

# CORRELATION BETWEEN THE ANTIBODY RESPONSE TOWARD SPECIFIC HCV PROTEINS AND HCV VIRAL LOAD

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## ABSTARCT

**Background:** Hepatitis C virus (HCV) is an RNA virus causing acute or chronic infection and affecting more than 2% of population worldwide. The first-line tests for diagnosis of HCV infection are 3rd or 4th generation enzyme immunoassays - ELISA and CIA. They indicate the presence of antibodies against HCV in serum. These tests are characterized by high sensitivity and specificity, but they cannot distinguish past, acute or chronic infection, and sometimes produce false positive results. Confirmatory tests, such as recombinant immunoblot-line immune assay (LIA), and quantitative PCR, are used to validate the positive antibody response. The recombinant immunoblot assay can be used to determine the specificity of antibody to HCV. The aim of the present study is to determine the correlation between the anti-HCV response in confirmatory immunoblot assay and the HCV viral load, measured by PCR.

**Materials and methods:** Twenty-nine anti-HCV positive sera were included in the study. Third generation ELISA assay was used for anti-HCV screening of the samples and for detection of anti-HCV antibodies against specific HCV proteins. Third generation line immunoassay INNO-LIA HCV Score, based on the principle of an enzyme immunoassay, was used as a confirmatory test. The HCV viral load was measured by quantitative PCR method – Abbott Real Time HCV (Abbott Molecular Inc., USA) with

linear sensitivity range from 1.08 Log<sub>10</sub> IU/ml (12 [IU/ml]) to 8.00 Log<sub>10</sub> IU/ml (100 000 000 [IU/ml]).

**Results:** HCV RNA was quantified in all studied samples. Ten of 29 serum samples (34%, Group I) were HCV RNA negative. The rest of the samples were HCV RNA positive as follows: 3 serums were with minimal viral load from < 12 to 10 000 IU/ml (10%, Group II); 3 serum samples –between 10 000 and 100 000 IU/ml (10%, Group III); 10 serum samples – between 100 000 and 1 000 000 IU/ml (34%, Group IV) and in 3 serum samples HCV RNA concentration was over 1 000 000 IU/ml (10%, Group V).

**Conclusion:** HCV screening strategies involving anti-HCV detection by ELISA combined with recombinant immunoblot assay can be the method of choice in laboratories with limited equipment and finances.

**Keywords:** *Hepatitis C virus, recombinant immunoblot assay, antibodies*

## INTRODUCION

Hepatitis C virus (HCV) is an RNA virus that belongs to the *Flaviviridae* family. HCV causes acute or chronic infection and affects more than 2% of the individuals worldwide. The virus is transmitted by blood transfusion or organ transplantations, injection drug use, vertically during pregnancy, or sexually. Because of its modes of transmission, current recommendations for HCV screening include the following categories: pregnant women; blood donors; healthcare workers; hemodialysis patients; patients undergoing surgery or chemotherapy; people living with HIV, sex workers. The time of appearance of serological and virological markers for HCV infection is not exactly defined yet. At present, the natural course of acute HCV infection is described as follows: initial eclipse phase when no serological and virological markers of HCV infection can be detected; appearance of HCV RNA, followed by the appearance of HCV core antigen in the absence of an antibody response, and finally development appearance of anti-HCV antibody response leading to HCV core antigen disappearance [WHO 2017]. Usually, the acute HCV infection is clinically silent and in up to 45% of the infected, spontaneous clearance occurs. In addition, in most patients, HCV infection progresses slowly and is diagnosed after the development of co-morbidity [Bruno S., C. Facciotto. 2008]. The diagnosis of HCV infection is initialized by the detection of anti-HCV antibodies in serum, as a marker of past or present infection.

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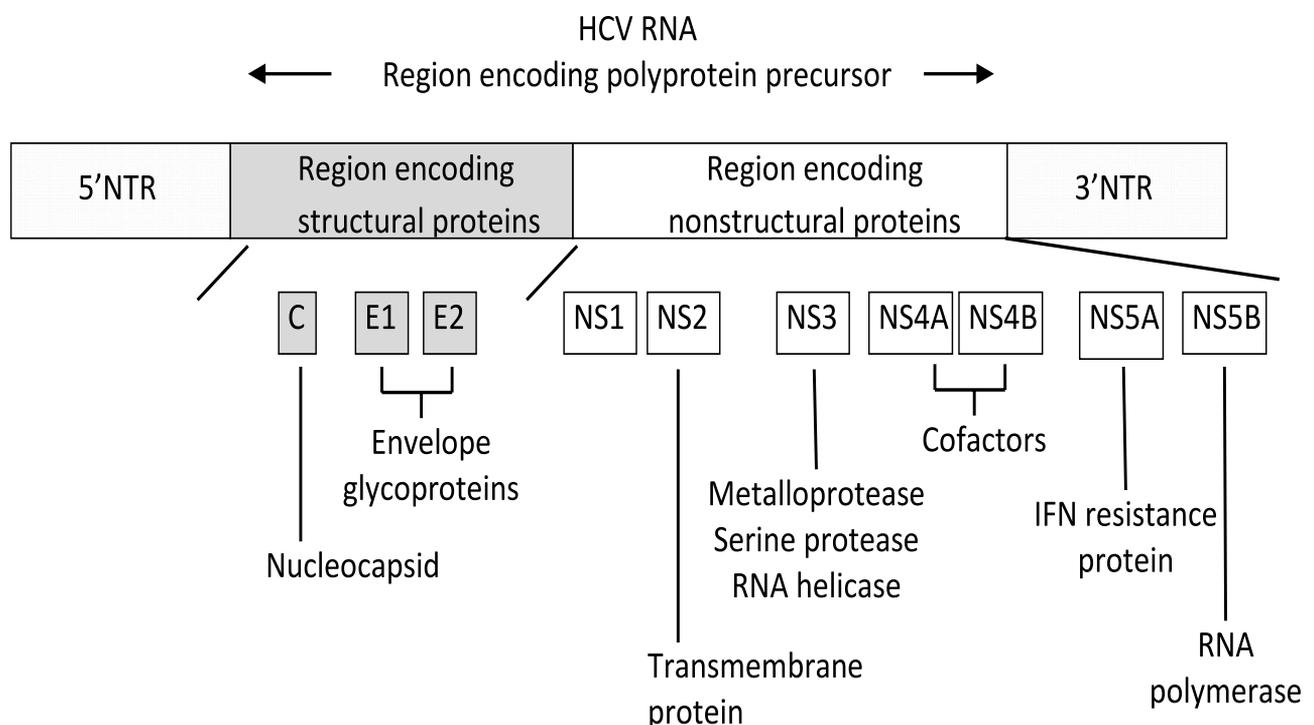
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## CORRELATION BETWEEN THE ANTIBODY RESPONSE TOWARD SPECIFIC HCV PROTEINS AND HCV VIRAL LOAD

At present, the first-line tests for diagnosis of HCV infection are 3rd or 4th generation serological assays based on immunoassay principles. They are available as rapid diagnostic tests (RDTs) or laboratory-based enzyme immunoassays (EIAs), chemiluminescence immunoassays (CLIAs) and electrochemiluminescence immunoassays (ECLs), and indicate the presence of antibodies against HCV (anti-HCV) in serum. These tests are characterized by high sensitivity and specificity, but they cannot distinguish past, acute or chronic infection, and sometimes produce false positive results. Anti-HCV assays have a lower positive predictive value in low-prevalence populations [Kamili S, et.al. 2012]. In this case a second anti-HCV test can be used to confirm the first positive test result. Therefore, the Center for Disease Control and Prevention (CDC) recommended that a positive result from an initial anti-HCV screening test should be followed by confirmatory tests such as recombinant immunoblot and quantitative PCR method [Kodani M, et. al., 2019]. The latter methods are used to validate the positive antibody response. Recombinant immunoblot assay allows tracking of the antibody response against specific HCV (structural and non-structural) proteins (Figure 1). The viral proteins

encoded by HCV genome, and used in INNO-LIA HCV test are as follows: core protein (C), NS4A, NS4B, E-protein and NS5A. The two envelope glycoproteins, E1 and E2, play pivotal role at different steps of HCV replicative cycle. They are essential for the host-cell entry by binding to receptors and inducing fusion with the host-cell membrane. The latter proteins are not genetically stable and their application in tests might be controversial. On the other hand, the core protein is an RNA-binding phosphoprotein that forms the viral nucleocapsid. It is genetically stable protein so it is used in many techniques for HCV detection. The nonstructural protein NS3 is a multifunctional enzyme with serine protease, NTPase, and RNA helicase activities. It is an essential replicative component of HCV. NS3 exhibits NTPase and helicase activity from its C-terminal helicase domain and serine protease activity from its N-terminal protease domain when it is bound by the HCV NS4A cofactor. NS4 protein is recruitment of other viral proteins and NS5 plays role in viral replication, modulation of cell signaling pathways and interferon response [Warkad SD, et.al., 2019]. The present study examines the correlation between the anti-HCV response in confirmatory immunoblot assay and the HCV viral load.

**Figure1.** HCV genome organization, structural and nonstructural viral proteins and their function (Usman A Ashfaq 2011)



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**MATERIALS AND METHODS:**

**Samples.** The sera of twenty-nine anti-HCV positive patients were included in the study. The samples were grouped, according to the detected HCV viral load as follows: Group I - negative for HCV RNA; Group II – from 12 [IU/ml] to 10 000 [IU/ml]; Group III - from 10 000 [IU/ml] to 100 000 [IU/ml]; Group IV - from 100 000 [IU/ml] to 1 000 000 [IU/ml]; and Group V > 1 000 000 [IU/ml].

**Detection of HCV antibodies (anti-HCV) by ELISA.**

Third generation ELISA assay was used for anti-HCV screening of serum samples (DiaPro, Italy). Samples were considered anti-HCV positive if the ratio between sample and calibrator optical density (OD) was greater than 1.1. Samples with a ratio less than 0.8 were considered negative.

**Detection of HCV antibodies (anti-HCV) by INNO-LIA HCV Score.**

For detection of anti-HCV against specific HCV proteins the 3<sup>rd</sup> generation line immunoassay INNO-LIA HCV Score, was used. Coated HCV antigens were derived from core region, E2 hypervariable region, NS3 helicase region, NS4A, NS4B and NS5A regions. The presence of anti-HCV was associated with the appearance of colored bands corresponding to coated HCV specific antigens. A reactivity rating was made separately for each sample using a reading card. The intensity of lines was proportional to the amount of captured HCV-specific antibodies from the sample and was rated as negative (-), weak positive (±) and moderate to strong positive 1+ to 4+ (from). Identification of the lines was done by alignment of the 3+ control line on the developed strip with the corresponding 3+ control line on the reading card. Results were reported as anti-HCV negative, when all HCV antigen lines were not reactive or if only one

antigen line except for NS3 line was weak positive,; and as anti-HCV positive if at least two HCV antigen lines had a weak ± or higher reactivity .

**Detection and quantification of serum HCV RNA.**

HCV viral load was measured by quantitative PCR method – Abbott Real Time HCV (Abbott Molecular Inc.,USA) with linear sensitivity from 1.08 Log<sub>10</sub> IU/ml (12 [IU/ml]) to 8.00 Log<sub>10</sub> IU/ml. Samples with lower (< 1.08 Log<sub>10</sub> IU/ml) or higher (> 8.00 Log<sub>10</sub> IU/ml) viral load were considered positive or. Only samples reported as “not detected” were consider negative.

**Results:** HCV RNA was quantified in all studied samples (Table 1). Ten out of 29 serum samples (34%, Group I) were HCV RNA negative. The rest of the samples were classified according to HCV RNA viral load as follows: 3 were with minimal viral load to up 10 000 IU/ml (10%, Group II); 3 –between 10 000 and 100 000IU/ml (10%, Group III); 10 – between 100 000 and 1 000 000 IU/ml (34%, Group IV) and 3 over 1 000 000 IU/ml (10%, Group V).

By INNO-LIA assay 11 (38%) of 29 tested samples were negative for the presence of HCV antibodies and 18 (62%) were positive (Table1). Reactivity with different intensity was detected against HCV antigens. The highest frequency of HCV antibody response to all HCV antigens was found in samples from Group IV, while the weakest antibody response was detected in samples from Group II. For the samples of Group I, 6 (60%) serum samples out of the 10, show positive INNO-LIA result. All samples of Group II were negative by INNO-LIA results interpretation. The number of positive samples increased concomitantly with HCV viral load – two (67%) of 3 positive samples in Group III, and 9 (90%) of 10 samples of Group IV.

Table 1. Group distribution of the tested samples and INNO-LIA reactivity interpretation according to HCV viral load groups

Groups	HCV RNA [IU/ml]		samples via group		Negative samples		Positive samples	
	≥	<	N	%	N	%	N	%
I	0		10	34%	4	40%	6	60%
II	< 12	10 000	3	10%	3	100%	-	
III	10 000	100 000	3	10%	1	33%	2	67%
IV	100 000	1 000 000	10	34%	1	10%	9	90%
V	> 1 000 000		3	10%	2	67%	1	33%
<b>Total</b>			<b>29</b>		<b>11</b>	<b>38%</b>	<b>18</b>	<b>62%</b>

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According to the presence of specific HCV antibodies the INNO-LIA results were as follows (Table 2). In Group I samples were detected HCV antibodies against all structural and non-structural HCV proteins – C1, C2, E2, NS3, NS4 and NS5, and the intensity of lines varied from weak positive ( $\pm$ ) to moderate (1+), except for one sample where the bands intensity was from moderate to strong positive (3+). The highest frequency was established against the nonstructural NS3. As mentioned above, samples from Group II generated the weakest responses, and specific antibodies were detected only against two HCV core proteins – C1 and C3, with a weak intensity of the reacting bands. In samples from Groups III, IV and

V, specific antibodies were detected against all HCV antigens. In Group III samples, the highest frequency was established for antibodies against the structural HCV E2, and the nonstructural NS3 antigens. In Group IV, HCV specific antibodies were detected against all antigens - C1, C2, E2, NS3, NS4 and NS5. The intensity of reacting bands ranged from moderate to strong positive and only one sample reacted with a weak positive antibody response against NS3. Finally, in Group V, decreased frequency of specific antibodies was established against all INNO-LIA comprised HCV antigens. Only one sample reacted against all HCV antigens and the intensity of the bands varied from weak to strong positive.

**Table 2.** Frequency of specific anti-HCV, detected by INNO-LIA assay

HCV Ag	I group (N=10)		II group (N=3)		III group (N=3)		IV group (N=10)		V group (N=3)	
	N	%	N	%	N	%	N	%	N	%
C1	4	40%	1	33%	1	33%	8	80%	1	33%
C2	4	40%	1	33%	1	33%	8	80%	1	33%
E2	1	10%	0	-	2	67%	7	70%	1	33%
NS3	5	50%	0	-	2	67%	9	90%	1	33%
NS4	4	40%	0	-	1	33%	8	80%	1	33%
NS5	4	40%	0	-	1	33%	7	70%	1	33%

### DISCUSSION

The prevalence of chronic HCV infection in Bulgaria is 0,9%, according to the cross-sectional study in adults (anti-HCV and RNA positive), conducted in 2018 [Sperle, I., et.al., 2020]. The morbidity due to acute HCV infection is 1,26‰ [Vladimirova N, et.al]. According to the World Health Organization Guidelines on Hepatitis B and C Testing, a serological assay for initial detection of evidence of past or present infection is recommended prior to supplementary nucleic acid testing (NAT) for evidence of viraemic infection [WHO 2017]. The Guidelines Development Group has recommended the use of secondary confirmatory serological assay instead HCV NAT technology in low HCV prevalence settings (< 0.4%), due to high percent of false positive anti-HCV results, and cost effectiveness,. INNO-LIA HCV Score is a 3th generation immunoblot assay for detection of specific antibodies against six HCV antigens derived from the core region, the E2 hypervariable region (HVR), the NS3 helicase region, the NS4A, NS4B, and NS5A regions.

Diagnosis of HCV infection is based on the detection of specific HCV antibodies in combination with detectible HCV RNA in cases of acute infection, and - on detection of antibodies in the absence of HCV RNA. In cases of resolved infection= Antibodies to HCV appear during the acute infection and persist up to 20 years after recovery [Takaki A, et.al., 2000]. The kinetics of HCV antibodies response is characterized initially with appearance of antibodies against viral capsid and non-structural NS3 proteins, followed by antibodies against NS4 and enveloped glycoproteins - E1 and E2, the last one acting as neutralizing antibodies [Santos, et.al., 2019]. It has been also established that human and murine humoral immune responses to HCV NS3 protein are serologically reactive during the early phase of HCV infection and therefore are routinely used in HCV antibody immunoassays [Bian Y, et.al., 2013]. In our study, the most of reacting serums were positive for antibodies against non-structural NS3 protein and two capsid antigens–C1 and C2. The weak antibody reactivity was measured in

HCV RNA negative samples, which is in agreement with previously conducted cross-sectional studies demonstrating that indeterminate and weak antibody reactivity predicted the absence of HCV viremia [Strasak AM, et al., 2011]. The anti-HCV positivity with no detectable serum HCV RNA can be due to past or occult HCV infection [De Marco L, et al., 2012]. It is worth mentioning, that 30% of people infected with HCV spontaneously clear the infection by a strong immune response [Grebely J, et al. 2012]. At the same time, for the samples of Group II, negative INNO-LIA results were determined, which can be explained with the early stage of HCV infection. The Increased frequency and HCV antibodies' band insensity were detected in Groups III and IV, with HCV increasing viral load. Group V, with the highest HCV viral load, was characterized by 67% INNO-LIA negative results. The only positive sample responded strongly against all INNO-LIA HCV antigens. It can be hypothesized that the absence of HCV antibodies was due to the diagnostic window period during which specific antibodies are not detected, but the HCV RNA has already reached peak serum levels between  $10^5$  IU/ml to  $10^7$  IU/ml [Bruno S., C. Facciotto. 2008]. Tested samples were anti-HCV positive by ELISA where the multiple-target response is measured [Warkad SD, et al., 2019]. Hence, the result could be explained with the weak antibody response, which cannot be detected against each specific antigen individually, but this should be further studied. Another explanation could be a possible immunosuppression, when the indeterminate immunoblot reaction can be observed occasionally in HCV RNA positive subjects [Makuria AT, et al. 2012]. Our study had a number of limitations. First of all, the number of tested samples is insufficient for drawing general conclusions about the correlation. Second, patients were with confirmed HCV status, but clinical and biochemical information about the course of the HCV infection was missing.

In conclusion, the outcome of this study will provide highlights for future studies on HCV diagnostic algorithms. HCV screening strategies involving anti-HCV detection by ELISA combined with recombinant immunoblot assay can be the method of choice in laboratories with limited equipment and finances.

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**Conflicts of Interest:** The authors declare no conflict of interest.

#### REFERENCES

1. WHO guidelines on hepatitis B and C testing. Geneva: World Health Organization; 2017. Licence: CC BY-NC-SA 3.0 IGO
2. Bruno S, Facciotto C. The natural course of HCV infection and the need for treatment. *Annals Hepatol.*, 2008; 7 (2): 114-119
3. Kamili S, Drobeniuc J, Araujo AC, Hayden TM. Laboratory diagnostics for hepatitis C virus infection. *Clin Infect Dis.* 2012 Jul;55 Suppl 1:S43-8. doi: 10.1093/cid/cis368. PMID: 22715213
4. Kodani M, Martin M, de Castro VL, Drobeniuc J, Kamili S. An Automated Immunoblot Method for Detection of IgG Antibodies to Hepatitis C Virus: a Potential Supplemental Antibody Confirmatory Assay. *J Clin Microbiol.* 2019;57(3):e01567-18. Published 2019 Feb 27. doi:10.1128/JCM.01567-18
5. Warkad SD, Song KS, Pal D, Nimse SB. Developments in the HCV Screening Technologies Based on the Detection of Antigens and Antibodies. *Sensors (Basel).* 2019;19(19):4257. Published 2019 Sep 30. doi:10.3390/s19194257
6. Ashfaq, U.A., Javed, T., Rehman, S. et al. An overview of HCV molecular biology, replication and immune responses. *Virology*, 161(2011). <https://doi.org/10.1186/1743-422X-8-161>
7. Sperle I, Nielsen S, Gassowski M et al. Prevalence of hepatitis C in the adult population of Bulgaria: a pilot study. *BMC Res Notes* 13, 326 (2020). <https://doi.org/10.1186/s13104-020-05158-3>
8. Vladimirova N, Kamenov G, Stoitsova S, Minkova A, Parmakova K, Acute infectious diseases in Bulgaria in 2019 (Main epidemiological indicators). *Epidemiology analysis 2019, NCIPD*
9. Takaki A, Wiese M, Maertens G, Depla E, Seifert U, Liebetrau A, Miller JL, Manns MP, Rehermann B. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med.* 2000 May;6(5):578-82. doi: 10.1038/75063. PMID: 10802716.
10. Santos, Vanessa Costa dos, Schinoni, Maria Isabel, Oliveira, Isabela Silva de, Atta, Maria Luiza Sousa, & Atta, Ajax Mercês. (2019). IgG1 and IgG4 antibodies against Core and NS3 antigens of hepatitis C virus. *Revista da Sociedade Brasileira de Medicina Tropical*, 52, e20180491. Epub March 07, 2019. <https://doi.org/10.1590/0037-8682-0491-2018>
11. Bian Y, Zhao S, Zhu S, Zeng J, Li T, Fu Y et al. (2013) Significance of Monoclonal Antibodies against the Conserved Epitopes within Non-Structural Protein 3 Helicase of Hepatitis C Virus. *PLoS ONE* 8(7): e70214. <https://doi.org/10.1371/journal.pone.0070214>
12. Strasak AM, Kim AY, Lauer GM, et al. Antibody dynamics and spontaneous viral clearance in patients with acute hepatitis C infection in Rio de Janeiro, Brazil. *BMC Infect Dis.* 2011; 11: 15. doi:10.1186/1471-2334-11-15
13. De Marco L, Manzini P, Trevisan M, Gillio-Tos A, Danielle F, Balloco C, et al. (2012) Prevalence and Follow-Up of Occult HCV Infection in an Italian Population Free of Clinically Detectable Infectious Liver Disease. *PLoS ONE* 7(8): e43541. <https://doi.org/10.1371/journal.pone.0043541>
14. Grebely J, Prins M, Hellard M, et al. Hepatitis C virus clearance, reinfection, and persistence, with insights from studies of injecting drug users: towards a vaccine. *Lancet Infect Dis.* 2012;12(5):408-414. doi:10.1016/S1473-3099(12)70010-5
15. Rando-Segura A, Buti M, Quer J, López-Martínez R, Rodríguez-

- Frías F. Viral Load Measurements in Individuals with Hepatitis C Virus Infection: on the European Association for the Study of the Liver Recommendations on Treatment of Hepatitis C 2018. *J Clin Microbiol.* 2019;58(1):e01379-19. Published 2019 Dec 23. doi:10.1128/JCM.01379-19
16. Pereira, Felicidade Mota, Zarife, Maria Alice Sant'ana, Reis, Eliana Almeida Gomes, & G. Reis, Mitermayer. (2014). Indeterminate RIBA results were associated with the absence of hepatitis C virus RNA (HCV-RNA) in blood donors. *Revista da Sociedade Brasileira de Medicina Tropical*, 47(1), 12-17. <https://doi.org/10.1590/0037-8682-0222-2013>
17. Makuria AT, Raghuraman S, Burbelo PD, Cantilena CC, Allison RD, Gibble J, Rehermann B, Alter HJ. The clinical relevance of persistent recombinant immunoblot assay-indeterminate reactions: insights into the natural history of hepatitis C virus infection and implications for donor counseling. *Transfusion.* 2012 Sep;52(9):1940-8. doi: 10.1111/j.1537-2995.2011.03524.x. Epub 2012 Feb 5. PMID: 22304422; PMCID: PMC3346857.
18. Zhang K, Wang L, Lin G, Li J: Is Anti-Hepatitis C Virus Antibody Level an Appropriate Marker to Preclude the Need for Supplemental Testing? *Intervirology* 2015;58:310-317. doi: 10.1159/000441474
19. Rafik M, Bakr S, Soliman D et al. Characterization of differential antibody production against hepatitis C virus in different HCV infection status. *Virology* 13, 116 (2016). <https://doi.org/10.1186/s12985-016-0572-9>