | 1.7 | IFNL4   | Cirrhosis(freq)   | CHC(freq) |  |   |       |                   | HCC(freq)  | CHC(freq) |  |   |       |                   |
| A C G C   |           |    |         |                   |           |    |         |                   |
| A C G T   | 3.11 (0.014) | 17.50 (0.059) | 6.25 | 0.012 | 0.237 [0.070-0.803] | 21.00 (0.053) | 8.29 (0.028) | 2.82 | 0.09 | 1.992 [0.878-4.519] |
| G C A C   | 27.60 (0.122) | 47.60 (0.161) | 0.90 | 0.343 | 0.782 [0.470-1.301] | 56.17 (0.140) | 47.60 (0.161) | 0.32 | 0.57 | 0.885 [0.581-1.348] |
| G C A T   | 27.32 (0.121) | 23.20 (0.078) | 3.59 | 0.058 | 1.753 [0.976-3.150] | 58.56 (0.146) | 23.20 (0.078) | 8.49 | 0.00 | 2.101 [1.265-3.491] |
| G C G C   | 37.66 (0.167) | 33.13 (0.112) | 4.56 | 0.033 | 1.730 [1.042-2.873] | 43.22 (0.108) | 33.13 (0.112) | 0.12 | 0.73 | 0.890 [0.461-1.717] |
| G C G T   | 26.63 (0.118) | 25.08 (0.085) | 2.31 | 0.128 | 1.562 [0.876-2.786] | 36.96 (0.092) | 25.08 (0.085) | 0.23 | 0.63 | 1.140 [0.670-1.941] |
| G T A C   | 46.53 (0.206) | 74.58 (0.252) | 0.67 | 0.403 | 0.835 [0.548-1.274] | 78.78 (0.197) | 74.58 (0.252) | 2.29 | 0.13 | 0.756 [0.527-1.087] |
| G T A T   | 30.69 (0.136) | 58.42 (0.197) | 2.35 | 0.126 | 0.688 [0.425-1.112] | 61.26 (0.153) | 58.42 (0.197) | 1.79 | 0.18 | 0.763 [0.513-1.135] |

Note: Multi-loci genotype frequency with a frequency <0.05 in both control and cases has been dropped. P value significant at Bonferroni's correction (P-value threshold of 0.0125) are in bold text and highlighted in red when positive association was found and in grey when a negative association was found.

OR (95% CI), Odds ratio with 95% confidence interval.

CIRRHOSIS: Global chi² is 18.330076 while df = 6; Fisher’s P value is 0.005516.

HCC: Global chi² is 13.963398 while df = 7; Fisher’s P value is 0.052061.

MC: Global chi² is 43.070274 while df = 7; Fisher’s P value is 0.348e-007.

NHL: Global chi² is 5.251823 while df = 6; Fisher’s P value is 0.512026.

Abbreviations: BD, blood donors; CHC, chronic hepatitis C virus infection; HCC, hepatocellular carcinoma; hepatic: cirrhosis and HCC; lymph: MC and NHL; MC, autoimmune lymphoproliferative mixed cryoglobulinaemia; NHL, non-Hodgkin lymphoma.
Recently, a new B-cell subpopulation, the CD19\(^+\) PD-L1\(^+\) regulatory B cell (Breg), has shown to require PD-L1 expression to regulate CD4\(^+\) PD-1\(^+\) T follicular helper (Tfh) cell expansion and differentiation and to mediate humoral immunity.\(^{54}\) These findings disclosed novel mechanisms by which PD-1–PD-L1 signalling regulates antibody production and helps to understand the role of this pathway in physiological states and in the altered humoral immunity found in some autoimmune diseases. Given that PD-L1\(^+\) B cells limit both memory B-cell development and plasma cell differentiation by interacting with Tfh cells,\(^{56,57}\) we analysed the expression of PD-1\(^+\) in CD4\(^+\) T cells and PD-L1\(^+\) in IgM\(^+\) CD19\(^+\) B lymphocytes from patients with HCV-related MC. We observed higher levels (\(\geq 10\%\)) of PD-1\(^+\) expression on CD4\(^+\) T cells and lower levels of PD-L1\(^+\) (\(\leq 1\%\)) on IgM\(^+\) CD19\(^+\) B cells in patients with HCV-related MC compared to patients with HCC without HCV-infection, suggesting an alteration in the control of B cell-T cell interaction in our MC cases. These data are consistent with a model in which the reduction in both CD4+PD-1\(^+\) + T cells and regulatory B cells (PD-L1\(^+\)) is less effective in MC cases.\(^{54,75}\) The favourable actions of CD19\(^+\) PD-L1\(^+\) B cells in humoral B-cell homeostasis and in the control of autoimmune diseases were further supported by the demonstration that these B cells are resistant to αCD20 B-cell depletion\(^{54}\) and that MC patients also benefit from αCD20 treatment.\(^{76}\)

This is the first study evaluating a possible association between PDCD1 polymorphisms and the risk of HCV-related lymphoproliferative disorders. These results should be considered descriptive, and larger studies are needed to confirm the model of IFNL4 and PDCD1 epistatic interactions in HCV-related MC and the independent contribution of the IFNL4[T] variant in the control of HCV infection and HCC development.

**FIGURE 4** Pairwise linkage disequilibrium (LD) relationships between the PD-1.3, PD-1.5, PD-1.7 and IFNL4 rs12979860 polymorphism. A–F, Results from linkage analysis conducted for the polymorphism of blood donors (A), CHC (B), cirrhosis (C), HCC (D), MC (E) and NHL (F) respectively.
Elimination of an infectious agent has been postulated to favour the development of autoreactivity.\(^7\) Based on our results we proposed that genes encoding immune proteins involved in the control of HCV infection, for example IFN\(\lambda\)4, need genes that encode other inhibitory proteins, for example PD-1, to inhibit the progression of the disease towards autoimmune MC. The previous study describing that the 15-year cumulative probability of developing cirrhosis and HCC was higher in MC(−) than in MC(+) patients (24.9% vs 14.2% and 20.3% vs 7.5% respectively),\(^7\) supports this model. In keeping with these findings, we found different haplotype associations between HCC and MC patients, although further studies across different populations and functional assessments of relevant polymorphisms are required to confirm the associations of pathogenic relevance.

In conclusion, our study found that IFNL4 and PDCD1 polymorphisms are both important in determining the risk of HCV-related MC, although we have yet to determine precisely how the proteins confer this risk. Our data also confirm and underline the important role of INF\(\lambda\)4 production as a risk factor for HCV persistence and HCC development. As a result of the importance of these genes in the immune response to hepatic infection, autoimmune disorders and malignancies, as well as their role in response to the emerging immune checkpoint treatment for HCC, our study adds new information to help understand the pathogenic role of host genetic variants in HCV-related diseases.

**FUNDING INFORMATION**
Mariangela De Zorzi, Laura Caggiari and Ombretta Repetto had fellowships funded by 5X1000_2010_MdS. Francesca Pezzuto is the recipient of a research fellowship awarded by FIRE/AISF ONLUS (Fondazione Italiana per la Ricerca in Epatologia) http://www.fondazionefegato.it/.

**ETHICS STATEMENTS**
This study was in accordance with the principles of Declaration of Helsinki and all subjects provided written informed consent. The study protocol was accepted by the Comitato Etico Indipendente of the Azienda Ospedaliero-Università Consorziale Policlinico di Bari, the Scientific Board and Ethics Committee of Fondazione G. Pascale Istituto Nazionale Tumori, the Comitato Etico Area Vasta Centro AO careggi, Florence and Committee for the Bio-banking Facility of the Centro di Riferimento Oncologico di Aviano.

**CONFLICT OF INTEREST**
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**ORCID**
Valli De Re https://orcid.org/0000-0001-6100-9373
Maria Lina Tornesello https://orcid.org/0000-0002-3523-3264
Mariangela De Zorzi https://orcid.org/0000-0002-3794-270X
Laura Caggiari https://orcid.org/0000-0003-1651-6653
Francesca Pezzuto https://orcid.org/0000-0002-9585-6834
Patrizia Leone https://orcid.org/0000-0002-0904-1074
Vito Racaneli https://orcid.org/0000-0002-8639-1940
Gianfranco Lauletta https://orcid.org/0000-0003-0152-9398
Stefania Zanussi https://orcid.org/0000-0003-0608-9766

**FIGURE 5** PD-1 and PD-L1 flow cytometry analysis. The lymphocyte population was selected on a forward-versus side-scatterplot for each patient. PBMCs from HCV-negative HCC (n = 2), HCV-associated HCC (n = 2) and HCV-associated MC cases (n = 2) were analysed by flow cytometry after staining with anti-CD4 and anti-PD-1 antibodies, or anti-CD19, anti-IgM and anti-PD-L1 antibodies. Representative dot plot of one case is shown in (A) and (B) respectively.
REFERENCES

1. Ryerson AB, Eheman CR, Altekruse SF, et al. Annual report to the nation on the status of cancer, 1975–2012, featuring the increasing incidence of liver cancer. Cancer. 2016;122:1312-1337.

2. De Re V, Caggiari L, Simula MP, De Vita S, Sansonno D, Dolcetti R. B-cell lymphomas associated with HCV infection. Gastroenterology. 2007;132:1205-1207.

3. Sansonno D, Carbone A, De Vita S, Sansonno D, Dolcetti R. Hepatitis C virus infection, cryoglobulinemia, and beyond. Rheumatology(Oxford). 2007;46:572-578.

4. Prokunina-Olsson L, Muchmore B, Tang W, et al. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet. 2013;45:164-171.

5. Xiao W, Jiang LF, Deng XZ, et al. PD-1/PD-L signal pathway participates in HCV F protein-induced T cell dysfunction in chronic HCV infection. Immunol Res. 2016;64:412-423.

6. Kim PS, Ahmed R. Features of responding T cells in cancer and chronic infection. CurrOpinImmunol. 2010;22:223-230.

7. Blank C, Gajewska TF, Makensen A. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. Cancer ImmunolImmunother. 2005;54:307-314.

8. Zamani MR, Aslani S, Salaminnejad A, Javan MR, Rezaei N, PD-1/PD-L and autoimmunity: A growing relationship. Cell Immunol. 2016;310:27-41.

9. Li Z, Li NA, Li F, et al. Immune checkpoint proteins PD-1 and TIM-3 are both highly expressed in liver tissues and correlate with their gene polymorphisms in patients with HBV-related hepatocellular carcinoma. Med Baltim. 2016;95:e5749.

10. El-Khoueiry AB, Sangro B, Yau T, et al. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. Lancet. 2017;389:2492-2502.

11. Goodman A, Patel SP, Kurzrock R. PD-1/PD-L1 immune-checkpoint blockade in B-cell lymphomas. Nat Rev Clin Oncol. 2017;14:203-220.

12. Cox MA, Nechaniotzky R, Mak TW. Check point inhibitors as therapeutics for infectious diseases. Curr Opin Immunol. 2017;48:61-67. 10.1016/j.coi.2017.07.016.

13. Pervolaraki K, Rastgou Taliem S, Albrecht D, et al. Differential induction of interferon stimulated genes between type I and type III interferons is independent of interferon receptor abundance. PLoS Pathog. 2018;14:e1007420.

14. Prokunina-Olsson L. Genetics of the human interferon lambda region. J Interferon Cytokine Res. 2019;39:599-608.

15. Bamford CG, Aranday-Cortes E, Filipe IC, et al. A polymorphic residue that attenuates the antiviral potential of interferon lambda 4 in hominid lineages. PLoS Pathog. 2018;14:e1007307.

16. Chinnaswamy S. Gene-disease association with human IFNL locus polymorphisms extends beyond hepatitis C virus infections. Genes Immun. 2016;17:265-275.

17. De Re V, De Zorzi M, Caggiari L, et al. HCV-related liver and lymphoproliferative diseases: association with polymorphisms of IL28B and TLR2. Oncotarget. 2016;7:37487-37497.

18. O’Brien TR, Pfeiffer RM, Paquin A, et al. Comparison of functional variants in IFNL4 and IFNL3 for association with HCV clearance. J Hepatol. 2015;63:1103-1110.

19. Ansari MA, Aranday-Cortes E, Ip CL, et al. Interferon lambda 4 impacts the genetic diversity of hepatitis C virus. eLife. 2019;8.

20. Vergara C, Thio CL, Johnson E, et al. Multi-ancestry genome-wide association study of spontaneous clearance of hepatitis C virus. Gastroenterology. 2019;156(1496–1507):e7.

21. Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. NatGenet. 2009;41:1105-1109.

22. Prokunina L, Castillo-Jémez C, Öberg B, et al. A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. Nat Genet. 2002;32:666-669.

23. Torre LA, Sauer AMG, Chen MS, Kagawa-Singer M, Jemal A, Siegel RL. Cancer statistics for Asian Americans, Native Hawaiians, and Pacific Islanders, 2016: Converging incidence in males and females. CA Cancer J Clin. 2016;66:182-202.

24. Petruzziello A, Loquercio G, Sabatino R, et al. Prevalence of Hepatitis C virus genotypes in nine selected European countries: A systematic review. J Clin Lab Anal. 2019;33.

25. European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu, European Association for the Study of the Liver. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J Hepatol. 2018;69:182-236.

26. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood. 2016;127:2375-2390.

27. Pezzuto F, Izzo F, Buonaguro L, et al. Tumor specific mutations in TERT promoter and CTNNB1 gene in hepatitis B and hepatitis C related hepatocellular carcinoma. Oncotarget. 2016;7(34):54253-54262.

28. Xiao W, Zhang Q, Deng XZ, et al. Genetic variations of IL-28B and PD-1 are in association with the susceptibility and outcomes of HCV infection in Southeast China. Infection, Genetics and Evolution. 2015;32:89-96.

29. Qian BP, Wang XQ, Qiu Y, Jiang H, Ji ML, Jiang J. An exon polymorphism of programmed cell death 1 gene is associated with both the susceptibility and thoracolumbar kyphosis severity of ankylosing spondylitis in a Chinese Han population. J OrthopSci. 2013;18:514-518.

30. Bertssias GK, Nakou M, Choulaki C, et al. Genetic, immunologic, and immunohistochemical analysis of the programmed death 1 programmed death ligand 1 pathway in human systemic lupus erythematosus. Arthritis Rheum. 2009;60:207-218.

31. Kong EK, Prokunina-Olsson L, Wong WH, et al. A new haplotype of PDCD1 is associated with rheumatoid arthritis in Hong Kong Chinese. Arthritis Rheum. 2005;52:1058-1062.

32. Nielsen C, Hansen D, Husby S, Jacobsen BB, Lillevang ST. Association of a putative regulatory polymorphism in the PD-1 gene with susceptibility to type 1 diabetes. Tissue Antigens. 2003;62:492-497.

33. Da LS, Zhang Y, Zhang CJ, et al. The PD-1 rs36084323 A > G polymorphism decrease cancer risk in Asian: A meta-analysis. PatholResPract. 2018;214:1758-1764.

34. Shamsdin SA, Karimi MH, Hosseini SV, et al. Associations of ICOS and PD-1 gene variants with colon cancer risk in the iranian population. Asian PacJ. Cancer Prev. 2018;19:693-698.

35. Tan D, Sheng L, Yi QH. Correlation of PD-1/PD-L1 polymorphisms and expressions with clinicopathologic features and prognosis of ovarian cancer. Cancer Biomark. 2018;21:287-297.

36. Salaminnejad A, Khoramshahi V, Azani A, et al. PD-1 and cancer: molecular mechanisms and polymorphisms. Immunogenetics. 2018;70:73-86.

37. Tang W, Chen S, Chen Y, et al. Programmed death-1 polymorphisms is associated with risk of esophagogastric junction adenocarcinoma
in the Chinese Han population: A case-control study involving 2,740 subjects. *Onco Targets*. 2017;8:39198-39208.

38. Li Y, Zhang HL, Kang S, Zhou RM, Wang N. The effect of polymorphisms in PD-1 gene on the risk of epithelial ovarian cancer and patients’ outcomes. *Gynecol Oncol*. 2017;144:140-145.

39. Zhou RM, Li Y, Wang N, Huang X, Cao SR, Shan BE. Association of programmed death-1 polymorphisms with the risk and prognosis of esophageal squamous cell carcinoma. *Cancer Genet*. 2016;209:365-375.

40. Zhang J, Zhao T, Xu C, Huang J, Yu H. The association between polymorphisms in the PDCD1 gene and the risk of cancer: A PRISMA-compliant meta-analysis. *Med Baltim*. 2016;95:e4423.

41. Ren H-T, Li Y-M, Wang X-J, et al. PD-1 rs2227982 polymorphism is associated with the decreased risk of breast cancer in Northwest Chinese women: A hospital-based observational study. *Med Baltim*. 2016;95:e3760.

42. Dong W, Gong M, Shi Z, Xiao J, Zhang J, Peng J. Programmed cell death-1 polymorphisms decrease the cancer risk: A meta-analysis involving twelve case-control studies. *PLoS One*. 2016;11:e0152448.

43. Ge J, Zhu L, Zhou J, et al. Association between co-inhibitory molecule gene tagging single nucleotide polymorphisms and the risk of colorectal cancer in Chinese. *J Cancer ResClin Oncol*. 2015;141:1533-1544.

44. Li Z, Li N, Zhu Q, et al. Genetic variations of PD1 and TIM3 are differentially and interactively associated with the development of cirrhosis and HCC in patients with chronic HBV infection. *Infection, Genetics and Evolution*. 2013;14:240-246.

45. Bayram S, Akkiz H, Ulger Y, Bekar A, Akgollu E, Yildirim S. Lack of an association of programmed cell death-1 PD1.3 polymorphism with risk of hepatocellular carcinoma susceptibility in Turkish population: a case-control study. *Gene*. 2012;511:308-313.

46. Sun RF, Yu QQ, Young KH. Critically dysregulated signaling pathways and clinical utility of the pathway biomarkers in lymphoid malignancies. *ChroniDis TransMed*. 2018;4:29-44.

47. Hude I, Sasse S, Engert A, Brockelmann PJ. The emerging role of immune checkpoint inhibition in malignant lymphoma. *Haematologica*. 2017;102:30-42.

48. Zhang G, Liu Z, Duan S, et al. Association of polymorphisms of programmed cell death-1 gene with chronic hepatitis B virus infection. *HumImmunol*. 2010;71:1209-1213.

49. Noureddin M, Rotman Y, Zhang F, et al. Hepatic expression levels of CD28 expression in chronic hepatitis B patients with advanced hepatocellular carcinoma. *Liver Int*. 2010;30:1379-1386.

50. Wang BJ, Bao JJ, Wang JZ, et al. Immunostaining of PD-1/PD-L in liver tissues of patients with hepatitis and hepatocellular carcinoma. *World J Gastroenterol*. 2011;17:3322-3329.

51. Rossini M, Li NA, Zhang P, et al. PD-1 mRNA expression is associated with clinical and viral profile and PD1 3’-untranslated region polymorphism in patients with chronic HBV infection. *Immunol Lett*. 2014;162:215-216.

52. Penna A, Pilli M, Zerbini A, et al. Dysfunctional and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology*. 2007;45:588-601.

53. Maddalena F, Iberbgu CC, Fernandez ML, et al. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol*. 2007;81:2545-2553.

54. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439:682-687.

55. Moreno-Cubero E, Larrubia JR. Specific CD8+ T cell response immunotherapy for hepatocellular carcinoma and viral hepatitis. *World J Gastroenterol*. 2016;22:6469-6483.

56. Fuller MJ, Callendret B, Zhu B, et al. Immunotherapy of chronic hepatitis C virus infection with antibodies against programmed cell death-1 (PD-1). *Proc Natl Acad Sci USA*. 2013;110:15001-15006.

57. Aloulou M, Fazillean U. Regulation of B cell responses by distinct populations of CD4 T cells. *Biomed J*. 2019;42:242-251.

58. Zhang Y, Zhu SL, Chen J, Li LQ. Meta-analysis of associations of interleukin-28B polymorphisms rs8099917 and rs12979860 with development of hepatitis virus-related hepatocellular carcinoma. *OncoTargetsTher*. 2016;9:3249-3257. https://doi.org/10.2147/OTT. S104904.

59. Bhushan A, Chinnaswamy S. Identifying causal variants at the interferon lambda locus in case-control studies: Utilizing non-synonymous variant rs117648444 to probe the role of IFN-lambda4. *Gene*. 2018;664:168-180. 10.1016/j.gene.2018.04.076.

60. Terczyńska-Dyla E, Biber S, Duong FHT, et al. Reduced IFN-γ activity is associated with improved HCV clearance and reduced expression of interferon-stimulated genes. *Nat Commun*. 2014;5:1-9.

61. Ifarrarairaegui M, Melero I, Sangro B. Immunotherapy of hepatocellular carcinoma: Facts and hopes. *Clin Cancer Res*. 2018;24:1518-1524.

62. Yao H, Wang H, Li C, Fang J-Y, Xu J. Cancer cell-intrinsic PD-1 and implications in combinatorial immunotherapy. *Front Immunol*. 2018;9:1774.

63. De Re V, De Vita S, Marzotto A, et al. Sequence analysis of the immunoglobulin antigen receptor of hepatitis C virus-associated non-Hodgkin lymphomas suggests that the malignant cells are derived from the rheumatoid factor-producing cells that occur mainly in type II cryoglobulinemia. *Blood*. 2000;96:3578-3584.

64. Boni C, Fiscaro P, Valdatta C, et al. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol*. 2007;81:4215-4225.

65. Fiscaro P, Valdatta C, Massari M, et al. Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B. *Gastroenterology*. 2010;138(682–93):693.

66. Hsu P-N, Yang T-C, Kao J-T, et al. Increased PD-1 and decreased CD28 expression in chronic hepatitis B patients with advanced hepatocellular carcinoma. *Liver Int*. 2010;30:1379-1386.

67. Zhang G, Li NA, Zhang P, et al. PD-1 mRNA expression is associated with clinical and viral profile and PD1 3’-untranslated region polymorphism in patients with chronic HBV infection. *Immunol Lett*. 2014;162:213-216.

68. Perna A, Pilli M, Zerbini A, et al. Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology*. 2007;45:588-601.

69. Radziewicz H, Ibebgwu CC, Fernandez ML, et al. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol*. 2007;81:2545-2553.

70. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439:682-687.

71. Vollmer-Hibbeler E, Larrubia JR. Specific CD8+ T cell response immunotherapy for hepatocellular carcinoma and viral hepatitis. *World J Gastroenterol*. 2016;22:6469-6483.

72. Nisperos-Castillo B, Zhu B, et al. Immunotherapy of chronic hepatitis C virus infection with antibodies against programmed cell death-1 (PD-1). *Proc Natl Acad Sci USA*. 2013;110:15001-15006.

73. Sansonno D, Lauletta G, De Re V, et al. Intrahepatic B cell clonal expansions and extrahepatic manifestations of chronic HCV infection. *EurJ Immunol*. 2004;34:126-136.

74. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly - TFH cells in human health and disease. *Nat Rev Immunol*. 2013;13:412-426.
virus-related mixed cryoglobulinemia: a long-term study. *Blood*. 2010;116:343-353.

77. Ercolini AM, Miller SD. The role of infections in autoimmune disease. *Clin Exp Immunol*. 2009;155:1-15.

78. Lauletta G, Russi S, Conteduca V, Sansonno L, Dammacco F, Sansonno D. Impact of cryoglobulinemic syndrome on the outcome of chronic hepatitis C virus infection: A 15-year prospective study. *Med Baltim*. 2013;92:245-256.

79. Hoffmann TW, Halimi J-M, Buchler M, et al. Association between a polymorphism in the human programmed death-1 (PD-1) gene and cytomegalovirus infection after kidney transplantation. *J Med Genet*. 2010;47:54-58.

**SUPPORTING INFORMATION**

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

**How to cite this article:** De Re V, Tornesello ML, De Zorzi M, et al. *PDCD1* and *IFNL4* genetic variants and risk of developing hepatitis C virus-related diseases. *Liver Int*. 2021;41:133-149. [https://doi.org/10.1111/liv.14667](https://doi.org/10.1111/liv.14667)
Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:
De Re, V; Tornesello, ML; De Zorzi, M; Caggiari, L; Pezzuto, F; Leone, P; Racanelli, V; Lauletta, G; Zanussi, S; Repetto, O; Gragnani, L; Rossi, FM; Dolcetti, R; Zignego, AL; Buonaguro, FM; Steffan, A

Title:
PDCD1 and IFNL4 genetic variants and risk of developing hepatitis C virus-related diseases

Date:
2021-01-01

Citation:
De Re, V., Tornesello, M. L., De Zorzi, M., Caggiari, L., Pezzuto, F., Leone, P., Racanelli, V., Lauletta, G., Zanussi, S., Repetto, O., Gragnani, L., Rossi, F. M., Dolcetti, R., Zignego, A. L., Buonaguro, F. M. & Steffan, A. (2021). PDCD1 and IFNL4 genetic variants and risk of developing hepatitis C virus-related diseases. LIVER INTERNATIONAL, 41 (1), pp.133-149. https://doi.org/10.1111/liv.14667.

Persistent Link:
http://hdl.handle.net/11343/272384

File Description:
Published version

License:
CC BY