



## Review

# Discussion on critical points for a tailored therapy to cure hepatitis C virus infection

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Hepatitis C virus (HCV) infects around 71 million people worldwide and in 2018 it is still a major health problem. Since 2011, anti-HCV therapy with availability of direct-acting antiviral drugs has revolutionized the clinical response and paved the way to eradication strategies. However, despite the high rate of sustained virological response, treatment failure may occur in a limited percentage of patients, possibly due to resistance-associated substitutions (RASs), either emergent or pre-existent even in minority viral populations. Clearly this problem may impair success of eradication strategies. With this background, several questions marks still exist around HCV treatment, including whether pan-genotypic treatments with complete effectiveness in any clinical conditions really exist outside clinical trials, the actual cost-effectiveness of genotyping testing, and utility of RAS detection in viral *quasispecies* by next generation sequencing approach. In this review, we describe these critical points by discussing recent literature data and our research experience. (**Clin Mol Hepatol 2019;25:30-36**)

**Keywords:** Direct-acting antivirals; Resistance-associated substitutions; Genetic variation; Deep sequencing

## INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus, infecting approximately 71 million people worldwide.<sup>1</sup> The World Health Organization estimated the elimination of HCV infection by 2030, but this assumption is limited mainly by global HCV epidemiology and financial availability of drugs in different countries.<sup>2</sup>

HCV displays a high genetic diversity and is classified now into eight genotypes,<sup>3</sup> at least 67 subtypes,<sup>4</sup> isolates and *quasispecies*.<sup>5</sup> Geographic distributions of HCV types vary according to epidemi-

ological factors. Even if data on HCV epidemiology are limited and restricted to hospital or regional levels, Kartashev et al. reported an update of genotype/subtype distribution based on data from 52 centers, routinely diagnosing HCV infection.<sup>6</sup> Between 2011 and 2015, the most prevalent was HCV genotype 1 (GT1), followed by GT3 and GT4 in West European (including Italy), Russian and Israeli regions.<sup>6-8</sup>

In the last seven years, therapy for HCV improved with the availability of several direct-acting antiviral (DAA) drugs, which allowed to skip the use of PEGylated-interferon (PEG-IFN) and ribavirin (RBV).<sup>9</sup> Indeed, DAA combinations confer good effective-

### Abbreviations:

DAA, direct-acting antiviral; HCV, hepatitis C virus; LiPA, line probe assay; NGS, next generation sequencing; PEG-IFN, PEGylated-interferon; RAP, substitutions at RAS position; RAS, resistance-associated substitutions; RBV, ribavirin; SOF, sofosbuvir; SVR, sustained virological response; VEL, velpatasvir; VOX, voxilaprevir; 3D, ombitasvir/paritaprevir/ritonavir plus dasabuvir

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Received : Jul. 18, 2018 / Revised : Nov. 22, 2018 / Accepted : Dec. 6, 2018

ness and safety for both treatment-naïve and previously treated patients in more than 95% of patients achieving sustained virological response (SVR).<sup>10</sup> Despite the high rate of SVR with DAAs, non-response to IFN-free regimens (5%) may be due to resistance-associated substitutions (RASs) specific for each genotype/subtype.<sup>10,11</sup> RAS are amino acid (AA) changes on DAA target regions, either pre-existing or selected by drug pressure and associated with a reduced susceptibility to the administered drugs.<sup>12,13</sup> When detected in more than 15% of the burden of the entire viral population, RASs play an important role for therapy outcome,<sup>10</sup> although the clinically meaningful cut-off is not validated yet. In this review, we focused our attention on the main questions in the field of clinical virology of HCV treatment nowadays (Table 1).

### FIRST QUESTION: “ARE CLINICAL TRIALS FULLY INFORMATIVE ON PAN-GENOTYPIC EFFECTIVENESS OF NEW REGIMENS IN THE REAL CLINICAL SETTING?”

DAAs target nonstructural proteins, including NS3/4A, NS5A and NS5B polymerases.<sup>14</sup> In 2011, the first-wave, first-generation of protease inhibitors boceprevir and telaprevir was approved in combination with PEG-IFN and RBV to treat chronic HCV GT1 infection. In 2013, a second-wave, first-generation of protease inhibitors, such as simeprevir and sofosbuvir, a nucleotide polymerase inhibitor, was approved or use in combinations, with or without PEG-IFN. Starting from 2014, IFN-free combinations were broadly used across European Union.<sup>15</sup> In Asian countries, such as Korea, boceprevir was approved in May 2014, while several DAA IFN-free regimens have been approved up to November 2015 for the treatment of chronic hepatitis C.<sup>13</sup> Some IFN-free regimens may provide broad coverage for all six major genotypes after 12 weeks of treatment,<sup>16</sup> however according to drugs availability on the different countries.<sup>17</sup> Lastly, in 2017, the US Food and Drug Administration approved a pan-genotypic therapy with a high ge-

netic barrier to resistance<sup>18</sup> that is the number of nucleotide changes requested to develop resistance.

However, efficacy of combination therapy appears to be influenced by HCV genotype/subtypes, carrying RAS at specific AA positions for each genomic region.<sup>12</sup> Several studies showed different regional prevalence of natural RASs.<sup>19-21</sup> For instance, AA sequences corresponding to targets of protease inhibitors are different in HCV GT3 compared with HCV GT1b. Indeed, HCV GT3 isolates carry the natural resistance D168Q in NS3 region. Also, the Q80K with high prevalence among HCV GT1a isolates reduced response in infected patients,<sup>19</sup> as well as the C316N variant associated with resistance to NS5B inhibitors was observed as natural polymorphism at different frequencies in isolates from Asia (54%), USA (28%) and Europe (20%).<sup>20</sup> The Y93H related to resistance to all NS5A inhibitors showed lower frequency in HCV GT1b in patients from China (5.08%), Europe (15%) and USA (9.3%).<sup>21</sup> This RAS was observed in half patients at baseline before asunaprevir plus daclatasvir treatment failure<sup>22</sup> and it was most important predictive factor of SVR in Asian countries.<sup>23</sup> Additionally, genetic variability of HCV types impacts on RAS selection.<sup>24</sup> Among IFN-free therapy, 3D±R regimen (ombitasvir/paritaprevir/ritonavir plus dasabuvir with or without RBV), showed different resistance profiles depending on subtypes. In fact, on one side, D168V in NS3, and M28T+Q30R in NS5A, and S556G in the NS5B region were detected on HCV GT1a strains at the time of failure.<sup>25</sup> On the other side, an HCV GT1b positive patient, naïve to all HCV treatment, carried the acquired NS5A Y93H and NS5B S556G RASs in two out of three regions at the time of 3D-R failure.<sup>26</sup>

Although new pan-genotypic combinations have been approved, solid, real-world data with these combinations in the diverse clinical scenarios and for all genotypes deserve to be collected. Also, in Table 2 we report some of clinical trials and SVR percentages according to genotypes/subtypes of HCV, showing that even in clinical trials small differences among genotypes exist. Such small differences could easily translate into major differences in absolute terms of patients who will fail when these regi-

**Table 1.** Major question marks

Question number	Question text
First	Are clinical trials fully informative on pan-genotypic effectiveness upon all HCV genotypes/subtypes in the real clinical setting?
Second	Is resistance testing still needed notwithstanding the high genetic barrier of current anti HCV drugs?
Third	Is next generation sequencing able to detect resistance-associated substitutions in minor viral populations useful for clinical practice?

HCV, hepatitis C virus.

mens are used extensively in clinical practice (Table 2).

Sofosbuvir/velpatasvir/voxilaprevir (SOF/VEL/VOX), a fixed-dose combination of NS5B, NS5A and NS3 protease inhibitors, was approved for treatment of HCV GT1-6 infections. Particularly, this regimen is indicated for NS5A inhibitor-naïve patients infected by HCV GT1a and HCV GT3. However, it is not recommended for patients with moderate-to-severe cirrhosis, because safety and efficacy have not been evaluated in patients with decompensated cirrhosis.<sup>27</sup> Glecaprevir (protease inhibitor) plus pibrentasvir (NS5A inhibitor) regimen showed a high barrier against resistance in NS3 and NS5A target regions,<sup>28</sup> and it is also suggested as a valid therapeutic option for patients with comorbidities, such as advanced stage chronic kidney disease.<sup>29</sup> However, a recent HCV GT6a *in vitro* model showed how virus “escapes”, with emergence of RASs for VEL (L31V in NS5A), and for SOF (S282T in NS5B), facilitating selection of L28S RAS in NS5A under pibrentasvir therapy.<sup>30</sup> So, HCV GT6a and HCV GT3a may have a lower genetic barrier against emergence of resistance to this drug when compared with HCV GT1a.<sup>30</sup> Finally, SOF/VEL with or without VOX are associated with high efficacy and improvement in patient-reported outcomes scores according to POLARIS-2 and POLARIS-3 clinical trials.<sup>31</sup>

DAA classes are characterized by different degree of genetic barrier against the emergence of viral resistance to drugs, which is related to viral characteristics, such as target region and genotypes/subtypes.<sup>24</sup> In fact, as discussed above, some HCV types are more prone to lower susceptibility to IFN-free regimens than others. In 2016, Polilli et al. reported a decrease in cost-effectiveness of DAA treatment due to decreased efficacy because drugs were chosen based on inaccurate genotyping by line probe assay (LiPA)

assay.<sup>32</sup> Cost of DAAs is still a limitation that may reduce cost-effectiveness of DAA treatment compared with PEG-IFN plus RBV.<sup>33</sup> If genotyping still has an impact on treatment choice and effectiveness of this treatment, then its importance for clinical practice is demonstrated. The seven major genotypes are globally distributed according to risk factors and new migration route in different countries. HCV GT1, HCV GT2 and HCV GT3 show a widespread distribution in almost all parts of the world. HCV GT4 is restricted to few countries, such as Middle East, Africa, Saudi Arabia, Egypt and Ethiopia. HCV GT5 has been reported in South Africa, HCV GT6 in the South-East Asia and HCV GT7 in the Central Africa.<sup>34</sup> In 2012 we created the South Italian Network for Rational Guidelines and International Epidemiology (SINERGIE) project to improve care delivery through integration of clinical, epidemiological, virological and biostatistics expertise.<sup>35</sup> In the Calabria Region, HCV GT1b was found to be the most prevalent (49.2%) followed by HCV GT2a/2c (22.4%), by HCV GT3 (7.4%) and HCV GT4 (6.2%). Therefore, the dynamics of HCV genotypes distribution showed overall a decrease of HCV GT1b and an increase of HCV GT4 from 2011 to 2013,<sup>36</sup> and, particularly, in a small town surveyed in 1996 and in 2010 it was shown that HCV GT2 became the most prevalent HCV type.<sup>37</sup> Given the diversity and evolution of genotypes in different contexts, determination of the infecting genotype in a patient cannot be avoided.

## SECOND QUESTION: “ARE RESISTANCE ASSAYS IMPORTANT IN CLINICAL PRACTICE?”

In this scenario, HCV genotype should be detected by NS5B se-

**Table 2.** Summary of best and worst response of hepatitis C virus genotypes/subtypes to the so called “pan-genotypic” regimens from selected clinical trials

Therapy	Treatment response				Treatment duration (weeks)	Treatment status	Clinical trials
	Best		Worst				
	SVR (N/N [%])	Genotype/subtype	SVR (N/N [%])	Genotype/subtype			
SOF/VEL/VOX	146/150 (97)	1	74/78 (95)	3	12	Experienced	POLARIS-1 <sup>27</sup>
SOF/VEL/VOX	54/53 (98)	1	51/54 (94)	2	12	Experienced	POLARIS-4 <sup>27</sup>
SOF/VEL	33/21 (97)	1b	44/52 (85)	2	12	Experienced	POLARIS-4 <sup>27</sup>
SOF/VEL/VOX	91/92 (99)	3	155/169 (92)	1	8	Naïve	POLARIS-2 <sup>31</sup>
SOF/VEL	170/172 (99)	1	86/89 (97)	3	12	Naïve	POLARIS-2 <sup>31</sup>
G/P	142/145 (98)	2	43/46 (93)	4	8	Naïve	SURVERIOR-1 <sup>16</sup>

SVR, sustained virological response; SOF, sofosbuvir; VEL, velpatasvir; VOX, voxilaprevir; G/P, glecaprevir/pibrentasvir; Experienced, previous direct-acting antiviral therapy; Naïve, previously not treated or treated only with PEGylated-interferon plus ribavirin.

quences analysis, which also allows detection of RASs. In fact, reverse hybridization LiPA is not able to determine the correct subtype.<sup>38</sup> Stelzl et al. reported a new real-time polymerase chain reaction assay including genotype and subtype specific primers for amplification of parts of 5'UTR, Core, and NS5B regions. This new assay allows correct determination of HCV GT1 to HCV GT4 and is able to discriminate HCV GT1a from HCV GT1b.<sup>39</sup> Viral genome sequencing is the gold standard for HCV type identification. In the last 5 years, during phylogenetic and molecular characterization of HCV, we compared results from routinely used LiPA assay and NS3/NS5B sequences analysis. Twenty-five isolates detected as HCV GT4a/4d/4d and 54 samples originally typed as HCV GT2a/2c by LiPA assay were truly classified by NS5B phylogenetic analysis.<sup>40,41</sup> In 2016, carrying out next generation sequencing (NGS) approach to detect RASs, we selected eight HCV GT1b positive patients. The HCV isolates were GT1b subtype by LiPA assay, except one classified as mixed infection HCV GT1b/4. In contrast, analyzing majority rule NS3 NGS consensus and NS5B Sanger sequences, all samples were classified as HCV GT1b by phylogenetic analysis. Additionally, analyzing the same isolates we found naturally occurring RASs, such as V36L for boceprevir, telaprevir and simeprevir (1/8), I132V for telaprevir (4/8), V170I for grazoprevir, and C316N for dasabuvir (2/8).<sup>42</sup>

Resistance testing for treatment of HCV is suggested for HCV GT1a at Q80K position on NS3 target region<sup>10</sup> and HCV GT1a/HCV3 at 28, 30, 31, 58 and 93 positions on NS5A target region,<sup>10,14</sup> in which the presence of RASs are clinically relevant for treatment choice and outcome during the first line therapy and retreatment.<sup>43</sup> Indeed, patients whose HCV harbors RASs on NS5A are very difficult to re-treat,<sup>18</sup> because these mutations persist for years after failure for genetic reasons not yet clarified.<sup>12</sup> This kind of situations opens a new possibility of transmission of HCV resistant strains. As already well demonstrated for human immunodeficiency virus, spreading of transmitted drug resistance can compromise treatment response, highlighting the importance to study local epidemics.<sup>44</sup> Obviously, emergent resistance to DAA in the setting of ongoing transmission is a significant obstacle against eradication of HCV infection. Last year, Cuypers et al. identified spread and transmission networks of HCV GT1a infection harboring Q80K by phylogenetic studies.<sup>45</sup> These analyses suggested cost-effectiveness of resistance testing in patients eligible for simeprevir therapy.<sup>45</sup>

Finally, RASs should be carefully interpreted in clinical practice from a virological point of view. In the diagnostic routine, such test needs a better access and an easy interpretation. At present,

geno2pheno (HCV) (g2p[HCV]) web-service supports the analysis of sequences of the target regions and can predict possible resistance against DAAs. This tool is easy to use, after the upload of sequence, the rules page allows to list RAS, substitutions at RAS position (RAPs) and fitness-associated substitutions related to drug response supported by scientific papers and clinical trials.<sup>46</sup>

### **THIRD QUESTION: "CAN NGS APPROACH IMPROVE DETECTION OF MINOR VIRAL POPULATION SO AS TO IMPROVE PREDICTION OF CLINICAL RESPONSE?"**

Currently, RASs detected at a lower frequency in minor populations are not believed to impact on SVR, because they usually reduce fitness compared with wild-type viruses.<sup>12,47</sup> However, detection of RASs, RAPs and fitness-associated substitutions on the three eligible target regions are affected by unavailability of a standardized commercial test and analysis based on population sequencing with a cut-off of 15%. It is currently unknown whether detection of mutations to below this cut-off may have an impact in clinical practice and many problems still exist.

First, in a limited number of laboratories in Europe and in other countries the test is hard-covered (*in house* assays), and a lack of a global consensus exists in the primers choice and reaction conditions to amplify eligible target regions.<sup>10</sup> Second, Sanger method is not enough to determine a tailored treatment due to high variability of virus. In fact, HCV is a viral *quasispecies*,<sup>48</sup> so the high replication rate ( $10^{12}$  virions per day) and an error-prone NS5B RNA-dependent RNA-polymerase contribute to generate mutations in HCV genome<sup>40</sup> and closely related viral variants during infection. In the new era of DAAs, drug pressure could select for minor viral populations carrying RASs plus fitness-associated substitutions which can influence the outcome of treatment.<sup>12</sup> Even if general data suggest that NGS sequencing (cut-off  $\geq 1\%$ ) does not improve resistance-based prediction compared with sequencing depths of  $\geq 15\%$ , several studies are ongoing to compare deep sequencing and direct sequencing and to develop specific NGS analysis workflow. In 2016, we reported a combination of Ion Personal Genome Machine (PGM) Sequencer in conjunction with a freely available software to analyze nucleotide mutations, ranging from 0.05% to 10.9%, in hot spot positions.<sup>42</sup> In the same year, PGM approach ( $< 20\%$ ) was used to assess the frequencies of RAS at baseline in HCV1a and HCV1b in DAA-naïve patients, showing deep sequencing as good method to detect RAS in the

minor viral population in an easier and complete way.<sup>49</sup>

Thirty-five phase I, II, and III studies were analyzed by Zeuzem et al. to determine NS5A RASs effect on outcomes of treatment.<sup>50</sup> A higher prevalence of RASs detection was observed at 1% sensitivity cut-offs, declining in sensitivity at 15%. However, this last cut-off was the most discriminant. Paolucci et al., in 2017, reported a study where deep sequencing with cut-off values of 0.5% and 1% detected 66.7% of RASs at baseline, whereas direct sequencing detected only 33.3%.<sup>51</sup> In recent clinical trials, such as POLARIS-1<sup>27</sup> and MAGELLAN,<sup>28</sup> NGS approach with a sensitivity threshold 1% and 2%, respectively, was used to detect RASs at baseline or at the time of virological failure to prove the impact of resistance during pan-genotypic treatment. In the POLARIS-1 study, five patients had selected RAS on NS3 or NS5A regions at failure using a sensitivity threshold of 1%.<sup>27</sup> The 2% of detection threshold identified V36M and M28G substitutions in NS3 and NS5A, respectively, in the MAGELLAN study.<sup>28</sup>

## CONCLUSION

Even if the latest generation of IFN-free regimens are better tolerated, randomized clinical trials are not fully informative in the clinical practice because patients are selected based on strict criteria and number of patients is still limited with regards to several research questions under study. In particular, it is necessary to increase the real-world data to demonstrate the potential role of RASs and polymorphisms detected below cut-off 15% in treated or experienced patients. If RAS became dominant under drug pressure, HCV infected patients will benefit from minor viral population analysis to optimize treatment regimens with a tailored approach. Since eradication is not a mere dream today, fine tuning of treatment is necessary to make this objective even more realistic.<sup>52</sup>

## Authors' contribution

All authors have contributed to the design and the writing of the review.

## Conflicts of Interest

No competing interests were disclosed.

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