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Hematological and Genetic Markers in the Rational Approach to Patients With HCV Sustained Virological Response With or Without Persisting Cryoglobulinemic Vasculitis

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BACKGROUND AND AIMS: Direct-acting antivirals (DAAs) usually lead to improvement/remission of cryoglobulinemic vasculitis (CV), although symptoms may persist/recur after a sustained virological response (SVR). We evaluated hematological and genetic markers in patients with HCV-SVR vasculitis with and without persisting/recurring symptoms to early predict the CV outcome.

APPROACH AND RESULTS: Ninety-eight patients with HCV-CV were prospectively enrolled after a DAA-induced SVR: Group A: 52 with complete clinical response; Group B: 46 with symptom maintenance/recurrence. Monoclonal B-cell lymphocytosis, t(14;18) translocation, and abnormal free light chains κ/λ ratios were detected by flow cytometry or nested-PCR or nephelometry in 4% Group A versus 17% Group B (P = 0.04) patients, 17% Group A versus 40% Group B patients (P = 0.02), and 17% Group A versus 47% Group B (P = 0.003) patients, respectively. At least 1 out of 3 clonality markers was altered/positive in 29% of Group A versus 70% of Group B patients (P < 0.0001). When available, pretherapy samples were also tested for t(14;18) translocation (detected in 12/37 [32%] Group A and 21/38 [55%] Group B) and κ/λ ratios (abnormal in 5/35 [14%] Group A and 20/38 [53%] Group B) (P = 0.0006), whereas at least one clonality marker was detected/altered in 16/37 (43%) Group A and 30/38 (79%) Group B (P = 0.002). CV-associated single-nucleotide polymorphisms were tested by real-time PCR. Among them, notch4 rs2071286 T minor allele and TT genotype showed a higher frequency in Group B versus Group A (46% vs. 29%, P = 0.01, and 17% vs. 2%, P = 0.006, respectively).

CONCLUSIONS: Hematological or genetic analyses could be used to foresee the CV clinical response after DAA therapy and could be valuable to assess a rational flowchart to manage CV during follow-up. (Hepatology 2021;74:1164-1173).

HCV is both hepatotropic and lymphotropic. HCV, together with liver damage, is therefore responsible for inducing lymphoproliferative disorders (LPDs), such as mixed cryoglobulinemia (MC) and non-Hodgkin's lymphoma

Abbreviations: CG, cryoglobulin; CLL, chronic lymphocytic leukemia; CV, cryoglobulinemic vasculitis; DAA, direct-acting antiviral; FLC, free light chains; HLA, human leukocyte antigen; IFN, interferon; LPD, lymphoproliferative disorder; MaSVE, Interdepartmental Center for Systemic Manifestations of Hepatitis Viruses; MBL, monoclonal B-cell lymphocytosis; MC, mixed cryoglobulinemia; NHL, non-Hodgkin's lymphoma; PBMC, peripheral blood mononuclear cell; RTX, rituximab; SNP, single-nucleotide polymorphism; SVR, sustained virological response.

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(NHL).[1,2] MC is the LPD that is more frequently associated with HCV infection; it is characterized by the clonal proliferations of B cells and by the presence of circulating immune complexes called cryoglobulins (CGs).[3] In the laboratory, CGs precipitate when the serum is cooled below 37°C,[4,5] and, in 30% of those with circulating CGs, they cause a systemic small/medium vessel vasculitis that determines multiorgan damage, called cryoglobulinemic vasculitis (CV).[5-7] Although CV is a benign condition, in 5%-10% of cases, it evolves into a frank NHL.[1,8]

The development of direct-acting antivirals (DAAs) completely changed HCV therapy, showing sustained virological response (SVR) rates higher than 90% and fewer side effects than previous interferon (IFN)-based regimens.[9] DAAs are effective and safe in patients with HCV and CV, showing no significant difference in SVR rates compared with patients with HCV without CV.[10-12] Furthermore, a high clinical and immunological effectiveness was confirmed in patients with HCV-CV.[10,13-16] Nevertheless, some patients with HCV-CV do not achieve a complete clinical and immunological response or, in some cases, a vasculitis recurrence during follow-up is observed.[13,17-20]

Monoclonal B-cell lymphocytosis (MBL) is a preclinical condition characterized by the expansion of monoclonal B cells of uncertain clinical significance that can be recognized in about 5% of the general population.[21] The diagnosis of MBL is based on the identification of a clonal lymphocyte population by immunophenotypic characterization. In 2005, an MBL subclassification in three different phenotypes was proposed, then adapted, by Shanafelt et al. in 2010[22]: (i) chronic lymphocytic leukemia (CLL)-like phenotype CD5+, CD19+, CD20(dim), CD23+; (ii) atypical CLL CD5+, CD19+, CD20(bright), CD23-; and (iii) non-CLL phenotype CD5- expressing CD20.[22] Based on prospective and retrospective studies, it is estimated that individuals who are MBL-positive have a 1%-2% annual risk of requiring CLL-specific treatment.[22]

The translocation (14;18) -t(14;18)- is interpreted as a mistake in the physiological VDJ genes rearrangement and is the hallmark of follicular lymphoma.[23,24] The rearrangement involves the translocation of the full BCL-2 gene, causing protein overexpression and the imbalance of the bcl2/bax ratio, leading to the inhibition of the physiological B-cell apoptosis. This implies a progressive increase of long-lasting B cells, which is a suitable condition for further lymphomagenic mistakes. The association between t(14;18) and HCV-related CV is well known,[25,26] and we showed a significantly higher prevalence of t(14;18) in peripheral blood mononuclear cell (PBMC) samples of patients infected with HCV, especially in HCV-related CV cases, as well as the clonal expansion of translocated B cells.[27-29]

Mature B cells produce immunoglobulins composed of two heavy chains and two light chains bound together. In physiological conditions, B cells produce a slight excess of light chains that can be detected as circulating free light chains (FLC) through an immunological test, measuring the κ and λ chain serum concentration and the κ/λ ratio.[30] This ratio depends on the balance between the production and the renal clearance of the light chains. In the last decade, a wide number of studies demonstrated that FLC ratio is altered in diseases

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like monoclonal gammopathy of undetermined significance, multiple myeloma, Waldenstrom macroglobulinemia, and light chain amyloidosis.[31,32] Although proposed as a useful test for the diagnosis of plasma proliferative diseases,[33] in 2014, the International Myeloma Working Group updated the myeloma diagnostic criteria, including the FLC assay.[34] An altered FLC profile has been correlated with CV and/or B-cell non Hodgkin's lymphoma in patients who are HCV-positive[35] and serum FLC ratio could be used as a surrogate marker for disease activity in CV.[36,37]

We still do not know exactly why only some patients infected with HCV develop MC and some of them develop symptoms. Different pathogenetic studies suggest that the virus triggers LPDs only on a particular host genetic background.[38] In fact, different single-nucleotide polymorphisms (SNPs) located on different genes were associated with a higher risk of HCV-related LPDs. Among them were SNPs in the Baff cytokine system[39] and SNPs in a short region of chromosome 6, as shown in a genome-wide association study performed in 2014.[40] Indeed, the minor allele of the notch 4 gene (rs2071286) and the minor allele of SNP rs9461776 of human leukocyte antigen (HLA) class II were associated with a higher risk of developing CV in participants infected with HCV[40]; the notch4 rs2071286 genotype was also associated with a higher risk of HCV-related lymphoma.[41]

DAAs improve or heal CV, however, its persistence/recurrence may occur after an SVR, and there are currently no tools available to aid clinicians in predicting the clinical outcome. Considering the knowledge on CV pathophysiology, we can hypothesize that CV persistence/recurrence could correlate with residual B-cell clonality and/or with a particular host genetic background.

Therefore, the aim of our study was the evaluation of B-cell clonality and genetic markers in patients with HCV-SVR vasculitis with and without persisting symptoms in order to early predict the CV clinical outcome.

Patients and Methods

The study was conceived using two different approaches. The first one was a prospective analysis of B-cell clonality persistence, conducted in the long posttherapy follow-up of patients with SVR and HCV-CV, that was implemented by the analysis of baseline frozen samples isolated from the same patients. The second approach was a genetic analysis of polymorphic variants previously associated with the risk of developing CV during HCV chronic infection.

Patients were enrolled in the study when their pretreatment condition conformed with described inclusion criteria.[14] Exclusion criteria also included coinfection with HIV or HBV, the presence of severe comorbidities not related to CV (i.e., nonhepatic or hematologic malignancies, including NHL), and the concomitant administration of therapies for vasculitis (i.e., rituximab [RTX] and plasma exchange cycles). Patients who previously underwent these treatments (at least 6 months prior) as well as patients taking low-medium doses of corticosteroids (0.1-0.5 mg/kg/day) or symptomatic drugs/measures (i.e., colchicine/low antigen diet) were also included.

Therefore, between January 2017 and July 2018, patients who had pretreatment HCV-related CV and obtained an SVR after IFN-free DAA treatment were enrolled at the outpatient clinic of the Interdepartmental Center for Systemic Manifestations of Hepatitis Viruses (MaSVE), Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy. PBMC samples and serum from the same patients were collected before the initiation of DAA treatment and were stored at -80°C in the MaSVE Center BioBank.

Patients were divided into two groups according to the absence/presence of CV symptoms at the end of the posttherapy follow-up:

- Group A: Patients with CV SVR with a complete clinical response
- Group B: Patients with CV SVR with symptom maintenance/recurrence

Clinical and immunological response was assessed following criteria.[14] In brief, we considered participants with the disappearance of all baseline symptoms as "full-complete responders," those with improvement of all the baseline symptoms as "complete responders," those with the disappearance or improvement of at least half of the baseline symptoms as "partial responders," and patients with the disappearance or improvement of fewer than half of the baseline symptoms as "nonresponders."

For the flow cytometry analysis, fresh EDTA peripheral blood samples obtained from all prospectively enrolled individuals were processed within 24 hours after

blood withdrawal. Samples were incubated for 5 minutes at room temperature in ammonium chloride and washed with phosphate-buffered saline pH 7.2 with 0.5% bovine serum albumin (PBS BSA). Samples were then incubated with the antibodies for 15 minutes at room temperature, washed with PBS BSA, and analyzed on a FACSCanto II (BD Biosciences, San Jose, CA) cytofluorimeter using FACSDIVA software (BD Biosciences). The following mix of antibodies was used: fluorescein isothiocyanate-phycoerythrin (FITC-PE) anti-κ- anti-λ (DAKO Agilent, Santa Clara, CA), PE-cyanine 7 (PE-cy7) anti-CD5 (BD Biosciences), allophycocyanin-anti-CD23 (APC-anti-CD23) (BD Biosciences), peridinin-chlorophyll-protein cy5.5 anti-CD20 (BD Biosciences), APC-H7-anti-CD45 (BD Biosciences), and HV-450-anti-CD19 (BD Biosciences). For each sample, up to 500,000 events were acquired according to the following gating strategy: lymphocytes were first gated on CD45 versus side scatter (SSC), and B cells were isolated by gating on CD19 versus SSC. B cells were examined on a CD19 versus CD5 plot and were further divided into CD5- and CD5+ subsets, and the κ/λ ratio was evaluated in both populations. CD20 versus CD19 plot was used to evaluate the CD20 expression intensity. The κ/λ ratio was considered abnormal when it was more than 3:1 or less than 1:3. CD19+CD5+CD23+CD20dim cells identify a "CLL-like MBL" population, CD19+CD5+CD23-CD20 bright cells identify an "atypical MBL," and CD19+CD5-CD23-CD20 bright cells identify a "CD5-MBL."

The t(14;18) translocation was detected in PBMCs isolated from fresh blood through "nested" PCR (major breakpoint region bcl-2/JH PCR) on genomic DNA, as described.[25] Sensitivity was approximately one rearranged cell in 10^5 to 10^6 normal cells. Amplification products were analyzed by ethidium bromide staining. The samples were tested in duplicate and negative samples were evaluated four times. t(14;18) was tested at baseline on frozen PBMCs available in the Masve Center BioBank and at the end of the posttherapy follow-up.

Circulating FLC and their ratio were assessed by means of Turbidimetric assay (Freelite Human Kappa and Lambda Free Kits, The Binding Site, Birmingham, UK) and were performed on the Optilite instrument (The Binding Site). The immunoassay consisted of two separate measurements, one to detect free κ chains (normal range: 3.3–19.4 mg/L) and the other to detect free λ chains (normal range: 5.7–26.3 mg/L). A ratio of κ/λ <0.26 or >1.65 is abnormal, according to the manufacturer's recommendations. Calibrators and controls were provided by the manufacturer and consisted of stabilized human sera containing polyclonal κ and λ FLC; calibrators and controls were diluted to the appropriate concentrations, following the manufacturer's instructions. Serum samples were tested at baseline on frozen serum samples available in the Masve Center BioBank and at the end of the posttherapy follow-up.

Five SNPs previously associated with LPDs were analyzed on genomic DNA from PBMCs: notch4 rs2071286, HLA rs9461776, baff gene rs12428930, baff promoter rs9514828, and baff receptor rs61756766. Genotyping was performed using specific TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) with supplied probes and primers on a Rotor Gene 6000 (Corbett Research, Sydney, Australia), as described.[41,42] The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. All subjects provided written informed consent and the protocol was approved by independent local ethic committee (approval code: BIO.16.014).

Results

Between January 2017 and July 2018, 98 patients with HCV-related CV that obtained an SVR after DAAs (35 male and 63 female, mean age 67 [58-73] were enrolled at the MaSVE Center; median post-therapy follow-up = 12 months [6-24]). Among the 98 patients, 52 (53%) participants not showing evidence of CV symptoms were included in Group A, and 46/98 (47%) with persistence or recurrence of CV symptoms were included in Group B. The main demographical, clinical, therapeutic, and virological characteristics were homogeneous in the two groups, as described in Table 1, except for the rheumatoid factor level, which was higher in Group B.

As reported in Table 1, 15 (15%) patients, 5 (10%) from Group A and 10 (22%) from Group B, received the anti-CD20 monoclonal antibody (RTX) before DAA therapy at least 10 months before the clonality marker analyses were performed.

The prospective analysis performed on the 98 patients showed an MBL in 2/52 (4%) of Group A and 8/46 (17%) of Group B patients, as shown in Fig. 1A.

Patients who were MBL-positive presented with all three phenotypes: 4/10 (40%) had NON-CLL phenotype (CD5-CD20+), 2/10 (20%) had atypical-CLL

TABLE 1. Main Demographical and Clinical Baseline Characteristic of 98 Patients With SVR-CV Stratified Based on the Clinical Outcome.

	General Population	Group A (Long-Lasting Responders)	Group B (Persistent/Recurrent Symptoms)	
	n = 98	n = 52	n = 46	*P* Value
Age (years)	67 (58-73)	64 (57-62)	66 (57-76)	
Sex (male/female)	35/63	19/33	16/30	
Metavir score[†]				
F0-F1	52 (53%)	26 (50%)	26 (57%)	
F2	15 (15%)	11 (21%)	4 (9%)	
F3	14 (14%)	6 (12%)	8 (17%)	
F4	17 (18%)	9 (17%)	8 (17%)	
HCV genotype				
1a	9 (9%)	4 (8%)	5 (11%)	
1b	52 (53%)	29 (56%)	23 (50%)	
2	21 (22%)	11 (21%)	10 (22%)	
3	10 (10%)	7 (13%)	3 (6%)	
4	5 (5%)	—	5 (11%)	
Mixed 1b+3	1 (1%)	1 (2%)	—	
ALT (U/L)[‡]	95 (±12)	100 (±9)	87 (±8)	
AST (U/L)[§]	55 (±10)	60 (±8)	52 (±13)	
Follow-up (month)	12 (6-24)	18 (6-24)	12 (6-24)	
Laboratory				
Cryocrit (%)	0.71 (±0.15)	0.57 (±0.16)	0.68 (±0.21)	
RF[‖]	104 (±30)	52 (±19)	170 (±60)	0.02
C4[¶]	0.16 (0.09-0.21)	0.17 (0.11-0.22)	0.13 (0.09-0.21)	
RTX	15 (15%)	5 (10%)	10 (22%)	
Previous IFN-based failed therapy	6 (6%)	3 (6%)	3 (7%)	

Note: Data are expressed as number, percentage, and, when required, mean and standard error of mean.
[†]Based on liver stiffness assessed by FibroScan.
[‡]ALT, alanine aminotransferase, normal range: 12-65 U/L.
[§]AST, aspartate aminotransferase, normal range: 15-37 U/L.
[‖]RF, rheumatoid factor (normal level <20 IU/mL).
[¶]C4, complement component C4 (normal range 0.1-0.4 g/L).
Abbreviation: RTX, rituximab.

phenotype (CD5+CD20^{BRIGHT}), and 4/10 (40%) had CLL-like phenotype (CD5+CD20^{DIM}).

t(14;18) analysis was performed in 46/52 (88%) of Group A patients and 45/46 (98%) of Group B patients. Eight/forty-six (17%) patients from Group A and 18/45 (40%) patients from Group B were t(14;18) positive (Fig. 1B).

The serum FLC κ/λ ratio was performed on 48/52 (92%) of Group A patients and 43/46 (93%) of Group B patients. In 8/48 (17%) of Group A and 20/43 (47%) of Group B serum samples, the FLC κ/λ ratio was above the upper limit of 1.65 (Fig. 1C). At least one clonality marker was altered/positive in 15/52 (29%) of Group A patients and 32/46 (70%) of Group B patients (Fig. 1D).

The persistence/recurrence of symptoms in the posttherapy follow-up required RTX administration in 12/46 (26%) patients from Group B. This was beneficial in all cases (data not shown). B-cell depleting treatment could not be administered to another Group B patient due to a previous intolerance experienced during a pre-DAA infusion. Among the 12 patients who underwent RTX after the SVR, all but 2 harbored at least 1 clonality marker.

Baseline PBMC and serum samples stored in the MaSVE Center BioBank were also evaluated. Seventy-five PBMC samples were available for t(14;18) detection: 37/75 of Group A and 38/75 of Group B. Results are shown in Fig. 2A: 12/37 (32%)

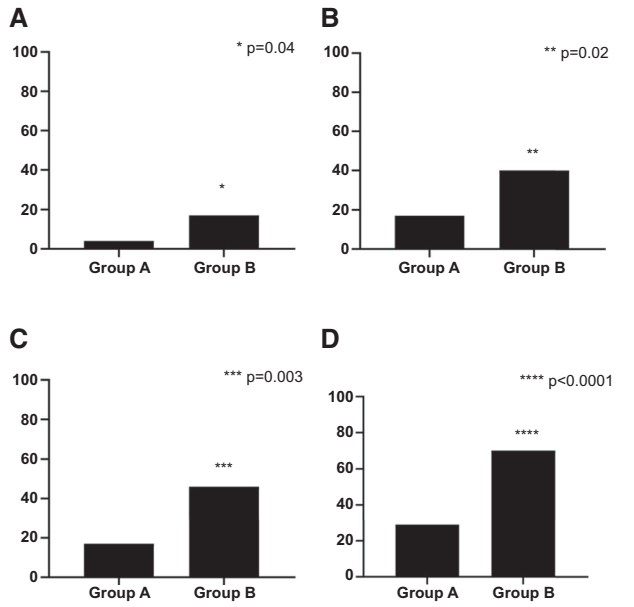

FIG. 1. Results of clonality marker analysis at the end of posttherapy follow-up. On the *y* axis, data are expressed as percentage. (A) Presence of MBL (B-cell surface κ/λ ratio) in Group A and Group B patients (4% vs. 17%, $P = 0.04$; Relative Risk (RR), 2.841; 95% CI, 0.8116-9.945). (B) Presence of t(14;18) in Group A and Group B patients (17% vs. 40%, $P = 0.02$; RR, 1.900; 95% CI, 1.030-3.503). (C) Alteration of serum FLC κ/λ ratio in Group A and Group B patients (16% vs. 46%, $P = 0.003$; RR, 2.222; 95% CI, 1.202-4.110). (D) Presence of at least one clonality marker in Group A and Group B patients (29% vs. 70%, $P < 0.0001$; RR, 2.273; 95% CI, 1.494-3.649).

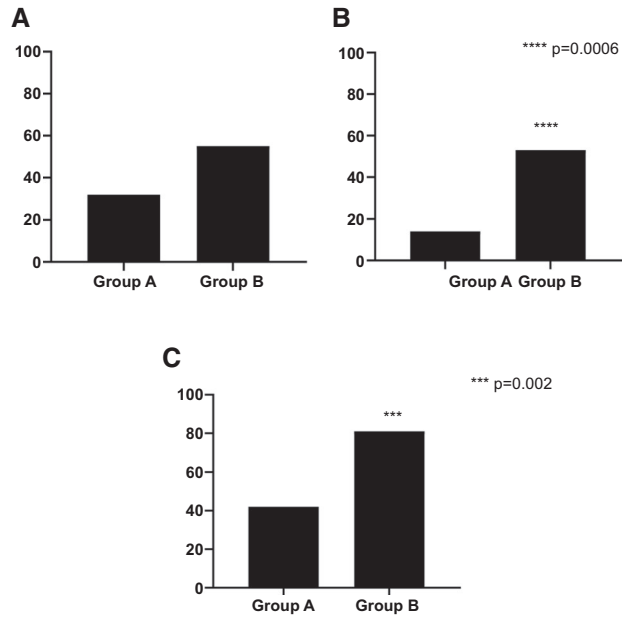

FIG. 2. Results of the clonality marker analysis in pretherapy available samples. On the *y* axis, data are expressed as percentage. (A) Presence of t(14;18) in Group A and Group B patients (32% vs. 55%). (B) Alteration of serum FLC κ/λ ratio in Group A and Group B patients (14% vs. 53%, $P = 0.0006$; OR, 6.667; 95% CI, 2.130-20.87). (C) Presence of at least one clonality marker in Group A and Group B patients (43% vs. 79%, $P = 0.002$; OR, 4.922; 95% CI, 1.830-13.76).

of Group A and 21/38 (55%) of Group B patients were t(14;18) positive.

Figure 2B shows results of the FLC κ/λ ratio analysis, performed on 35 serum samples of patients from Group A and 38 patients from Group B. Five/thirty-five (14%) of Group A patients showed a κ/λ altered ratio: in 4 samples, the ratio was higher than 1.65, whereas in one sample, it was lower than 0.26. Concerning Group B patients, 20/38 (53%) had an abnormal ratio (over the upper limit of 1.65).

At least one clonality marker was detected/altered in 16/37 (43%) of Group A and 30/38 (79%) of Group B patients (Fig. 2C).

Regarding the Baff system SNPs, we did not observe any difference in the allele frequency between the two groups (data not shown).

The analysis of HLA rs9461776 showed that the heterozygous genotype A/G and G minor allele were more frequent in Group B than in Group A (56% vs. 31% and 33% vs. 22%, respectively). The homozygous minor genotype G/G showed similar frequency between the two groups (6% in Group A vs. 4% in Group B) (Fig. 3A).

The analysis of notch4 rs2071286 showed a significantly higher frequency for T minor allele (46% vs. 29%, $P = 0.01$; OR, 2.17; 95% CI, 1.18-3.9) and TT genotype (17% vs. 2%, $P = 0.006$) in Group B versus Group A (Fig. 3B). We found a significant association between rs2071286 SNPs and Group B patients with the dominant and recessive model of penetrance (C/C vs. C/T+T/T for the dominant model of penetrance: $P = 0.04$; OR, 0.37; 95% CI, 0.1434-0.9158; for the recessive model of penetrance T/T vs. C/T+C/C: $P = 0.02$; OR, 9.33; 95% CI, 1.099-79.28).

The presence of B-cell clonality or genetic markers did not affect the SVR, as all the patients were virological responders.

Discussion

We here reported a dual approach analysis (of B-cell clonality and genetic markers) of patients who

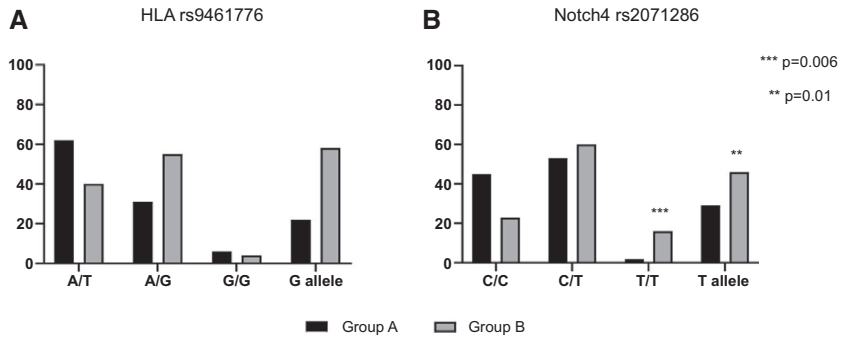

FIG. 3. Results of the genotyping analysis of HLA class II rs9461776 and Notch4 rs2071286. On the *y* axis, data are expressed as percentage. (A) Distribution of polymorphic variant of the HLA class II rs9461776. A/G was present in 31% Group A vs. 56% Group B, and G minor allele was present in 22% Group A and 33% Group B. The homozygous minor genotype G/G was present in 6% Group A vs. 4% Group B. (B) Distribution of polymorphic variant of the Notch4 rs2071286. Homozygous haplotype T/T: 2% Group A vs. 16% Group B; $P = 0.006$. T minor allele frequency: 29% Group A vs. 47% Group B ($P = 0.01$; OR, 2.17; 95% CI, 1.18-3.9).

were HCV-positive with CV that underwent effective antiviral treatment and were followed up with after the SVR in order to monitor the clinical outcome of vasculitis.

As reported, although the majority of patients with CV obtained a long-lasting clinical response after viral eradication, some of them experienced a symptom persistence,[13,17,18] whereas others showed a CV relapse after a transient improvement/healing.[19,20,43,44] The vasculitis symptoms recurrence has been closely associated to events such as infections or cancer onset[20] and even to flu vaccination.[43] These conditions or other unknown/unnoticed events, characterized by immune system stimulation, might reactivate persistent pathological B-cell clones expressing stereotyped B-cell receptors.[45]

In addition, a very recent study suggested that CV relapse despite SVR is more frequent after DAAs than after IFN-based therapy.[46] The authors attributed their observation to IFN antiproliferative activity. Further studies will be useful in order to definitively confirm this finding.

We performed a residual B-cell clonality evaluation by using three methods (flow cytometry, κ/λ ratio in serum and t(14;18) in peripheral B cells) in patients with CV stratified based on the remission/persistence or relapse of vasculitis in a long posttherapy follow-up. We also performed an analysis on available pretherapy samples. In Group B, the prospective approach revealed that all three clonality markers were significantly more frequent. Similarly, the analysis of baseline samples showed a significantly higher percentage of an altered serum κ/λ ratio and a higher frequency of t(14;18) in those with symptoms maintenance/recurrence. Taken together, the B-cell clonality analyses showed a clear correlation between the presence of residual B-cell clonal expansion and CV symptoms persistence/recurrence.

Visentini et al. evaluated the persistence of B-cell clonality through cytofluorimetry techniques in 45 patients with CV treated with DAAs and followed up with from 9 to 38 months after therapy.[47] Among the 45 patients with CV, 8 had lymphoma. Interestingly, a circulating B-cell clone persisted in all of the patients with lymphoma as well as in 27% of patients with CV without an overt malignancy. Although a stratification of patients based on the clinical outcome was not part of the scope, among 3 patients with clinical relapse, 2 showed the persistence of a B-cell clone, which was consistent with our findings.[47]

An MBL analysis was recently reported by Pozzato et al. in a study describing the hematological response of 67 patients with HCV and CV, 6 of whom had a frank NHL.[48] At baseline, 9/30 (30%) patients with CV showed an MBL that persisted at week 24 of follow-up in 7/30 (23%) of cases. The authors did not correlate MBL maintenance to the CV clinical outcome, as their analysis was aimed at evaluating the hematological response in patients with SVR.[48] Overall, Pozzato et al. reported a posttreatment MBL rate higher than the one resulting from our analysis. It is conceivable to attribute this discrepancy to the

larger population we evaluated (98 compared with 30 patients) and to the longer follow-up (a mean of 12 months compared with 24 weeks). Although MBL analysis was conducted in patients with CV by other authors with different aims from ours, these previous reports stressed the importance of MBL detection in the clinical characterization of CV.

The prospective analysis showed that t(14;18) and the alteration of serum κ/λ ratio were significantly associated with CV persistence/recurrence. The baseline analysis approach completely confirmed these results for κ/λ ratio assessment, whereas the presence of t(14;18) in Group B was extremely close to the statistical significance but did not reach it, conceivably because of a smaller amount of pretherapy sampling compared with the prospective one.

However, among the different clonality markers, FLC κ/λ ratio proved to be the most strongly associated with the persistence/relapse of CV after HCV eradication. In fact, the κ/λ ratio was already identified as a disease activity index in HCV-related CV[35,49] and as a predictive marker of RTX treatment outcome.[37]

Interestingly, the analysis performed on therapy baseline samples showed an abnormal FLC κ/λ ratio in a significantly higher percentage of patients with CV with persistence/relapse of symptoms after treatment. This finding could have a notable impact on clinical practice because it could define an early predictor of vasculitis outcome to evaluate before the initiation of antiviral therapy. In fact, the FLC κ/λ ratio is a routine test that is usually available in all diagnostic laboratories, as it is cheap and noninvasive and therefore easy to perform.

In our experience, because of the frequency of non-CV-specific manifestations, the decision to perform an RTX cycle in Group B patients complaining of consistent symptoms during the follow-up after SVR was supported by the B-cell clonal analysis results.

In addition, we observed a significant association between notch4 rs2071286 minor allele and CV persistence/relapse after the SVR, suggesting that this SNP could represent an early biomarker for CV persistence/recurrence in the posttherapy follow-up.

An international genome-wide association study showed that Notch4 rs2071286 was associated with an increased risk of developing CV during HCV chronic infection,[40] and a further study confirmed this strong association for HCV-related NHL.[41]

On this basis, Artemova et al. observed that a combination of long HCV duration and increased genetic risk caused by the presence of minor alleles of Notch4 rs2071286 and HLA rs9461776 were associated with, on average, at least 11 weeks longer persistence of CGs after a DAA-induced SVR.[50] However, vasculitis symptom behavior was not taken into account by the authors, as immunological response was the only evaluated parameter.[50]

The significant association we found between notch4 rs2071286 minor allele and persistence/relapse of CV after an SVR suggests that this allelic variant could be used to establish an early genetic test to assess the risk for CV persistence/recurrence in the posttherapy follow-up.

At the moment, there are no certain clinical clues that could predict CV maintenance/recurrence after DAAs. Overall, based on clinical experience, the only criterion to somehow anticipate a poor or transient vasculitis response seems to be the disease severity.[13,14,18,19] In the era of very powerful anti-HCV drugs ensuring a high success rate with extremely rare side effects, the management of extrahepatic manifestations such as CV is the residual challenge in this field. Therefore, matching the need for tests with a certain prognostic value for CV outcome is a priority in HCV research.

In fact, it is relatively common to stop the follow-up that is after SVR after a DAA-induced SVR, referring the patient to the family practitioner. We are of the opinion that this should be avoided in the case of patients with CV, even if they have experienced a clinical response, as it could be transitory.

Our results suggest that either hematological or genetic analyses could be used to foresee CV clinical response after DAA therapy and could be valuable for the assessment of a rational flowchart to manage patients with CV during follow-up, thereby selecting the best frequency and type of checkups as well as the most appropriate therapeutic strategy (i.e., B-cell depletion with Rrtuximab) and preventing any further evolution toward malignancy.

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