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Resistance-Associated Substitutions/Variants Correlate to Therapeutic Outcomes of Novel Direct-Acting Antivirals in Different HCV Genotype Treated Individuals

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Additional information is available at the end of the chapter

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Abstract

The expanded classification of hepatitis C virus (HCV) genome into various genotypes and numerous subtypes significantly correlates to therapeutic outcomes of interferon-free direct-acting antivirals (DAAs) in HCV treated patients. In particular, genotypes 3 and 4 are still harder to treat, and higher sustained virologic response (SVR) rates are not achieved in some difficult-to-treat specific populations (i.e., HCV subtype 1a patients, compensated and decompensated cirrhotic patients, HCV/HIV co-infection, and prior treatment failure with pegylated interferon plus ribavirin and first-generation protease inhibitor based therapeutic regimens). Furthermore, the pre-existing and treatment-emergent resistance associated substitutions (RAS) at specific amino acid positions within the viral quasispecies may increase the chances of viral breakthrough (HCV RNA remains lower limit of quantification, but increased to 100 IU/mL or 1.log₁₀ during DAAs therapy), viral relapse (undetectable viral load at the end of treatment but positive within the follow-up of 6 months), and discontinuation of therapy in treated individuals. Although the clinical importance of RAS is not entirely elucidated, it is believed that such substitutions decrease the therapeutic efficacy of DAAs in treated individuals. Similarly, the emergence of multiclass hepatitis C virus resistance to interferon-free DAAs failure in real-world experiences demands eagerly tailored second-line anti-hepatitis C therapies. This book chapter comprehensively overviews the clinical correlation of HCV genotypes, viral quasispecies and harboring RAS to treatment outcomes of revolutionary interferon-free DAAs in hepatitis C-treated patients.

Keywords: hepatitis C virus, quasispecies, direct-acting antivirals, NS3/4A serine protease inhibitors, NS5A inhibitors, NS5B inhibitors, antiviral drug resistance, resistance-associated amino acid variants, resistance-associated substitutions, viral breakthrough, viral relapse, virologic failure

1. Introduction

The diverse genetic heterogeneity of hepatitis C virus genome, poor fidelity of virus replication enzyme (an RNA-dependent RNA polymerase enzyme (RdRp) encoded by NS5B protein in hepatitis C viral genome) and rapid HCV genome replication rate classify hepatitis C genome into various genotypes (GT) or clades (seven genotypes) and numerous subtypes (at least 67 subtypes) [1, 2]. Such type of huge genetic diversity, a hallmark of single strand RNA viruses is amazing because of the discovery of the virus by molecular cloning methods and further nucleotide sequences from the plasma of a chimpanzee as compared to the isolation/characterization of other human RNA viruses [3, 4]. Afterwards, complete HCV genome were isolated and sequenced from different HCV isolates from various parts of the world [5]. The polymerase enzyme lacks proofreading mechanism of viral genome which generates closely related but diverse population of viral variants known as viral quasispecies even within the infected individuals (at a rate of approximately 1 mutation/replication cycle) [6, 7]. The propagation of HCV infection is a highly dynamic process due to a few hours of viral half-life, rapid replication rate *in vivo* and an error-prone nature of NS5B encoded viral replication enzyme [8, 9]. The viral progeny is produced by a rate of an estimated 10 trillion copies per day which exist as quasispecies of numerous closely related viral variants within a single patient [10]. Although, HCV based acquired immunity is developed after primary hepatitis C infection by constant mutation; however; HCV intends to escape such natural/acquired host immune barriers of viral detection/elimination and propagates/maintain persistent infection [10].

2. Hepatitis C virus genome heterogeneity

Hepatitis C viral genome varies 30–50% at genotype level and 15–30% among different subtypes [11]. However, this variation also exists within a specific genotype at nucleotide sequence level where a difference of 1–5% is reported in a single infected patient [12]. These nucleotide variants may be a possible cause of the origination of pre-existing or treatment emergent resistance-associated variants or substitutions (RAV or RAS) in treated subjects. The sequence variability is uniformly and equally distributed throughout the viral genome; however, not reported in highly conserved genome region (e.g., 5'UTR, 3'UTR, and core region) and some hyper variable (HVR) region in E2 protein [13]. HVR1 in E2 protein is also demonstrated a predisposing factor for persistent viral infection [14]. Geographical distribution of hepatitis C genotypes also varies where genotype 1 (subtype 1a/1b) is frequently prevailed in the United States and Western Europe, followed by genotype 2 and 3 infection [15]. However,

the other genotypes are found in distinct regions, where genotype 3 is the most common in South Asia, genotype 4 in Central Africa (almost endemic in Egypt), genotype 5 in South Africa and genotype 6 in Southeast Asia [15].

2.1. HCV genotype testing

HCV genotype testing is very important to predict the overall treatment duration as well as the outcome of direct-acting antivirals in treated individuals. For this reason, it is performed at baseline to identify patients to initiate therapy and select appropriate regimens. In principle, the nucleotide variations to certain targeted genes (e.g., core, E1, NS5A, and NS5B) of viral genome as well as untranslated regions (e.g., 5' UTR) are performed by sequencing reaction [12, 45]. An ideal approach to perform HCV genotyping includes polymerase chain reaction (PCR) amplification of targeted gene and sequencing, or PCR amplification and hybridization with genotype-specific probes, or real-time reverse transcription PCR (RT-PCR) approach. No food and drug administration (FDA) approved methods exist to determine HCV genotypes and various institutes and laboratories have developed their own specific protocols.

Some reference methods demonstrate the amplification and direct sequencing of NS5B or 5'UTR regions, their alignments and phylogenetic analysis. However, the methods are time consuming, expensive and require equipment/software usually used in research laboratories. Similarly, those are used to epidemiological studies where exact genotype is needed. HCV genotype testing by such methods is advantageous because it reveals genomic variability, and the presence of quasiespecies during the natural progression of the disease and overall response to antiviral therapy. Commercially available kits are used to perform HCV genotyping in clinical practice which employ PCR amplification and hybridization with genotype-specific probes.

Currently and most widely used methods include reverse-hybridization line probe INNO-LiPA HCV II assay® (Innogenetics, Ghent, Belgium), simplified direct sequencing Trugene 5'NC HCV Genotyping assays® (Siemens AG), and the Abbott Real-Time HCV Genotype II Assay® (Abbott Laboratories). These assays are generally very reliable with high degree of concordance and the margin of incorrect typing is rare (i.e., <3%). However, the mixed genotypes are detected but uncommon and 5% specimens cannot be genotyped due to low viral load, PCR amplification threshold and very high genome sequence variations.

In principle, INNO-LiPA HCV II is a reverse hybridization line probe assay which uses specific oligonucleotide probes to capture 5' UTR of hepatitis C genome. The current version of the assay (i.e., INNOLiPA version 2.0) is a next-generation line probe assay which detects 5'UTR and core region of viral genome while INNO-LiPA HCV version 2.0 Siemens AG® identifies HCV GT1 subtypes (1a, 1b, 1c etc.,) in clinical and commercial studies. The Trugene 5'C HCV Genotyping Kit (Siemens AG®) analyzes 5' UTR and compare with the genomic libraries of HCV genotypes. The Abbott Genotype II Assay (Abbott Laboratories®) is based on Real-Time PCR method which quantifies viral mRNA and identify hepatitis C GT 1 (subtype 1a, 1b) GT 2 (subtype 2a, 2b) 3, 4, 5, and 6 [45].

2.2. Therapeutic outcomes of DAAs against various HCV genotypes

Since 2010, the treatment strategies for HCV infection have been revolutionized after the advent of interferon-free direct-acting antivirals (**Table 1** enlists the FDA approved and recommended interferon-free direct-acting antivirals for hepatitis C infected patients. The table also concisely demonstrates the patient category, recommended dose and treatment duration with special recommendations) [2, 16, 40]. Such therapeutic regimens achieve higher sustained virologic response rates in treated individuals along with favor tolerability, fewer side effects

Treatment regimens	Dose (mg/day)	Treatment duration (weeks)	Treatment recommendations
Daclatasvir [DCV] (Daklinza®)	60	12 8 (when HCV RNA level is <6 million IU/mL. 24 (for compensated or decompensated cirrhosis with or without RBV, liver transplant, HCV/HIV co-infection and no baseline NS5A mutations) 8 (for acute hepatitis C patients)	<ul style="list-style-type: none"> a. GT1 (Subtype 1a & 1b), GT2, GT3, GT4, and GT5/6 treatment naïve, treatment experienced, without or with compensated or decompensated cirrhosis patients. b. PEG-IFN/RBV, PEG-IFN/RBV plus SOF and DAAs experienced patients. c. NS3 PIs inhibitor + PEG-IFN/RBV experienced patients. d. GT1, GT2, GT3, GT4, GT5, and GT6 treatment naïve/experienced kidney or liver transplant recipients with or without compensated cirrhosis. e. Acute hepatitis C patients.
Sofosbuvir [SOF] (Sovaldi®)	400	12 24 (without RBV for compensated cirrhosis, liver transplant, HCV/HIV co-infection and no basal Q80K mutations) 8 (for acute hepatitis C patients)	<ul style="list-style-type: none"> a. GT1 (Subtype 1a & 1b), GT2, GT3, GT4, and GT5/6 treatment naïve, treatment experienced, without or with compensated cirrhosis patients. b. PEG-IFN/RBV treatment experienced patients. c. NS3 PIs inhibitor + PEG-IFN/RBV experienced patients. d. GT1, GT2, GT3, GT4, GT5, and GT6 treatment naïve/experienced kidney or liver transplant recipients with or without compensated cirrhosis. e. Acute hepatitis C patients.
Simeprevir [SMV] (Olysio®)	150	12 24 (without RBV for compensated cirrhosis or no basal Q80K mutations)	<ul style="list-style-type: none"> a. GT1 (Subtype 1a & 1b), and GT4 treatment naïve, treatment experienced, without or with compensated cirrhosis patients. b. PEG-IFN/RBV treatment experienced patients. c. No basal Q80K mutations.
Ledipasvir/sofosbuvir [LDV/SOF] (Harvoni®)	90/400	12 24 (with or without RBV for compensated or decompensated	<ul style="list-style-type: none"> a. GT1 (Subtype 1a & 1b), GT4, GT5/6 treatment naïve, treatment experienced, without or with

Treatment regimens	Dose (mg/day)	Treatment duration (weeks)	Treatment recommendations
		cirrhosis, liver transplant, HCV/HIV co-infection, and SOF/NS5A-based treatment failed) 8 (for acute hepatitis C patients)	<ul style="list-style-type: none"> b. compensated cirrhosis and decompensated cirrhotic patients. c. SOF or NS5A- based treatment failure. d. PEG-IFN/RBV treatment experienced patients. e. NS3 PIs inhibitor + PEG-IFN/RBV experienced patients. f. GT1, GT4, GT5, and GT6 treatment naïve/experienced kidney or liver transplant recipients with or without compensated cirrhosis. f. Acute hepatitis C patients.
Dasabuvir, ombitasvir, paritaprevir, ritonavir [DSV/OMV/PTV/r] (Viekira Pak®)	500/25/150/100	12 24 (with weight-based RBV for compensated cirrhosis)	<ul style="list-style-type: none"> a. GT1 (Subtype 1a & 1b), and GT4 (without dasabuvir) treatment naïve, treatment experienced, without or with compensated cirrhotic patients. b. PEG-IFN/RBV treatment experienced patients. c. HCV along with chronic kidney disease patients.
Sofosbuvir/velpatasvir [SOF/VEL] (Epclusa®)	400/100	12 24 (without RBV for decompensated cirrhosis, liver transplant, HCV/HIV co-infection, and SOF/NS5A-based treatment failure) 8 (for acute hepatitis C patients)	<ul style="list-style-type: none"> a. GT1 (Subtype 1a & 1b), GT2, GT3, GT4, and GT5/6, treatment naïve, treatment experienced, without or with compensated cirrhosis and decompensated cirrhotic patients. b. SOF or NS5A- based treatment failure. c. PEG-IFN/RBV treatment experienced patients. d. NS3 PIs inhibitor + PEG-IFN/RBV experienced patients. e. GT1, GT2, GT3, GT4, GT5, and GT6 treatment naïve/experienced kidney or liver transplant recipients with or without compensated cirrhosis. f. Acute hepatitis C patients.
Elbasvir/grazoprevir [EBR/GZR] (Zepatier®)	50/100	12 16 (for baseline NS5A RASs for elbasvir)	<ul style="list-style-type: none"> a. GT1 (Subtype 1a & 1b), and GT4 treatment naïve, treatment experienced, without or with compensated cirrhosis patients. b. PEG-IFN/RBV treatment experienced patients. c. HCV along with chronic kidney disease patients. d. No baseline NS5A RAS for elbasvir.
Sofosbuvir/velpatasvir/voxilaprevir [SOF/VEL/VOX] (Vosevi®)	400/100/100	12	<ul style="list-style-type: none"> a. GT1 (Subtype 1a & 1b), GT2, GT3, GT4, and GT5/6 treatment naïve, treatment experienced, without or

Treatment regimens	Dose (mg/day)	Treatment duration (weeks)	Treatment recommendations
Glecaprevir/pibrentasvir [GLE + PIB] (Mavyret®)	300/120	12 8 (without cirrhosis) 16 (NS5A-based treatment failure without prior treatment of NS3 PIs inhibitor)	with compensated cirrhosis patients b. NS5A alone or SOF/NS5A-based treatment failure. a. GT1 (Subtype 1a & 1b), GT2, GT3, GT4, and GT5/6 treatment naïve, treatment experienced, without or with compensated cirrhosis patients. NS5A alone or NS3-based treatment failure but not both.

¹The data shown in the table was derived from phase III clinical trials of IFN-free DAA regimens approved and recommended by the US FDA for the treatment of hepatitis C. RBV = ribavirin, PEG-IFN = pegylated interferon, GT = genotype, RAS = resistance-associated substitutions, and PIs = protease inhibitors.

Table 1. Recommended therapeutic regimens for hepatitis C virus infection¹ [34].

and fewer drug-drug interactions [16]. However, there are certain challenges to meet while achieving the global goal of HCV eradication soon. In parallel to that high therapy costs, treatment access to poor countries, real-world clinical data, and the emergence of resistance-associated variants are big challenges to coup [2, 16].

Interestingly, the new DAA regimens attain higher SVR rates in all genotypes patients (i.e., genotype 1–6) but still the therapeutic efficacy varies at genotypes level as well as subtypes level and even in harder to treat specific populations (e.g., HCV GT1 subtype 1a, genotype 3 & 4 patients with compensated and decompensated cirrhosis, chronic kidney disease and severe liver-impairment patients and HCV/HIV coinfecting patients) [16]. DAA regimens alone, in combination (e.g., Olysio®, Sovaldi®, Daklinza® with or without ribavirin) or as a fixed-dose combination (Harvoni®, Viekira Pak®, Eplclusa®, Zepatier®, Vosevi®, Mavyret®) achieve higher SVR rates (>95%) in GT1, 2, 5 and 6 treated patients. However, the GT 3 patients exhibited SVR rates ≤90–95% as most of the clinical studies performed for the approval of DAA regimens [16]. Similarly, the viral relapse, virologic breakthrough and treatment discontinuation were prominent in cirrhotic patients.

It was also demonstrated that single or dual DAA regimens could not achieve higher SVR rates in HCV genotype 3 patients and addition of another DAAs (i.e., triple DAA regimens) is highly recommended to achieve higher SVR rates for this genotype. HCV genotype 4 patients with or without cirrhosis also achieved compromised SVR rates (≤85–95%) in clinical studies of approved regimens [16]. Due to this reason, the newly approved regimens are cautiously recommended in compensated or decompensated cirrhotic patients. These mechanisms or phenomena are involved for the variable therapeutic response of all oral DAAs to various HCV genotypes or subtypes are not fully elucidated. However, the remarkable viral genome heterogeneity, high viral load, disease progression and in particular the emergence of viral escape mutants are considered the predisposing factors in this prospect [16–18]. The incoming

sections pragmatically overview the molecular kinetics of the emergence of RAS, their effect on treatment response and possible ways to prevent them.

3. The clinical dynamics of RAS for various HCV genotypes

The antiviral drug resistance is a commonly observed phenomenon in chronically infected HCV patients who are recommended to take telaprevir, boceprevir (first-generation NS3/4A protease inhibitors (PIs)) or simeprevir (second-generation PIs) as therapeutic regimens to treat the infection [19]. This problem may also arise in HCV-infected individuals during or after the treatment completion when administered to telaprevir, boceprevir or simeprevir as monotherapy or in combination with pegylated interferon (PEG-IFN) and ribavirin (RBV) [20]. Interestingly, it is rarely reported in those infected patients who are administered to asunaprevir, paritaprevir, grazoprevir (second-generation PIs) and non-nucleoside polymerase inhibitors (NNIs, e.g., dasabuvir) and nucleotide RNA polymerase inhibitors (e.g., sofosbuvir) [17, 18]. Similarly, the development and approval of next-wave interferon-free DAA regimens (e.g., ledipasvir, daclatasvir, ombitasvir, elbasvir, velpatasvir, voxilaprevir, glecaprevir, and pibrentasvir) for chronic hepatitis C and difficult to treat specific populations have shown promise in clinical trials while achieving higher SVR rates, improved adverse event profile, fewer drug-drug interactions and a strong barrier to antiviral drug resistance [18]. Nevertheless, the viral escape mutants are often emerged against one particular drug in interferon-free DAA combination regimens, although the frequency of emergence is lower (Table 2).

Numerous genetic variants or different HCV isolates (termed as quasispecies) are persistently produced in HCV-infected individuals due to the high mutation rate of the viral genome (10^{-5} – 10^{-4} nucleotide per replication cycle) and poor fidelity of the virus replication enzyme (i.e., RNA-dependent RNA polymerase) during HCV replication [21, 22]. Some variants develop sophisticated mutations which may have the tendency to alter the conformation of the binding sites of NS3/4A serine protease, NS5A, and NS5B inhibitors in their targeted active sites and ultimately decrease their therapeutic efficacy [23, 24]. These pre-existing genome variants have a fitness advantage with specific antivirals and may become the dominant viral quasispecies during or after the treatment completion [23, 24]. HCV quasispecies mostly exhibit an attenuated replication and usually displaced by the wild-type HCV genome after stopping the exposure to direct-acting antivirals [23, 24].

At HCV genotypes level, the genotype 1 is the most studied GT regarding the DAAs resistance profile [18]. Genotype 1 infected patients are more prone to develop RAS during or after the treatment completion or exist with pre-existing RAS before the start of therapy [18]. At subtype levels, subtype 1a demonstrates the least genetic barrier to drug resistance than 1b [18]. Genotype 3 and to somehow genotype 4 are still harder to treat and SVR rates are not achieved very significantly in some specific populations (compensated cirrhotic or decompensated cirrhotic patients, treatment experienced patients with first-generation PIs, HCV/HIV co-infection, liver transplant, renal impairment and dialysis patients) as compared

DAA	RAS ² (alone or in combination)	RAS effect on treatment response ³
Telaprevir [31] (Incivek®)	R155, A156, V36, T54, D168, R155K/T, V36M, V36M + R155K, T54A/S, V36A/L, A156S/T, V36G/I, I132V, R155G/M, A156V/F/N or D168N	Telaprevir was discontinued by the US FDA after the advent and recommendation of new IFN-free DAA regimens for HCV-infected individuals; however, the treatment experienced patients with first-generation protease inhibitors (telaprevir, boceprevir) still express baseline and treatment emergent RAS and are treated with newer IFN free DAA regimens to achieve higher SVR12 rates.
Boceprevir [31] (Victrelis®)	For HCV subtype 1a: V36M, T54S, R155K, V36A, T54A, V55A, V55I, V107I, R155T, A156S, V158I, D168N, I/V 170T, I/V170F For HCV subtype 1b: T54A, T54S, V55A, A156S, I/V170A, V36A, V36M, T54C, T54G, V107I, R155K, A156T, A156V, V158I, I/V170T, M175L	Boceprevir was discontinued by the US FDA after the advent and recommendation of new IFN-free DAA regimens for HCV-infected individuals; however, the treatment experienced patients with first-generation protease inhibitors (telaprevir, boceprevir) still express baseline and treatment emergent RAS and are treated with newer IFN free DAA regimens to achieve higher SVR12 rates.
Daclatasvir [46] (Daklinza®) [ALLY-1] [ALLY-2] [ALLY-3] [ALLY-3+] [COMMAND-4] [HALLMARK-DUAL] [HALLMARK-QUAD] [UNITY-1] [UNITY-2]	Pre-existing or treatment-emergent substitutions: M28T, Q30H/K/R, L31M/V, H54R, H58D/P, Y93C/N, P32, A30K/S, L31i, S62A/L/P/R/T, Y93H, A112T, L159F, E237G, Q355H, S282T + Q355H	HCV genotype 1a patients with RAS M28, Q30, L31 or Y93: a. SVR 12 with NS5A polymorphism 76% (13/17) a.1 without cirrhosis 100% (11/11) a.2 with cirrhosis 33% (2/6) b. SVR 12 without polymorphism 95% (142/149) b.1 without cirrhosis 99% (100/101) b.2 with cirrhosis 88% (42/48) HCV genotype 3 patients with RAS Y93H: a. SVR 12 with NS5A polymorphism 54% (7/13) a.1 without cirrhosis 67% (6/9) a.2 with cirrhosis 25% (1/4) b. SVR 12 without NS5A polymorphism 92% (149/162) b.1 without cirrhosis 98% (125/128) b.2 with cirrhosis 71% (24/34)
Sofosbuvir [47] (Sovaldi®) [BOSON] [FISSION] [FUSION] [NEUTRINO] [PHOTON-1,2] [POSITRON] [VALENCE]	Treatment-emergent substitutions: S282T, L159F, V321A, L320F	The cutoff value was below 1% while detecting treatment-emergent RAS against sofosbuvir in different clinical trials, so no significant change in SVR12 of different treated groups was demonstrated.
Simeprevir [48] (Olysio®) [ATTAIN] [C212] [OPTIMIST-1] [OPTIMIST-2] [PROMISE] [QUEST-1] [QUEST-2] [RESTORE]	Treatment-emergent substitutions: F43, Q80, S122, R155, A156, D168, D168E, D168V, Q80R, R155K, Q80X + D168X, R155X + D168K, Q80K, S122A/G/I/T, S122R, R155Q, D168A, D168F, D168H, D168T, I170T	HCV genotype 1a patients with any RAS F43, Q80, S122, R155, A156, or D168 95% (110/116) D168E 15% (17/116) D168V 10% (12/116) Q80R 4% (5/116) R155K 77% (89/116) Q80X + D168X 4% (5/116) R155X + D168K 13% (15/116) Q80K, S122A/G/I/T, S122R, R155Q, D168A, D168F, <10%

DAA	RAS ² (alone or in combination)	RAS effect on treatment response ³
		D168H, D168T, I170T HCV genotype 1b patients with any RAS F43, Q80, S122, R155, A156, or D168 86% (70/81) D168E 17% (14/81) D168V 60% (49/81) Q80R 12% (10/81) R155K 0% (0) Q80X+ D168X 14% (11/81) R155X+D168K 4% (3/81) Q80K, S122A/G/I/T, S122R, R155Q, D168A, D168F, <10% D168H, D168T, I170T
Ledipasvir/sofosbuvir [49] (Harvoni®) [ION-1] [ION-2] [ION-3] [ION-4]	NS5A RAS: K24R, M28T/V, Q30R/H/K/L, L31M, Y93H/N, Q30R, Y93H/N, L31M, L31V/M/I, H58D/P, Y93H/C NS5B RAS: L159, V321, D61G, A112T, E237G, S473, M289I, S282T, L320V/I, V321I + L31M, Y93H, Q30L	Virologic relapse rate with or without baseline NS5A polymorphism: <ol style="list-style-type: none"> Treatment naive GT1 patients with baseline NS5A polymorphism = 6% at week 8 and 1% at week 12. Treatment naive GT1 patients without baseline NS5A polymorphism = 5% at week 8 and 1% at week 12. Treatment experienced GT1 patients with baseline NS5A polymorphism = 22% at week 12 and 0% at week 24. Decompensated cirrhotic GT1 patients with baseline NS5A polymorphism = 7% at week 12 and 5% without polymorphism. Limited data for GT 2, 3, 4, 5 or 6 patients with baseline NS5A polymorphism.
Dasabuvir, ombitasvir, paritaprevir, ritonavir [50] (Viekira Pak®) [PEARL-II] [PEARL-III] [PEARL-IV] [RUBY-I] [SAPPHIRE-I] [SAPPHIRE-II] [TURQUOISE-I] [TURQUOISE-II] [TURQUOISE-III]	Treatment-emergent substitutions: NS3 RAS: V36A/M/T, F43L, V55I, Y56H, Q80K/L, I132V, R155K, A156G, D168, P334S, S342P, E357K, V406A/I, T449I, P470S NS4A RAS: V23A NS5A RAS: K24R, M28A/T/V, Q30E/K/R, H/Q54Y, H58D/P/R, Y93C/H/N NS5B RAS: G307R, C316Y, M414I/T, E446K/Q, A450V, A553I/T/V, G554S, S556G/R, G558R, D559G/I/N/V, Y561H	<ol style="list-style-type: none"> Virologic failure with NS3 Q80K polymorphism = 38% Virologic failure with ombitasvir associated NS5A polymorphism = 22% Virologic failure with dasabuvir associated NS5B polymorphism = 05%
Sofosbuvir/velpatasvir [51] (Epclusa®) [ASTRAL-1] [ASTRAL-2] [ASTRAL-3] [ASTRAL-4] [ASTRAL-5] [POLARIS-2] [POLARIS-3] [POLARIS-4]	Treatment-emergent substitutions: NS5A RAS: Y93N, K24M/T, L31I/V, Q30R, L31M, H58P NS5B RAS : L314F/I/P	Overall viral relapse rates with pre-existing NS5A RAS in patients without cirrhosis/compensated cirrhosis and decompensated cirrhosis = 15% <ol style="list-style-type: none"> Viral relapse in GT1 patients with compensated cirrhosis = 1%. Viral relapse in GT3 patients with compensated cirrhosis = 33%. No viral relapse in GT2, 4, 5 and 6 compensated cirrhotic patients. Viral relapse in GT1 patients with decompensated cirrhosis = 2%. Viral relapse in GT3 patients with decompensated cirrhosis = 15%.

DAA	RAS ² (alone or in combination)	RAS effect on treatment response ³
Elbasvir/grazoprevir (Zepatier®) [41–43, 52] [C-EDGE CO-STAR] [C-EDGE co-infection] [C-EDGE treatment-experienced] [C-EDGE treatment-naïve] [C-SURFER]	Treatment-emergent substitutions: NS5A RAS: M28A/G/T, Q30H/K/R/Y, L31F/M/V, H58D, Y93H/N/S, L28M, L28S/T, M31I/V, P58D NS3 RAS: V36L/M, Y56F/H, V107I, R155I/K, A156G/T/V, V158A, D168A/G/N/V/Y, A156M/T/V, V170I	6. No viral relapse in GT2, 4, 5 and 6 decompensated cirrhotic patients. SVR12 rates in GT1a patients without baseline NS5A polymorphism; With 12 weeks treatment = 98% With 16 weeks treatment = 100% SVR12 rates in GT1a patients with baseline NS5A polymorphism; With 12 weeks treatment = 70% With 16 weeks treatment = 100% No impact on SVR12 in GT1a patients with NS3 Q80K polymorphism SVR12 rates in GT1b patients with baseline NS5A polymorphism = 94% SVR12 rates in GT1b patients without baseline NS5A polymorphism = 99% No impact on SVR12 in GT1b patients with NS3 Q80K polymorphism SVR12 rates in GT4 patients with baseline NS5A polymorphism = 100% SVR12 rates in GT4 patients without baseline NS5A polymorphism = 95% SVR12 rates in GT4 patients with baseline NS3 polymorphism = 100% SVR12 rates in GT4 patients without baseline NS3 polymorphism = 96%
Sofosbuvir/velpatasvir/voxilaprevir (Vosevi®) [53] [POLARIS-1] [POLARIS-2] [POLARIS-3] [POLARIS-4] [POLARIS-5]	NS5A RAS: Q30T, L31M, Y93H/N, V36A, E92K, A30K NS3 RAS: Q41K, V55A, R155M, M28T NS5B RAS: S282T	Overall SVR12 rates in patients with or without baseline NS3 and NS5A polymorphism = 97% Overall SVR12 rates in patients with NS5B polymorphism = 95%
Glecaprevir/pibrentasvir (Mavyret®) [54] [ENDURANCE-1] [ENDURANCE-2] [ENDURANCE-3] [ENDURANCE-4] [EXPEDITION-1] [EXPEDITION-2] [EXPEDITION-4] [MAGELLAN-1 (Part-2)] [SURVEYOR-II] (Part-3) [SURVEYOR-II] (Part-4)	Treatment-emergent substitutions: NS3 RAS: Y56H/N, Q80K/R, A156G, Q168L/R, A166S NS5A RAS: M28A/G, A30G/K, L31F, P58T, Y93H, L31M, Q30K/R, H58D, P29Q/R	Baseline NS3 and NS5A polymorphism in GT1, 2, 4, 5 and 6 patients had no impact on treatment response Overall SVR rates in G3 patients without cirrhosis but with NS5A A30K polymorphism = 78% Overall SVR rates in GT3 patients with baseline NS5A Y93H polymorphism = 100%

²The data for resistance associated substitutions mentioned against interferon free regimens in table 2 was derived from phase III clinical trials and clinical trials registered to ClinicalTrials.gov.

³The treatment outcomes data for interferon-free regimens in RAS detected, pre-existing or treatment-experienced RAS was retrieved from phase III clinical trials and clinical trials registered to ClinicalTrials.gov.

Table 2. Resistance-associated substitutions associated with interferon-free DAAs regimens and their overall impact on treatment response.

to other viral genotypes (**Table 2**) [16, 17]. Interestingly, the RAS associated with GT 3 and 4 patients are not responsible for the failure to achieve higher SVRs in specific populations as the clinical studies demonstrated [17]. However, the limited number of patients in those clinical trials and possible biasness are some major limitations of these studies, which further demands to extensively elucidate in large patient populations [17].

Many studies demonstrate that such variants/substitutions reduce the chances to achieve higher SVR rates as well as are a potential cause of viral relapse, virologic breakthrough and treatment discontinuation in treated individuals [17, 18]. Although the ratio of viral escape mutants (also known as resistance-associated variant (RAV) and RAS) to emerge is low with the administration of second generation (e.g., simeprevir and sofosbuvir) and next-wave direct-acting antivirals (e.g., daclatasvir, ledipasvir, dasabuvir, ombitasvir, paritaprevir, elbasvir, grazoprevir, velpatasvir, glecaprevir, pibrentasvir, and voxilaprevir) in clinical studies; however, their impact on treatment response is still significant [17, 18]. Similarly, the treatment experienced patients with first-generation DAAs (i.e., telaprevir and boceprevir) having no therapeutic response or with virologic relapse and viral breakthrough exist in real-world clinical settings and when treated with the second and next-wave DAAs still express the pre-existing or treatment emergent RAVs [17, 18]. NS3/4A, NS5A and NS5B baseline polymorphism and pre-existing and treatment-emergent RAS are also big hurdle even the patients are administered with next-wave direct-acting antivirals [17, 18]. The phase III clinical studies explicit the emergence of these RAS with variable SVRs in different genotype treated individuals, although the data are limited (**Table 2** concisely overviewed the baseline and treatment-emergent RAS and their impact on therapeutic outcome of FDA approved anti-hepatitis C regimens in different HCV genotypes) [17, 18].

One good example is the resistance variants of NS5A protein which can pre-exist in the viral quasispecies population (both in treatment-naïve and treatment-experienced patients) as well as emerge during or after treatment completion (i.e., treatment-emergent RAS) [18]. Similarly, the detection of resistance variants with currently available laboratory techniques is difficult as the viral variants usually replicate at low levels; however; the next-generation sequencing (NGS) techniques make it feasible to do at a certain cutoff level [18]. HCV quasispecies can be detected at low levels in approximately 1% patients, which are resistant to protease or non-nucleoside polymerase inhibitors (NNIs) and have never been treated with these specific antivirals before [18]. For this reason, such therapeutic regimens are administered cautiously in patients who are previously resistant (i.e., patients treated with PEG-IFN/RBV and dual therapies based on PEG-IFN/RBV plus first-generation PIs, first-generation NS5A and NS5B inhibitor resistant which could not achieve SVR rates after treatment completion and patients with virologic relapse, virologic breakthrough and treatment discontinuation) and detected with viral escape mutants [17, 18]. First-generation NS5A inhibitors (i.e., daclatasvir and ledipasvir) have low genetic barrier to resistance while the next-wave NS5A-targeting molecules (e.g., elbasvir, grazoprevir) are potent inhibitors with pan-genotypic drug efficacy against HCV genotypes 1 to 6 and various subtypes [17, 18].

4. Viral resistance substitutions against first-generation direct-acting antivirals

The patients who take telaprevir or boceprevir as monotherapy may develop antiviral resistance within a few days during treatment [20, 23]. The minor resistant populations against these drugs exist at baseline in all HCV-infected individuals and are selected rapidly with telaprevir or boceprevir monotherapy [20]. Similarly, notable drug-drug interactions with many human immunodeficiency virus (HIV) antiretrovirals and calcineurin inhibitors also decrease the therapeutic activity of telaprevir and boceprevir monotherapy (due to severe drug adverse events, numerous possible drug-drug interactions and rapid emergence of RAVs, the first-generation PIs have been discontinued by the FDA to treat hepatitis C patients in the US and other parts of the world) [20, 31]. R155 is the most overlapping position in NS3/4A serine protease (a protein involved in HCV translation and also potential drug active site for the design and development of protease inhibitors), where different mutations may produce and confer resistance to nearly all protease inhibitors, (An exception is MK-5172) [25–29]. *In vivo* mutations at four positions (V36A/M/L, T54A, R155K/M/S/T, and A156S/T) and only one *in vitro* (i.e., replicon system) mutation (A156) has detected and characterized against telaprevir [30]. These mutations either alone (V36A/M, T54A, R155K/T, A156S) or as double mutations (A156T/V, V36M + R155K, V36M + 156T) confer low to high resistance barrier against telaprevir by altering the catalytic active sites of NS3/4A serine protease [30]. The pattern of resistance against telaprevir also differs significantly among HCV subtypes. The clinical studies reveal that antiviral resistance occurs much more frequently in HCV genotype 1a infected patients as compared to genotype 1b either using telaprevir alone or in combination with PEG-IFN α plus RBV [31]. It is due to a single nucleotide polymorphism at position R155K in NS3/4A serine protease, where codon AGA encodes R in HCV subtype 1a versus 1b (where codon CGA also encodes R) [30, 31]. In HCV genotype 1a isolates, only a single nucleotide substitution is required to change R to K at position 155 while 2 nucleotide changes require in subtype 1b [50]. Some studies also demonstrate that subtype 1a display higher fitness advantage than genotype 1b isolates, which is a predisposing factor in developing viral escape mutants and viral breakthroughs to other positions within NS3/4A catalytic subunit and other genomic regions of 1a isolates [30, 31].

5. RAS against second-generation direct-acting antivirals

Q80R/K polymorphism is responsible for low-level resistance to a macrocyclic protease inhibitor, simeprevir. The clinical studies predict Q80K variants up to 50% in HCV genotype 1a-infected patients (which is approximately 20% in Europe and 50% in the United States) and almost 1% of 1b isolates [32]. Lower SVR rates and a slow viral decline have reported in HCV genotype 1a patients treated with simeprevir-based triple therapy in phase III clinical studies (20% lower in HCV genotype 1a than 1b) [33]. Q80K polymorphism and NS3 genotype testing prior to therapy is highly recommended for HCV subtype 1a patients to avoid any adverse events, low virologic response and treatment discontinuation during therapy [32, 33].

The viral variants associated with NS3 PIs may detect by first synthesizing cDNA by reverse transcription reaction, followed by performing polymerase chain reaction (PCR) and then sequencing reaction [32, 33]. Q80K polymorphism and viral variants testing against NS3 PIs have been launched in the USA by Quest Diagnostics® and LabCorp® [32, 33].

6. RAS against next-wave interferon-free DAAs

The first-generation NS5A inhibitors (e.g., daclatasvir) lead initially to higher SVR rates in treated patients, but the emergence of viral resistance occurs rapidly indicating its relatively lower genetic barrier to resistance [35]. The viral resistant mutants were found very commonly at amino acid residue Q30E and Y93N of NS5A protein in subtype 1a patients and confer the highest level of drug resistance [18]. Some studies demonstrate that these mutations are responsible for increasing the EC₅₀ (i.e., the concentration of a drug which produces therapeutic response halfway between the baseline and maximum after a certain period of time) of daclatasvir in treated patients [18]. Similarly, L31 and Y93 substitution positions express the greatest aptitude for resistance to daclatasvir, where double mutations sometime increase the EC₅₀ of DCV to a far greater degree. However, viral resistance substitutions were reported less frequently at position L31 and Y93 in HCV subtype 1b patients [18, 44]. From the clinical point of view, these substitutions against DCV are also considered to be responsible for resistance to other NS5A inhibitors as discussed below.

Ledipasvir in combination with sofosbuvir, as a fixed-dose combination was approved for GT1 patients with or without cirrhosis [18]. The fixed-dose combination also demonstrates pan-genotypic clinical efficacy in patients with GT3, 4, 5, and 6 patients. The approval was based on the achievement of SVR rates $\geq 95\%$ in GT 1 treatment-naïve and treatment-experienced patients without cirrhosis. SVR rates were achieved $>78\%$ in decompensated cirrhotic patients awaiting liver transplant while 100% SVR rates were demonstrated for liver transplant recipients with fixed-dose LDV/SOF plus RBV. The addition of RBV did not significantly impact SVR rates in patients without cirrhosis; however, the addition is mandatory for GT1 and 4 decompensated cirrhotic patients as well as liver transplant recipients for 24-weeks. In phase III clinical trials, Q30R, Y93H/N, and L31M were the most commonly detected RAS in subtype 1a treatment failure patients while only one mutation Y93H was detected for 1b. However, the impact of these baseline RAVs was very limited on the overall therapeutic outcome of the regimens. Similarly, LDV shows strong therapeutic activity against SOF- induced mutants (e.g., S282T) as no drug cross-resistance between these two drugs were reported in clinical studies and vice versa. Another advantage of this fixed-dose combination (FDC) is to confer antiviral activity against RAVs associated with other NS5B NNIs and NS3 PIs [18].

Ombitasvir (OMV) another NS5A inhibitor was approved in combination with paritaprevir (PTV), r (ritonavir) and dasabuvir (DSV) for the treatment of difficult to treat GT1 specific populations as achieved higher SVR rates (~100%) in treatment naïve (1a subtype) and treatment-experienced patients (IFN-based 1b subtype) [18]. Similarly, OMV plus PTV/r without DSV were recommended to treat GT4 chronic hepatitis C (CHC) patients as DSV clinically

ineffective against GT4 patients. However, the drug combination is strictly prohibited to administer in patients with decompensated or moderate to severe hepatic impairment. Despite being the multiprotein targeting regimens with the chances to develop mutations, the pooled analysis showed high genetic barrier to drug resistance. Both pre-existing and treatment based RAVs were reported in virologic failure experienced patients; however, interestingly baseline RAVs did not impact the overall efficacy of treatment. OMV monotherapy for 12 weeks in treatment-naïve GT1 patients also generated variants in both subtypes but without any baseline RAVs. The most surviving variants in GT1 subtype 1a patients were reported at amino acids positions M28, Q30, and Y93; however, only one substitution Y93H was noticed in GT1 subtype 1b patients although with 77-fold more drug resistance. Due to this reason, OMV is always recommended in combination with PTV/r/DSV or PTV/r [18].

A fixed-dose combination (FDC) of elbasvir/grazoprevir (Zepatier®) (50 mg/100 mg) one a day has been approved by the United States Food and Drug Administration (US FDA) for the treatment of HCV GT 1 & 4 infected patients with chronic kidney diseases and HCV/HIV-co-infection. However, the treatment is recommended with cautions in some specific populations including viral subtype 1a, prior treatment experienced with NS3 PIs, and NS5A associated RASs at position M28, Q30, L31, or Y93 [18]. Similarly, the treatment regimen is prescribed with many precautions in subtype 1a patient with prior testing of NS5A associated RAVs, because it determines the overall treatment duration and the inclusion of ribavirin to therapy [18]. This regimen achieved higher SVR rates in all patient arms (~97%) in particular previous non-responders to IFN-based therapies as well as in individuals with severe renal impairment (94%). The low therapeutic outcomes were revealed for GT1 subtype 1a patients with substitutions at positions M28T, Q30, L31, or Y93 after 12-weeks drug administration in clinical studies. Another interesting fact also revealed that those mutations against elbasvir also decrease the therapeutic efficacy of other NS5A inhibitors. However, elbasvir was found fully active against the mutations generated by grazoprevir (NS3/4A PIs) while used in combination. Moreover, the mutations existing against SOF-based therapeutic regimens are harmless to elbasvir [18].

RAVs associated with NS5A inhibitors do not impair replication fitness during the treatment as compared to viral resistant mutants of NS3 PIs and consequently do not disappear during follow-up examinations at the end of therapy [18, 35]. Viral resistance mutants against NS5A inhibitors persist even after 1 year follow-up studies in treated individuals but interestingly no cross-resistance has been reported between DCV and other DAAs as yet [18]. For this reason, the prior testing of NS5A variants in such patients before the treatment initiation is essential to determine overall treatment duration and inclusion of RBV in therapy.

7. Clinical significance of viral escape mutants

The clinical importance of RAVs is still not clear, but some studies have revealed that these mutations are commonly shared between first and second-generation direct-acting antivirals and to less extent for next-wave DAAs [36, 37]. Similarly, the clinical relevance of the viral escape mutants is also not completely understood. However, numerous studies demonstrate

that these pre-existing variants may reduce the chances to achieve higher SVR rates with DAA-based triple therapies if the patients are individually less sensitive to PEG-IFN α plus RBV treatment [38, 39]. Due to this overlapping resistance profile, one protease inhibitor cannot be substituted for the other, and even a combination of two protease inhibitors does not make sense to be used in the cases of viral breakthroughs and treatment relapse in infected patients [37]. As a result, if an HCV-infected patient fails to respond one PI, the retreatment with other direct-acting antivirals may seem very difficult [38, 39]. PEG-IFN α and RBV are considered an integral part of telaprevir- or boceprevir-based triple therapies, as some studies suggest that RAVs are not associated with less sensitivity to interferon and ribavirin-based combination therapies [40]. Interestingly, if the patient response is weak toward PEG-IFN α /RBV therapeutic regimen, the risks to develop viral resistant mutants are significantly higher [40]. HCV genome sequencing to determine the sequences of RAVs before or during therapy have no rational because it has no practical consequences. The exception is testing for Q80K variants in HCV genotype 1a patients which are recommended before simeprevir administration in the US prescribing information [32, 33]. It is uncertain that the test is cost effective in other parts of the world where genotype 1a is not highly prevalent, and Q80K polymorphism is rare. In QUEST-1 clinical trials, 41% HCV genotype 1a patients had this particular variant and their SVR rates were not significantly increased as compared to placebo when treated with simeprevir [32, 33]. However, the SVR rates were almost similar to HCV genotype 1b patients without Q80K variants in HCV genotype 1a patients [32, 33]. Interestingly, if Q80K variants detect at baseline, even then the chances to achieve optimal SVR rates will be higher provided that simeprevir is a part of the therapeutic regimen [32, 33]. In this scenario, a combination of next-wave DAAs (i.e., sofosbuvir and daclatasvir) with a very high resistance barrier and weak antiviral (e.g., ribavirin) activity may lead to high SVR rates. However, such drugs cannot be combined with first-generation DAAs (telaprevir or boceprevir) due to lack of clinical data and potential drug-drug interactions via the Pgp transporter proteins [18]. If viral escape mutants emerge during or after therapy in treated patients, for how long will they persist and which type of adverse effects would produce is not clearly understood. Some studies have reported that viral escape mutants revert to wild type within 1–2 years after the completion of treatment with first-generation PIs; however, RAS associated with NS5A inhibitors may persist for long time even after the treatment completion [18, 40]. NS5A baseline polymorphism and NS5A RAS detection by cloning sequencing is strongly recommended before the start of treatment in patients with persistent NS5A variants.

8. Prevention to viral escape mutants

The emergence of viral escape mutants against direct-acting antivirals has an adverse impact on treatment failure, when retreated with the same or other DAA-based combination therapies (Table 2) [18]. Phase, III follow-up studies of teleprevir and boceprevir-based triple therapies, revealed this fact where a rapid decline of viral escape mutants was detected (below the limit of detection, i.e., >20% of quasispecies) by using population sequencing techniques [31, 40]. However, these resistance mutants were detectable after several years in a single patient

treated with telaprevir or boceprevir by using cloning sequencing techniques within smaller phase 1b studies [31]. Similarly, one study related to the retreatment of 5 HCV-infected patients with simeprevir-based triple therapy (who developed early simeprevir resistance during monotherapy and demonstrated SVR rates in only 3 out of 5 patients), also indicated a possible effect of low-level persistence of viral escape mutants [31, 32].

Adherence to the dose of medication (especially for PIs) and compliance with fertility rules are two significant ways which may adopt during therapy to avoid viral escape mutants [17, 18]. Similarly, it may be managed by alternative treatment strategies and by improving the pharmacokinetics profile of the newly developed direct-acting antivirals. Currently, the approvals and recommendations of next-wave all oral interferon-free regimens have shifted the treatment paradigms for difficult to treat “specific” populations including the patients found resistant to first- and second-generation PIs and first-generation NS5A and NS5B inhibitors (**Table 1**) [34, 41]. Interferon free combination regimens where, one drug with higher therapeutic activity but lower genetic barrier to drug resistance and other with strong barrier to drug resistance but with lower therapeutic activity may reduce the chances of viral relapse and viral breakthroughs in treated individuals [17, 18, 34]. Furthermore, some non-nucleoside analog inhibitors with low antiviral efficacy but the high barrier to drug resistance are also in investigational trials to be a valuable part of oral interferon-free regimens to treat patients who are previously resistant to first- and second-generation DDA-based triple therapies [17, 18].

The clinical data improvising the failure of IFN free DAAs in treated individuals is still limited from the phase III clinical trials of the regimens and retreatment statistics are not sufficient to accomplish standard recommendations [17]. However, some currently available retreatment data for treatment-failure regimens is briefly mentioned here. For NIs-based (e.g., sofosbuvir) retreatment patient, 24 weeks treatment with addition of RBV is recommended, unless contraindicated. Sofosbuvir based triple or quadruple therapeutic regimens for 12 or 24 weeks along with RBV are also considerable if applicable. Similarly, for treatment failure of SOF and SMV, preferable retreatment options include a combination of LDV/SOF or SOF/DCV for 24 weeks in cirrhotic patients and along with RBV for 12 weeks. For SOF plus RBV failure, the retreatment strategies include SOF-based triple regimens including PEG-IFN and RBV for 12 weeks or alone RBV for 24 weeks. For SOF/LDV failure with NS5B variants, retreatment with PEG-IFN/RBV plus SOF was recommended for 12 weeks. Some retreatment strategies have been reported from real-world clinical practice studies, where the treatment failure of DCV-based regimens was retreated with SOF plus SMV and with or without RBV for 12 weeks. Despite achieving higher SVR rates, the retreatment strategies are still deficient in scientific evidences to support their recommendations [17].

9. Conclusions

The pre-existing or treatment-emergent resistance-associated variants in hepatitis C-treated patients decrease the overall cure rates (i.e., higher SVR rates) of direct-acting antivirals and other anti-hepatitis C regimens. These variants may cause viral relapse, viral breakthrough

and treatment failure during or after the completion of therapy. The clinical impact of resistance-associated variants/substitutions is significant on the overall treatment outcome as the clinical studies predict variable SVR rates in different HCV genotype patients. The detection of resistance-associated variants is of utmost importance prior to initiation of therapy, to decide treatment duration as well as to choose retreatment or alternate treatment plan for previously treatment failure patients with first- and second-generation DAAs or to some extent new-wave DAAs. The discovery and development of interferon free combination regimens with pan-genotypic drug efficacy provide optimism to treat such difficult-to-treat populations where one drug with high antiviral efficacy and other one with strong barrier to drug resistance achieves highly significant sustained virologic response rates in treated individuals.

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Conflict of interest

None.

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