RESEARCH ARTICLE

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B-cell Clonality in HCV-Induced Patients Treated with Oral Direct-Acting Antiviral Agents

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Abstract

Background: Hepatitis C virus (HCV) is a worldwide' health problem as Egypt has a very high prevalence (14.7%) that may affect the B-Lymphocytes, and in some cases leading to an expansion of monoclonal B-cell detected by immunoglobulin heavy chain (IgH) gene rearrangement. Therefore, we aimed to assess the occurrence of IgH gene rearrangement in Egyptian chronic HCV patients and studying the effect of oral direct-acting antiviral (DAAs) therapy on regression of the clonality markers. **Methods:** 78 Egyptian patients with chronic HCV infection were included in this study and polymerase chain reaction (PCR) analysis was used to detect IgH rearrangement based on standardized PCR protocols of the BIOMED-2 international guidelines study. **Results:** Clonal IgH showed a significant increase of HCV-RNA expression and correlated with increased alanine transaminase (ALT) in all patients, while a significant increase of kappa and lambda free light chain observed only in clonal IgH with lymphoproliferative disorders (LPD) patients. A total of 37.17% (29/78) IgH clonality was detected in all patients (7.69% with LPD and 29.48% without LPD). 37% of these IgH clonality disappeared with HCV eradication after DAAs regimen. **Conclusions:** we concluded that different DAAs regimen with or without RBV is safe and effective for the treatment of Egyptian patients, but its effect is partially and not completely in the eradication of IgH clonality. Also, using IgH rearrangement in patients with chronic HCV is helpful as indicator in patients at high risk for prediction of LPD.

Keywords: Clonality- HCV- LPD- DAAs- IgH rearrangement

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Introduction

Hepatitis C virus (HCV) is major health problem worldwide. Egypt has a very high prevalence of HCV infection that reached 14.7% according to Egypt Demographic and Health Survey 2008 (EDHS, 2008). Saleh et al., (2019) HCV is a lymphotropic and hepatotropic virus that may affect the B-lymphocytes compartments, with the occurrence of B-cell lymphoproliferative disorders (B-LPD). Nasser et al., (2018) Epidemiological studies confirmed an association between HCV infection and a broad spectrum of B-LPDs such as mixed cryoglobulinemia (MC) and different B-cell non-Hodgkin's lymphoma (B-NHL) subtypes. Frosi, (2021) However, the understanding of the mechanisms linking HCV active infection to lymphoma development is still limited.

Monoclonal B-cell lymphocytosis (MBL) is an asymptomatic condition characterized by the presence of a clonal B-cell population in the peripheral blood (PB) with less than 5×10^9/L B-cells and no other signs of LPD (Galigalidou et al., 2021).

This clonal process might be caused by chronic antigenic stimulation leading to a strong antibody response, which undergoes other oncogenic events in some cases and eventually leading to an expansion of the monoclonal B-cell that can be detected by immunoglobulin heavy chain (IgH) gene rearrangement (Schiavinato et al., 2017). B-cells may produce a slightly excess of light chains, and these chains can be detected as circulating free light chains (FLC) through an immunological test, measuring the serum concentration of kappa (κ) and lambda (λ) light chains and the κ/λ ratio (Gragnani et al., 2021).

Later, interferon (IFN)-α-based antiviral treatment was confirmed to be efficacious for treating HCV-associated lymphomas (Schiavinato et al., 2017; Othman et al., 2013).

However, HCV therapy was completely changed after the development of direct-acting antivirals (DAAs) which showed sustained virological response (SVR) rates more than 90% and its side effects is fewer than previous (IFN)-based regimens (Gragnani et al., 2021; Cacoub et

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al., 2022).

Therefore, the present study aimed to assess the occurrence of IgH gene rearrangement in Egyptian chronic HCV patients and to study the effect of oral DAAs antiviral therapy on regression of clonality marker.

Materials and Methods

Patients

Our study is a prospective cohort study of 78 Egyptian patients with chronic HCV infection recruited from The Specialized Medical Hospital, Mansoura University, Mansoura, Egypt. 12 of these patients had LPD associated HCV (Group A) which diagnosed according to WHO 2016 criteria. The rest 66 patients without LPD (Group B). Patients with HIV or HBV co-infection were excluded. Written consent was obtained from all patients and the study design was carried out in accordance with Declaration of Helsinki. The patients have been followed up for 6 months after receiving the therapy. All patients received either a combination therapy of sofosbuvir (SOF) 400 mg with daclatasvir (DCV) 60 mg for 12 weeks with or without ribavirin (RBV) at a dose adjusted according to body weight, or a combination of (Ombitasvir + Paritaprevir + Ritonavir) and RBV for 12 weeks. The choice of antiviral therapy combination was made at the decision of each physician.

Laboratory investigations:

The level of HCV-RNA (viral load) in the serum was quantified at baseline, during antiviral therapy, 12- and 24-weeks after treatment withdrawal using the Step One™ Real-Time PCR System (Applied bio system) with a lower detection limit of 15 IU/ml. The response to antiviral therapy could be summarized as follows:

• Rapid virological response (RVR), i.e., negative for HCV RNA after 4 weeks of therapy.

• Sustained virological response (SVR), i.e., negative for HCV RNA 24 weeks after the end of treatment.

• Relapse: patients who achieved undetectable HCV RNA levels at the end of antiviral therapy and then subsequently relapsed with positive HCV RNA after treatment withdrawal.

Detection of IgH rearrangement

Genomic DNA was extracted from peripheral blood samples using the Qiagen QIAamp DNA mini prep kit (Cat No: 51104; Qiagen, Santa Clarita, California, USA) according to the manufacturer's instructions. PCR analysis was performed based on standardized PCR protocols of the BIOMED-2 international guidelines study and three sets of VH primers were used, the three VH FR regions (FR1, FR2, and FR3). The primers used for IGH VH-JH PCR (Van Dongen et al., 2003) are shown in Table 1.

PCR for each sample performed into 3 tubes (A, B and C). The reaction mix for each tube was: 12.5 ul of Hot Start master mix (Qiagen), 0.1 ul of each sense primer, 0.1 ul of JH anti-sense primer, 2 ul of DNA and finally the reaction volume was completed to 25 ul with nuclease free water. The amplification was performed using 9700 GeneAmp thermal cycler (Applied Biosystems, California, USA). The PCR conditions are initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 90 seconds, then a final extension at 72°C for 10 minutes.

Heteroduplex analysis

To avoid serious false-positive PCR results, PCR products were denatured at 94ºC for 5 minutes then incubated at 4ºC for 60 minutes. Then were immediately loaded on a 3% agarose gel electrophoresis. These results interpreted as positive if one or two prominent bands were visualized within the expected size range for each set.

Free light chain quantification (serum kappa and lambda light chains)

FLC quantification was carried out by using fully automated chemistry analyzer BT3500. The assay of κ and λ light chains is based on immune turbidimetric measurement. The FLC quantitation consisted of two separate assays: one to detect free κ light chains with normal range (160 – 450 mg/dl) and the other to detect free λ light chains with normal range (110 – 240 mg/dl). The normal range for a serum FLC ratio is 0.26–1.65.

Statistical analysis

Data were entered and analyzed using IBM-SPSS software (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp). Qualitative data were expressed as N (%). Quantitative data were initially tested for normality using Shapiro-Wilk's test with data being normally distributed if $p>0.050$. Quantitative data were expressed as mean, standard deviation (SD), and standard error (SE) or median, range (maximum - minimum), and Interquartile range (IQR = 75^{th} percentile – 25^{th} percentile). For any of the used tests, results were considered as statistically significant if p value ≤ 0.050.

Results

This study involved 78 patients with chronic HCV infection who had received oral DAAs antiviral therapy for 3-6 months. Within group A, ten out of the twelve cases with LPD (83.3%) showed SVR after 12 months of antiviral therapy, whereas all 66 in group B (non-LPD cases, 100%) showed SVR after the 12 months of antiviral therapy (p=0.022). Table 2 illustrate the baseline clinical and laboratory data in both groups. Significant higher frequency of moderately enlarged spleen and higher proportion of positive FR2 was observed in group A when compared with group B. Also, a significance higher FIB4-score, HCV-RNA, ALT, TSH, Free-kappa & lambda light chain observed in group A with lower platelet count in the same group when compared to group B. No statistical differences detected in the other parameters between both groups.

We stratified each group according to clonality (Table 3 and 4) and we found that clonal IgH in both groups showed a significantly increased HCV-RNA expression and ALT. In addition, a significant kappa and

Table 1. Primers of IgH at Different FR Regions

Primer	Sequence	Size
IgH FR1 sense		
VH1-FR1	GGCCTCAGTGAAGGTCTCCTGCAAG	
VH2-FR1	GTCTGGTCCTACGCTGGTGAAACCC	
VH3-FR1	CTGGGGGGTCCCTGAGACTCTCCTG	310-360
VH4-FR1	CTTCGGAGACCCTGTCCCTCACCTG	
VH5-FR1	CGGGGAGTCTCTGAAGATCTCCTGT	
VH6-FR1	TCGCAGACCCTCTCACTCACCTGTG	
IgH FR2 sense		
VH1/7-FR2	CTGGGTGCGACAGGCCCCTGGACAA	
VH2-FR2	TGGATCCGTCAGCCCCCAGGGAAGG	
VH3-FR2	GGTCCGCCAGGCTCCAGGGAA	250-295
VH4-FR2	TGGATCCGCCAGCCCCCAGGGAAGG	
VH5-FR2	GGGTGCGCCAGATGCCCGGGAAAGG	
VH6-FR2	TGGATCAGGCAGTCCCCATCGAGAG	
IgH FR3 sense		
VH1-FR3	TGGAGCTGAGCAGCCTGAGATCTGA	
VH2-FR3	CAATGACCAACATGGACCCTGTGGA	
VH3-FR3	TCTGCAAATGAACAGCCTGAGAGCC	
VH4-FR3	GAGCTCTGTGACCGCCGCGGACACG	100-170
VH5-FR3	CAGCACCGCCTACCTGCAGTGGAGC	
VH6-FR3	GTTCTCCCTGCAGCTGAACTCTGTG	
VH7-FR3	CAGCACGGCATATCTGCAGATCAG	
JH consensus anti-sense	CTTACCTGAGGAGACGGTGACC	

lambda FLC was observed with clonal IgH only in LPD group. The laboratory parameters over the course of treatment in the studied cases were improved (data not shown).

As shown in Table 5 and Figure 1, only 2/6 patients (33.33%) within the LPD group who had positive clonal IgH turned negative after completing therapy, whereas 9/23 patients (39.13%) of the non-LPD group who had positive clonal IgH became negative after completing their therapy.

Discussion

This study was conducted to detect IgH gene rearrangement and to assess the response to treatment in 78 Egyptian patients with genotype-4 HCV infection, 12 patients with LPD and 66 patients without LPD. A statistically significant difference in DAAs regimens between both patients' groups was observed (p=0.01). In group of patients with LPD, most of treatment used was SOF+DCV+RBV for 12 weeks and it was received by 50 % of patients. The other treatment was SOF+DCV for 12 weeks received by 8.3% patients, SOF+DCV for 24 weeks received by 8.3%, and33.3% patients received (Ombitasvir+Paritaprevir+Ritonavir) + RBV for 12. While in the group without LPD, the most treatment used that being received by 50% of the patients in this group was SOF+DCV for 12 weeks. The remaining patients

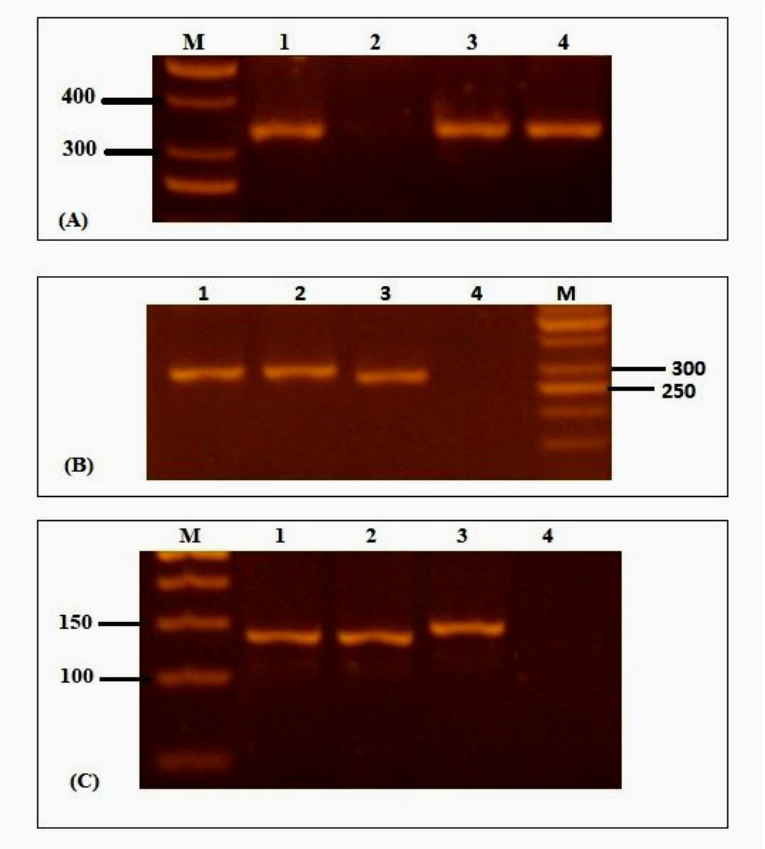

Figure 1. Agarose Electrophoresis Analysis of IgH. (A), FR1 clonality; M is 50 bp ladder, 1 and 2 is positive FR1 clonality turned negative after treatment; 3 and 4 is a positive FR1 clonality remain positive after treatment; (B), FR2 clonality; M is 50 bp ladder, 1 and 2 is a positive FR2 clonality remain positive after treatment, 3 and 4 is positive FR2 clonality turned negative after treatment; (C), FR3 clonality; M is 50 bp ladder, 1 and 2 is a positive FR3 clonality remain positive after treatment, 3 and 4 is positive FR3 clonality turned negative after treatment.

Table 2. Baseline Characteristics of the Studied HCV Patients with and without LPD

	Group A (n=12)	Group B (n=66)	P value
Age (years), Median (range)	55 (48.3-60.5)	50 (43-55.3)	0.108
Gender, N (%)			
Male	8 (66.6%)	28 (42.42%)	0.121
Female	4 (33.3%)	38 (57.57%)	
Spleen size, N (%)			
Normal sized	5 (41.7%)	43 (65.2%)	0.003
Mildly enlarged	3 (25%)	22 (33.3%)	
Moderately enlarged	4 (33.3%)	1 (1.5%)	
FIB-4 score, Median (range)	2.95 (1.36-4.71)	1.74 (1.20-2.31)	0.042
PCR HCV RNA (IU/ml), Median (range)	1.6×10^6 (1.148×10^6 -2.99×10^6)	5.74×10^5 (1.83×10^5-2.45×10^6)	0.026
SVR12 achieved, N (%)	10 (83.3%)	66 (100%)	0.022
ALT (IU/L), Median (range)	68 (46 - 98.5)	45.5 (32.75 -63)	0.029
AST (IU/L), Median (range)	61.5 (42.25-74.5)	45 (33.75-59)	0.06
TSH (IU/mL), Median (range)	3.35 (1.55-4.85)	1.9 (1.22-2.5)	0.006
WBC (k/ul), Median (range)	7.95 (5.78-11.18)	6.65 (5.29-7.93)	0.2
Hemoglobin level (g/dl), Median (range)	13.05 (11.43- 14.83)	12.65 (11.4 -13.83)	0.58
Lymphocyte %, Median (range)	37.75 (30.5-48.25)	39 (35.75-46.5)	0.62
Platelet count, Median (range)	145 (87.5-166.75)	189.5 (151.75-243.5)	0.005
Kappa (mg/dL), Median (range)	402 (202-454.75)	228 (204.75-285.25)	0.029
Lambda (mg/dL), Median (range)	252.5 (191.5-287.5)	179.5 (141.75-219.5)	0.01
Free-kappa–lambda ratio, Median (range)	1.4 (1.0-1.8)	1.3 (1.2-1.4)	0.39
IgH rearrangement, N (%)	6 (50%)	23 (34.85%)	
FR1 positivity	4 (33.3%)	7 (10.6%)	0.06
FR2 positivity	6 (50%)	12 (18.2%)	0.026
FR3 positivity	3 (25%)	6 (9.1%)	0.137

FIB-4, Fibrosis-4 score; SVR, sustained virological response; p value significant if < 0.05.

included 37.9% patients received SOF+DCV+RBC for 12 weeks, 3% received SOF+DCV for 24 weeks, 9.1% patients received (Ombitasvir+Paritaprevir+Ritonavir) + RBV for 12 weeks.

SVR of 12 weeks was 100% achieved in the non-LPD patients after completing treatment and was 83.3% (10/12) in the LPD patients reaching a statistically significant of P = 0.022. Other studies from different population reported a variation range of SVR12 in HCV-infected patients with or without associated LPD from 86% to 100% (Schiavinato et al., 2017; Arcaini et al., 2016; Fontaine et al., 2015; Crespo et al., 2017). This variation could be

Table 3. Baseline Characteristics According to IgH Clonality in HCV associated LPD Group

	Positive clonal IgH (N = 6)	Negative clonal IgH (N = 6)	P value
Age (years), Median (range)	58 (49-63)	50 (26-67)	0.228
FIB-4 score, Median (range)	3.12 (1.1-6.47)	2.95 (0.88-11.26)	0.873
PCR HCV RNA (IU/ml), Median (range)	2.82×10^6(1.69×10^6-5.34×10^6)	1.16×106 (1.44×10^4-1.51×10^6)	0.004
ALT (IU/L), Median (range)	90.5 (56-167)	49 (20-79)	0.025
AST (IU/L), Median (range)	65.5 (37-90)	61.5 (13-73)	0.522
TSH (IU/mL), Median (range)	4.25 (2.3-6.04)	2.07 (1.20-4.4)	0.077
WBC count 103 /Ul, Median (range)	9.47 (3.0-43.5)	7.95 (2.3-9.0)	0.522
Hemoglobin level (g/dl), Median (range)	13.5 (8.8-16.2)	13.05 (11.4-16.3)	0.936
Lymphocyte %, Median (range)	42.75 (30.0-82.5)	33.5 (28-50)	0.15
Platelet count, Median (range)	119 (63-257)	148.5 (50-195)	0.749
Kappa (mg/dL), Median (range)	454.5 (379-572)	202 (97-449)	0.01
Lambda (mg/dL), Median (range)	268.5 (247-336)	192 (97-305)	0.045
Free-kappa–lambda ratio, Median (range)	1.6 (1.0-1.8)	1.2 (0.7-1.8)	0.104

p value significant if < 0.05.

Table 4. Baseline Characteristics according to IgH Clonality in HCV non-LPD Group

	Positive clonal IgH (N=23)	Negative clonal IgH (N=43)	P value
Age (years), Median (range)	49 (26-79)	50 (21-65)	0.681
FIB-4 score, Median (range)	1.47 (0.72-9.91)	1.81 (0.39-9.65)	0.316
PCR HCV RNA (IU/ml), Median (range)	2.7×10^6(621-1.0×10^6)	3.7×10^5 (3500-4.2×10^6)	0.0001
ALT (IU/L), Median (range)	59 (21-94)	40 (18-131)	0.0001
AST (IU/L), Median (range)	47 (30-85)	38 (19-122)	0.114
TSH (IU/mL), Median (range)	1.9 (0.6-4.3)	1.9 (0.18-5.6)	0.647
WBC count 10^3 /uL, Median (range)	7.0 (3.4-10.6)	6.6 (1.2-11.8)	0.647
Hemoglobin level (g/dl), Median (range)	13.5 (8.2-16.5)	12.6 (9.5-15.7)	0.27
Lymphocyte %, Median (range)	38 (25-591)	40 (29.1-70.0)	0.069
Platelet count, Median (range)	198 (61.0-335.0)	186 (43-290)	0.412
Kappa (mg/dL), Median (range)	234 (189-428)	225 (107-300)	0.135
Lambda (mg/dL), Median (range)	192 (105-338)	172 (80-278)	0.144
Free-kappa–lambda ratio, Median (range)	1.3 (0.6-2.2)	1.28 (1-2.1)	0.757

p value significant if < 0.05.

Table 5. Frequency of IgH Rearrangement before and after Treatment

		IgH rearrangement clonality		P value
	Negative	Positive		
		Remain positive after therapy	Turned negative after therapy	
Group A (n=12)	6 (50%)	4 (33.33%)	2 (16.66%)	0.5
FR1	-	4	0	
FR2	-	4	2	
FR3	-	3	0	
Group B (n=66)	43 (65.15%)	14 (21.21%)	9 (13.63%)	0.004
FR1	-	5	2	
FR2	-	8	4	
FR3	-	3	3	

p value significant if < 0.05.

due to the presence of patients who were difficult to treat as might be due to other comorbidity factors.

In our study, there was a statistically significantly higher FIB4-score, ALT and TSH, Free-kappa and lambda light chain and a statistically significantly lower platelet count (P value = 0.042, 0.026, 0.029, 0.006, 0.029, 0.01, 0.005 respectively) in group A vs. group B. In the meantime, the laboratory parameters progression during follow up showed a decline in liver enzymes (ALT, AST) from baseline to week 24. The improvement of liver function parameters (ALT, AST, and albumin) in HCV patients after treatment support the efficacy and tolerability of direct antiviral agents. This decrease in levels were in parallel with other studies that reported a post-treatment reduction in liver function test and serum albumin, indicating the impact of DAAs in improving liver markers (Trifan et al., 2021; Ali et al., 2020; Sharma et al., 2018; Hashim et al., 2020; Ahmed at al., 2018; Elsharkawy et al., 2017). Also, hemolytic anemia is known to occur with RBV treatment, but in our patients, we didn't observe any differences in the hemoglobin levels between baseline and after treatment in both groups. This suggests the safety of adding RBV in the treatment for our patients. On the other hand, we detected an increase in platelets count in group A after SVR 155.81 ± 12.96 could be related to the prominent improvement in liver fibrosis after DAA treatment.

While the median levels of kappa and lambda FLC were higher in HCV patients with LPD compared to HCV patients without LPD (p = 0.029 and p = 0.010, respectively), but κ/λ ratio did not reach statistically differences (p= 0.39). And the median levels of kappa and lambda chains were higher in monoclonal positive patients compared to monoclonal negative patients in LPD patients (p= 0.010 and p =0.045, respectively) without observing differences in the κ/λ ratio (p=0.104).

The overall IgH clonality detected in all the studied patients was 37.17% (29/78), in which 6 (7.69%) patients were detected in the LPD group A and the remaining 23 (29.48%) patients were detected in the non-LPD group B. The different prevalence of the total clonality in our study and other previous studies might related to the different numbers of samples, ethnic background, and the different techniques used for detection of IgH clonality.

In group A, we found that clonality detected in 6 patients who represent 50% of group A [all 6 patients had clonality in FR2 region (100%), 4 patients had clonality

in FR1 region (66.66%), and 3 patients had clonality in FR3 region (50%)]. While in group B, in which clonality detected in 23 patients that represent 34.84% of this group [7 patients had FR1 clonality (30.43%), 12 patients had FR2 clonality (52.17%) and 6 patients had FR3 region clonality (26.08%)]. This distribution showed that the highest frequency of clonality in our patients was in FR2 region.

In patients with IgH gene rearrangement, there was a significant correlation in the baseline PCR HCV-RNA, ALT and kappa and lambda FLC in group A (P= 0.004, 0.025, 0.010 and 0.045 respectively), while in group B there was only a highly statistically significant difference detected in the baseline PCR HCV-RNA and ALT between patients with or without clonality in group B (p= 0.0001).Other parameters did not show any statistically differences or association in both groups. This is in agreement with Abdelgawad et al., (2015) study who reported a high viral load and ALT in positive patients and Inokuchi et al., (2009) who suggested that HCV-RNA is an independent factor associated with the presence of clonal B lymphocytes among other markers for lymphoproliferation but in controversy with De Re et al., (2012) who did not detect any association between the presence of clonal B cell and liver enzymes. These variables may be related to the ethnic background, severity of HCV infection and genetic and environmental factors.

In our cohort of study, we observed that 37% (11/29) of IgH clonality regressed with HCV eradication with DAAs regimen (2 patients (6%) in group A and 9 patients (31%) in group B). The regression of clonal IgH in group A was non-significant (p=0.5) whereas a significant positive clonality was observed in group B (P=0.004). This lower percentage of clonality regressed after treatment was also observed in previous studies (Schiavinato et al., 2017; Gragnani et al., 2021; Visentini et al., 2019).

These results showed that IgH clonality was not completely regressed after using oral DAAs in patients with HCV. As a result, we conclude that using IgH gene rearrangement together with serum κ and λ FLC in patients with chronic HCV is important to screen for high-risk patients developing LPD disease. Also, different DAAs regimen with or without RBV is safe and effective for the treatment of chronic HCV patients, but its effect in clonal regression is not absolute. Therefore, multidisciplinary management team shared with expert hematologists is highly warranted in this setting for treatment of those type of patients.

Author Contribution Statement

All authors contributed equally in this study.

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