J Clin Immunol (2013) 33:420–426
DOI 10.1007/s10875-012-9827-4

ORIGINAL RESEARCH

Differential Reactivity to IMPDH2 by Anti-rods/rings Autoantibodies and Unresponsiveness to Pegylated Interferon-alpha/Ribavirin Therapy in US and Italian HCV Patients

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Received: 13 September 2012 / Accepted: 17 October 2012 / Published online: 26 October 2012

Abstract
Purpose Autoantibodies to cytoplasmic structures called rods and rings (RR) are primarily specific to patients with hepatitis C virus (HCV) infection treated with pegylated interferon-alpha/ribavirin (IFN/R). Our aim is to examine anti-RR antibodies specificity and correlation with the response to IFN/R therapy in two independent cohorts (US and Italy) of HCV patients.
Methods Sera from the US cohort (n=47) and the Italian cohort (n=46) pre-selected for anti-RR antibodies were analyzed by immunofluorescence and radioimmunoprecipitation. The prevalence and titers of anti-RR were analyzed for correlation with the response to IFN/R therapy.
Results In the US cohort, anti-RR antibodies were more frequently non-responders to IFN/R (71 % vs 29 % responders). Titers in responder patients (n=11) were ≤1:3200, whereas titers in non-responder patients (n=27) reached 1:819,200 (p=0.0016). In the Italian cohort, anti-RR titers ranged from 1:200 to >1:819,200 and only relapsers had the highest anti-RR titers. Radioimmunoprecipitation demonstrated that anti-RR autoantibodies were mainly anti-inosine monophosphate dehydrogenase 2 (IMPDH2) - 96 % in the Italian cohort vs. 53 % in the US cohort.
Conclusions In the two cohorts analyzed, the anti-IMPDH2 response as a component of the anti-RR response is much more prominent in the Italian cohort. The reason for the difference between the US and Italian cohorts is unclear but it possibly illustrates the heterogeneity in response and the overall negative correlation between the production of these autoantibodies and response to IFN/R therapy. Patients with high titer anti-RR antibodies are either relapsers (Italian) or non-responders/relapsers (US).

Keywords IMPDH2 · hepatitis C · autoantibody

Abbreviations

ANA	Anti-nuclear antibody
CTPS1	Cytidine triphosphate synthetase 1
DON	6-diazo-5-oxo-L-norleucine

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HCV	Hepatitis C virus
IFN/R	Pegylated interferon-alpha/ribavirin
IFN-α	Pegylated interferon-alpha
IIF	Indirect immunofluorescence
IMPDH2	Inosine monophosphate dehydrogenase 2
IP	Immunoprecipitation
NR	Non-responders
RBV	Ribavirin
RR	Rods and rings
SVR	Sustained virological response

Introduction

Hepatitis C virus (HCV) is an enveloped positive single-stranded RNA virus classified in the genus *Hepacivirus* of the family *Flaviviridae* [1]. It was identified in 1989 and is a major cause of chronic liver disease, frequently leading to liver cirrhosis and eventually to hepatocellular carcinoma [2–4]. An estimated 170 million people are infected worldwide and there is no vaccine available for this viral infection [5]. The virus genome encodes a polyprotein of approximately 3000 amino acids that is flanked by 5′ and 3′ non-coding regions [6, 7]. Translation of the polyprotein is mediated by an internal ribosome entry site embedded within the 5′ non-coding regions, and the individual viral proteins are produced upon cleavage of the polyprotein by host and viral proteases. These include three structural proteins (core, E1, and E2), the p7 protein, and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), which are involved in virion assembly and viral RNA replication [6, 7]. The virus is classified into eleven different genotypes and more than a hundred subtypes, and it also manifests itself in sera or tissues of patients with the appearance of quasi species [8, 9]. The current treatment of HCV is a combination of pegylated interferon alpha (IFN-α) and ribavirin (RBV, IFN/R). Although the addition of RBV to the IFN-α treatment has significantly improved patient outcomes, only half of the patients infected with HCV respond to therapy [10]. Since the ultimate goal in treatment is to achieve a sustained virological response, new drugs have been approved for the treatment of HCV [11].

Patients with HCV may also develop aberrant immunological alterations, such as autoantibody production, autoimmune thyroid disorders, B cell lymphomas, and mixed cryoglobulinemia [12–14]. HCV patients can produce non-organ specific autoantibodies such as anti-nuclear antibodies (ANA), anti-smooth muscle antibodies, and anti-LKM1 antibodies with much higher prevalence than healthy people [15, 16]. Since HCV enters hepatocytes through binding of its envelope protein E2 with CD81, which is also expressed in B-lymphocytes, Pileri et al. proposed that HCV binding to B-lymphocytes lowers the B cell activation threshold and therefore facilitates the production of autoantibodies [17]. Autoantibodies are commonly used to help with the clinical diagnosis of certain autoimmune conditions such as systemic lupus erythematosus, scleroderma, and polymyositis [18, 19], but data on the association of autoantibodies in chronic HCV hepatitis or response to treatment are still controversial [20–22].

A novel autoantibody that recognizes distinct cytoplasmic rods and rings (RR) structures was first identified in sera of HCV patients that were screened using commercial ANA slides [23]. Localization of two specific enzymes, inosine monophosphate dehydrogenase 2 (IMPDH2) and cytidine triphosphate synthetase 1 (CTPS1), to these structures was identified in our previous study [23]. RR were induced in cultured cancer cells when exposed to IMPDH2 inhibitors RBV, or CTPS1 inhibitor 6-diazo-5-oxo-L-norleucine (DON) or acivicin [23]. Anti-RR antibodies were specific to HCV patients treated with IFN-α and RBV but not prior to treatment [24]. The aim of the present study is to identify the clinical significance of anti-RR autoantibodies developed during IFN/R therapy in HCV infected patients.

Materials and Methods

Human Sera and Autoantibodies

One cohort consists of 47 anti-RR antibody positive patient samples collected from the outpatient clinic for chronic liver diseases at the University of Florida (US). The second cohort consists of 46 sera pre-screened positive for anti-RR obtained from three hospital clinics in north-eastern Italy. Patients infected with HCV-1 and HCV-4 genotypes were generally treated for 48 weeks, while HCV-2 and HCV-3 patients were treated for 24 weeks. Patients were classified as: non-responders (NR) if HCV RNA was still detectable at week 24 of therapy; relapsers if HCV RNA was detected after the end of treatment in patients with a virological response; sustained virological responders (SVR) if HCV RNA was undetectable in the 24 weeks after the completion of therapy. The US cohort was only classified into two groups (NR/relapsers and SVR) and not separated into distinct NR and relapsers. The study conforms to Institutional Review Board requirements in all institutions. None of the patients were treated with telapravir in addition to IFN/R therapy.

Indirect Immunofluorescence (IIF)

IIF analysis of autoantibodies in human sera was performed as described, using commercial HEp-2 ANA slides (INOVA Diagnostics, San Diego, CA) [23]. To determine anti-RR

titer, samples were two-fold serially diluted in PBS containing 0.1 % bovine serum albumin, with a starting dilution of 1:50 and ending dilution of 1:819,200. Titer analysis for anti-RR was determined by two readers (WCC and SJC) and endpoint titer was defined by more than 50 % of cells with detectable RR staining. Fluorescent images were captured with a Zeiss Axiovert 200M microscope fitted with a Zeiss AxioCam MRm camera using a 40x (0.75 NA) objective. The majority of anti-RR positive samples had titer below 1:100,000. Titers over 1:100,000 were determined with the aid of a Zeiss AxioCam MRm camera.

Immunoprecipitation (IP)

IP of lysate from [^{35}S]-methionine-labeled K562 cells for the analysis of the proteins recognized by human autoimmune sera was performed as previously described [23, 25]. Anti-IMPDH2 autoantibodies were determined using reference sera [23].

Statistical Analysis

The statistical analysis was performed using the GraphPad Prism 5 software for Windows (La Jolla, CA). The one-way analysis of variance (ANOVA), Mann–Whitney Test, and Fisher's exact test were performed. A p value of <0.05 was considered statistically significant.

Results

Autoantibodies to Cytoplasmic Rods and Rings in the US and Italian Cohorts

HCV patients from both US and Italian cohorts were initially selected for anti-RR reactivity in local laboratories. The anti-RR reactivities for all samples were subsequently confirmed in the corresponding author's laboratory using INOVA HEp-2 slides at 1:50 dilution of sera. IIF staining of HEp-2 cells typically showed 1 to 2 distinct cytoplasmic rods (~3–10 μm in length, arrows, Fig. 1a–c) and/or rings (2–5 μm diameter, arrowheads) per cell. Both US and Italian patients recognized the same RR as routinely determined by costaining using rabbit anti-IMPDH2 antibodies (data not shown). It was previously demonstrated that IMPDH2 is a component of RR and rabbit anti-IMPDH2 antibodies costain RR detected by sera of HCV patients [23]. This confirmed that the structures recognized by Italian and US patients were the same. The titers for anti-RR were determined side by side using the same lot of slides and reagents. Determination of the ultrahigh anti-RR titers was accomplished with the aid of the Zeiss AxioCam camera, otherwise RR were visualized by eye at titers as high as 1:102,400. Figure 1a–c shows representative anti-RR staining from HCV patient with titers at 1:400, 1:102,400, and 1:819,200, respectively. The highest dilution used was 1:819,200, and three patients in each cohort had titers higher than this upper limit. The US cohort had titers that ranged from 1:50 to >1:819,200 with a median titer of 1:1,200, while the Italian cohort had titers that ranged from 1:200 to >1:819,200 with a median titer of 1:25,600 (p=0.0003, Fig. 1d).

Prevalence of Anti-IMPDH2 Antibodies by IP

It was previously shown that IMPDH2 is a component of RR and some anti-RR autoantibodies recognize IMPDH2 as an autoantigen [23] although the prevalence of anti-IMPDH2 has not been determined. Therefore, sera from the two cohorts were tested by IP for the prevalence of anti-55 kDa IMPDH2. Figure 1e,f shows representative IP of ^{35}S-methionine labeled K562 cell lysate using selected patients from both US and Italian cohorts. Not all anti-RR positive sera immunoprecipitated IMPDH2 (arrow in Fig. 1e,f). In the Italian cohort, 44 anti-RR positive patients (96 %) immunoprecipitated IMPDH2 and only 2 patients with anti-RR antibodies did not have the 55 kDa band corresponding to IMPDH2 [23]. Additional controls show that none of the patients without anti-RR antibodies immunoprecipitated the IMPDH2 band (data not shown). However, in the US cohort, only 25 anti-RR positive patients (53 %) had the IMPDH2 band, while the other 22 anti-RR-positive patients did not immunoprecipitate IMPDH2. Patients were classified by IMPDH2 band intensity from 0 to 4 (weakest to strongest, Fig. 1g). Patients in the Italian cohort immunoprecipitated IMPDH2 with stronger intensity compared to patients in the US cohort.

Comparison of IIF Titer versus Anti-IMPDH2 IP

Anti-RR titer evaluated by IIF was correlated to the intensity of the IMPDH2 band in IP for both cohorts (Fig. 2a). Patients in the US and Italian cohort with higher anti-RR titers immunoprecipitated the IMPDH2 band with a higher intensity (p<0.0001 and 0.0034, respectively). This strong correlation validates the finding that anti-55 kDa IMPDH2 is the predominant target in the anti-RR response in these patients. This is clearly true in the Italian cohort with 96 % anti-RR positive sera immunoprecipitated the 55 kDa IMPDH2. It is interesting that a significantly lower percentage (53 %) of patients immunoprecipitated IMPDH2 in the US cohort. It remains to be determined what autoantigenic component(s) is undetected by IP in those 47 % who are negative for anti-IMPDH2. Since RR are relatively large

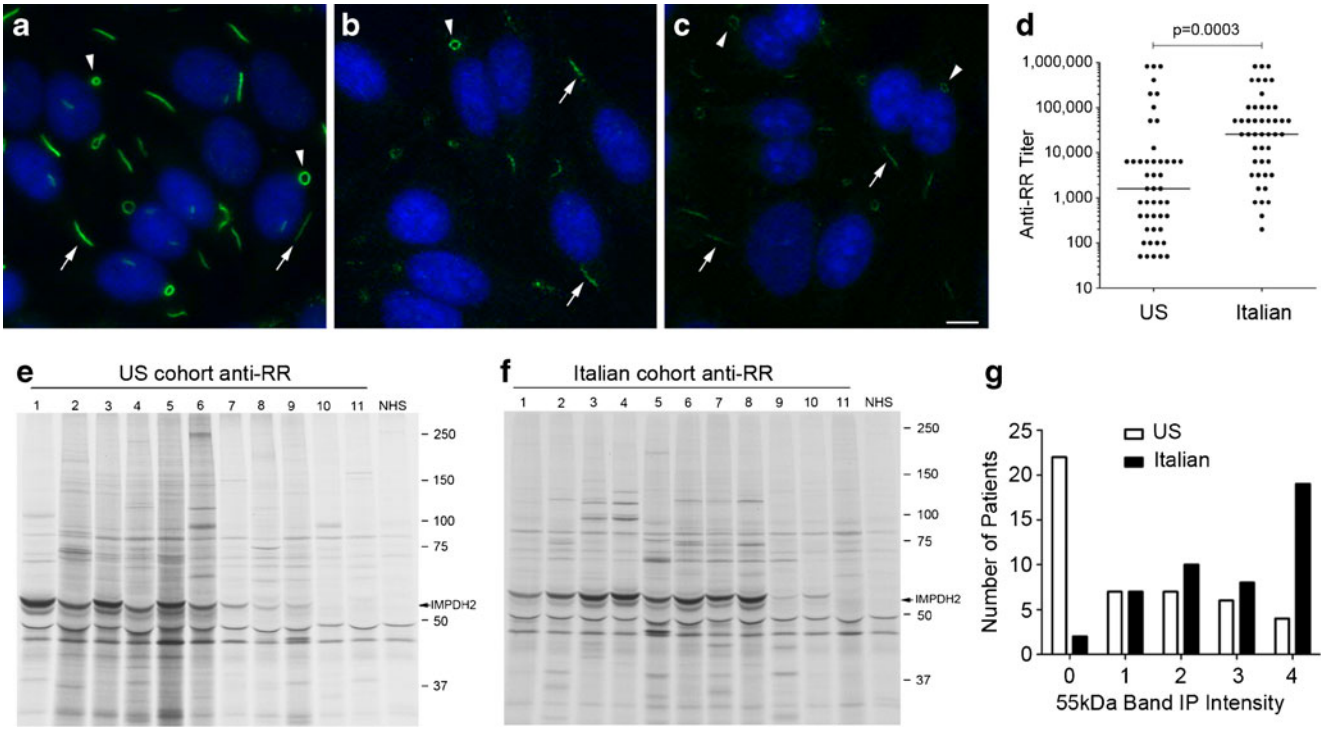

Fig. 1 Anti-RR antibody titer and prevalence of the immunoprecipitated 55 kDa IMPDH2 in two cohorts of HCV patients with autoantibodies to cytoplasmic rods and rings. **a**–**c** HEp-2 ANA slide stained with HCV patient sera with various titers. Representative images of rods and rings (*RR, green; arrows, rods; arrowheads, rings*) from sera diluted at 1:400 (**a**), 1:102,400 (**b**), and 1:819,200 (**c**). Nuclei were counterstained with DAPI (*blue*). Scale bar, 10 μm. **d** Anti-RR titers of US and Italian cohorts determined using serial two-fold dilutions started from 1: 50 of sera on HEp-2 ANA slides. Each dot represents a single positive anti-RR patient; patients that were negative for anti-RR antibodies are not represented. P=0.0003 by Mann–Whitney. **e**, **f** IP of ^{35}S-methionine labeled K562 cell extract was performed with all anti-RR positive patient sera from (**e**) US and (**f**) Italian cohorts. Numbers correspond to individual samples. The 55 kDa band corresponding to IMPDH2 is indicated by *arrow*. **g** Semi-quantitative analysis of all patients from both cohorts, grading the intensity of the 55 kDa band detected in IP using a scale from 0 (negative) to 4 (strongest). NHS, normal human serum

subcellular structures, compared to mitochondria for example, it is reasonable that there are many proteins localized to RR and whether some of these yet-to-be-defined RR proteins are targeted by these lower titer anti-RR autoantibodies remains to be determined.

Correlation of Anti-RR/IMPDH2 Prevalence and Titer with Therapeutic Outcome

Next, we analyzed whether the titers of anti-RR antibodies are different between US versus Italian patients and whether they are associated with the response to IFN/R therapy. A correlation was observed between anti-RR titers and response to treatment in the US cohort, and in fact patients classified as NR/relapsers had significantly higher anti-RR titers compared to SVR (median =1:3,200 vs 1:100; p= 0.0016, Fig. 2b). In the US cohort there were a total of 11 SVR and 27 NR/relapser patients, while in the Italian cohort there were 6 NR, 28 SVR and 11 relapsers. In the Italian cohort, relapsers had significantly higher titers when compared to NR and SVR (p=0.0040 and p=0.015, respectively, Fig. 2b). When the intensity of the IMPDH2 IP band was compared between SVR and NR/relapsers in the US cohort, 8 out of 9 patients with strong (3+4+) IP band intensity were NR/relapsers (Fig. 2c). For patients in the Italian cohort, relapsers had a significantly stronger 55 kDa band IP intensity compared to SVR (p=0.031) or NR (p=0.016) patients (Fig. 2c).

Discussion

In a previous study, we described anti-RR autoantibodies produced in an Italian cohort of HCV patients treated with IFN/R, but not in untreated HCV patients or in patients with other liver diseases [24]. The production of anti-RR was not significantly affected by a number of factors examined, including sex, age, HCV genotype, viral RNA copy, alanine aminotransferase, anti-nuclear antibodies, anti-smooth muscle antibodies, anti-liver/kidney/microsome antibodies, steatosis, cirrhosis, and diabetes [24]. The only significant difference observed was that these anti-RR antibodies were more often detected in non-responders/relapsers than in responder patients (33 % vs 11 %; p=0.037) [24]. Although

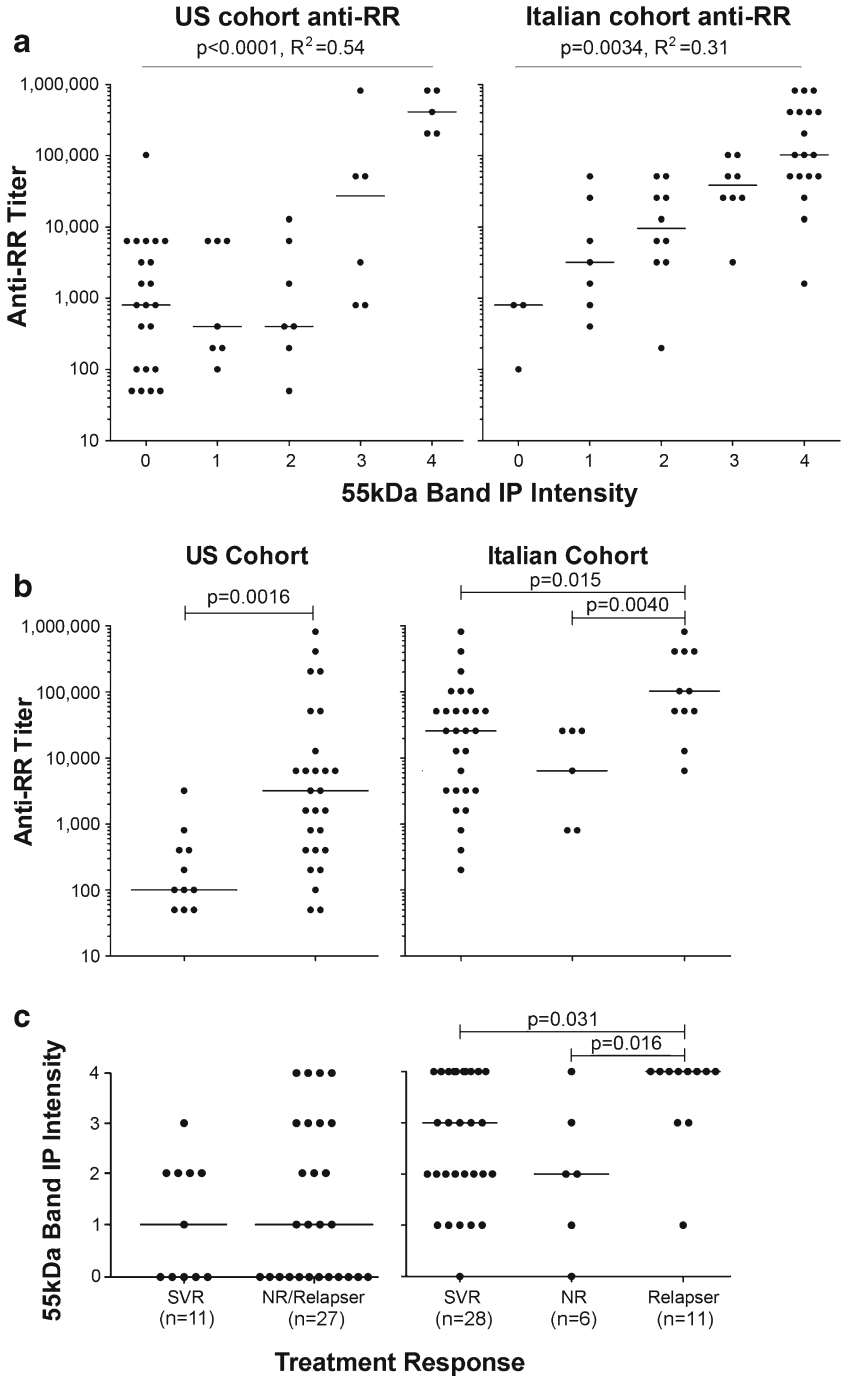

Fig. 2 Correlation of anti-RR IIF titer, intensity of the 55 kDa IMPDH2 IP band and IFN/R treatment outcome. **a** The intensity of the 55 kDa IMPDH2 IP band was correlated to anti-RR titer by IIF for each patient, for the US and the Italian cohort. Each dot represents one individual. Statistics analysis was determined by ANOVA. **b** Patients were divided according to their response to treatment. The US cohort was divided into two groups, NR and SVR, while the Italian cohort was divided into NR, SVR, and relapsers. Anti-RR titer (**b**) and 55 kDa IP band intensity (**c**) were correlated to treatment response for each cohort. The intensity of the 55 kDa IP band was visually graded from 0 (negative) to 4 (strongest). *Lines* the median; *NR* non-responders; *SVR* sustained virological responders. *P* values in (**b**) and (**c**) are by Mann–Whitney

the difference in prevalence of anti-RR antibodies was observed, the sample size was too small and anti-RR titer was not examined. Another recent study by Seelig et al. on anti-RR antibodies did not examine correlation of antibodies with clinical and therapeutic response [26]. Thus, the present study focuses on the higher number of patients with anti-RR autoantibodies collected in the two cohorts and further defines the autoantibody specificity and titer.

Our report on the characterization of the structure and function of RR showed that RR are highly enriched in two CTP/GTP biosynthetic enzymes, CTPS1 and IMPDH2, and are induced by inhibitors specific to these enzymes [23]. Thus, the HCV treatment with both IFN and RBV appears to trigger the immune response to RR in a subset of HCV patients. In the present study, anti-RR produced by patients treated with RBV, a potent inhibitor of IMPDH2, are shown to produce antibodies to IMPDH2 predominantly. However, it is notable that the two cohorts examined displayed a difference in IMPDH2 antibody expression, with 96 % of the Italian cohort showing IP of the 55 kDa IMPDH2 compared to 53 % of patients in the US cohort. One may consider whether there are differences in the selection of

patients in the two cohorts. To the best of our knowledge, the selection was comparable. There is no obvious difference in the therapy regimen or manufacturer of IFN/RBV between the US and Italian cohorts. Given that primarily only ANA slides from INOVA are consistent in the detection of anti-RR as previously reported [23], and both cohorts were screened for anti-RR reactivity using INOVA ANA slides, the only explanations for these differences thus far are the genetic backgrounds and environmental factors associated with the two cohorts.

In the US cohort, NR/relapser patients had higher anti-RR titers than SVR patients. This new information is not surprising, as we showed in the previous smaller Italian cohort [24] that anti-RR antibodies were more often detected in NR/relapsers than in SVR patients (33 % vs 11 %, p=0.037). In the current Italian cohort, relapsers had higher levels of anti-RR antibodies than both SVR and NR. With the working hypothesis that RBV induces the formation of RR structures in certain cells in the treated patients and a subset of HCV patients produce autoantibodies to RR, it is intriguing that relapser and NR/relapser patients are those with the highest titers of anti-RR. The small number of NR patients in the current Italian cohort that do not show high RR titers is an obvious discrepancy, but since there are only six patients in this group, the data may not be representative.

The mechanism for the production of anti-RR antibodies remains unknown but we speculate that a subset of patients develop anti-RR antibodies from exposure to RR structures induced by RBV during IFN/R therapy. It is also reasonable to consider that RBV binding to IMPDH2 may induce conformational changes such that it becomes antigenic. Pileri et al. proposed that HCV binds to CD81 on B-lymphocytes through its envelope protein E2, thus lowering the activation threshold on the B cells and contributing to the production of autoantibodies [17]. B cell activation, in addition to RR formation by RBV, may play a role in the development of anti-RR antibodies. However, it should be noted that general B-cell activation cannot explain observations in the present study because the reactivity of autoantibodies are highly restricted to IMPDH. Alternatively, the observation that higher-titered anti-RR/IMPDH2 response is associated with NR/relapsers suggests the possibility that these autoantibodies may interfere with IFN/R therapy, although the mechanism remains unclear.

Acknowledgements Natasha Deming, Pabina Dhawan, and Jennifer R. Bess are acknowledged for the early work helping in the screening of HCV patient sera for anti-RR positive samples.

Disclosures The authors declare that they have no conflicts of interest.

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