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Original Article

CD5/CD20 expression on circulating B cells in HCV-related chronic hepatitis and mixed cryoglobulinemia

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ABSTRACT

The role of $CD5^+$ B cells in patients with HCV infection and HCV-related disorders, including mixed cryoglobulinemia (MC), has been addressed in previous reports with conflicting results. We established a correlation between CD5/CD20 expression on circulating B lymphocytes, characterizing monoclonal B cell lymphocytosis (MBL), and clinical features in a cohort of 45 patients with chronic HCV hepatitis [without MC: 23 patients (MC- group); with MC: 22 patients (MC+ group)], and 45 HCV-negative healthy subjects as controls. By flow cytometry analysis, three B cells phenotypes were singled out: 1) $CD5^+CD20^{dim}$ (CLL-like phenotype); 2) $CD5^+CD20^{bright}$ (atypical phenotype); and 3) $CD5^-CD20^+$ phenotype. $CD5^+CD20^{bright}$ cells were reduced in MC- patients ($p = 0.049$). $CD5^+CD20^{dim}$ B cells were significantly higher in group B than in the control group ($p = 0.003$). ROC curve analysis in MC+ patients showed the highest positive likelihood ratio at $\geq 7.35\%$ ($p = 0.008$) for CLL-like phenotype and at $\leq 63.6\%$ ($p = 0.03$) for the $CD5^-CD20^+$ B cell phenotype. HCV infection was associated with a higher frequency of CLL-like (odds ratio = 16, $p = 0.002$) and a lower frequency of atypical (odds ratio: 3.1, $p = 0.02$) and $CD5^-CD20^+$ (odds ratio: 11, $p = 0.01$) phenotypes. The association with higher levels of CLL-like phenotype progressively increased from group of MC- patients (odds ratio: 9.3, $p = 0.04$) to the group of MC+ patients (odds ratio: 25.1, $p = 0.0003$).

Conclusions: The occurrence of a CLL-like pattern may allow to identify HCV-infected patients at risk of developing MC and eventually non-Hodgkin lymphoma, who should require a closer surveillance and a longer follow-up.

1. Introduction

CD5 lymphocyte receptor, also known as lymphocyte antigen T1/Leu-1, is a scavenger-like receptor initially used to identify T cells, but subsequently detected on a subset of normal B-cells (B-1a lymphocytes) secreting polyreactive antibodies, as well as on the surface of leukemic B-cells [1,2]. CD5 is considered a modulator of antigen receptor signaling. Its expression on T lymphocytes regulates T cell receptor (TCR) response and is directly proportional to the avidity of antigen-specific receptors [3]. B cells modulate CD5 expression levels through the use of exon 1 for transcription [4]. According to this mechanism, CD5 can be expressed on B-cell surface or retained in the cytoplasm as a truncated form.

In B cell chronic lymphocytic leukemia (B-CLL) the surface expression of CD5 results predominant, suggesting that this marker confers a survival benefit on B cells [5]. $CD5^+$ B cells have also been considered as the bridge linking innate and acquired immune responses, characterized by their early onset in ontogeny and the production of low-affinity IgM rheumatoid factor (RF) molecules [6]. Their increased frequency has also been found to be associated with the

Abbreviations: AID, autoimmune disease; aOR, adjusted odds ratio; B-CLL, B cell chronic lymphocytic leukemia; B-NHL, B cell non-Hodgkin lymphoma; BSA, bovine serum albumin; CH, chronic hepatitis; CMV, cytomegalovirus; DAAs, direct-acting antiviral agents; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; MBL, monoclonal B cell lymphocytosis; MC, mixed cryoglobulinemia; OR, odds ratio; PBS, phosphate-buffered saline solution; RF, rheumatoid factor; ROC, receiver operating characteristic; SD, standard deviation; SVR, sustained virological response; TCR, T cell receptor.

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production of circulating autoantibodies in autoimmune diseases (AIDs), such as rheumatoid arthritis and Sjögren's syndrome, as well as virus-related autoimmune manifestations [7–11].

Hepatitis C virus (HCV) is a major hepatotropic virus causing chronic hepatitis, cirrhosis and primary liver tumors, but also a lymphotropic virus associated with benign lymphoproliferative diseases such as mixed cryoglobulinemia (MC) and with a subset of B-cell non-Hodgkin lymphomas (B-NHLs). MC is a small-vessel vasculitis characterized by the production of serum monoclonal or polyclonal IgM molecules with RF activity that react with polyclonal IgG, leading to the formation of cryoprecipitating immune complexes. MC is also characterized by the selection of B cell clones sustaining autoimmunity and with potential malignant evolution [12–14]. However, cryoglobulin-related illness, known as cryoglobulinemic vasculitis, appears in a minority of patients (10 to 15%) [15,16].

A clonal expansion of peripheral $CD5^+$ B cells has been previously reported in HCV infection [11,17]. $CD5^+$ B cells have been shown to produce a monoclonal RF with WA cross-reactive idiotype [18–20], and are likely responsible for the production of other autoantibodies associated with HCV infection. The expansion of $CD5^+CD81^+$ B cells both in the liver and peripheral blood of HCV chronically infected patients suggests a virus-induced clonal expansion [21], and is possibly related to the development of HCV-associated autoimmunity and lymphoproliferation [22]. However, the relations between HCV and immune system are not completely understood and markers capable of identifying patients that could develop a lymphoproliferative disorder are not currently available.

A condition named monoclonal B cell lymphocytosis (MBL) has been described in the peripheral blood of otherwise healthy individuals. Its prevalence increases with age to a variable extent, depending on the detection methods [23–25]. MBL has been associated both with an increased risk of developing an overt hematological malignancy, mainly B-CLL, and with senescence of the immune system [26,27], and is possibly related to persistent viral infections such as HCV and CMV, as well as to prolonged exposure to autoantigens [28–30]. As specified below, MBL may exhibit three different cellular phenotypes, and all of them can be detected in patients with HCV chronic infection more frequently than in the general population [31].

Based on these premises, we studied the frequencies of three B cell subsets based on CD5/CD20 expression in patients with HCV-related chronic hepatitis, with and without MC, compared with healthy subjects. Our aim was to establish whether the expression of a particular B cell phenotype could help to characterize patients with chronic HCV infection and identify those potentially evolving into a lymphoproliferative disease.

2. Materials and methods

2.1. Patients and controls

Forty-five HCV chronically infected patients were included in this study: in 23 of them showing a clinical picture of chronic hepatitis (CH), cryoglobulins were not detected (MC- group), whereas in the remaining 22 patients chronic hepatitis was associated with MC (MC+ group). Forty-five healthy subjects were included as controls.

All patients and controls were Caucasians, tested HBV- and HIV-negative and were followed-up at our Department. Written informed consent was obtained from all of them. The study was approved by the local ethical committee and was conducted in accordance with the Declaration of Helsinki.

2.2. Flow cytometry

Anonymized peripheral blood samples in EDTA were maintained at room temperature and then tested by flow cytometry within 6 hours from collection. One hundred µl of each peripheral blood sample were incubated for 30 minutes with 0.3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) solution to avoid nonspecific interactions. Samples were then incubated in the dark with suitable antibodies for 15 minutes at room temperature. The following antibodies by Immunotech (Marseille, France) were used: phycoerythrin-Texas Red (ECD)-conjugated anti-CD19, fluorescein isothiocyanate (FITC)-conjugated anti-CD5, and R phycoerythrin-Cyanin 5.1 (PC5)-conjugated anti-CD20. Then, 2 ml of IOTest3 Lysis Solution (Immunotech) were added to lyse red blood cells, and the mixture was incubated for 10 minutes in the dark. Following several washes with PBS plus BSA solution, up to 500,000 events were recorded for each sample on a Coulter Epics XL-MCL instrument (Beckman Coulter, Brea, CA, USA). The lymphocyte population was identified gating low forward and side scatter (FSC/SSC) $CD19^+$ cells, which were then separated into $CD5^+$ and $CD5^-$ subsets. Gated $CD19^+$ cells were analyzed in a dot plot comparing the expression of CD5 versus CD20 to identify different B cells subsets, as described below.

According to the diagnostic criteria for MBL [32] and their recent revision [33], leaving aside clonal expansion, B cells were distinguished into three phenotypes: 1) $CD5^+CD20^{dim}$ (CLL-like phenotype); 2) $CD5^+CD20^{bright}$ (atypical phenotype); and 3) $CD5^-CD20^+$ phenotype. The gating procedure was calibrated on a sample from a patient with long-lasting diagnosis of B-CLL. Absolute B cell counts were calculated from flow cytometry and complete blood count data (Fig. 1).

2.3. Statistical analysis

Demographic characteristics and laboratory data were summarized as counts, proportions, and mean ± SD or median with range, as appropriate. Normal distribution of data was assessed by Kolmogorov-Smirnov test. Differences among groups were evaluated by t, Mann-Whitney, ANOVA, Kruskal-Wallis, Fisher, and Spearman correlation tests according to the nature of data. Receiver operating characteristic (ROC) curves were used to estimate the threshold value with the highest positive likelihood ratio for each B cell phenotype. We performed a forest plot of odds ratios for levels of B cell subsets in patients affected with CH or MC, and a logistic regression analysis to evaluate the impact of age on B cell phenotypes. P values < 0.05 were considered significant. Analyses were performed using IBM SPSS Statistics 20.0 (IBM Corp., Armonk, NY) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

3. Results

Table 1 summarizes the main evaluation parameters of patients with and without MC, and controls. Female sex prevailed in patients MC+ group. White blood cells and absolute lymphocyte counts were roughly similar in both groups. Increased levels of RF were a hallmark of MC+ patients. All patients were anti-HCV positive and 25 of them were viremic (14/23 and 11/22 patients in MC- group and in MC+ group, respectively). On the contrary, 9 MC- patients and 11 MC+ patients were treated with either pegylated interferon-based regimens or with all-oral direct antiviral agents (DAAs) including sofosbuvir/ribavirin, sofosbuvir/ledipasvir, sofosbuvir/simeprevir, ombitasvir/paritaprevir/ritonavir/dasabuvir according to viral genotype and grade of liver fibrosis. HCV RNA was undetectable in all of them.

The results of flow cytometry analyses defining CD20 and CD5 expression on B lymphocytes are summarized in Table 2. MC+ patients showed a lower frequency of circulating $CD20^+$ cells than controls ($p = 0.02$). We identified three different patterns of CD5/CD20 expression on B cells surface. $CD5^+CD20^{bright}$ (atypical phenotype) cells were reduced in MC- patients ($p = 0.049$) as compared with controls. On the contrary, the percentages of $CD5^+CD20^{dim}$ B cells (CLL-like phenotype) were significantly higher in MC+ patients (8.0 ± 13.3, mean ± SD) as compared with control group ($p = 0.003$).

ROC curve analyses regarding the three B cell phenotypes showed a

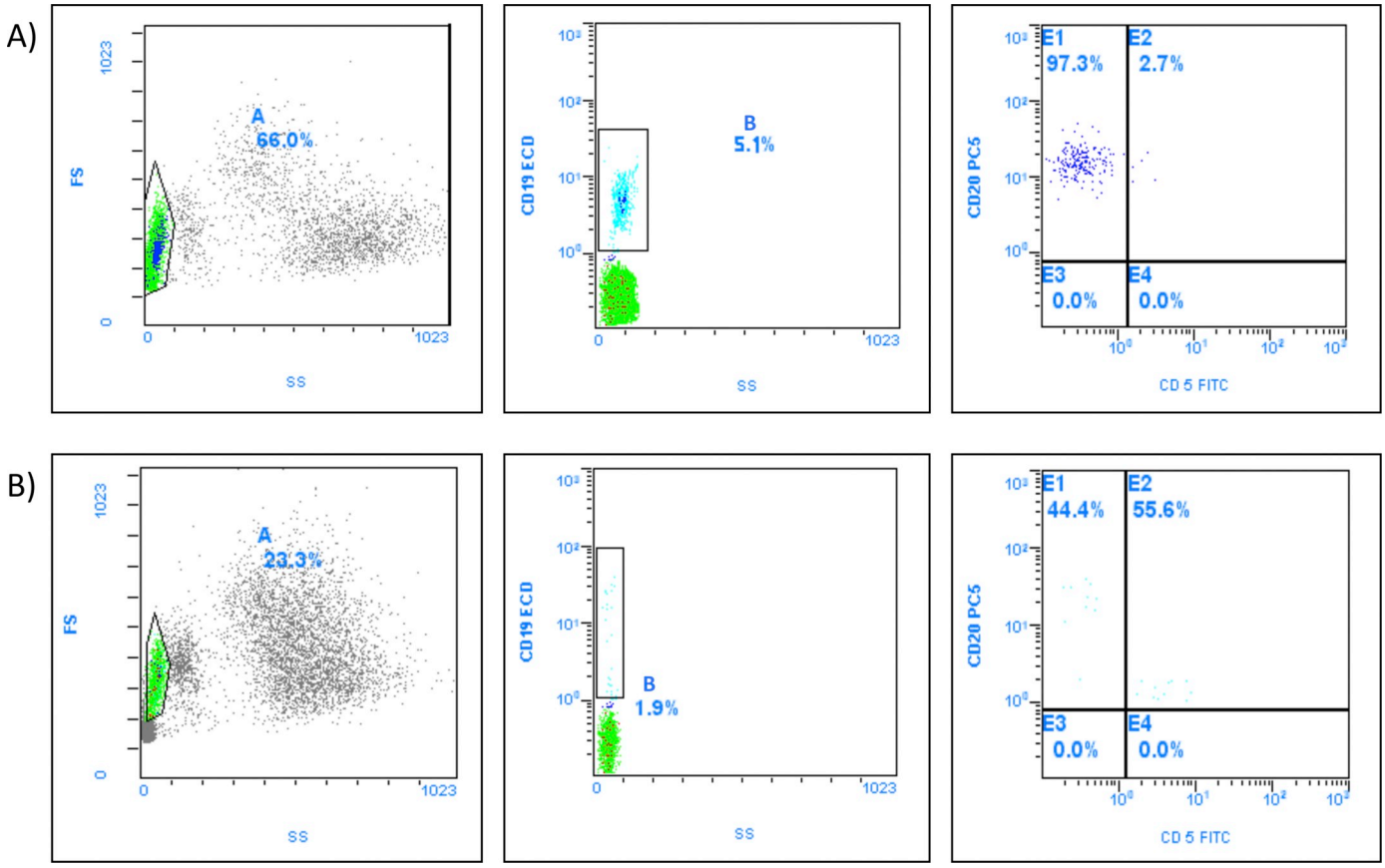

Fig. 1. Representative flow cytometry dot plots for quantification of peripheral B cells MBL phenotypes in a control subject (A) and a patient with HCV-related mixed cryoglobulinemia (B). Lymphomonocytes are detected by morphological characteristics (Forward and Side Scatter; gate A) and CD19 positivity (gate B). Gated $CD19^+$ cells have been evaluated for expression of CD20 and CD5 to identify different B cells subsets.

significant area under the curve for CLL-like phenotype in MC+ patients (Table 3). The threshold value with the highest positive likelihood ratio for this group of patients was ≥7.35%. Moreover, a cut-off ≤ 63.6% was established as significant for $CD5^-CD20^+$ B cells phenotype, whereas for atypical pattern the cut-off ≤ 1.5%, although not significant, was however considered, as it represents the value with the highest positive likelihood ratio. As shown in Fig. 2, the number of patients with frequency of $CD5^+CD20^{dim}$ CLL-like phenotype higher

Table 1
Summary of the main demographic and pathologic characteristics of patients with chronic hepatitis, without (MC- group) and with (MC+ group) MC as compared with controls

	Controls (45)	Chronic HCV hepatitis			
		MC- group (23)	p	MC+ group (22)	p
Age, (yrs, mean ± SD)	54.3 ± 16.4	67.5 ± 10.5	0.001	68.1 ± 10.1	0.001
Sex, (F/M)	27/18	12/11		18/4	
Alt, (nv: 12-78 IU/L; mean ± SD)	28.3 ± 10.3	75.5 ± 102.8	< 0.0001	89.8 ± 143.1	0.02
Total bilirubin, (nv: 0.20-1.00 mg/dL; mean ± SD)	0.55 ± 0.25	0.62 ± 0.34		0.84 ± 0.65	0.03
Serum creatinine, (nv: 0.67-1.17 mg/dL)	0.83 ± 0.2	0.70 ± 0.16	0.009	0.90 ± 0.24	
Liver fibrosis[a]	ND				
F0-F2		12		13	
F3-F4		11		9	
Anti-HCV positive, (n, %)	0	23 (100)		22 (100)	
Serum HCV RNA					
Positive, (n %)	0	14 (60.9)		11 (50)	
Titer, (median Log IU/ml; range)		6.2 (5.3–7.3)		6.2 (4.9–7.6)	
HCV genotype	ND				
G1		16		12	
G2		7		10	
WBC, (cell/μl; mean ± SD)	6664.9 ± 1883.3	5378.7 ± 2080.4	0.01	5558.1 ± 1719.9	0.04
Lymphocytes, (%; mean ± SD)	28.8 ± 8.0	31.6 ± 7.1		27.3 ± 9.6	
Lymphocytes, (cell/μl; mean ± SD)	1857.8 ± 605.3	1647.0 ± 568.7		1520.3 ± 692.7	
Rheumatoid factor, (nv: 10-15 U/mL; mean ± SD)	5.9 ± 2.9	5.2 ± 4.0		102.2 ± 204.5	< 0.0001

[a] Evaluated on the basis of liver biopsy or transient elastography (Fibroscan).

Table 2
CD5 and CD20 expression on peripheral B lymphocytes by flow cytometry in HCV-positive patients without (MC- group) and with (MC+ group) MC, and controls.

	Controls	MC- group	p	MC+ group	p
	(45)	(23)		(22)	
$CD20^+$, (%, mean ± SD)	9.2 ± 4.5	7.7 ± 4.7		6.6 ± 5.3	0.02
$CD20^+$, (cells/μl, mean ± SD)	173.2 ± 103.5	128.0 ± 90.1		99.3 ± 94.5	0.004
$CD5^+CD20^{bright}$, (%, mean ± SD)	2.7 ± 3.5	1.5 ± 2.6	0.049	2.1 ± 4.1	0.05
$CD5^+CD20^{dim}$, (%, mean ± SD)	1.3 ± 2.3	2.7 ± 4.1		8.0 ± 13.3	0.003
$CD5^-CD20^+$, (%, mean ± SD)	88.0 ± 16.4	85.5 ± 24.3		76.5 ± 26.4	

Table 3
ROC curve analyses regarding CLL-like, atypical, and $CD5^-$ B cell phenotypes in HCV-chronically infected patients without (MC- group) and with (MC+ group) MC.

	Area	95% CI	P value	Cut-off	Sensitivity	Specificity	Positive likelihood ratio
CLL-like#							
MC- GROUP	0.58	0.43 – 0.73	0.28	≥ 7.35	17.4	97.8	7.8
MC+ GROUP	0.70	0.56 – 0.84	0.008	≥ 7.35	36.4	97.8	16.4
Atypical*							
MC- GROUP	0.64	0.50 – 0.78	0.06	≤ 1.25	69.6	60.0	1.7
MC+ GROUP	0.64	0.50 – 0.80	0.06	≤ 1.5	72.7	66.7	2.1
CD5‡							
MC- group	0.57	0.42 – 0.73	0.31	≤ 61.0	13.0	97.8	5.8
MC+ group	0.64	0.50 – 0.79	0.03	≤ 63.6	27.3	97.8	12.3

For further analyses cut-off values were set at: # ≥ 7.35; * ≤ 1.5 (reversed ROC analysis); ‡ ≤ 63.6 (reversed ROC analysis).

than the cut-off progressively increases in both patients' groups, with statistical significance as compared with controls (p = 0.04 and p = 0.0003, respectively). At the same time, we observed a higher number of patients with atypical and $CD5^-CD20^+$ phenotypes lower than the cut-off.

HCV infection seems to increase the risk of higher levels of CLL-like B cells (OR: 16) and lower frequency of atypical and $CD5^-CD20^+$ phenotypes (OR: 3.1 and 11, respectively) (Fig. 3A). In addition, the strength of the association with higher levels of CLL-like phenotype progressively increases from MC- (OR: 9.3; p = 0.04) to MC+ patients (OR: 25.1; p = 0.0003) (Fig. 3B). At the same time, the finding of lower $CD5^-CD20^+$ phenotype was associated to the occurrence of MC (OR: 16.5; p = 0.004), whereas a lower atypical B cell phenotype was associated with the occurrence of chronic hepatitis without MC (OR: 3.2; p = 0.04) (Fig. 3C and D).

No correlation was found between the patients' age, a clinical parameter previously shown to be associated with MBL [21–23], and CLL-like B cell levels, neither overall (r = 0.1, p = 0.22) nor in each patient's group (MC+ group: r = -0.2, p = 0.43 and MC- group: r = -0.4, p = 0.07) (Fig. 4A). Similar results were found when considering B cells with atypical phenotype (Fig. 4B).

Binary logistic regression analysis of CLL-like, atypical, and $CD5^-CD20^+$ cell phenotypes as the first, second, and third block was also performed to assess disease prediction capability. The analysis showed that CLL-like B cells level is the main parameter that correlates with the occurrence of MC (p = 0.003, aOR = 25.14, Nagelkerke R^2 = 0.27). The introduction of atypical B cells level in the model resulted not significant in group B (p = 0.26, aOR = 2.0, Nagelkerke R^2 = 0.29). No effects were also observed when introducing $CD5^-$ B cells phenotype in the model as third factor; on the contrary, they worsened the model (Table 4).

As reported above, 11 patients with chronic hepatitis and MC achieved a sustained virological response (SVR) after antiviral therapy. In Table 5 the main clinical and laboratory parameters related to liver disease and cryoglobulinemic vasculitis at baseline and after SVR are summarized. The length of follow-up after SVR ranged from 29 to 47 months. In 5 cases (3 treated with Peg-IFN/RBV and 2 with DAAs), production of cryoglobulins and clinical signs of cryoglobulinemic vasculitis persisted despite SVR. In particular, in the 2 patients treated with DAAs, CLL-like cells were absent at baseline vs 19.5% ± 14.8 at the end of treatment (mean ± SD). CLL-like phenotype was also expanded in 3 cryoglobulinemic patients experiencing long-lasting SVR after Peg-IFN/RBV-based therapy. Conversely, a reduction of $CD5^+CD20^{dim}$ B cells was observed in two additional HCV-positive cryoglobulinemic patients who achieved SVR after DAAs therapy associated with MC disappearance (13.8% ± 15.7 at baseline vs 4.9% ± 2.3 at the end of treatment, mean ± SD).

4. Discussion

CD5 lymphocyte receptor plays a role in the modulation of antigen-receptor signaling on B and T cells, probably exerting a pro-survival effect in some conditions [5,34–37]. A possible pathogenetic role of $CD5^+$ B-cells has been reported in several AIDs as well as in lymphoproliferative diseases such as CLL [1,6–11]. $CD5^+$ B cell expansion has also been described in the course of HCV chronic infection [11,17].

MC is an AID closely associated to chronic HCV infection, characterized by the production of monoclonal (more frequently than polyclonal) IgM molecules with RF activity that react with polyclonal IgG, leading to the formation of cryoprecipitating immune complexes, sustained by B cell clonal expansions with potential evolution to B-NHL [38]. The role of $CD5^+$ B cells in the course of HCV infection has been investigated with variable results. A previous report from our group on MC suggested that HCV stimulates $CD5^+$ B cells (B-1a cells) to produce polyspecific IgM molecules bearing the WA cross-reactive idiotype which characterize soluble, non-cryoprecipitating immune complexes. In addition, monospecific IgM are probably secreted by $CD5^-$ B cells as a result of somatic mutations after preselection in germinal centers [19].

We hypothesized that, following HCV infection of hepatocytes, a variety of cellular and humoral immune responses occur aimed at eliminating the virus. In the proposed model, $CD5^+$ (B-1) B cells are responsible for the primary response. IgM derived from $CD5^+$ B cells bind with relatively low affinity either to the immunizing virus or to various self-antigens such as $F(ab')_2$ and Fc fragments of IgG. These polyreactive antibodies enhance the early recruitment of the virus to secondary lymphoid organs, and this results in a specific protective response. On the basis of established features in the marked difference of $CD5^-$ (B-2) B cells to acquire somatic mutations and to undergo

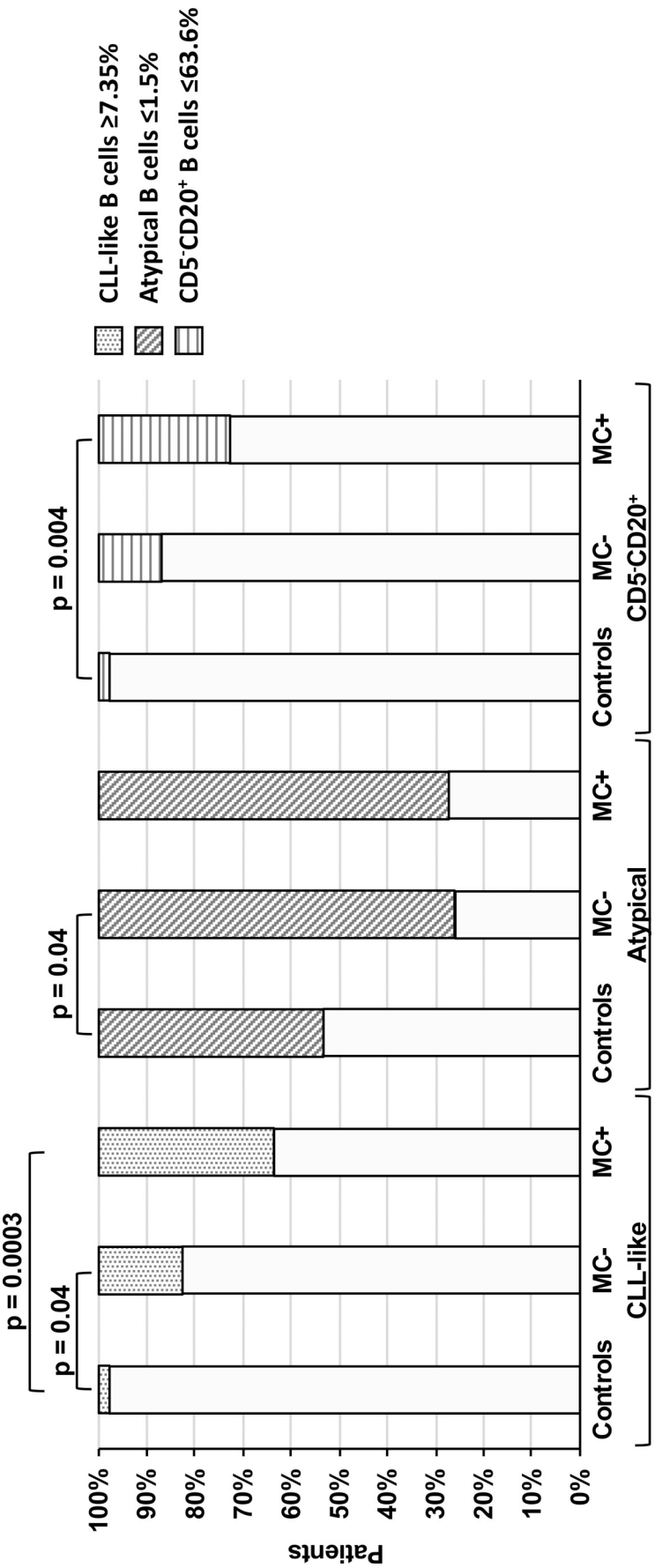

Fig. 2. Frequencies of patients with levels of CLL-like, atypical, and $CD5^-CD20^+$ B cell phenotypes in MC-, MC+ and controls. According to ROC curve analysis, we considered as cut-off the threshold value with the highest positive likelihood ratio for each group (CLL-like ≥ 7.35%; atypical ≤ 1.5%; $CD5^-CD20^+$ ≤ 63.6%).

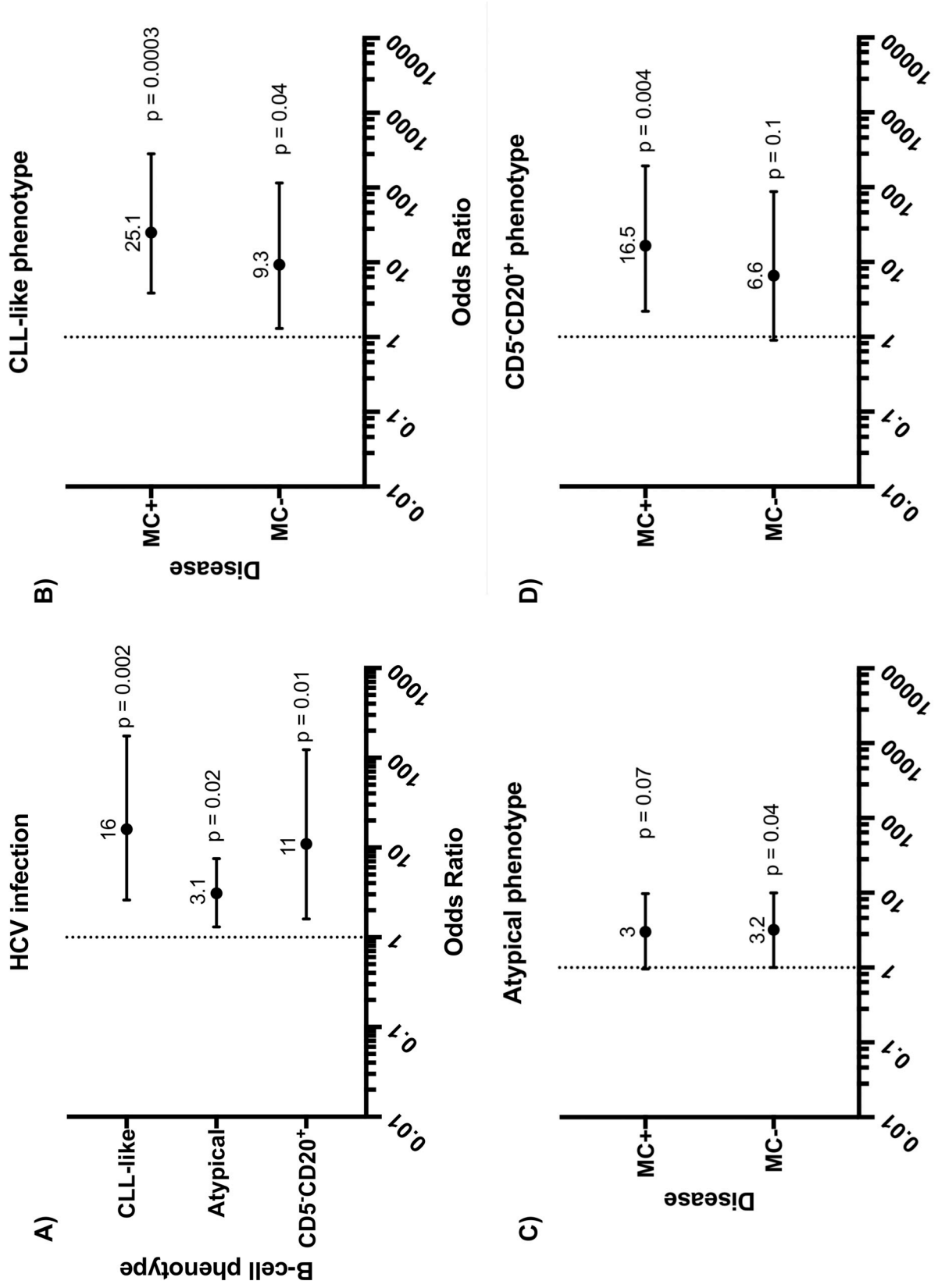

Fig. 3. Association between the occurrence of CLL-like, atypical, and $CD5^{-}CD20^{+}$ phenotypes and HCV infection (A). Impact of CLL-like (B), atypical (C), and $CD5^{-}CD20^{+}$ (D) phenotypes on the occurrence of CH with and without MC.

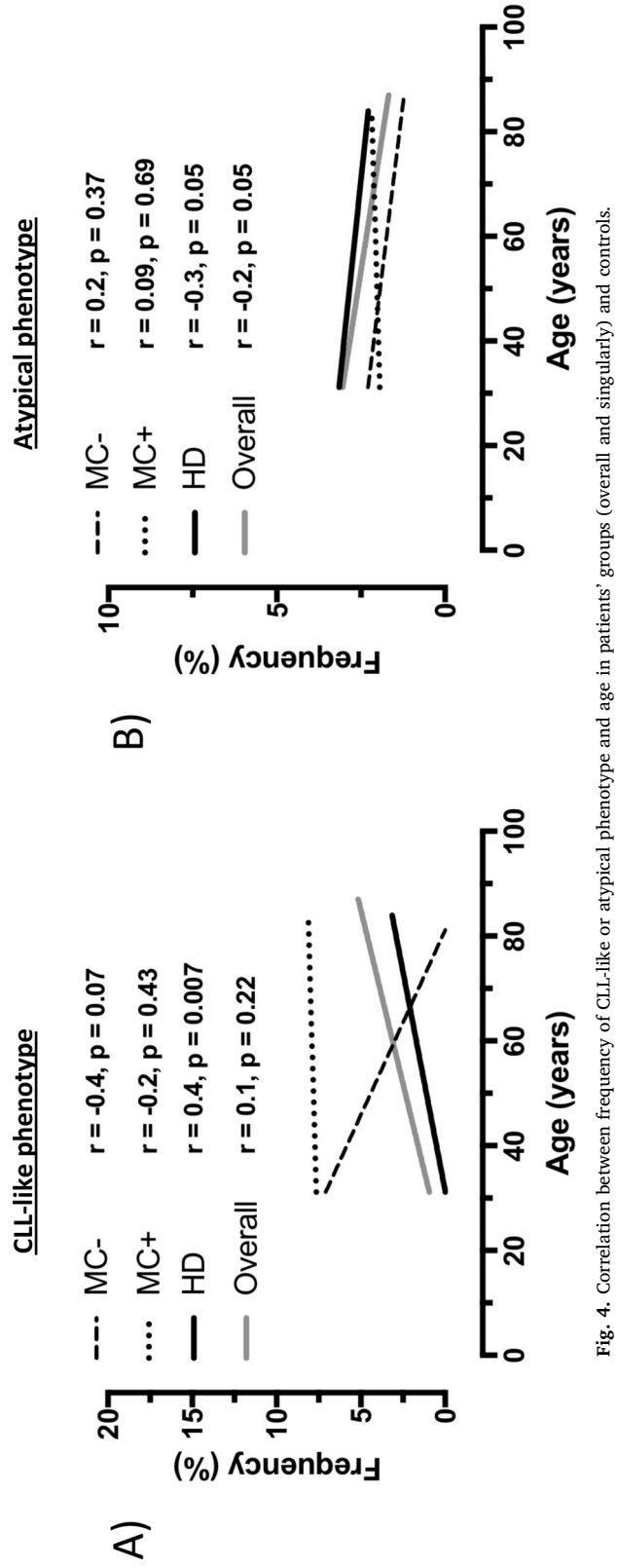

Fig. 4. Correlation between frequency of CLL-like or atypical phenotype and age in patients' groups (overall and singularly) and controls.

Table 4
Binary logistic regression analysis considering CLL-like, atypical, and $CD5^-$ B cell phenotypes to evaluate disease prediction capability.

	Phenotype	MC- group			MC+ group		
		p	aOR	N-R^2	p	aOR	N-R^2
Step 1	CLL-like	0.05	9.26	0.10	0.003	25.1	0.27
Step 2	CLL-like	0.11	8.80	0.12	0.007	20.2	0.29
	Atypical	0.30	1.77		0.26	2.0	
Step 3	CLL-like	0.12	6.63	0.14	0.03	12.8	0.31
	Atypical	0.50	1.48		0.38	1.7	
	$CD5^-CD20^+$	0.23	4.46		0.27	4.2	

aOR: adjusted odds ratio, N-R^2: Nagelkerke R squared.

isotype switching, these cells are possibly stimulated to proliferate and differentiate into antibody-secreting cells within germinal centers. In addition to specific anti-HCV antibodies, high-affinity RF molecules are likely secreted [12]. However, the prevalence of $CD5^+$ instead of $CD5^-$ B cell clones in the course of chronic HCV infection, with or without MC, is still a debating issue.

MBL is a condition described in peripheral blood of otherwise healthy subjects, associated with an increased risk of developing a clear lymphoproliferative disease and with immune senescence phenomena [23–25]. We assessed the frequencies of different B-cell phenotypes in patients with chronic HCV infection with and without cryoglobulinemia and controls. Our results showed a clear prevalence of $CD5^+CD20^{dim}$ B cells phenotype (CLL-like phenotype) in the patients with MC, whereas $CD5^+CD20^{bright}$ (atypical phenotype) B-cells were reduced in chronic HCV infection without MC.

On the basis of cut-offs estimated by ROC curve analyses, we observed that the frequency of CLL-like phenotype progressively increases from patients with chronic hepatitis without MC to those with MC. In addition, the frequency of atypical and $CD5^-$ B cells was lower than cut-off. MC is considered a lymphoproliferative disease characterized by the expansion of pathogenic B cell clones. When these clones have been analyzed, an increased use of IgHV genes like IgHV1-69 has been reported [39,40]. In our series, a CLL-like B cell pattern higher than a cut-off value of 7.35% was associated with atypical and $CD5^-CD20^+$ B cell levels lower than the cut-off values (1.5% and 63.6%, respectively), thus defining an HCV-related lymphoproliferative disease setting. Given that cryoprecipitation can be observed in about 40% of HCV chronically-infected patients, but only 12-15% of them eventually develop a clinically overt cryoglobulinemic syndrome [15], a simple flow-cytometry analysis (rather than a more complex clonal analysis of B cells) could help to identify HCV positive patients at risk of progression to a lymphoproliferative disorder such as MC.

Currently available DAAs are highly effective in eradicating HCV infection. Although these drugs have also been shown to achieve SVR in MC, the persistence of MC has been repeatedly reported, even after a long-term follow-up [41–44]. Persistence of cryoglobulins despite viral clearance is an intriguing issue with many open questions [45]. It can be speculated that such persistence could be related to stage and severity of vasculitis, irreversible organ damage, late disappearance of immune complexes (requiring longer follow-up), different composition of immune complexes as well as to an incomplete suppression of B-cell clones [46]. Our results indicate that the levels of $CD5^+CD20^{dim}$ B cells increased after therapy withdrawal in 2 MC patients treated with DAAs with persisting cryoglobulin production despite SVR, as well as in 3 patients treated with IFN-based regimens. On the contrary, in 2 patients with cryoglobulin disappearance associated with SVR after DAAs, $CD5^+CD20^{dim}$ B cells levels decreased at the end of therapy. No relapse of MC was observed in patients with cryoglobulins disappearance associated with SVR.

We acknowledge that the main limitations of the present study include the relatively low number of patients, the difference among

Table 5
Changes over time of clinical and laboratory features in 11 cryoglobulinemic patients who achieved SVR following antiviral therapy.

	Before treatment							Time since SVR (months)	After treatment				
	HCV GT	HCV RNA	Liver fibrosis	C4 (g/L)	RF (U/mL)	Cryocrit (%)	Clinical features		C4 (g/L)	RF (U/mL)	Cryocrit (%)	Clinical features	MC outcome
pIFN/RBV													
Pt. #1	2	634162	F2	10.03	431	3%	P; A	47	0.22	245	1%	P; A	Persistence
Pt. #2	2	296800	F2	12	146	4%	P; A; U	36	10	81	3%	P; A; U	Persistence
Pt. #3	2	45200000	F2	3	158	3%	P; A; PN	40	0.13	62	1%	P; A; PN	Persistence
Pt. #4	2	2017000	F1	29	20	2%	P; A; PN	36	19	< 20	absent	none	Remission
DAAs													
Pt. #5	1	5710000	F4	2	50	1%	P; A	30	1.5	28	absent	none	Remission
Pt. #6	1	4321000	F4	2	35	3%	P; A	29	0.02	30	3%	P; A	Persistence
Pt. #7	1	1140000	F4	7	530	1%	P; A; GN	30	11	255	absent	none	Remission
Pt. #8	2	219300	F4	0.006	230	1%	P; A; GN	30	3	177	1%	P; A; GN	Persistence
Pt. #9	1	3982000	F3	13	136	2%	P; A; W	29	20	14	absent	none	Remission
Pt. #10	2	5728162	F3	0.08	38	2%	P; A; W	32	0.1	< 10	absent	none	Remission
Pt. #11	1	3422000	F2	0.05	94	1%	P; A; W	34	0.15	12	absent	none	Remission

GT: Genotype; RF: Rheumatoid Factor; SVR: Sustained Virological Response; MC: Mixed Cryoglobulinemia; pIFN/RBV: Peg-Interferon plus Ribavirin; DAAs: Direct-acting antivirals.
A: Arthralgias; W: Weakness; GN: Glomerulonephritis; P: Purpura; PN: Peripheral Neuropathy; U: leg Ulcers.

treatment regimens, the duration of follow-up that in 64% of the patients (7/11) was less than 3 years, and the lack of B-cell pattern detection at different time points. However, the expression of CLL-like B cell phenotype could help to identify HCV-infected cryoglobulinemic patients requiring a closer surveillance and a longer follow-up due to their higher susceptibility of progression to MC and eventually NHL. Additional multicenter studies along the same lines are therefore warranted, including a higher number of cases and adequate validation setting, in order to establish the real impact of $CD5^{+}CD20^{dim}$ B cell phenotype in the management of these patients.

Author contributions

S.R., F.D., and G.L. designed and supervised the study. A. Vincenti, A. Vinella, and MA.M. performed experiments and data analysis. G.L. and F.P. recruited subjects, collected samples and clinical data. S.R. and G.L. performed statistical analyses, created figures, and wrote the manuscript. All authors reviewed the paper draft and gave final approval of the version to be published.

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Disclosure

All authors declare no conflict of interest.

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