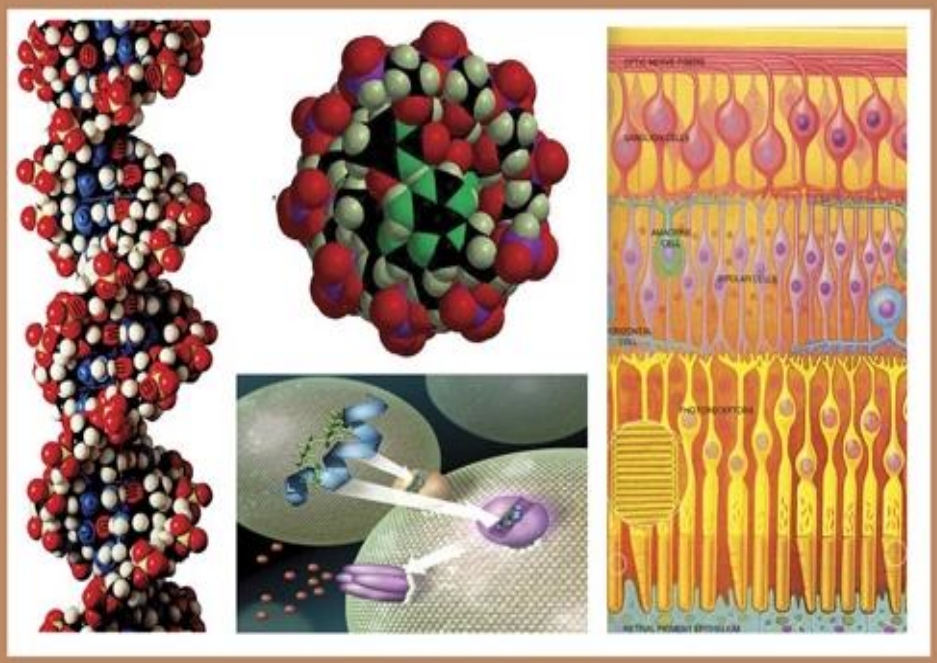
EGYPTIAN ACADEMIC JOURNAL OF

BIOLOGICAL SCIENCES

C

PHYSIOLOGY & MOLECULARBIOLOGY



ISSN
2090-0767

WWW.EAJBS.EG.NET

Vol. 13 No. 1 (2021)

Citation: *Egypt.Acad.J.Biolog.Sci. (C.Physiology and Molecular biology) Vol. 13(1) pp93-107 (2021)*
DOI: 10.21608/EAJBSC.2021.164320

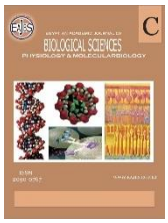
Egypt. Acad. J. Biolog. Sci., 13(1):93-107 (2021)

Egyptian Academic Journal of Biological Sciences

C. Physiology & Molecular Biology

ISSN 2090-0767

www.eajbsc.journals.ekb.eg



Risk Assessment of Hepatitis C Virus Resistance to Ns3/4a Protease Inhibitors (Pis)

Shaia Saleh R. Almalki

Department of Laboratory Medicine,Faculty of Applied Medical Sciences,Albaha University,Albaha- Saudi Arabia

*E. Mail: almalkishaia@hotmail.com

ARTICLE INFO

Article History

Received:2/2/2021

Accepted:12/4/2021

Keywords:

Protease inhibitors, HCV, resistance, substitution, NS3(Non-structural).

ABSTRACT

Hepatitis C virus (HCV) persistently infects more than 175-185 million people globally. Drug résistance to anti-HCV DAAs therapeutics particularly NS3/4A has been a major concern for efficacy of HCV treatment due to generation of HCV quasispecies. Risk determination of the resistance of HCV to anti-NS3/4A inhibitors was accomplished using ViPR algorithm that computes and analyzes resistance-associated amino acid substitutions in HCV NS3 protein target site and reveal altered response to NS3/4A PIs. Spot comparison and data visualization was carried out using ncbi.nlm.nih.gov protein sequence graphics and R-package for Ubuntu version 12.04. Resistance to multiple NS3/4A PIs (telaprevir, simeprevir, faldaprevir and asunaprevir) was exhibited by HCV genotype 1 variant D1194A/E/T/G-NS3 (a substitution hot spot) while the risk of resistance to telaprevir (V1062 L NS3 variant) and simeprevir (S1148R variant) was demonstrated by HCV genotype 2. Genotype 3 & 4 revealed resistance against only telaprevir. Another almost pan-genotype (genotype 2,3,4,5 and unclassified) substitution hot spot was identified as anti-telaprevir variant V1062 L. Single substitution conferring multiple anti-NS3/4A PIs resistance was observed in D1194A NS3 variant (anti-simeprevir, anti-faldaprevir, anti-asunaprevir in genotype 1), Q1106K NS3 variant (anti-simeprevir, anti-faldaprevir in genotype 5 and 6) and T1080S NS3 variant (anti-faldaprevir, anti-telaprevir in genotype 5 and unclassified NS3 sequence). In addition to that, variant S1148G exhibited increased sensitivity to simeprevir in case of genotype 1. Because of a higher degree of HCV genomic variability, NS3 variants with decreased susceptibility to NS3/4A PIs exists, and therefore the resistance profile along with potency, adverse effect of PIs is noteworthy and should be well-considered while developing different protease inhibitors for effective treatment of HCV patients.

INTRODUCTION

Hepatitis C virus (HCV) is a hepatotropic bloodborne pathogen that persistently infects more than 175-185 million people corresponding to a 3-5% overall prevalence of HCV chronic infection globally **(**Petruzziello *et al.,* 2016; Romano *et al.,* 2010). Based on complete genomic analysis HCV has been categorized into seven genotypes and various sub-genotypes (Khodabandehloo and Roshani, 2014) which are causative agents of serious chronic end-stage hepatic diseases such as progressive liver fibrosis leading to cirrhosis and hepatocellular carcinoma that may eventuates in liver failure (Perz *et al.,* 2006; Thorgeirssion *et al.,* 2002; Bruix, *et al.,* 2004, Hu *et al.,* 2005).

Chronicity of infection is well-established with the help of HCV genomic variability in conjunction with numerous potential immune evasion mechanisms of the virus. The currently available standard therapeutic option for the clinical management of HCV chronic infection is a combination therapy of weekly injection of pegylated interferon-α2 (pegIFN-α2) and daily oral ribavirin doses (Manns *et al.,* 2001; Strader *et al.,* 2004). On the basis of sustained viral response (SVR) this combination therapy remains ineffective in about half of the cases of treated HCV infected patients and even less than 50% in treated cases of HCV genotype-1 infected patients in developed countries (Manns, 2001, Fried *et al.,* 2002; Hadziyannis *et al.,* 2004). Apart from its poor SVR this standard therapeutic approach is allied with significant long-term adverse effects of both the elements of the combination encompassing depression, fatigue and hemolytic anemia (Ascione *et al.,* 2002; Schaefer *et al.,* 2003; Toniutto *et al.,* 2005; Lebray *et al.,* 2005). Both components of this therapeutic combination are categorized as indirect antiviral agents as they don't specifically target any HCV protein or genomic (RNA) element. Therefore, unmet clinical requirements for medicine to treat HCV infection with fewer adverse effects led to the discovery of various direct-acting antiviral drugs (DAAs) (Asselah *et al.,* 2016). DAAs are three new classes of drugs that specifically target the virus itself to reduce the length of treatment, adverse effects and to enhance the SVR. The HCV NS3/4A multifunctional protein has a membrane-targeted serine protease activity at the amino-terminal while helicase activity at the carboxy-terminal domain (Courcambeck *et al.,* 2004). NS3/4A serine protease one of the main therapeutic targets for currently available antiviral drugs (Keating *et al.,* 2015). NS3/4A is to perform proteolytic cleavage at 4 non-structural sites (NS) in order to produce NS3, NS4A, NS5A and NS5B proteins (Shiryaev *et al.,* 2012). NS3/4A plays a significant role in HCV replication by performing dsRNA unwinding and evasion of immune attack by hampering the interferon regulatory factor 3 (IFR3) activation process (Foy *et al.,* 2003; Gale *et al.,* 2005). NS3/4A protease inhibitors (PIs) belong to a class of DAAs that interferes with the HCV replication, maturation and immune evasion to combat HCV infections (Lamb *et al.,* 2017; Morsica *et al.,* 2017; Gale, 2005). Although with the advent of DAAs and pan-genotypic antiviral activities of NS3/4A PIs, therapeutic option for clinical management of HCV-infected patients has enormously improved, however, the effectiveness of anti-HCV drugs are facing an emerging resistance associated with substitutions (RAASs) in target protein molecules (Ng *et al.,* 2018; Pawlotsky *et al.,* 2016; Sulkowski *et al.,* 2015; Zeuzem *et al.,* 2014). Emergence of actual antiviral drug resistance during longstanding treatment of the infected patients with particular drug(s) is noteworthy. That is the reason why the phenomenon of resistance is highly emphasized while developing a therapeutic candidate (Dalmau *et al.,* 2005). Emergence of resistant strains of the virus plays a role in confining the longstanding efficacy of antiviral drugs and the main factors behind resistance in viruses are specific to their enzymes such as reverse transcriptase and RNA-dependent RNA polymerase for RNA virus etc (Daar *et al.,* 2005, De Francesco *et al.,* 2005). The role of mutations in HCV protease has recently surfaced as a key factor in emergence of drug-resistant viral strains. RAASs usually cause the weakening of inhibitor binding that leads to decreased activity of inhibitors against a target enzyme. Although HCV NS3-4A

protease inhibitors (PIs) are very effective antiviral drug molecules that prompts the reduction in HCV viral load during treatment of infected patients, however, their susceptibility to RAASs at protease active site must be remarkably taken into consideration (Lawitz *et al.*, 2016, Lawitz *et al.*, 2018). Revealing molecular mechanisms of antiviral drug resistance or sensitivity is considered highly crucial for drug designing, development and appropriate production of effective antiviral drugs for future applications, and therefore the present study was aimed to study the substitutions in amino acids (mutation) in NS3/4A serine protease of HCV that are responsible for conferring antiviral drug resistance against existing NS3/4A protease inhibitors (PIs).

MATERIALS AND METHODS

Determination of anti-NS3/4A inhibitors resistance risk assessment was executed on multiple datasets generated after retrieving NS3 protein sequences of HCV at the end of 2019 from one of the Bioinformatics Resource Centers (BRC) known as ViPR (https:// www. viprbrc.org).

Retrieval of NS3 Sequences And Datasets Creation:

NS3 protein sequences of all the genotypes of HCV (genotype 1-7) were retrieved using the Sequence Retrieval System(SRS) of ViPR. NS3 protein sequences of unclassified strains of HCV as per ViPR genotype classification SOP (https://www.viprbrc.org/SOP_HCV_typing.pdf) were also included in the study. Sample attributes (Ascitic fluid, blood, cell supernatant, plasma and serum), host attributes (male and female), infection type (chronic and experimental), virus attributes (all the sub-genotype of HCV) and time attribute (year 2011 to 2019) were taken into consideration while using SRS. A sum total of n=3839 NS3 protein sequences of HCV genotypes was retrieved, purified and eight different sequence datasets were created to run the anti-NS3/4A inhibitors resistance risk assessment analysis (Figs. 1b & 1c).

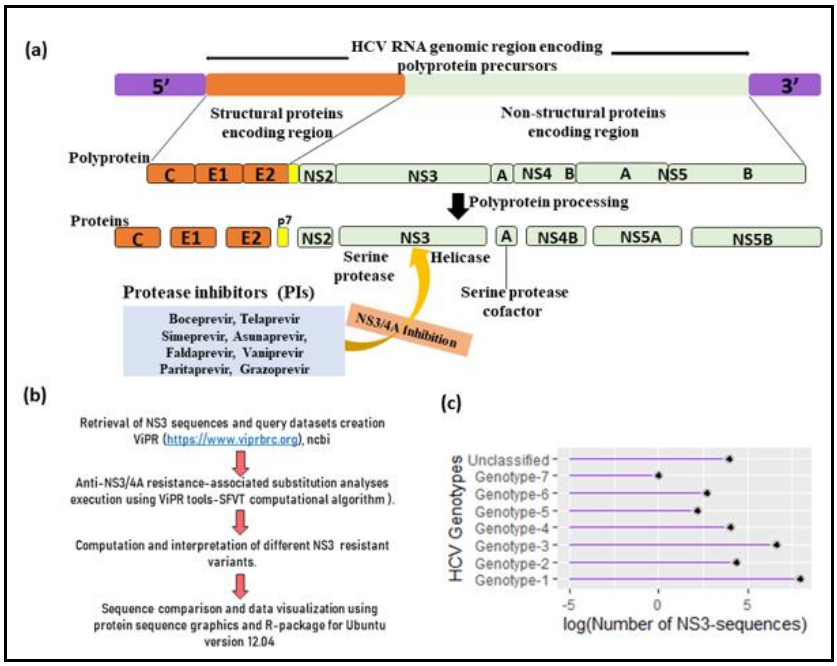


Fig. 1. FDA approved anti-NS3/4A PIs, their target site and RAAS analyses tool. a-NS3/4A inhibition target site for NS3/4A PIs, b-methods and RAASs analysis tools and c-logarithmic value of NS3- protein sequences of all the HCV genotypes and unclassified.

Determination of Anti-NS3/4A Inhibitors Resistance Risk Assessment:

NS3/4A protein inhibitors (PIs) one of the three main categories of DAAs that encompass boceprevir, telaprevir, simeprevir, asunaprevir, grazoprevir and paritaprevir (Figure 1a). These PIs are boosted by another PI ritonavir. First generation NS3/4A PIs (boceprevir and telaprevir) in combination with PegIFN- alpha and ribavirin was approved by FDA in 2011 for treating of HCV-1 chronic infection. Because of the various drug-drug interactions, these combinations were replaced with different PIs as well as other DAAs and approved by FDA from 2013 to 2016 (Ghany *et al.,* 2011; Geddawy *et al.,* 2017). Since these FDA-approved NS3/4A PIs have been in use since 2011 for treating infections caused by different genotypes of HCV, therefore in the present study determination of altered response to these PIs due to changes at amino acid level over time has been accomplished using ViPR algorithm on all the datasets of retrieved NS3 sequences. Anti- NS3/4A protein inhibitors (PIs) resistance risk assessment was determined using ViPR antiviral resistance risk assessment tool that leverages sequence feature variant type (SFVT) computational algorithm to evaluate the Anti-NS3/4A protein inhibitors (PIs) resistance-associated substitution in query sequence datasets (Fig. 1b). This analysis tool reveals mainly two resistance risk categories such as increased resistance to anti-viral drug (when query sequence has a particular substitution-allied with enhanced drug resistance) and increased sensitivity to antiviral drugs (when query sequence has a particular substitution-allied with enhanced drug sensitivity). Mutations with no effect on sensitivity and/or resistance to anti-viral agents were excluded from result of the present study. Spot comparison and data visualization was carried out using ncbi.nlm.nih.gov protein sequence graphics and R-package for Ubuntu version 12.04.

RESULTS AND DISCUSSION

The risk determination of anti-NS3/4A protein inhibitor/s resistance was accomplished for all the HCV genotypes and NS3 unclassified sequences in terms of resistance-associated amino acid substitution (RAAS) at the level of single and multiple anti-NS3/4A protein inhibitor/s resistance-associated amino acid substitutions. Total number of fifteen (n=15) resistance-associated NS3 substitutions among seven HCV genotypes as well as in unclassified HCV NS3 sequences have been identified (Fig. 2). The RAAS frequency in the case of HCV genotype 1 was found to be n=23 (14 single anti-NS3/4A protein inhibitor resistance-associated amino acid substitution and 9 multi anti-NS3/4A protein inhibitors resistance-associated amino acid substitution). Single anti-NS3/4A resistance-associated amino acid substitutions were in response to four different NS3/4A protease inhibitors(PIs) namely boceprevir, telaprevir, asunaprevir and simeprevir (Table 1). Primary NS3 protease substitutions across HCV genotype 1variants conferring anti-boceprevir resistance was recognized to be V1196A while that anti-telaprevir was identified to be I1158V and V1062A/L. The main NS3 protease substitutions conferring anti-simeprevir and anti-asunaprevir were noticed to be D1194E/T and D1194G respectively while variant S1148G conferred sensitivity to simeprevir (Table 1,and Fig. 2). The most frequently identified NS3-RAASs in case of HCV genotype 1 were observed against simeprevir (%f =50) followed by telaprevir (%f =21.4). The frequency of NS3-RAASs against boceprevir (%f =14.3) and asunaprevir

(%f =14.3) was equal. Various NS3-RAASs conferring resistance against multiple (2-3) anti-NS3/4A PIs have also been spotted in genotype 1 (Table 2). Noteworthy NS3 protease substitutions imparting resistance against faldaprevir + asunaprevir, faldaprevir + telaprevir and Faldaprevir + Simeprevir were recognized as R1181K, T1080S and, Q1106K/R and A1182V respectively (Fig. 4 & Table 2). In this case single position substitution has been noticed to play a significant role in developing resistance against two types of NS3/4A PIs simultaneously. Apart from single position substitution conferring resistance attributes to genotype 1 against single or double NS3/4A PIs, single position substitution imparting resistance to multiple PIs was determined too. D1194A NS3 substitution was assessed to be a key factor in emergence of resistance against 3 types of NS3/4A PIs (simeprevir, faldaprevir and asunaprevir) (Table 2, and Fig. 2). The most often noted NS3 substitutions were Q1106K/R and A1182V (%f =44.4) followed by R1181K (%f =22.2) and T1080S (%f =22.2) while the least frequent but the most potent NS3 substitution was observed to be D1194A (%f =11.1). A NS3 substitution D1194A/E/T/G in genotype 1 (Fig. 3) has been marked to play a considerable role in emergence of resistance to many antiviral agents such as simeprevir, faldaprevir and asunaprevir (Tables 1&2). D1194E NS3 substitution has also been identified in genotype 5 to confer resistance to simeprevir. Resistance-associated substitution was accounted against only two NS3/4A PIs(telaprevir and simeprevir) in case of genotype 2 but no any mutation in genotype 2 NS3 protein sequences was identified to play a role emergence of resistance against multiple targeted protein inhibitors. A sum total of substitutions in n=46 different variants of genotype 2 was spotted. V1062 L (%f =53.3) and S1148R (%frequency =46.7) were recognized to be associated with the resistance to telaprevir and simeprevir. Thus both HCV genotypes 1 & 2 are in process of developing resistance to simeprevir. V1062L (anti-telaprevir) has been noticed across genotypes 2, 3,4,5 and unclassified to be associated with resistance to telaprevir. But V1062L has been equally observed in genotypes 5 & 4 followed by unclassified sequence and genotype 3. HCV genotype 3 & 4 have exhibited resistance to only telaprevir while genotype 5 showed against telaprevir and simeprevir. Unclassified genotype sequences unraveled NS3 substitutions play an important role in evolution of resistance to simeprevir, faldaprevir and telaprevir NS3/4A PIs (Table 1&2). Multiple RAAS analyses for genotypes 5 & 6 have also been accomplished and it was observed that a Q1106K NS3 substitution was involved in a resistance associated phenomenon to faldaprevir and simeprevir PIs. Its presence in genotype 5 was more frequent than in genotype 6. T1080S was associated with resistance allied with telaprevir and faldaprevir in both genotype 5 and unclassified HCV sequences. In addition to the RAASs, increased sensitivity to NS3/4A PIs was assessed too. Only a substitution at position 1148 in NS3 sequences of genotype 5 and unclassified HCV sequences was identified to confer increased sensitivity to a simeprevir protease inhibitor. S1148A/G was the only substitution to impart increased sensitivity to simeprevir. Various other substitutions with an unknown effect on NS3/4A PIs, which were excluded from the result section**.**

Drug résistance to anti-HCV therapeutics particularly NS3/4A has been a major concern for efficacy of HCV treatment. In recent past, though lots of progress has been done in the development of anti-HCV therapeutics and their clinical viability, emerging viral

variants with multiple substitutions in protease pose a new challenge for the success of NS3/4A PIs (Gane *et al.,* 2016; Ng *et al.,* 2018). NS3/4A PIs are very much effective in declining the viral load in HCV-infected patients but due to the susceptibility to resistance-associated substitutions around NS3 protease active sites, their activities are reduced. (Lawitz *et al.,* 2016, 2018). In the present study risk assessment of resistance to different FDA-approved NS3/4A PIs due to amino acid substitutions in NS3 sequences of all the HCV-genotypes has been accomplished. Total number of fifteen (n=15) different resistance-associated NS3 substitutions among seven HCV genotypes and in the unclassified HCV NS3 sequences were identified (Fig. 2). These amino acid substitutions in different HCV variants were found to be involved in evolution of resistance against various NS3/4A PIs in clinical use such as boceprevir, telaprevir, asunaprevir, simeprevir and faldaprevir and therefore their clinical viability is to be challenged (Gane *et al.,* 2016). HCV genotype 1 was found to show maximum number of variants with single anti-NS3/4A protein inhibitor resistance-associated amino acid substitution and multianti-NS3/4A protein inhibitors resistance-associated amino acid substitution. Single anti-NS3/4A resistance-associated amino acid substitutions were in response to four different NS3/4A protease inhibitors(PIs) namely boceprevir, telaprevir, asunaprevir and simeprevir (Table 1). Evident drug resistance-associated substitution at the level of single amino acid in NS3 serine protease domain has been reported by Lin *et al,* (Lin *et al.,* 2004) for double protease inhibitors. Primary NS3 protease substitutions across genotype 1 variants conferring anti-boceprevir resistance were recognized to be V1196A. Tong et al, reported the same substitution V170A along with two other T54A and A156S mutations which conferred low to moderate grade of resistance to SCH 503034 NS3 PI about less than 20 fold (Tong *et al.,* 2006). V170A mutation was found to be most dominant and consistent during various continuous selections in his study where positon 170 has been given with reference to query sequences while in the current study all the substitution positions have been considered with reference to reference sequences. Primary NS3 protease substitutions across HCV genotype 1variants conferring anti-telaprevir were identified to be I1158V and V1062A/L which is validated by the study of Jian et al, on telaprevir resistant NS3 variants reporting the varying degree of anti-telaprevir resistance conferred by various NS3 substitutions including I1158V and V1062A/L (Jiang *et al.,* 2013). Anti-telaprevir resistant NS3 HCV genotype 1 variants were also reported by Kieffer *et al.,* (Kieffer *et al.,* 2012). HCV genotype 1 NS3 protease variant exhibiting anti-asunaprevir resistance was spotted as D/A at position 1194 in NS3 reference sequence considered in the present study (Table 1, Fig. 3) that is validated by an investigation reporting a high degree of anti-asunaprevir resistance (16-280 fold) due to various predominant substitutions at D1194 amino acid residue in genotype 1 (D168A/G/H/V/Y-position 168 here is with reference to query sequence) (McPhee *et al.,* 2012). NS3 R147K, D168G, I170T, R155K and D168A variants were also reported to be involved in anti-asunaprevir resistance emergence (McPhee *et al.,* 2012, Souman et al., 2014). The HCV genotype 1 variants D1194E/T conferring anti-simeprevir resistance (Fig. 3) has also been demonstrated by Lenz et al (Lenz *et al.,* 2010). The D1194A genotype 1 NS3 variant was assessed to be a key factor in emergence of resistance against multiple NS3/4A PIs (simeprevir, faldaprevir and asunaprevir) which is well-demonstrated by Lenz et al too (Lenz *et al.,* 2010).

However, variant S1148G exhibited increased sensitivity to simeprevir in case of genotype 1 but S1148R variant in genotype 2 revealed resistance to simeprevir that is validated by the finding of Lenz et al (Lenz *et al.,* 2010) suggesting that this amino acid residue position is not stable only for drug resistance. Role of D1194 substitution in emergence of antiviral drug resistance was also reported by Courcambeck et al (Courcambeck *et al.,* 2006). HCV genotype 5 D1194E NS3 variant was also identified to confer resistance to simeprevir. These drug-resistant variant analyses unravel a mutation hot spot (D1194 in NS3 reference sequence) in case genotype 1 that demonstrates multiple D1194 variants conferring resistance to single and multiple NS3/4A PIs (Fig. 3). The most frequently identified NS3-RAASs in case of HCV genotype 1 were against simeprevir (%f =50) followed by telaprevir (%f =21.4). NS3-RAAS against boceprevir (%f =14.3) and asunaprevir (%f =14.3) were equally frequent. Another almost pan-genotype (genotype 2,3,4,5 and unclassified) substitution hot spot was identified as anti-telaprevir variant V1062 L which corroborates with other resistance associated NS3 substitution analysis studies (Lin *et al.,* 2005; Lin *et al.,* 2004; Kieffer *et al.,* 2012; Jiang *et al.,* 2013). HCV genotypes 2 & 5 showed resistance to simeprevir and telaprevir. while genotypes 3 & 4 developed resistance against only telaprevir. Unclassified genotype sequences unraveled NS3 substitutions that play role in developement of resistance to simeprevir, faldaprevir and telaprevir NS3/4A PIs (Tables 1&2). Multiple RAAS analyses for genotypes 5 & 6 unravel variant Q1106K conferring resistance to faldaprevir and simeprevir PIs while another NS3 variant T1080S was associated with resistance allied with telaprevir and faldaprevir in both genotype 5 and unclassified HCV sequences (Lin *et al.,* 2005; Lin *et al.,* 2004, Kieffer *et al.,* 2012, Jiang *et al,* 2013). HCV quasispecies generation owing to replication rapidity and low polymerase fidelity have posed a challenge to the development and efficacy of DAAs particularly NS3/4A PIs (Halfon *et al.,* 2011). And because of higher degree of HCV genomic variability, NS3 variants with decreased susceptibility to NS3/4A PIs exist naturally even before the commencement of treatment but generally at a very low level. Therefore, resistance profile of PIs should be considered remarkably while developing different protease inhibitors for effective treatment of HCV patients.

Table 1. Characteristics of single anti-NS3/4A protein inhibitor resistance-associated amino acid substitutions (RAASs).

HCV-Genotype 1							
GBPA No.	**RSA no.**	**RSP**	**AIRS**	**QSP**	**AIQS**	**VT no.**	**PIQS**
VIPR_ALG4_908273241_1_1893.1	AAA72945.1	1148	S	122	G	14	IS-Simeprevir
VIPR_ALG4_AST22949_1_591.1	AAA72945.1	1148	S	122	G	14	IS-Simeprevir
VIPR_ALG4_575502871_1_575.1	AAA72945.1	1196	V	170	A	13	IR-Boceprevir
VIPR_ALG4_568110881_1_575.1	AAA72945.1	1196	V	170	A	13	IR-Boceprevir
VIPR_ALG4_908269165_1_543.1	AAA45676.1	1158	I	132	V	8	IR-Telaprevir
VIPR_ALG4_ATY34994_1_695.1	AAA72945.1	1194	D	168	E	12	IR-Simeprevir
VIPR_ALG4_ATY34994_1_695.1	AAA72945.1	1194	D	168	E	12	IR-Simeprevir
VIPR_ALG4_597512356_1_1893.1	AAA72945.1	1194	D	168	T	12	IR-Simeprevir
VIPR_ALG4_530656976_1_498.1	AAA45676.1	1194	D	160	G	14	IR-Asunaprevir
VIPR_ALG4_597512292_1_1893.1	AAA72945.1	1194	D	168	E	12	IR-Simeprevir
VIPR_ALG4_597512292_1_1893.1	AAA72945.1	1062	V	36	A	10	IR-Telaprevir
VIPR_ALG4_336039226_1_500.1	AAA72945.1	1194	D	160	T	12	IR-Simeprevir
VIPR_ALG4_568110888_1_575.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_333611678_1_498.1	AAA45676.1	1194	D	160	G	14	IR-Asunaprevir
HCV-Genotype 2							
GBPA No.	**RSA No.**	**RSP**	**AIRS**	**QSP**	**AIQS**	**VT no.**	**PIQS**
VIPR_ALG4_ATY35030_1_664.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_ATY35030_1_664.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_544168870_1_513.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_544168870_1_513.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_1152728397_3440_5332.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1152728397_3440_5332.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_1152728395_3436_5328.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_1152728379_3433_5325.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1152728379_3433_5325.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_576294944_3431_5323.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_393714879_3431_5323.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_401712478_1_432.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_401712478_1_432.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_544168874_1_513.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_544168874_1_513.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_1152728331_3565_5457.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_ATY35031_1_654.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_ATY35031_1_654.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_401712476_1_432.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_401712476_1_432.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_1152728359_3444_5336.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1152728359_3444_5336.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_544168876_1_513.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_544168876_1_513.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_401712474_1_432.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_401712474_1_432.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_1152728369_3461_5353.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1152728369_3461_5353.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_ATY35029_1_676.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_ATY35029_1_676.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_401712520_1_432.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_401712520_1_432.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_1152728313_3426_5318.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_509263121_3431_5323.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1152728311_3430_5322.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_ATY35005_1_657.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_ATY35005_1_657.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_1152728355_3457_5349.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1152728355_3457_5349.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_1152728371_3441_5333.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1152728371_3441_5333.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_ATY35003_1_657.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_ATY35003_1_657.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_1152728391_3442_5334.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1152728391_3442_5334.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
VIPR_ALG4_544168878_1_513.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_544168878_1_513.1	AAA72945.1	1148	S	122	R	14	IR-Simeprevir
HCV-Genotype 3							
GBPA No.	**RSA No.**	**RSP**	**AIRS**	**QSP**	**AIQS**	**VT no.**	**PIQS**
VIPR_ALG4_1152728501_3428_5320.1	AAA72945.1	1062	V	36	L	**10**	IR-Telaprevir
HCV-Genotype 4							

GBPA No.	RSA No.	RSP	AIRS	QSP	AIQS	VT no.	PIQS
VIPR_ALG4_475628336_1_1893.1	AAA72945.1	1062	V	36	L	**10**	IR-Telaprevir
VIPR_ALG4_475628354_1_1893.1	AAA72945.1	1062	V	36	L	**10**	IR-Telaprevir
VIPR_ALG4_ARR74221_22_607.1	AAA72945.1	1062	V	36	L	**10**	IR-Telaprevir
VIPR_ALG4_ATY35035_1_585.1	AAA72945.1	1062	V	36	L	**10**	IR-Telaprevir
VIPR_ALG4_1009028115_3310_5202.1	AAA72945.1	1062	V	36	L	**10**	IR-Telaprevir
HCV-Genotype 5							
GBPA No.	**RSA No.**	**RSP**	**AIRS**	**QSP**	**AIQS**	**VT no.**	**PIQS**
VIPR_ALG4_1026671954_1_1893.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1009028101_3356_5248.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1009028101_3356_5248.1	AAA72945.1	1148	S	122	A	14	IS-Simeprevir
VIPR_ALG4_1009028103_3356_5248.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1009028103_3356_5248.1	AAA72945.1	1148	S	122	A	14	IS-Simeprevir
VIPR_ALG4_1026671962_1_1893.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_1026671962_1_1893.1	AAA72945.1	1194	D	168	E	12	IR-Simeprevir
VIPR_ALG4_751660976_3424_5316.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
HCV-Genotype Unclassified							
GBPA No.	**RSA No.**	**RSP**	**AIRS**	**QSP**	**AIQS**	**VT no.**	**PIQS**
VIPR_ALG4_ARB18146_1_3424_5316.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir
VIPR_ALG4_844573227_1_376.1	AAA72945.1	1106	Q	18	L	14	IR-Faldaprevir
VIPR_ALG4_ASE05938_1_363.1	AAA72945.1	1148	S	119	R	14	IR-Simeprevir
VIPR_ALG4_ASE05936_1_363.1	AAA72945.1	1062	V	36	L	10	IR-Telaprevir

GBPA No.= GenBank accession number (VIPR), RSA no.= Reference sequence accession number, RSP= position in reference sequences, AIRS= amino acid in reference sequence, QSP= position in query sequences, AIQS = amino acid in query sequences, VT no. = Number of variant types, PIQS= phenotypic(s) in query sequences. IR-= Increased resistance to, IS-= Increased sensitivity to.

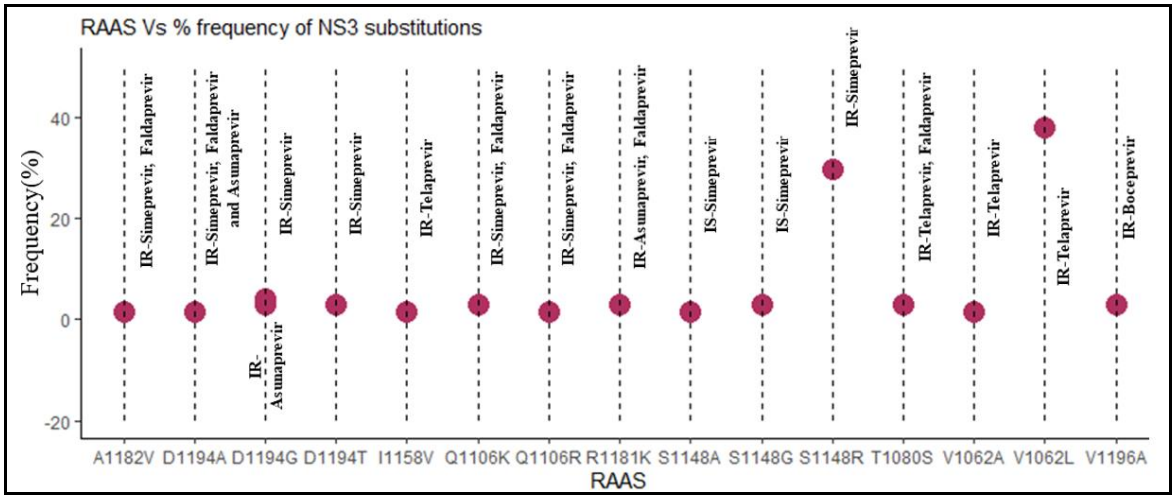


Fig. 2. Resistance associated NS3 substitutions' frequency and the increased resistance/sensitivity to different NS3/4A protein inhibitors. IR/IS- = Increased resistance/sensitivity.

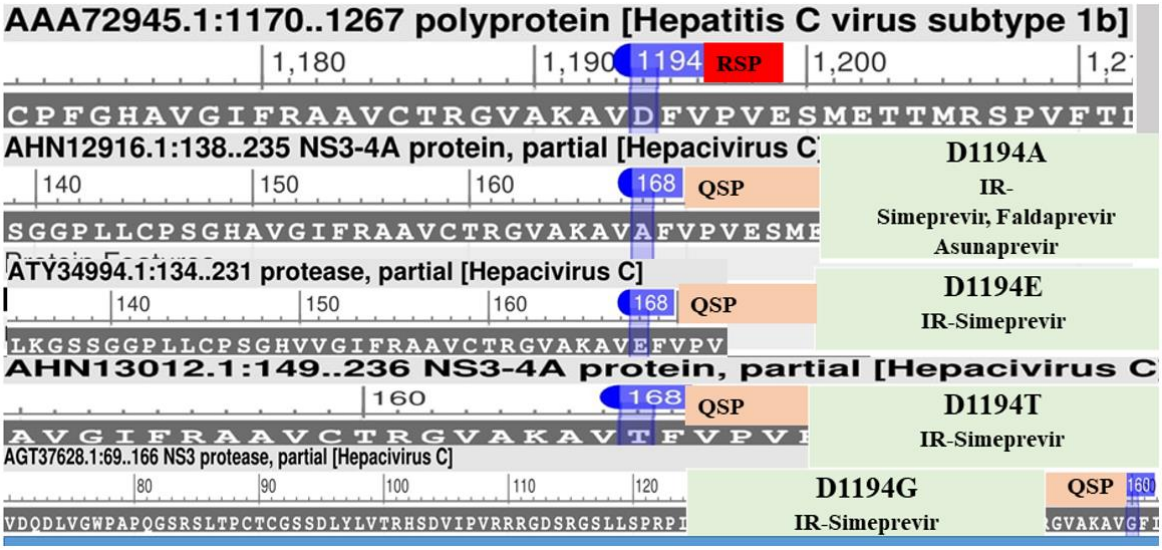


Fig. 3. D1194A/E/T/G NS3 substitutions exhibited by HCV genotype I involved in multiple anti-NS3/4A protein inhibitors resistance phenomena. RSP and QSP are the respective position in reference and query sequence respectively.

Table 2. Characteristics of multianti-NS3/4A protein inhibitors resistance-associated amino acid substitutions (RAASs).

HCV-Genotype 1							
GBPA No.	**RSA no.**	**RSP**	**AIRS**	**QSP**	**AIQS**	**VT no.**	**PIQS**
VIPR_ALG4_333611772_1_498.1	AAA45676.1	1181	R	147	K	10	IR-Faldaprevir, IR-Asunaprevir
VIPR_ALG4_568110975_1_575.1	AAA72945.1	1080	T	54	S	8	IR-Faldaprevir, IR-Telaprevir
VIPR_ALG4_ATY34994_1_695.1	AAA72945.1	1106	Q	80	R	14	IR-Faldaprevir, IR-Simeprevir
VIPR_ALG4_597512164_1_1893.1	AAA72945.1	1194	D	168	A	12	IR-Simeprevir, IR-Faldaprevir IR-Asunaprevir
VIPR_ALG4_597512292_1_1893.1	AAA72945.1	1106	Q	80	K	14	IR-Faldaprevir, IR-Simeprevir
VIPR_ALG4_575502871_1_575.1	AAA72945.1	1080	T	54	S	8	IR-Faldaprevir, IR-Telaprevir
VIPR_ALG4_908268421_1_1893.1	AAA72945.1	1106	Q	80	K	14	IR-Faldaprevir, IR-Simeprevir
VIPR_ALG4_333611598_1_498.1	AAA72945.1	1182	A	148	V	9	IR-Faldaprevir, IR-Simeprevir
VIPR_ALG4_333611772_1_498.1	AAA45676.1	1181	R	147	K	10	IR-Faldaprevir, IR-Asunaprevir
HCV-Genotype 5							
GBPA No.	**RSA No.**	**RSP**	**AIRS**	**QSP**	**AIQS**	**VT No.**	**PIQS**
VIPR_ALG4_751660976_3424_5316.1	AAA72945.1	1106	Q	80	K	14	IR-Faldaprevir, IR-Simeprevir
VIPR_ALG4_1026671962_1_1893.1	AAA72945.1	1106	Q	80	K	14	IR-Faldaprevir, IR-Simeprevir
VIPR_ALG4_1009028103_3356_5248.1	AAA72945.1	1106	Q	80	K	14	IR-Faldaprevir, IR-Simeprevir
VIPR_ALG4_1026671954_1_1893.1	AAA72945.1	1106	Q	80	K	14	IR-Faldaprevir, IR-Simeprevir
VIPR_ALG4_1009028101_3356_5248.1	AAA72945.1	1106	Q	80	K	14	IR-Faldaprevir, IR-Simeprevir
HCV-Genotype 6							
GBPA No.	**RSA No.**	**RSP**	**AIRS**	**QSP**	**AIQS**	**VT no.**	**PIQS**
VIPR_ALG4_751660980_3436_5328.1	AAA72945.1	1106	Q	80	K	14	IR-Faldaprevir, IR-Simeprevir
VIPR_ALG4_1026671966_1_1893.1	AAA72945.1	1106	Q	80	K	14	IR-Faldaprevir, IR-Simeprevir
VIPR_ALG4_1026671960_1_1893.1	AAA72945.1	1106	Q	80	K	14	IR-Faldaprevir, IR-Simeprevir
HCV-Genotype Unclassified							
GBPA No.	**RSA No.**	**RSP**	**AIRS**	**QSP**	**AIQS**	**VT no.**	**PIQS**
VIPR_ALG4_ASE05969_1_363.1	AAA72945.1	1080	T	51	S	8	IR-Faldaprevir, IR-Telaprevir
VIPR_ALG4_ASE05955_1_363.1	AAA72945.1	1080	T	51	S	8	IR-Faldaprevir, IR-Telaprevir

GBPA No.= GenBank accession number(VIPR), RSA no.= Reference sequence accession number, RSP= position in reference sequences, AIRS= amino acid in reference sequence, QSP= position in query sequences, AIQS = amino acid in query sequences, VT no. = Number of variant types, PIQS= phenotypic(s) in query sequences.

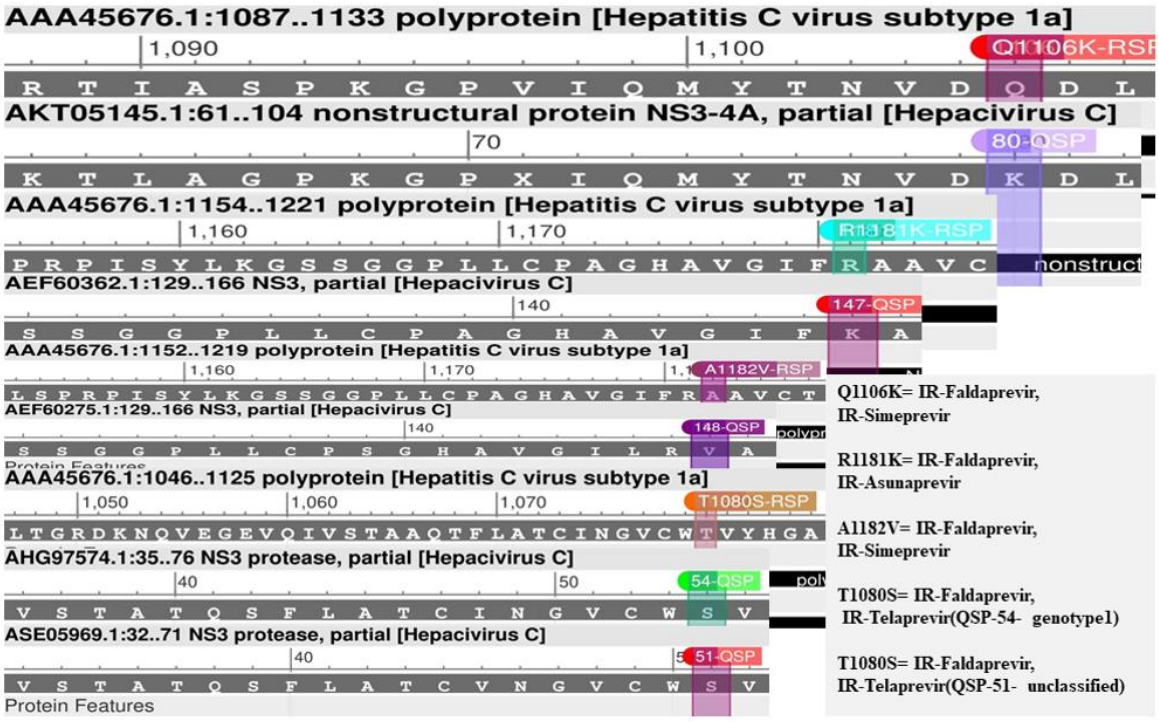


Fig. 4. NS3 substitutions exhibited by different HCV genotypes involved in multiple anti-NS3/4A protein inhibitors resistance phenomena. Q1106K-genotype 1,5,6 & unclassified HCV sequences, T1080S-genotype 1 & unclassified, R1181K and A1182V-genotype 1. RSP and QSP are the respective position in reference and query sequence respectively.

Conclusion:

With an advancement in molecular biology in the recent past, many novel anti-HCV therapeutics have been developed that target certain HCV viral proteins including NS3/4A PIs but the emerging viral variants with multiple substitutions in protease pose a new challenge to success of NS3/4A PIs. Single and multiple RAASs were determined in the present study that encompasses anti-telaprevir and anti-simepevir across most of the genotypes with considerable frequency. In addition to this anti-asunaprevir, anti-faldaprevir and anti-boceprevir associated variants were also recognized. A few cases of increased sensitivity to simeprevir have also been determined. HCV quasispecies generation owing to replication rapidity and low polymerase fidelity have posed a challenge to the development and efficacy of DAAs particularly NS3/4A PIs. Thus, because of a higher degree of HCV genomic variability, NS3 variants with decreased susceptibility to NS3/4A PIs exist naturally even before the commencement of treatment generally but at a very low level. Therefore, resistance profile along with potency, adverse effect and oral administration frequency of PIs should be considered remarkably while developing different protease inhibitors.

Acknowledgement:

The author would like to thank NCBI and viprbrc.org for providing sequence data and various analysis and visualization tools. The authors extend their appreciation to the deputyship for Research and innovation, Ministry of education in Saudi Arabia for funding this research work through the project number MOE-BU-1-2020.

Conflict of interest:

No conflict of interest

REFERENCES

Ascione, A., Luca, M., Costanzo, G., Picciotto, F., Lanza, A., Canestrini, C., Morisco, F., Tuccillo, C. and Caporaso, N., 2002. Incidence of side effects

during therapy with different types of alpha interferon: a randomised controlled trial comparing recombinant alpha 2b versus leukocyte interferon in the therapy of naive patients with chronic hepatitis C. *Current pharmaceutical design*, *8*(11), pp.977-980.

Asselah, T., Boyer, N., Saadoun, D., Martinot-Peignoux, M. and Marcellin, P., 2016. Direct-acting antivirals for the treatment of hepatitis C virus infection: optimizing current IFN-free treatment and future perspectives.*LiverInternational*, *36*, pp.47-57.

Bruix, J., Boix, L., Sala, M. and Llovet, J.M., 2004. Focus on hepatocellular carcinoma. *Cancer cell*, *5*(3), pp.215-219.

Courcambeck, J., Bouzidi, M., Perbost, R., Jouirou, B., Amrani, N., Cacoub, P., Pèpe, G., Sabatier, J.M. and Halfon, P., 2006. Resistance of hepatitis C virus to NS3-4A protease inhibitors: mechanisms of drug resistance induced by R155Q, A156T, D168A and D168V mutations. *AntiviralTherapy*, *11*(7), p.847.

Daar, E.S. and Richman, D.D., 2005. Confronting the emergence of drug-resistant HIV type 1: impact of antiretroviral therapy on individual and population resistance. *AIDS Research & Human Retroviruses*, *21*(5), pp.343-357.

Dalmau, D., Klimkait, T. and Telenti, A., 2005. Opinion paper. Resistance to new anti-HIV agents: problems in the pathway of drug registration. *Antivir Ther*, *10*, pp.867-872.

De Francesco, R. and Migliaccio, G., 2005. Challenges and successes in developing new therapies for hepatitis C. *Nature*, *436*(7053), pp.953-960.

Foy, E., Li, K., Wang, C., Sumpter, R., Ikeda, M., Lemon, S.M. and Gale, M., 2003. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science*, *300*(5622), pp.1145-1148.

Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Gonçales Jr, F.L., Häussinger, D., Diago, M., Carosi, G., Dhumeaux, D. and Craxi, A., 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *New England Journal of Medicine*, *347*(13), pp.975-982.

Gale, M. and Foy, E.M., 2005. Evasion of intracellular host defence by hepatitis C virus, *Nature*, *436*(7053), pp.939-945.

Gane, E., Ben Ari, Z., Mollison, L., Zuckerman, E., Bruck, R., Baruch, Y., Howe, A.Y.M., Wahl, J., Bhanja, S., Hwang, P. and Zhao, Y., 2016. Efficacy and safety of grazoprevir+ ribavirin for 12 or 24 weeks in treatment-naïve patients with hepatitis C virus genotype 1 infection. *Journal of viral hepatitis*, *23*(10), pp.789-797.

Geddawy, A., Ibrahim, Y.F., Elbahie, N.M. and Ibrahim, M.A., 2017. Direct acting anti-hepatitis C virus drugs: clinical pharmacology and future direction. *Journal of translational internal medicine*, *5*(1), pp.8-17.

Ghany, M.G., Nelson, D.R., Strader, D.B., Thomas, D.L. and Seeff, L.B., 2011. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases . *Hepatology*, *54*(4),

pp.1433-1444.

Hadziyannis, S.J., Sette, H., Morgan, T.R., Balan, V., Diago, M., Marcellin, P., Ramadori, G., Bodenheimer, H., Bernstein, D., Rizzetto, M. and Zeuzem, S., 2004. Peginterferon-α2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Annals of internal medicine*, *140*(5), pp.346-355.

Halfon, P. and Locarnini, S., 2011. Hepatitis C virus resistance to protease inhibitors. *Journal of hepatology*, *55*(1), pp.192-206.

Hu, J. and Ludgate, L., 2007. HIV–HBV and HIV–HCV coinfection and liver cancer development. In *Aids-Associated Viral Oncogenesis* (pp. 241-252). Springer, Boston, MA.

Jiang, M., Mani, N., Lin, C., Ardzinski, A., Nelson, M., Reagan, D., Bartels, D., Zhou, Y., Nicolas, O., Rao, B.G. and Müh, U., 2013. In vitro phenotypic characterization of hepatitis C virus NS3 protease variants observed in clinical studies of telaprevir. *Antimicrobial agents and chemotherapy*, *57*(12), pp.6236 -6245.

Keating, G.M., 2015. Ledipasvir/Sofosbuvir: a review of its use in chronic hepatitis C. *Drugs*, *75*(6), pp.675-685.

Khodabandehloo, M. and Roshani, D., 2014. Prevalence of hepatitis C virus genotypes in Iranian patients: a systematic review and meta-analysis. *Hepatitis monthly*, *14*(12).

Kieffer, T.L., De Meyer, S., Bartels, D.J., Sullivan, J.C., Zhang, E.Z., Tigges, A., Dierynck, I., Spanks, J., Dorrian, J., Jiang, M. and Adiwijaya, B., 2012. Hepatitis C viral evolution in genotype 1 treatment-naive and treatment-experienced patients receiving telaprevir-based therapy in clinical trials. *PloS one*, *7*(4).

Lamb, Y.N., 2017. Glecaprevir/pibrentasvir: first global approval. *Drugs*, *77*(16), pp.1797-1804.

Lawitz, E., Yang, J.C., Stamm, L.M., Taylor, J.G., Cheng, G., Brainard, D.M., Miller, M.D., Mo, H. and Dvory-Sobol, H., 2018. Characterization of HCV resistance from a 3-day monotherapy study of voxilaprevir, a novel pangenotypic NS3/4A protease inhibitor. *Antivir Ther*, *23*(4), pp.325-334.

Lawitz, E.J., O'Riordan, W.D., Asatryan, A., Freilich, B.L., Box, T.D., Overcash, J.S., Lovell, S., Ng, T.I., Liu, W., Campbell, A. and Lin, C.W., 2016. Potent antiviral activities of the direct-acting antivirals ABT-493 and ABT-530 with three-day monotherapy for hepatitis C virus genotype 1 infection. *Antimicrobial agents and chemotherapy*, *60*(3), pp.1546-1555.

Lebray, P., Nalpas, B., Vallet-Pichard, A., Broissand, C., Sobesky, R., Serpaggi, J., Fontaine, H. and Pol, S., 2005. The impact of haematopoietic growth factors on the management and efficacy of antiviral treatment in patients with hepatitis C virus. *Antivir Ther*, *10*(6), pp.769-76.

Lenz, O., Verbinnen, T., Lin, T.I., Vijgen, L., Cummings, M.D., Lindberg, J., Berke, J.M., Dehertogh, P., Fransen, E., Scholliers, A. and Vermeiren, K., 2010. In vitro resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. *Antimicrobial agents and chemotherapy*, *54*(5), pp.1878-1887.

Lin, C., Gates, C.A., Rao, B.G., Brennan,

D.L., Fulghum, J.R., Luong, Y.P., Frantz, J.D., Lin, K., Ma, S., Wei, Y.Y. and Perni, R.B., 2005. In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. *Journal of Biological Chemistry*, *280*(44), pp.36784-36791.

Lin, C., Lin, K., Luong, Y.P., Rao, B.G., Wei, Y.Y., Brennan, D.L., Fulghum, J.R., Hsiao, H.M., Ma, S., Maxwell, J.P. and Cottrell, K.M., 2004. In Vitro Resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061 structural analysis indicates different resistance mechanisms. *Journal of Biological Chemistry*, *279*(17), pp.17508-17514.

Manns, M.P., McHutchison, J.G., Gordon, S.C., Rustgi, V.K., Shiffman, M., Reindollar, R., Goodman, Z.D., Koury, K., Ling, M.H., Albrecht, J.K. and International Hepatitis Interventional Therapy Group, 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *The Lancet*, *358*(9286), pp.958-965.

McPhee, F., Friborg, J., Levine, S., Chen, C., Falk, P., Yu, F., Hernandez, D., Lee, M.S., Chaniewski, S., Sheaffer, A.K. and Pasquinelli, C., 2012. Resistance analysis of the hepatitis C virus NS3 protease inhibitor asunaprevir. *Antimicrobial agents and chemotherapy*, *56*(7), pp.3670-3681.

Morsica, G., Andolina, A., Merli, M., Messina, E., Hasson, H., Lazzarin, A., Uberti-Foppa, C. and Bagaglio, S., 2017. NS3 protease resistance-associated substitutions in liver tissue and plasma samples from patients infected by hepatitis C virus genotype 1A or 1B. *Archives of virology*, *162*(8), pp.2271-2277.

Ng, T.I., Tripathi, R., Reisch, T., Lu, L., Middleton, T., Hopkins, T.A., Pithawalla, R., Irvin, M., Dekhtyar, T., Krishnan, P. and Schnell, G., 2018. In vitro antiviral activity and resistance profile of the next-generation hepatitis C virus NS3/4A protease inhibitor glecaprevir. *Antimicrobial agents and chemotherapy*, *62*(1), pp.e01620-17.

Pawlotsky, J.M., 2016. Hepatitis C virus resistance to direct-acting antiviral drugs in interferon-free regimens. *Gastroenterology*, *151*(1), pp.70-86.

Perz, J.F., Armstrong, G.L., Farrington, L.A., Hutin, Y.J. and Bell, B.P., 2006. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *Journal of hepatology*, *45*(4), pp.529-538.

Petruzziello, A., Marigliano, S., Loquercio, G., Cozzolino, A. and Cacciapuoti, C., 2016. Global epidemiology of hepatitis C virus infection: An up-date of the distribution and circulation of hepatitis C virus genotypes. *World journal of gastroenterology*, *22*(34), p.7824.

Romano, K.P., Ali, A., Royer, W.E. and Schiffer, C.A., 2010. Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding. *Proceedings of the National Academy of Sciences*, *107*(49), pp.20986-20991.

Schaefer, M., Schmidt, F., Folwaczny, C., Lorenz, R., Martin, G.,

Schindlbeck, N., Heldwein, W., Soyka, M., Grunze, H., Koenig, A. and Loeschke, K., 2003. Adherence and mental side effects during hepatitis C treatment with interferon alfa and ribavirin in psychiatric risk groups. *Hepatology*, *37*(2), pp.443-451.

Shiryaev, S.A., Thomsen, E.R., Cieplak, P., Chudin, E., Cheltsov, A.V., Chee, M.S., Kozlov, I.A. and Strongin, A.Y., 2012. New details of HCV NS3/4A proteinase functionality revealed by a high-throughput cleavage assay. *PloS one*, *7*(4).

Soumana, D.I., Ali, A. and Schiffer, C.A., 2014. Structural analysis of asunaprevir resistance in HCV NS3/4A protease. *ACS chemical biology*, *9*(11), pp.2485-2490.

Strader, D.B., Wright, T., Thomas, D.L. and Seeff, L.B., 2004. Diagnosis, management, and treatment of hepatitis C. *Hepatology*, *39*(4), pp.1147-1171.

Sulkowski, M., Hezode, C., Gerstoft, J., Vierling, J.M., Mallolas, J., Pol, S., Kugelmas, M., Murillo, A., Weis, N., Nahass, R. and Shibolet, O., 2015. Efficacy and safety of 8 weeks versus 12 weeks of treatment with grazoprevir (MK-5172) and elbasvir (MK-8742) with or without ribavirin in patients with hepatitis C virus genotype 1 mono-infection and HIV/hepatitis C virus co-infection (C-WORTHY): a randomised, open-label phase 2 trial. *The Lancet*, *385*(9973), pp.1087-1097.

Thorgeirsson, S.S. and Grisham, J.W., 2002. Molecular pathogenesis of human hepatocellular carcinoma. *Nature genetics*, *31*(4), pp.339-346.

Tong, X., Chase, R., Skelton, A., Chen, T., Wright-Minogue, J. and Malcolm, B.A., 2006. Identification and analysis of fitness of resistance mutations against the HCV protease inhibitor SCH 503034. *Antiviral research*, *70*(2), pp.28-38.

Toniutto, P., Fabris, C., Fumo, E., Apollonio, L., Caldato, M., Avellini, C., Minisini, R. and Pirisi, M., 2005. Pegylated versus standard interferon-α in antiviral regimens for post-transplant recurrent hepatitis C: Comparison of tolerability and efficacy. *Journal of gastroenterology and hepatology*, *20*(4), pp.577-582.

Zeuzem, S., Jacobson, I.M., Baykal, T., Marinho, R.T., Poordad, F., Bourlière, M., Sulkowski, M.S., Wedemeyer, H., Tam, E., Desmond, P. and Jensen, D.M., 2014. Retreatment of HCV with ABT-450/r–ombitasvir and dasabuvir with ribavirin. *New England Journal of Medicine*, *370*(17), pp.1604-1614.