

RESEARCH ARTICLE

Geno2pheno_[HCV] – A Web-based Interpretation System to Support Hepatitis C Treatment Decisions in the Era of Direct-Acting Antiviral Agents

Prabhav Kalaghatgi¹, Anna Maria Sikorski^{2,3}, Elena Knops², Daniel Rupp³, Saleta Sierra^{2*}, Eva Heger², Maria Neumann-Fraune², Bastian Beggel¹, Andreas Walker⁴, Jörg Timm⁴, Hauke Walter⁵, Martin Obermeier⁵, Rolf Kaiser², Ralf Bartenschlager³, Thomas Lengauer¹

1 German Center for Infection Research (DZIF)–Saarbrücken Partner Site, Department of Computational Biology and Applied Algorithmics, Max Planck Institute for Informatics, 66123, Saarbrücken, Germany, **2** German Center for Infection Research (DZIF)–Cologne-Bonn Partner Site, Institute of Virology, University of Cologne, 50935, Cologne, Germany, **3** German Center for Infection Research (DZIF)–Heidelberg Partner Site, Department of Infectious Diseases, Molecular Virology, Heidelberg University, 69120, Heidelberg, Germany, **4** Institute for Virology, University Hospital Düsseldorf, Heinrich Heine University, 40225, Düsseldorf, Germany, **5** MVZ Medizinisches Infektiologiezentrum Berlin (MIB), 13353, Berlin, Germany

* saleta.sierra-aragon@uk-koeln.de



OPEN ACCESS

Citation: Kalaghatgi P, Sikorski AM, Knops E, Rupp D, Sierra S, Heger E, et al. (2016) Geno2pheno_[HCV] – A Web-based Interpretation System to Support Hepatitis C Treatment Decisions in the Era of Direct-Acting Antiviral Agents. *PLoS ONE* 11(5): e0155869. doi:10.1371/journal.pone.0155869

Editor: Luis Menéndez-Arias, Centro de Biología Molecular Severo Ochoa (CSIC-UAM), SPAIN

Received: February 17, 2016

Accepted: May 5, 2016

Published: May 19, 2016

Copyright: © 2016 Kalaghatgi et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files. Additional sequence data can be found under the GenBank Accession numbers KP409203-KP409213.

Funding: The development of geno2pheno_[HCV] is funded by the German Center for Infection Research (DZIF, grant no. 8000 802-3; DZIF-TTU Hepatitis 05.805), HIV-GRADE e.V.; Resina HIV-HEP-MASTER (IIA5-2013-2514AUK375); and the MSD-PEPSI Project.

Abstract

The face of hepatitis C virus (HCV) therapy is changing dramatically. Direct-acting antiviral agents (DAAs) specifically targeting HCV proteins have been developed and entered clinical practice in 2011. However, despite high sustained viral response (SVR) rates of more than 90%, a fraction of patients do not eliminate the virus and in these cases treatment failure has been associated with the selection of drug resistance mutations (RAMs). RAMs may be prevalent prior to the start of treatment, or can be selected under therapy, and furthermore they can persist after cessation of treatment. Additionally, certain DAAs have been approved only for distinct HCV genotypes and may even have subtype specificity. Thus, sequence analysis before start of therapy is instrumental for managing DAA-based treatment strategies. We have created the interpretation system geno2pheno_[HCV] (g2p_[HCV]) to analyse HCV sequence data with respect to viral subtype and to predict drug resistance. Extensive reviewing and weighting of literature related to HCV drug resistance was performed to create a comprehensive list of drug resistance rules for inhibitors of the HCV protease in non-structural protein 3 (NS3-protease: Boceprevir, Paritaprevir, Simeprevir, Asunaprevir, Grazoprevir and Telaprevir), the NS5A replicase factor (Daclatasvir, Ledipasvir, Elbasvir and Ombitasvir), and the NS5B RNA-dependent RNA polymerase (Dasabuvir and Sofosbuvir). Upon submission of up to eight sequences, g2p_[HCV] aligns the input sequences, identifies the genomic region(s), predicts the HCV geno- and subtypes, and generates for each DAA a drug resistance prediction report. g2p_[HCV] offers easy-to-use and fast subtype and resistance analysis of HCV sequences, is continuously updated and freely accessible under <http://hcv.geno2pheno.org/index.php>. The system was partially validated with respect to the NS3-protease inhibitors Boceprevir, Telaprevir and Simeprevir

Competing Interests: The authors have declared that no competing interests exist.

by using data generated with recombinant, phenotypic cell culture assays obtained from patients' virus variants.

1. Introduction

Infection with hepatitis C virus (HCV) is a major health problem worldwide. It is estimated that 130 to 150 million individuals are chronically infected with this virus [1]. Epidemiological studies have shown that persistent infection with HCV leads to a significantly increased risk of developing severe liver diseases, most notably liver cirrhosis and hepatocellular carcinoma (HCC) [2]. The incidence of HCC in HCV infected individuals is 15 to 20 fold higher than in HCV-negative individuals and, as a consequence, more than 350,000 people die from hepatitis C-related liver diseases each year [3].

HCV is an enveloped RNA virus with a positive-sense single stranded genome and belongs to the family *Flaviviridae* [4]. HCV infections are highly dynamic processes that are maintained by rapid production of new virions and continuous cell-to-cell spread. Model-based approaches suggest a virion production rate of 10^{12} virions/day [5,6]. Moreover, genome amplification by the HCV NS5B RNA-dependent RNA polymerase (RdRp) is characterized by a high error rate ($\sim 10^{-3}$ errors per round of replication [7,8]), due to the lack of a proof-reading mechanism. These two properties result in the high genomic variability of HCV that is reflected in the existence of seven distinct genotypes (1 to 7) with a pairwise nucleotide divergence (percentage of non-homologous genomic sites) of at least 30% and at least 67 distinct subtypes (e.g. 1a, 1b, . . .) with a pair-wise nucleotide divergence of at least 20% [9,10].

The face of HCV therapy has changed dramatically since 2011. Novel direct-acting antiviral agents (DAAs), designed to inhibit distinct steps in the HCV replication cycle have been approved in the EU and the US. Currently, three classes of DAAs are available: inhibitors of the NS3 protease, the NS5A replicase factor and the NS5B RdRp. The amino-terminal domain of NS3 associates with NS4A to form the NS3-4A serine-type protease complex that catalyzes the cleavage of the HCV polyprotein. NS5A plays multiple roles in the HCV replication cycle such as induction of the membranous replication factory, acting as a cofactor for HCV RNA replication, and supporting the assembly of infectious virus particles. The RdRp in NS5B is responsible for viral RNA amplification. DAAs lay the foundation for all-oral, interferon-free treatment regimens [11,12]. However, the specific DAA eligibility, resistance prevalence and efficacy of treatment depend on the HCV geno- and subtypes [13–18]. Treatment failure with DAAs has been associated with the selection of resistance-associated variants (RAVs) that become majoritary during therapy either by de novo generation or a consequence of selection from variants present at baseline [19–21]. Indeed, resistance mutations for different DAAs are detected in therapy-naïve patients [16,21,22]. In addition, resistance mutations in NS3 were shown to persist for months after cessation of therapy and even for years in case of NS5A resistant variants [20,21,23–25], thus reducing the success rate with subsequent treatments and also increasing the risk to spread new infections with DAA-resistant HCV variants [11]. The number of treatment failures and drug resistant variants is expected to increase within the next years through selection pressure imposed by DAA-based therapy [11]. The characterization of these variants and their impact on first-line and re-treatment strategies remains a great challenge.

We have developed geno2pheno_[HCV] (g2p_[HCV]), a web-service that supports the analysis of HCV sequence data with respect to geno- and subtypes and possible resistance against

licensed DAAs. g2p_[HCV] is a new member of the geno2pheno family, a set of web-based interpretation tools for analyzing sequences of hepatitis B virus and human immunodeficiency virus [26–28]. The subtyping algorithm of g2p_[HCV] accounts for all geno- and subtypes recognized by the International Committee for Taxonomy of Viruses [10]. The drug resistance analysis is based on a comprehensive set of rules that were collected from clinical and *in vitro* studies and were reviewed and carefully weighted by an expert panel. g2p_[HCV] can be freely accessed via an easy-to-use web interface and affords the export of the analysis in PDF format to facilitate communication and storage of results. g2p_[HCV] may be used by researchers and may help physicians in developing personalized treatment schedules. To evaluate g2p_[HCV], we used a selected number of HCV variants from patients suffering from therapy failure and conducted phenotypic assays to monitor drug sensitivity and replication fitness.

2. Materials and Methods

2.1. Geno2pheno[HCV] prediction tool

2.1.1. Reference sequence set. For subtyping, a reference alignment of 191 reference sequences for seven genotypes including 82 assigned subtypes and 35 unassigned subtypes was obtained in February 2015 from the International Committee for Taxonomy of Viruses [10]. From this reference alignment we extracted genomic regions relevant for drug resistance. These include: the protease domain of NS3 (up to amino acid position 181 of NS3), the amphipathic α -helix and the D1 domain of NS5A (up to amino acid position 213 of NS5A), and the complete NS5B region. For each region and subtype we defined one sequence to be the default reference sequence (e.g. H77 for 1a, HCV-J for 1b, etc.). The default reference is used for subtyping and for reporting genetic variants.

2.1.2. Query Sequence Processing. g2p only processes nucleotide sequences. Mixtures of nucleotides at an individual position can be included if they are coded as indicated by the International Union of Pure and Applied Chemistry (IUPAC) [<http://www.bioinformatics.org/sms/iupac.html>]. A query sequence that is submitted as input to g2p is processed as follows: (1) the genomic region is identified, (2) the geno- and subtypes are identified, (3) the nucleotide sequence is translated into an amino acid sequence and a list of amino acid variants is extracted, (4) the amino acid variants are subjected to the rule set to perform the drug resistance analysis. We now describe these processing steps in more detail.

2.1.2.1. Identification of the genomic region: To identify all the genomic regions present in the query sequence, g2p aligns the query sequence against the multiple sequence alignments of the NS3, NS5A and NS5B regions. For each genetic region, the system computes the alignment length and alignment quality. Alignment length is the number of columns in the multiple sequence alignment that do not contain any gaps. If the alignment length is less than 100, the query sequence is found to be of poor quality and it is not analyzed any further. Otherwise, the sequence similarity between the aligned query sequence and each reference sequence is computed. Sequence similarity is defined as the number of aligned characters that match divided by the alignment length. If sequence similarity is less than 65% the query sequence most likely contains many sequencing errors and is not analyzed any further. To summarize, regions of the query sequence are identified that correspond to NS3, NS5A and NS5B, and then all the query regions that satisfy the quality checks are analyzed.

2.1.2.2. Geno- and subtypes prediction: it is carried out individually for each query region and is based on homology. The geno- and subtypes of the query region are determined by the geno- and subtypes of the reference sequence with which the query sequence has the highest sequence similarity (proportion of matching characters). For subtype 1a sequences, also the clade classification is provided [16,29]. Sequence similarities against all reference sequences are

displayed in the results page. This method was validated with 177 sequences (see [Materials and Methods](#), section Subtyping validation).

2.1.2.3. The query region is translated into the corresponding amino acid sequence:

Depending on the settings (flag 3 selection) in the input page (use H77 or use the most similar reference sequence), all substitutions with respect to the corresponding reference are extracted. Nucleotide ambiguities of the query sequence are processed accordingly and might result in several possible amino acids present at a single position (denoted by, e.g. I170IV). Note that for the amino acid positions we always refer to the sequence H77 as a numbering reference (GenBank Accession number AF011751). At the end of this step, for each query region, the system generates a list of amino acid substitutions.

2.1.2.4. Rule set: An extensive literature survey was performed in order to obtain a comprehensive summary of the knowledge on drug resistance to NS3 inhibitors (Asunaprevir, Boceprevir, Grazoprevir, Paritaprevir, Simeprevir, and Telaprevir), NS5A inhibitors (Daclatasvir, Elbasvir, Ledipasvir, and Ombitasvir), and NS5B inhibitors (Dasabuvir and Sofosbuvir) [20,30–73]. The final rule set was selected by a panel of experts. Each rule is represented by a Boolean expression (see next paragraph) and is associated with a list of geno- and subtypes to which the rule applies, a summarizing drug resistance prediction (see [Table 1](#)), and references to the relevant literature and the levels of evidence. The levels of evidence were established similarly to the classification system used by the European HIV Drug Resistance Guidelines: I = based on at least one prospective randomized study using surrogate markers e.g. viral load; II = based on at least one retrospective study; III = expert opinion based on scientific evidence derived from other clinical and in vitro observations.

Each resistance rule is either a simple rule like pos₁AA₁ (e.g. 155K) or one of the following compound rules:

1. pos₁AA₁ or pos₁AA₂ or . . . or pos₁AA_n (e.g. 168A or 168H or 168T or 168Y)
2. pos₁AA₁ and pos₂AA₂ and . . . and pos_nAA_n (e.g. 28M and 31F)

where AA_i indicates the amino-acid observed in the query at position pos_i.

2.1.3. Applying resistance rules to the query sequence. For the major geno- and subtypes of HCV (1a, 1b, 2a, 2b, 3a, 4a and 4d), the procedure for determining drug resistance is as follows. Flag 4 in the input page allows for the selection of resistance rule set. The default option enables the specific resistance rules that are applicable for the geno- and subtypes and region of the query. If the option “ignore subgenotype for drug resistance prediction” is selected, then all resistance rules applicable to the region of the query will be used.

Table 1. Description of the summarizing drug resistance prediction.

Rank	Resistance prediction	Description
1	resistant	The provided sequence contains at least one of the so far known resistance-associated mutations for this drug class (see “Rules”-tab).
2	possibly resistant	The provided sequence contains at least one mutation related to resistance but whose specific fold change/therapy failure association has not been determined yet (see “Rules”-tab).
3	substitution on scored position	The provided sequence contains mutation(s) in (an) amino acid residue (s) related to resistance but whose specific fold change/therapy failure association has not been determined yet (see “Rules”-tab).
4	susceptible	The provided sequence contains none of the so far known resistance-associated mutations for this drug class (see “Rules”-tab).
5	not licensed for genotype	The drug is not licensed for the predicted genotype.

doi:10.1371/journal.pone.0155869.t001

The application of each rule in the list to the amino acid substitutions present in the query results in one of the following cases. The cases are listed in increasing order of susceptibility to the drug corresponding to the rule.

1. The rule applies fully. All amino acid substitutions required by the rule are present in the query. The associated drug resistance prediction is either “possibly resistant” or “resistant”. This is determined by the corresponding entry in the rules table.
2. The rule applies partially. This occurs when the resistance rule is a complex rule of type 2 and only some of the variants are present in the query. The associated drug prediction is “rule applies partially”.
3. There is a substitution at a resistance conferring position but the observed substitution is not known to confer resistance. The associated drug prediction is “substitution on scored position”.

For rare geno- and subtypes there is not sufficient clinical or phenotypic-assay based evidence to confidently make resistance predictions for any observed substitution. However it is possible that a substitution in a rare geno- and subtype may be of clinical importance if it is known to confer resistance in a closely related common subtype. In order to report such substitutions in rare geno- and subtypes g2p first identifies the common geno- and subtypes that is most similar to the query by homology. For instance if the query has been geno- and subtyped as 4k, a rare geno- and subtypes, then the common geno- and subtypes will be 4d, the most similar common geno- and subtypes. Subsequently it is checked which rules fully apply for the common geno- and subtypes. The associated drug prediction is “resistance-associated mutation (RAM) in related common geno- and subtypes”.

2.1.4. Validation of subtyping. 1684 non-recombinant full genome sequences annotated with geno- and subtypes were downloaded from the Los Alamos HCV Sequence Database [74]. All sequences that are contained in the genotyping reference set were removed. Additionally, the sequences with the accession numbers AY878650, AY878651, KC197235, and KC197240 were also excluded (due to likely incorrect geno- and subtypes annotations). From the remaining set of sequences at most 20 sequences for each geno- and subtypes were randomly selected. This resulted in a test set of 177 full genome sequences covering the following 33 subtypes: 1a, 1b, 1c, 2a, 2b, 2c, 2i, 2j, 2k, 2m, 3a, 3b, 3i, 4a, 4d, 4f, 4l, 4m, 4n, 4o, 4r, 5a, 6a, 6b, 6e, 6f, 6i, 6l, 6m, 6n, 6o, 6t, and 6v.

We tested our homology-based subtyping approach for different lengths of the query sequence (50, 100, 200, 300, 500, 700, and 900 base-pairs) constructed at randomly selected genomic positions to see which length of the input sequence would allow for accurate subtyping. We further expanded the test set by introducing random nucleotide mutations to the sequences at different error rates: 0%, 10%, 20%, or 30% of the sequence positions. An error rate of x% means that nucleotides at x% of randomly selected positions were substituted with an arbitrary other nucleic acid.

2.2. Phenotypic resistance determination

For validation of the g2p predictions we used 11 samples from 11 patients included in the PEPSI Study. The samples displayed different patterns of resistance-associated mutations (RAMs). Phenotypic resistance assays to the protease inhibitors boceprevir (BOC), telaprevir (TVR) and simeprevir (SMV) was conducted by using a method described elsewhere [75]. In brief, HCV RNA was isolated from the blood samples using the Magna Pure Systems (Roche) according to the manufacturer’s protocol. RT-PCR (One Step RT-PCR Kit, Qiagen, Hilden,

Germany) was performed as previously described [76], but with HCV subtype-specific primers. NS3/protease amplicons were purified and inserted into the subgenomic HCV replicon pFKi341-PiLucNS3-3'_ET [77] that was modified to contain ClaI and AscI restriction sites [75]. These were used to insert NS3-specific amplicons obtained with patient sera. Insert sequences were checked by sequencing (GenBank Accession Numbers: KP409203-KP409213). Then, Replicon-encoding plasmid vectors were used for *in vitro* transcription and replicon RNAs were transfected into Huh7-Lunet cells by electroporation [78]. Different concentrations of the drugs were added to the transfected cells and replication was determined by luciferase assay [78]. All measurements were performed in triplicate. IC₅₀ values were calculated using the GraphPad Prism software package by applying non-linear regression fit curves. The mean IC₅₀ value was then normalized to the IC₅₀ values of the corresponding reference construct and expressed as mean fold-change IC₅₀ value.

2.2.1. Ethics statement. All patients enrolled in the PEPSI Study gave their written consent allowing the use of their blood samples for scientific purposes. The PEPSI Study has been approved by the ethics committee of the Medical Council North Rhine (Ärztchamber Nordrhein, Germany), No. 2012048.

3. Results and Discussion

3.1. Geno2pheno_[HCV] web interface

The web-service geno2pheno_[HCV] (<http://hcv.geno2pheno.org/index.php>) was created to predict clinically relevant phenotypes based on viral sequence data. The web interface provides several pages, namely the input, results, rules, reference, contact and team pages (Fig 1).

3.1.1. Input page. In the input page a user can enter (1) a sequence identifier that is displayed throughout the data analysis, (2) up to eight query sequences in FASTA format, (3) the H77 flag that specifies whether the list of amino acid substitutions should be listed with respect to H77 or with respect to the subtype specific reference, (4) the subtype flag which determines whether only the rules specific for the subtype inferred from the input sequence should be used or whether all rules should be used, (5) the alignment width used for the graphical representation of the alignment, (6) the CSV flag which allows the user to download the results as a CSV file, and (7) the action menu which allows the user to load a set of sample sequences or start the analysis.

3.1.2. Results page. Upon pressing the action button “Align and Predict” in the input page, g2p_[HCV] performs the analysis and automatically switches to the result page. The result page offers one subpage for each query sequence with labels ranging from “1” up to “8”. Each of these so-called sequence pages further contains three subpages, the alignment subpage, the prediction subpage, and the subtype subpage.

The alignment sub-page provides visual representation of the nucleotide and amino acid sequence alignments of the NS3, NS5A, and NS5B regions of the query to the respective reference sequence. The alignment contains visual markers for RAMs to indicate mutations with respect to the selected reference sequence.

The prediction subpage contains the following three tables: (1) sequence information, (2) drug resistance prediction, and (3) detailed mutation information.

3.1.2.1. The sequence information table: it contains the sequence identifier (extracted from the FASTA header and combined with the identifier provided at the input page), the predicted subtype (in parentheses we provide the sequence similarity at the nucleotide level to the closest reference sequence) and the clade classification for subtype 1a sequences, the amino acid positions covered by the query, (in parentheses we indicate whether there are positions relevant to drug resistance that are not covered by the query), the list of amino acid substitutions

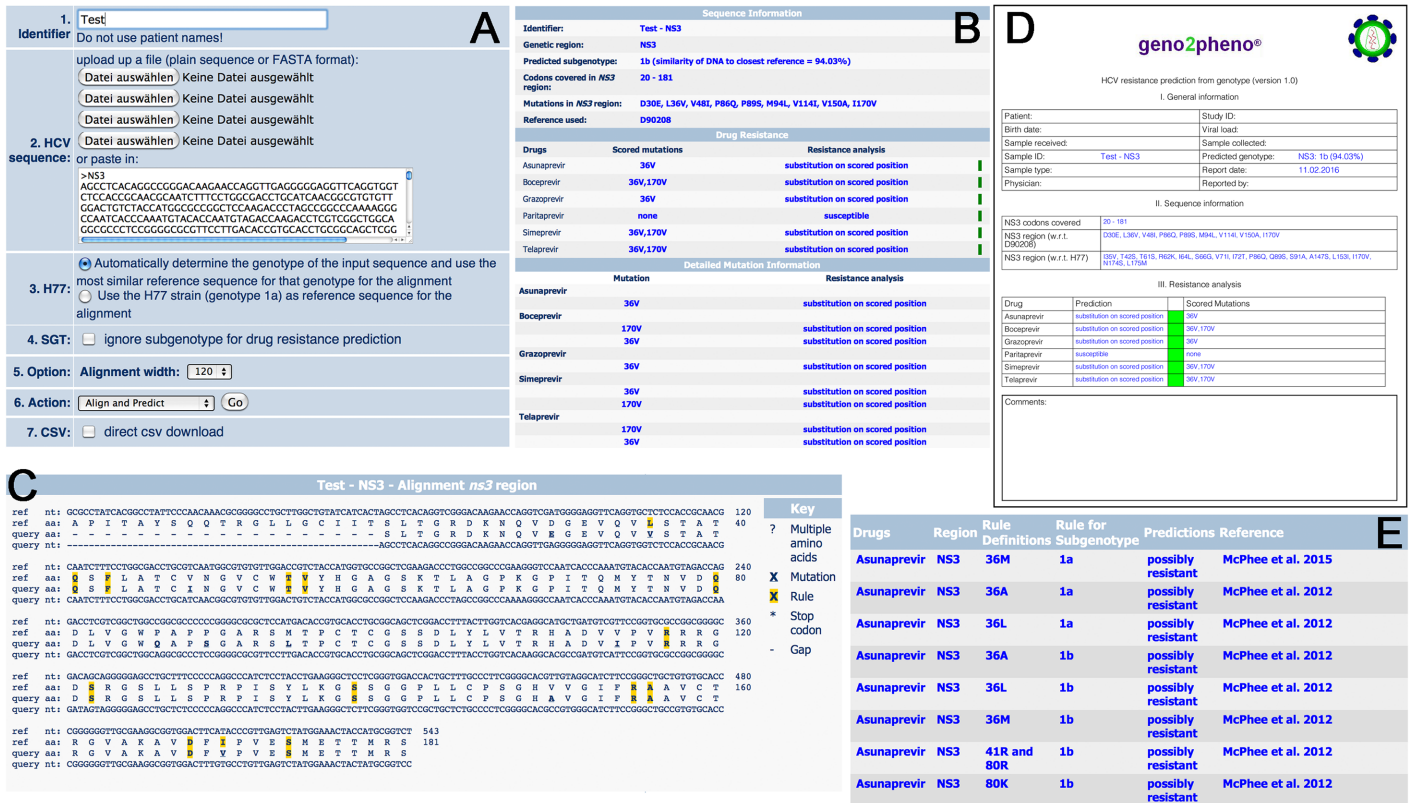


Fig 1. Multiple snapshots of the geno2pheno_[HCV] web interface. (A) The input page that allows the uploading of the sequence data and the configuration of the analysis. (B) The prediction sub-page that summarizes the subtype and drug resistance analysis. (C) The alignment sub-page (D) The drug resistance rule set as reference. (E) The PDF output to facilitate communication and storage of results.

doi:10.1371/journal.pone.0155869.g001

with respect to the selected reference, and the GenBank accession number of the selected reference.

3.1.1.2.2. The drug resistance table: it contains the summary result of the drug resistance prediction. It has one row for each drug associated with the genomic region(s) identified in the query sequence. Each row contains the overall resistance prediction (see Table 1 for a detailed description) and the list of amino acid substitutions relevant for the prediction (so called scored mutations). The overall resistance prediction is shown in the right column as a colored square: green for “substitution on scored position” and “susceptible”, yellow for “possibly resistant” and red for “resistant”. The drug overall resistance prediction is the worst prediction among the resistance predictions corresponding to all scored mutations for that drug. For example if there are three scored mutations for a drug with the resistance predictions “resistant”, “possibly resistant” and “substitution on scored position” then the overall resistance prediction is “resistant”. If no scored mutations are found for a drug then the overall resistance prediction is “susceptible”. If the drug is not licensed for the subtype of the query then, instead of a resistance prediction, the message “drug not licensed for subtype” is displayed, and no color is provided for the overall resistance.

3.1.1.2.3. The detailed mutation information table: it contains one row for each scored mutation and lists its resistance prediction.

The genotype sub-page contains the list of sequence similarities of the query sequence with respect to all reference sequences. The list is sorted in the order of decreasing sequence similarity

and may be helpful in assessing the reliability of the subtyping result. The genotype subpage also includes a “Download PDF” button to get a full report PDF that can be filled in for medical records.

3.1.3. Rules page. The complete set of rules used for the drug resistance predictions is provided in the rules page. Each row corresponds to a rule associated with drug resistance. The entries of each row are (1) drug for which the rule is applicable, (2) target HCV protein, (3) the resistance rule provided as a Boolean expression, (4) the list of geno- and subtypes for which the rule is applicable, (5) the resistance prediction, (6) a list of scientific references from which this rule was derived and, (7) the evidence level qualifying the amount of clinical and phenotypic evidence that supports this rule.

3.1.4. References, contact, and team page. The Reference page contains the full description of all references cited in the Rules page. The Contact page provides contact information regarding g2p_[HCV]. Please do not hesitate to let us know if you find our service useful or if you run into any issues using our service. The Team page lists all the institutions and collaborators instrumental in the creation, maintenance and updating of g2p_[HCV].

3.2. Subtyping validation

A test set of 177 full genome HCV sequences of 33 different subtypes was compiled from the Los Alamos HCV sequence database. The detailed results of the subtyping validation are provided in [S1](#) and [S2](#) Figs. In short, we found that subtyping results are reliable if the sequence length is at least 300 base pairs (irrespective of genomic location). This resulted in 100% accuracy on our test set for the genomic regions encoding NS3 and NS5A independent of the error rate. Thus, even after flipping 30% of the bases the correct genotype could always be inferred. Subtyping accuracy for NS5B with a sequence length of at least 300 bases amounted to 97.1% to 98.3% depending on the error rate. We also analyzed the sequence similarity to the closest subtype which is provided as a quality criterion for the subtype predictions. For sequence lengths of at least 300 and error rates of at most 10%, 4202 of 4248 (98.9%) predictions exceeded a sequence similarity of 80%. Only 3 of the 4202 (0.07%) predictions that exceeded a sequence similarity of 80% were incorrect. The true subtypes for the incorrect predictions are 6n, 6o, and 6n; and the predicted subtypes are 6o, 6a, and 6e, respectively. The remaining 3999 (99.93%) predictions were correct. On the other hand, 46 of 4248 predictions had a sequence similarity that was less than 80%. Of these 22 (47%) predictions were correct and 24 (53%) were incorrect. Thus, homology-based subtyping of at least 300 base-pairs long sequences was found to be reliable on our test set for cases where the sequence similarity to the closest subtype exceeded 80%.

3.2.1. Validation of predictions of drug resistance by using a phenotypic assay.

NS3-specific sequences were amplified from 11 patient sera and the amplicons were inserted into a subgenomic HCV replicon of the isolate Con1 engineered to allow easy transfer of NS3 amplicons via unique restriction sites. In this way, for each patient sample an NS3-specific library contained in a subgenomic replicon was generated and transfected into Huh7-Lunet cells. Their resistance to the NS3 protease inhibitors BOC, TVR and SMV was determined ([Fig 2](#)) and is expressed as “Fold-Change” (FC; [Table 2](#)). These results were compared to the predictions obtained with geno2pheno_[HCV]. We found that phenotypic resistance determination with the replicon system correlated well with the corresponding genotypic resistance prediction by geno2pheno_[HCV]. For clinical purposes it is important that samples detected to be highly resistant ($FC \geq 10$) by phenotypic assays were also predicted as such by geno2pheno_[HCV].

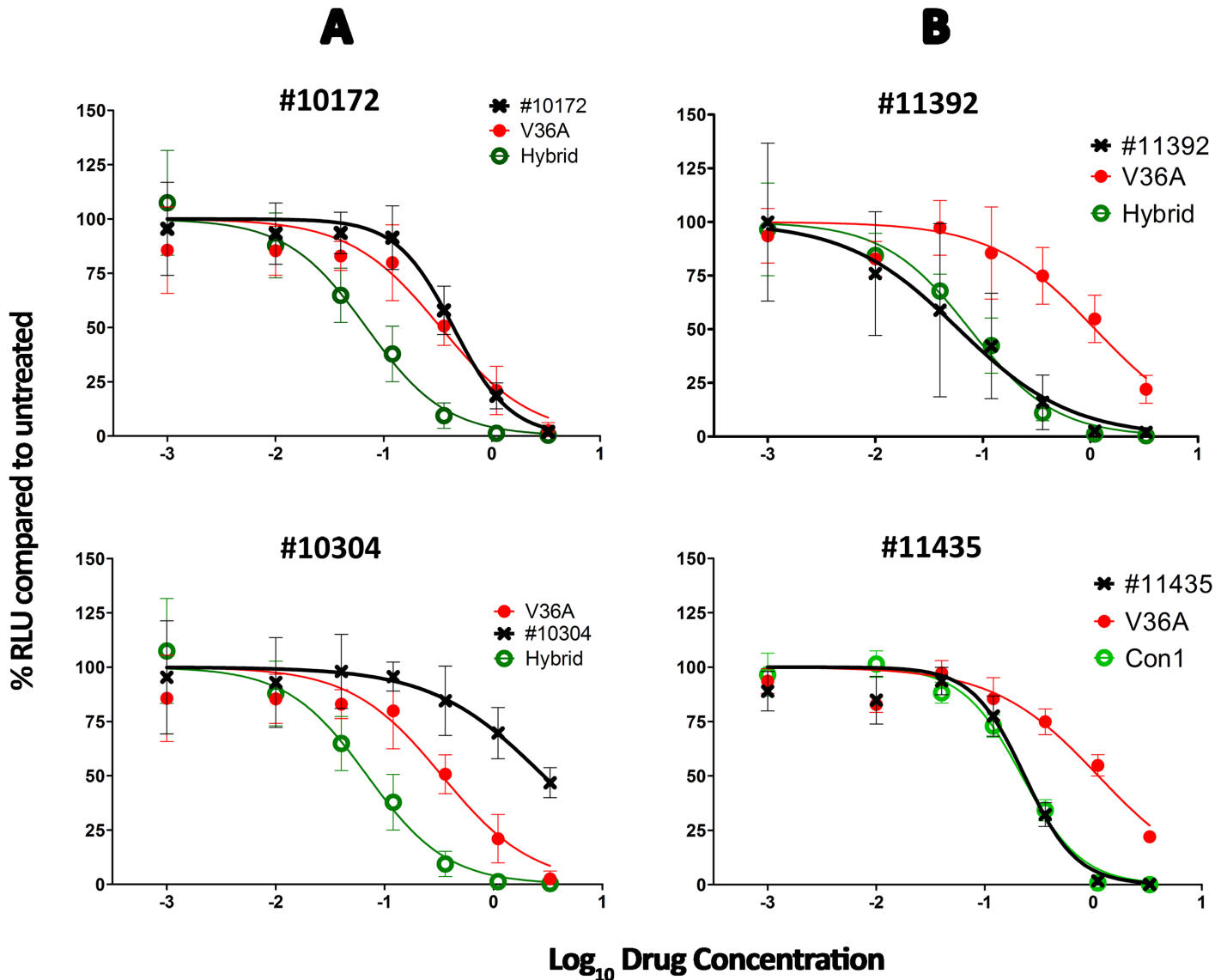


Fig 2. Examples of phenotypic measurements of PI susceptibility. The curves in green correspond to the susceptible controls (*Hybrid /Con1*), those in red to the resistant construct 36A, and the black ones to the specific sample. A) Samples #10172 and 10304 are resistant to BOC; B) Samples #10172 and 10304 are susceptible to TVR.

doi:10.1371/journal.pone.0155869.g002

3.3. Use cases

The first version of the geno2pheno_[HCV] was made available for scientific use in March 2011 and has been regularly updated. The current g2p_[HCV] version (Oct 21st, 2015) is based on a rule set that incorporates state-of-the-art knowledge, is hand curated by the authors, and is regularly updated to account for novel developments. g2p_[HCV] can be useful in a variety of scenarios. In the following we describe two typical use cases.

3.3.1. Case 1: patient 15170. A virus from a treatment-naïve patient was subtyped as 1a. Planned treatment for this patient was a combination therapy of Sofosbuvir plus Simeprevir. Resistance analysis performed with g2p_[HCV] revealed the presence of the Q80K mutation in the NS3 region. The Q80K mutation has been associated to lower response and also led to

Table 2. Phenotypic FC determinations.

Sample/Library	RAMs	BOC		TVR		SMV	
		g2p	FC assay	g2p	FC assay	g2p	FC assay
Con1	132V	S	1.0	S	1.0	S	1.0
Hybrid	-	S	1.0	S	1.0	S	1.3
36A	36A+132V	R	2.4	R	7.5	PR	1.0
9712	36A+132L+170V	R	1.5	PR	2.2	R	1.3
10172	36L	R	6.3	PR	6.3	PR	2.0
10304	36M+155K+170V	R	63.4	R	21.2	R	117.3
10615	80L	S	1.3	S	1.0	S	1.2
11392	122G+174S	S	0.3	PR	0.8	S	0.7
11429	132V	S	0.7	S	0.9	S	0.5
11435	41H+117H+132V	S	1.5	PR	1.0	S	0.4
11610	36M+80K+155K+174S	R	31.8	R	25.9	R	5.5x10 ⁷
12292	80K+132V+174S	S	1.8	PR	1.4	R	2.2
12476	174S	S	1.1	PR	2.8	S	2.0
12516	80K	S	1.6	S	0.5	R	9.1

g2p: S = susceptible; PR = possibly resistant; R = resistant; FC assay: fold change calculated from the phenotypic resistance assay

doi:10.1371/journal.pone.0155869.t002

SMV resistance in phenotypic assays [79–82]. In addition, SMV fold changes of resistance up to 11 have been detected *in vitro* [37,38,50]. Due to this evidence another treatment strategy was chosen and the patient was subjected to a 12-week treatment with Sofosbuvir plus Ledipasvir, as the patient sequence did not show any resistance to these two drugs. The patient achieved sustained virological response.

3.3.2. Case 2: patient 16083. A virus from a treatment naïve HCV was subtyped as 1a. The patient was then planned for a combination therapy of Paritaprevir, Ombitasvir and Dasabuvir. Resistance analysis followed by interpretation with g2p_[HCV] revealed the presence of the Q80K mutation in the NS3 region and 444D+556G in the NS5B region. The mutations 444D and 556G are described to confer resistance to Dasabuvir [64]. Consequently, the patient started treatment of Sofosbuvir plus Ledipasvir. 12 weeks after starting treatment (last value available), the viral load was still below the limit of detection.

3.4. Statistics of site usage

We tracked the number of unique queries per day that were submitted to g2p_[HCV] since its launch in March 2011. We found that geno2pheno_[HCV] is a popular tool which received an average of 4600 queries per month in 2015. See Fig 3 for the cumulative queries per quarter from March 2011 up till December 2015.

3.5. Conclusion

To our knowledge, we present the first and only freely available web-service that provides an analysis of HCV sequence data with respect to subtype and simultaneously drug resistance. The service can interpret baseline drug resistance mutations and can be helpful in optimizing antiviral therapy.

We are committed to continuously updating g2p_[HCV] when novel drugs or resistance patterns are available. In addition, our access to phenotypic resistance determination assays will

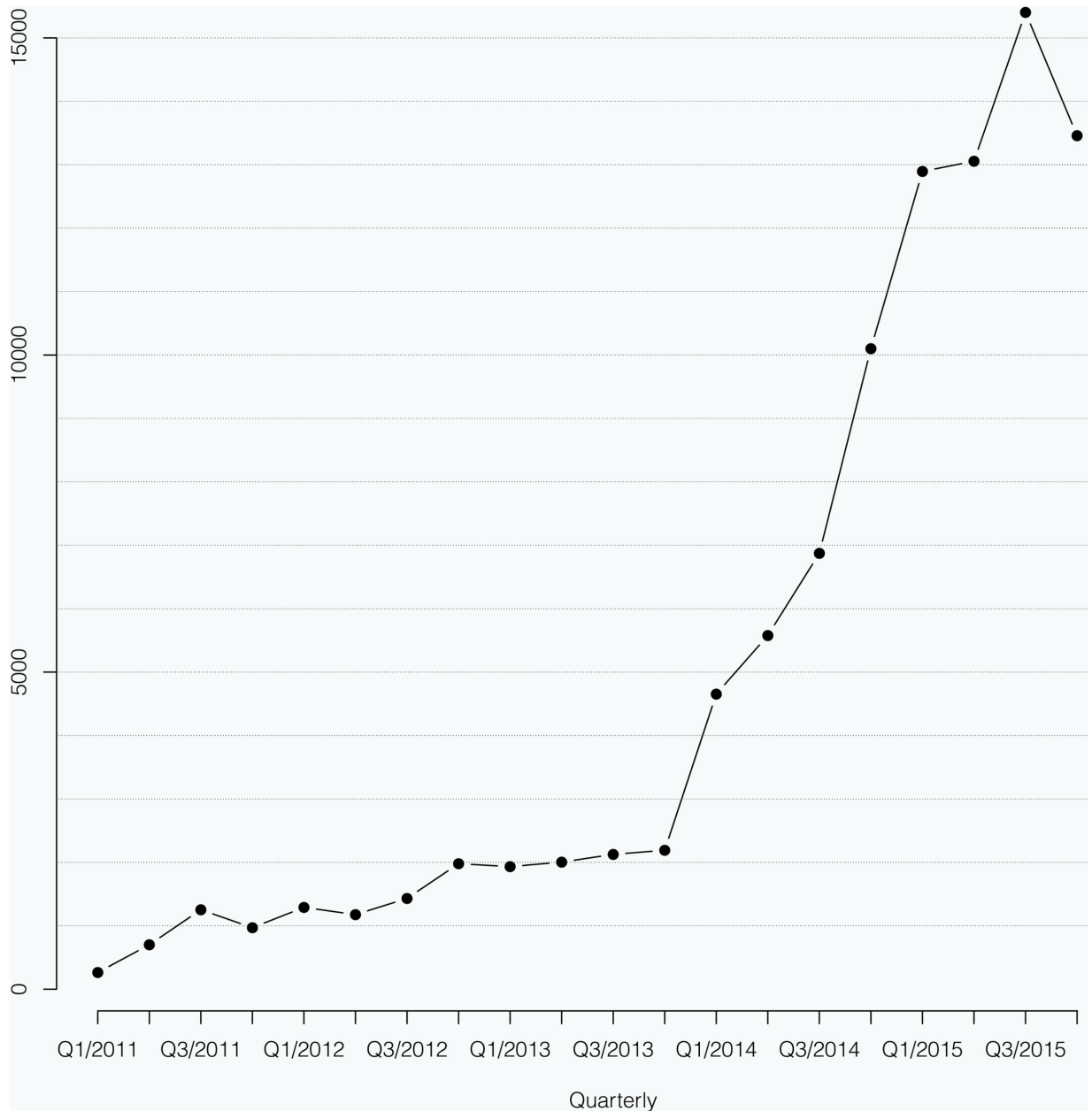


Fig 3. Cumulative number of queries per quarter that was submitted by users since the launch of the web-server g2p_[HCV].

doi:10.1371/journal.pone.0155869.g003

permit us to further validate the system but also to test mutations in target genes whose role in resistance is not clearly elucidated.

For the future, we also see high potential in the integration of additional host markers into g2p_[HCV] to further improve treatment recommendations. g2p_[HCV] can freely be accessed at <http://hcv.geno2pheno.org/index.php>.

Supporting Information

S1 Fig. Sequences subtyped against sequence length for each setting of genetic region and error rate. Each panel plots the number of sequences that were subtyped correctly (pink) and

incorrectly (blue). For each genetic region, contiguous sequences of the specified length were randomly sampled and x% (error rate) of sequence characters were substituted with another nucleotide. The subtype for this sequence was given by subtype of the reference sequence that was most similar (%matches) to the sequence. Results are shown for 100 sequences constructed for each setting of error rate, sequence length and genetic region.

(TIF)

S2 Fig. Similarities between the sequences and the reference of the correct subtype. Each panel shows the similarity between the query sequence and the reference of the correct subtype, against sequence length for each setting of genetic region and error rate. Cases where the sequence was subtyped correctly are shown in pink and the rest are shown in blue. For each genetic region, contiguous sequences of the specified length were randomly sampled and x% (error rate) of sequence characters were substituted with another nucleotide. The subtype for this sequence was given by subtype of the reference sequence that was most similar (%matches) to the sequence. Results are shown for 100 sequences constructed for each setting of error rate, sequence length and genetic region.

(TIF)

Acknowledgments

The authors thank all the patients and treating sites collaborating in the PEPSI Study.

Author Contributions

Conceived and designed the experiments: PK EK SS TL MNF EH RK. Performed the experiments: PK AMS DR BB RB TL. Analyzed the data: PK EK SS BB AW JT HW MO RB TL. Wrote the paper: PK BB SS.

References

1. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology*. 2013; 57: 1333–1342. doi: [10.1002/hep.26141](https://doi.org/10.1002/hep.26141) PMID: [23172780](https://pubmed.ncbi.nlm.nih.gov/23172780/)
2. El-Serag HB. Hepatocellular carcinoma. *N Engl J Med*. 2011; 365: 1118–1127. doi: [10.1056/NEJMra1001683](https://doi.org/10.1056/NEJMra1001683) PMID: [21992124](https://pubmed.ncbi.nlm.nih.gov/21992124/)
3. Toshikuni N, Arisawa T, Tsutsumi M. Hepatitis C-related liver cirrhosis—strategies for the prevention of hepatic decompensation, hepatocarcinogenesis, and mortality. *World J Gastroenterol*. 2014; 20: 2876–2887. doi: [10.3748/wjg.v20.i11.2876](https://doi.org/10.3748/wjg.v20.i11.2876) PMID: [24659879](https://pubmed.ncbi.nlm.nih.gov/24659879/)
4. Bartenschlager R, Lohmann V, Penin F. The molecular and structural basis of advanced antiviral therapy for hepatitis C virus infection. *Nat Rev Microbiol*. 2013; 11: 482–496. doi: [10.1038/nrmicro3046](https://doi.org/10.1038/nrmicro3046) PMID: [23748342](https://pubmed.ncbi.nlm.nih.gov/23748342/)
5. Guedj J, Dahari H, Rong L, Sansone ND, Nettles RE, Cotler SJ, et al. Modeling shows that the NS5A inhibitor daclatasvir has two modes of action and yields a shorter estimate of the hepatitis C virus half-life. *Proc Natl Acad Sci U S A*. 2013; 110: 3991–3996. doi: [10.1073/pnas.1203110110](https://doi.org/10.1073/pnas.1203110110) PMID: [23431163](https://pubmed.ncbi.nlm.nih.gov/23431163/)
6. Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, et al. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science*. 1998; 282: 103–107. PMID: [9756471](https://pubmed.ncbi.nlm.nih.gov/9756471/)
7. Ogata N, Alter HJ, Miller RH, Purcell RH. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. *Proc Natl Acad Sci U S A*. 1991; 88: 3392–3396. PMID: [1849654](https://pubmed.ncbi.nlm.nih.gov/1849654/)
8. Lutchman G, Danehower S, Song BC, Liang TJ, Hoofnagle JH, Thomson M, et al. Mutation rate of the hepatitis C virus NS5B in patients undergoing treatment with ribavirin monotherapy. *Gastroenterology*. 2007; 132: 1757–1766. PMID: [17484873](https://pubmed.ncbi.nlm.nih.gov/17484873/)
9. Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology*. 2005; 42: 962–973. PMID: [16149085](https://pubmed.ncbi.nlm.nih.gov/16149085/)

10. Smith DB, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, et al. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. *Hepatology*. 2014; 59: 318–327. doi: [10.1002/hep.26744](https://doi.org/10.1002/hep.26744) PMID: [24115039](https://pubmed.ncbi.nlm.nih.gov/24115039/)
11. Schinazi R, Halfon P, Marcellin P, Asselah T. HCV direct-acting antiviral agents: the best interferon-free combinations. *Liver Int*. 2014; 34 Suppl 1: 69–78. doi: [10.1111/liv.12423](https://doi.org/10.1111/liv.12423) PMID: [24373081](https://pubmed.ncbi.nlm.nih.gov/24373081/)
12. Holmes JA, Thompson AJ. Interferon-free combination therapies for the treatment of hepatitis C: current insights. *Hepat Med*. 2015; 7: 51–70. doi: [10.2147/HMER.S55864](https://doi.org/10.2147/HMER.S55864) PMID: [26586968](https://pubmed.ncbi.nlm.nih.gov/26586968/)
13. Pawlotsky JM, Feld JJ, Zeuzem S, Hoofnagle JH. From non-A, non-B hepatitis to hepatitis C virus cure. *J Hepatol*. 2015; 62: S87–99. doi: [10.1016/j.jhep.2015.02.006](https://doi.org/10.1016/j.jhep.2015.02.006) PMID: [25920094](https://pubmed.ncbi.nlm.nih.gov/25920094/)
14. EASL. EASL Recommendations on Treatment of Hepatitis C 2015. *J Hepatol*. 2015; 63: 199–236. doi: [10.1016/j.jhep.2015.03.025](https://doi.org/10.1016/j.jhep.2015.03.025) PMID: [25911336](https://pubmed.ncbi.nlm.nih.gov/25911336/)
15. AASLD-IDS (2015) HCV Guidance: Recommendations for Testing, Managing, and Treating Hepatitis C.
16. De Luca A, Di Giambenedetto S, Lo Presti A, Sierra S, Prosperi M, Cella E, et al. Two Distinct Hepatitis C Virus Genotype 1a Clades Have Different Geographical Distribution and Association With Natural Resistance to NS3 Protease Inhibitors. *Open Forum Infectious Diseases*. 2015; 2: 1–9.
17. McCown MF, Rajyaguru S, Kular S, Cammack N, Najera I. GT-1a or GT-1b subtype-specific resistance profiles for hepatitis C virus inhibitors telaprevir and HCV-796. *Antimicrob Agents Chemother*. 2009; 53: 2129–2132. doi: [10.1128/AAC.01598-08](https://doi.org/10.1128/AAC.01598-08) PMID: [19273674](https://pubmed.ncbi.nlm.nih.gov/19273674/)
18. Cento V, Mirabelli C, Salpini R, Dimonte S, Artese A, Costa G, et al. HCV genotypes are differently prone to the development of resistance to linear and macrocyclic protease inhibitors. *PLoS One*. 2012; 7: e39652. doi: [10.1371/journal.pone.0039652](https://doi.org/10.1371/journal.pone.0039652) PMID: [22792183](https://pubmed.ncbi.nlm.nih.gov/22792183/)
19. Pawlotsky JM. Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus. *Hepatology*. 2011; 53: 1742–1751. doi: [10.1002/hep.24262](https://doi.org/10.1002/hep.24262) PMID: [21374691](https://pubmed.ncbi.nlm.nih.gov/21374691/)
20. McPhee F, Hernandez D, Yu F, Ueland J, Monikowski A, Carifa A, et al. Resistance analysis of hepatitis C virus genotype 1 prior treatment null responders receiving daclatasvir and asunaprevir. *Hepatology*. 2013; 58: 902–911. doi: [10.1002/hep.26388](https://doi.org/10.1002/hep.26388) PMID: [23504694](https://pubmed.ncbi.nlm.nih.gov/23504694/)
21. Karino Y, Toyota J, Ikeda K, Suzuki F, Chayama K, Kawakami Y, et al. Characterization of virologic escape in hepatitis C virus genotype 1b patients treated with the direct-acting antivirals daclatasvir and asunaprevir. *J Hepatol*. 2013; 58: 646–654. doi: [10.1016/j.jhep.2012.11.012](https://doi.org/10.1016/j.jhep.2012.11.012) PMID: [23178977](https://pubmed.ncbi.nlm.nih.gov/23178977/)
22. Itakura J, Kurosaki M, Takada H, Nakakuki N, Matsuda S, Gondou K, et al. Naturally occurring, resistance-associated hepatitis C virus NS5A variants are linked to interleukin-28B genotype and are sensitive to interferon-based therapy. *Hepatol Res*. 2015; 45: E115–121. doi: [10.1111/hepr.12474](https://doi.org/10.1111/hepr.12474) PMID: [25564756](https://pubmed.ncbi.nlm.nih.gov/25564756/)
23. Wang C, Sun JH, O'Boyle DR 2nd, Nower P, Valera L, Roberts S, et al. Persistence of resistant variants in hepatitis C virus-infected patients treated with the NS5A replication complex inhibitor daclatasvir. *Antimicrob Agents Chemother*. 2013; 57: 2054–2065. doi: [10.1128/AAC.02494-12](https://doi.org/10.1128/AAC.02494-12) PMID: [23403428](https://pubmed.ncbi.nlm.nih.gov/23403428/)
24. Sullivan JC, De Meyer S, Bartels DJ, Dierynck I, Zhang EZ, Spanks J, et al. Evolution of treatment-emergent resistant variants in telaprevir phase 3 clinical trials. *Clin Infect Dis*. 2013; 57: 221–229. doi: [10.1093/cid/cit226](https://doi.org/10.1093/cid/cit226) PMID: [23575197](https://pubmed.ncbi.nlm.nih.gov/23575197/)
25. Barnard RJ, Howe JA, Ogert RA, Zeuzem S, Poordad F, Gordon SC, et al. Analysis of boceprevir resistance associated amino acid variants (RAVs) in two phase 3 boceprevir clinical studies. *Virology*. 2013; 444: 329–336. doi: [10.1016/j.virol.2013.06.029](https://doi.org/10.1016/j.virol.2013.06.029) PMID: [23876458](https://pubmed.ncbi.nlm.nih.gov/23876458/)
26. Neumann-Fraune M, Beggel B, Kaiser R, Obermeier M. Hepatitis B virus drug resistance tools: one sequence, two predictions. *Intervirology*. 2014; 57: 232–236. doi: [10.1159/000361076](https://doi.org/10.1159/000361076) PMID: [25034493](https://pubmed.ncbi.nlm.nih.gov/25034493/)
27. Beerenwinkel N, Daumer M, Oette M, Korn K, Hoffmann D, Kaiser R, et al. Geno2pheno: Estimating phenotypic drug resistance from HIV-1 genotypes. *Nucleic Acids Res*. 2003; 31: 3850–3855. PMID: [12824435](https://pubmed.ncbi.nlm.nih.gov/12824435/)
28. Lengauer T, Sander O, Sierra S, Thielen A, Kaiser R. Bioinformatics prediction of HIV coreceptor usage. *Nat Biotechnol*. 2007; 25: 1407–1410. PMID: [18066037](https://pubmed.ncbi.nlm.nih.gov/18066037/)
29. Pickett BE, Striker R, Lefkowitz EJ. Evidence for separation of HCV subtype 1a into two distinct clades. *J Viral Hepat*. 2011; 18: 608–618. doi: [10.1111/j.1365-2893.2010.01342.x](https://doi.org/10.1111/j.1365-2893.2010.01342.x) PMID: [20565573](https://pubmed.ncbi.nlm.nih.gov/20565573/)
30. Lin C, Lin K, Luong YP, Rao BG, Wei YY, Brennan DL, et al. In vitro resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms. *J Biol Chem*. 2004; 279: 17508–17514. PMID: [14766754](https://pubmed.ncbi.nlm.nih.gov/14766754/)
31. Lin C, Gates CA, Rao BG, Brennan DL, Fulghum JR, Luong YP, et al. In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. *J Biol Chem*. 2005; 280: 36784–36791. PMID: [16087668](https://pubmed.ncbi.nlm.nih.gov/16087668/)

32. Sarrazin C, Kieffer TL, Bartels D, Hanzelka B, Muh U, Welker M, et al. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. *Gastroenterology*. 2007; 132: 1767–1777. PMID: [17484874](#)
33. He Y, King MS, Kempf DJ, Lu L, Lim HB, Krishnan P, et al. Relative replication capacity and selective advantage profiles of protease inhibitor-resistant hepatitis C virus (HCV) NS3 protease mutants in the HCV genotype 1b replicon system. *Antimicrob Agents Chemother*. 2008; 52: 1101–1110. PMID: [18086851](#)
34. Tong X, Bogen S, Chase R, Girijavallabhan V, Guo Z, Njoroge FG, et al. Characterization of resistance mutations against HCV ketoamide protease inhibitors. *Antiviral Res*. 2008; 77: 177–185. doi: [10.1016/j.antiviral.2007.11.010](#) PMID: [18201776](#)
35. Zhou Y, Bartels DJ, Hanzelka BL, Muh U, Wei Y, Chu HM, et al. Phenotypic characterization of resistant Val36 variants of hepatitis C virus NS3-4A serine protease. *Antimicrob Agents Chemother*. 2008; 52: 110–120. PMID: [17938182](#)
36. Susser S, Welsch C, Wang Y, Zettler M, Domingues FS, Karey U, et al. Characterization of resistance to the protease inhibitor boceprevir in hepatitis C virus-infected patients. *Hepatology*. 2009; 50: 1709–1718. doi: [10.1002/hep.23192](#) PMID: [19787809](#)
37. Bae A, Sun SC, Qi X, Chen X, Ku K, Worth A, et al. Susceptibility of treatment-naive hepatitis C virus (HCV) clinical isolates to HCV protease inhibitors. *Antimicrob Agents Chemother*. 2010; 54: 5288–5297. doi: [10.1128/AAC.00777-10](#) PMID: [20855726](#)
38. Lenz O, Verbinnen T, Lin TI, Vijgen L, Cummings MD, Lindberg J, et al. In vitro resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. *Antimicrob Agents Chemother*. 2010; 54: 1878–1887. doi: [10.1128/AAC.01452-09](#) PMID: [20176898](#)
39. Kieffer TL, De Meyer S, Bartels DJ, Sullivan JC, Zhang EZ, Tigges A, et al. Hepatitis C viral evolution in genotype 1 treatment-naive and treatment-experienced patients receiving telaprevir-based therapy in clinical trials. *PLoS One*. 2012; 7: e34372. doi: [10.1371/journal.pone.0034372](#) PMID: [22511937](#)
40. Lagacé L, White PW, Bousquet C, Dansereau N, Do F, Llinas-Brunet M, et al. In vitro resistance profile of the hepatitis C virus NS3 protease inhibitor BI 201335. *Antimicrob Agents Chemother*. 2012; 56: 569–572. doi: [10.1128/AAC.05166-11](#) PMID: [22024816](#)
41. Lam JT, Jacob S. Boceprevir: a recently approved protease inhibitor for hepatitis C virus infection. *Am J Health Syst Pharm*. 2012; 69: 2135–2139. doi: [10.2146/ajhp110500](#) PMID: [23230035](#)
42. McPhee F, Sheaffer AK, Friberg J, Hernandez D, Falk P, Zhai G, et al. Preclinical Profile and Characterization of the Hepatitis C Virus NS3 Protease Inhibitor Asunaprevir (BMS-650032). *Antimicrob Agents Chemother*. 2012; 56: 5387–5396. doi: [10.1128/AAC.01186-12](#) PMID: [22869577](#)
43. Susser S, Schelhorn S, Lange C, Welsch C, Vermehren J, Perner D, et al. Ultratiefe Pyrosequenz-Analyse (UDPS) von neu beschriebenen seltenen Resistenzvarianten der Hepatitis C Virus NS3 Protease bei Patienten, die mit Telaprevir oder Boceprevir behandelt wurden. *Z Gastroenterol*. 2012; 50.
44. Wang C, Huang H, Valera L, Sun JH, O'Boyle DR 2nd, Nower PT, et al. Hepatitis C virus RNA elimination and development of resistance in replicon cells treated with BMS-790052. *Antimicrob Agents Chemother*. 2012; 56: 1350–1358. doi: [10.1128/AAC.05977-11](#) PMID: [22214777](#)
45. Wyles DL. Beyond telaprevir and boceprevir: resistance and new agents for hepatitis C virus infection. *Top Antivir Med*. 2012; 20: 139–145. PMID: [23154254](#)
46. Coburn CA, Meinke PT, Chang W, Fandozzi CM, Graham DJ, Hu B, et al. Discovery of MK-8742: an HCV NS5A inhibitor with broad genotype activity. *ChemMedChem*. 2013; 8: 1930–1940. doi: [10.1002/cmdc.201300343](#) PMID: [24127258](#)
47. Hernandez D, Zhou N, Ueland J, Monikowski A, McPhee F. Natural prevalence of NS5A polymorphisms in subjects infected with hepatitis C virus genotype 3 and their effects on the antiviral activity of NS5A inhibitors. *J Clin Virol*. 2013; 57: 13–18. doi: [10.1016/j.jcv.2012.12.020](#) PMID: [23384816](#)
48. Lawitz E, Mangia A, Wyles D, Rodriguez-Torres M, Hassanein T, Gordon SC, et al. Sofosbuvir for previously untreated chronic hepatitis C infection. *N Engl J Med*. 2013; 368: 1878–1887. doi: [10.1056/NEJMoa1214853](#) PMID: [23607594](#)
49. Lawitz E, Poordad F, Membreno FE, Hyland H, Ding X, Hebner C, et al. Once Daily Sofosbuvir/Ledipasvir Fixed Dose Combination with or without Ribavirin Resulted in ≥95% Sustained Virologic Response In Patients with HCV Genotype 1, Including Patients with Cirrhosis: the LONESTAR trial; 2013 November 1–5, 2013; Washington, DC, USA. pp. Abstract 215.
50. Lenz O, Vijgen L, Berke JM, Cummings MD, Fevery B, Peeters M, et al. Virologic response and characterisation of HCV genotype 2–6 in patients receiving TMC435 monotherapy (study TMC435-C202). *J Hepatol*. 2013; 58: 445–451. doi: [10.1016/j.jhep.2012.10.028](#) PMID: [23142061](#)
51. Wang C, Valera L, Jia L, Kirk MJ, Gao M, Fridell RA. In vitro activity of daclatasvir on hepatitis C virus genotype 3 NS5A. *Antimicrob Agents Chemother*. 2013; 57: 611–613. doi: [10.1128/AAC.01874-12](#) PMID: [23089758](#)

52. Afdhal N, Reddy KR, Nelson DR, Lawitz E, Gordon SC, Schiff E, et al. Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection. *N Engl J Med*. 2014; 370: 1483–1493. doi: [10.1056/NEJMoa1316366](https://doi.org/10.1056/NEJMoa1316366) PMID: [24725238](https://pubmed.ncbi.nlm.nih.gov/24725238/)
53. Wong KA, Worth A, Martin R, Svarovskaia E, Brainard DM, Lawitz E, et al. Characterization of Hepatitis C virus resistance from a multiple-dose clinical trial of the novel NS5A inhibitor GS-5885. *Antimicrob Agents Chemother*. 2013; 57: 6333–6340. doi: [10.1128/AAC.02193-12](https://doi.org/10.1128/AAC.02193-12) PMID: [23877691](https://pubmed.ncbi.nlm.nih.gov/23877691/)
54. Howe AY, Black S, Curry S, Ludmerer SW, Liu R, Barnard RJ, et al. Virologic resistance analysis from a phase 2 study of MK-5172 combined with pegylated interferon/ribavirin in treatment-naïve patients with hepatitis C virus genotype 1 infection. *Clin Infect Dis*. 2014; 59: 1657–1665. doi: [10.1093/cid/ciu696](https://doi.org/10.1093/cid/ciu696) PMID: [25266289](https://pubmed.ncbi.nlm.nih.gov/25266289/)
55. Lok AS, Gardiner DF, Hezode C, Lawitz EJ, Bourliere M, Everson GT, et al. Randomized trial of daclatasvir and asunaprevir with or without PegIFN/RBV for hepatitis C virus genotype 1 null responders. *J Hepatol*. 2014; 60: 490–499. doi: [10.1016/j.jhep.2013.10.019](https://doi.org/10.1016/j.jhep.2013.10.019) PMID: [24444658](https://pubmed.ncbi.nlm.nih.gov/24444658/)
56. McPhee F, Hernandez D, Zhou N, Yu F, Ueland J, Monikowski A, et al. Virological escape in HCV genotype-1-infected patients receiving daclatasvir plus ribavirin and peginterferon alfa-2a or alfa-2b. *Antivir Ther*. 2014; 19: 479–490. doi: [10.3851/IMP2729](https://doi.org/10.3851/IMP2729) PMID: [24448487](https://pubmed.ncbi.nlm.nih.gov/24448487/)
57. Murakami E, Imamura M, Hayes CN, Abe H, Hiraga N, Honda Y, et al. Ultradeep sequencing study of chronic hepatitis C virus genotype 1 infection in patients treated with daclatasvir, peginterferon, and ribavirin. *Antimicrob Agents Chemother*. 2014; 58: 2105–2112. doi: [10.1128/AAC.02068-13](https://doi.org/10.1128/AAC.02068-13) PMID: [24468783](https://pubmed.ncbi.nlm.nih.gov/24468783/)
58. Tong X, Le Pogam S, Li L, Haines K, Pisco K, Baronas V, et al. In vivo emergence of a novel mutant L159F/L320F in the NS5B polymerase confers low-level resistance to the HCV polymerase inhibitors mericitabine and sofosbuvir. *J Infect Dis*. 2014; 209: 668–675. doi: [10.1093/infdis/jit562](https://doi.org/10.1093/infdis/jit562) PMID: [24154738](https://pubmed.ncbi.nlm.nih.gov/24154738/)
59. Svarovskaia ES, Dvory-Sobol H, Parkin N, Hebner C, Gontcharova V, Martin R, et al. Infrequent development of resistance in genotype 1–6 hepatitis C virus-infected subjects treated with sofosbuvir in phase 2 and 3 clinical trials. *Clin Infect Dis*. 2014; 59: 1666–1674. doi: [10.1093/cid/ciu697](https://doi.org/10.1093/cid/ciu697) PMID: [25266287](https://pubmed.ncbi.nlm.nih.gov/25266287/)
60. Zeuzem S, Jacobson IM, Baykal T, Marinho RT, Poordad F, Bourliere M, et al. Retreatment of HCV with ABT-450/r-ombitasvir and dasabuvir with ribavirin. *N Engl J Med*. 2014; 370: 1604–1614. doi: [10.1056/NEJMoa1401561](https://doi.org/10.1056/NEJMoa1401561) PMID: [24720679](https://pubmed.ncbi.nlm.nih.gov/24720679/)
61. Chayama K, Hayes CN. HCV Drug Resistance Challenges in Japan: The Role of Pre-Existing Variants and Emerging Resistant Strains in Direct Acting Antiviral Therapy. *Viruses*. 2015; 7: 5328–5342. doi: [10.3390/v7102876](https://doi.org/10.3390/v7102876) PMID: [26473914](https://pubmed.ncbi.nlm.nih.gov/26473914/)
62. Donaldson EF, Harrington PR, O'Rear JJ, Naeger LK. Clinical evidence and bioinformatics characterization of potential hepatitis C virus resistance pathways for sofosbuvir. *Hepatology*. 2015; 61: 56–65. doi: [10.1002/hep.27375](https://doi.org/10.1002/hep.27375) PMID: [25123381](https://pubmed.ncbi.nlm.nih.gov/25123381/)
63. Jensen SB, Serre SB, Humes DG, Ramirez S, Li YP, Bukh J, et al. Substitutions at NS3 Residue 155, 156, or 168 of Hepatitis C Virus Genotypes 2 to 6 Induce Complex Patterns of Protease Inhibitor Resistance. *Antimicrob Agents Chemother*. 2015; 59: 7426–7436. doi: [10.1128/AAC.01953-15](https://doi.org/10.1128/AAC.01953-15) PMID: [26392503](https://pubmed.ncbi.nlm.nih.gov/26392503/)
64. Kati W, Koev G, Irvin M, Beyer J, Liu Y, Krishnan P, et al. In vitro activity and resistance profile of dasabuvir, a nonnucleoside hepatitis C virus polymerase inhibitor. *Antimicrob Agents Chemother*. 2015; 59: 1505–1511. doi: [10.1128/AAC.04619-14](https://doi.org/10.1128/AAC.04619-14) PMID: [25534735](https://pubmed.ncbi.nlm.nih.gov/25534735/)
65. Krishnan P, Beyer J, Mistry N, Koev G, Reisch T, DeGoey D, et al. In vitro and in vivo antiviral activity and resistance profile of ombitasvir, an inhibitor of hepatitis C virus NS5A. *Antimicrob Agents Chemother*. 2015; 59: 979–987. doi: [10.1128/AAC.04226-14](https://doi.org/10.1128/AAC.04226-14) PMID: [25451055](https://pubmed.ncbi.nlm.nih.gov/25451055/)
66. McPhee F, Suzuki Y, Toyota J, Karino Y, Chayama K, Kawakami Y, et al. High Sustained Virologic Response to Daclatasvir Plus Asunaprevir in Elderly and Cirrhotic Patients with Hepatitis C Virus Genotype 1b Without Baseline NS5A Polymorphisms. *Adv Ther*. 2015; 32: 637–649. doi: [10.1007/s12325-015-0221-5](https://doi.org/10.1007/s12325-015-0221-5) PMID: [26155891](https://pubmed.ncbi.nlm.nih.gov/26155891/)
67. Osinusi A, Townsend K, Kohli A, Nelson A, Seamon C, Meissner EG, et al. Virologic response following combined ledipasvir and sofosbuvir administration in patients with HCV genotype 1 and HIV co-infection. *JAMA*. 2015; 313: 1232–1239. doi: [10.1001/jama.2015.1373](https://doi.org/10.1001/jama.2015.1373) PMID: [25706232](https://pubmed.ncbi.nlm.nih.gov/25706232/)
68. Pilot-Matias T, Tripathi R, Cohen D, Gaultier I, Dekhtyar T, Lu L, et al. In vitro and in vivo antiviral activity and resistance profile of the hepatitis C virus NS3/4A protease inhibitor ABT-450. *Antimicrob Agents Chemother*. 2015; 59: 988–997. doi: [10.1128/AAC.04227-14](https://doi.org/10.1128/AAC.04227-14) PMID: [25451053](https://pubmed.ncbi.nlm.nih.gov/25451053/)
69. Schnell G, Tripathi R, Beyer J, Reisch T, Krishnan P, Lu L, et al. Hepatitis C Virus Genotype 4 Resistance and Subtype Demographic Characterization of Patients Treated with Ombitasvir plus

- Paritaprevir/Ritonavir. *Antimicrob Agents Chemother.* 2015; 59: 6807–6815. doi: [10.1128/AAC.01229-15](https://doi.org/10.1128/AAC.01229-15) PMID: [26282418](https://pubmed.ncbi.nlm.nih.gov/26282418/)
70. EMA DAKLINZA: SUMMARY OF PRODUCT CHARACTERISTICS. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/003768/WC500172848.pdf, Latest accessed: Dez 8, 2015
 71. EMA EXVIERA: SUMMARY OF PRODUCT CHARACTERISTICS. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/003837/WC500182233.pdf, Latest accessed: Dez 8, 2015
 72. EMA VIEKIRAX: SUMMARY OF PRODUCT CHARACTERISTICS. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/003839/WC500183997.pdf, Latest accessed: Dez 8, 2015
 73. EMA HARVONI: SUMMARY OF PRODUCT CHARACTERISTICS. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/003850/WC500177995.pdf, Latest accessed: Dez 8, 2015
 74. Kuiken C, Yusim K, Boykin L, Richardson R. The Los Alamos HCV Sequence Database. *Bioinformatics.* 2005; 21: 379–384. PMID: [15377502](https://pubmed.ncbi.nlm.nih.gov/15377502/)
 75. Qi X, Bae A, Liu S, Yang H, Sun SC, Harris J, et al. Development of a replicon-based phenotypic assay for assessing the drug susceptibilities of HCV NS3 protease genes from clinical isolates. *Antiviral Res.* 2009; 81: 166–173. doi: [10.1016/j.antiviral.2008.11.002](https://doi.org/10.1016/j.antiviral.2008.11.002) PMID: [19063924](https://pubmed.ncbi.nlm.nih.gov/19063924/)
 76. Sierra S, Kaiser R, Lubke N, Thielen A, Schuelter E, Heger E, et al. Prediction of HIV-1 coreceptor usage (tropism) by sequence analysis using a genotypic approach. *J Vis Exp.* 2011.
 77. Friebe P, Lohmann V, Krieger N, Bartenschlager R. Sequences in the 5' nontranslated region of hepatitis C virus required for RNA replication. *J Virol.* 2001; 75: 12047–12057. PMID: [11711595](https://pubmed.ncbi.nlm.nih.gov/11711595/)
 78. Schmitt M, Scrima N, Radujkovic D, Caillet-Saguy C, Simister PC, Friebe P, et al. A comprehensive structure-function comparison of hepatitis C virus strain JFH1 and J6 polymerases reveals a key residue stimulating replication in cell culture across genotypes. *J Virol.* 2011; 85: 2565–2581. doi: [10.1128/JVI.02177-10](https://doi.org/10.1128/JVI.02177-10) PMID: [21209117](https://pubmed.ncbi.nlm.nih.gov/21209117/)
 79. Lenz O, Verbinnen T, Fevery B, Tambuyzer L, Vijgen L, Peeters M, et al. Virology analyses of HCV isolates from genotype 1-infected patients treated with simeprevir plus peginterferon/ribavirin in Phase IIb/III studies. *J Hepatol.* 2015; 62: 1008–1014. doi: [10.1016/j.jhep.2014.11.032](https://doi.org/10.1016/j.jhep.2014.11.032) PMID: [25445400](https://pubmed.ncbi.nlm.nih.gov/25445400/)
 80. Sulkowski M, Ghalib R, Rodriguez-Torres M, Younossi Z, Corregidor A, Fevery B, et al. Once-daily simeprevir (TMC435) plus sofosbuvir (GS-7977) with or without ribavirin in HCV genotype 1 prior null responders with metavir F0-2: Cosmos study subgroup analysis. *J Hepatol.* 2014; 60: S4.
 81. Lawitz E, Rodriguez-Torres M, Younossi ZM, Corregidor A, Sulkowski MS, DeJesus E, et al. Simeprevir plus sofosbuvir with/without ribavirin in HCV genotype 1 prior null-responder/treatment-naive patients (Cosmos study): primary endpoint (SVR12) results in patients with metavir F3-4 (Cohort 2). *J Hepatol.* 2014; 60: S524.
 82. Fried MW, Buti M, Dore GJ, Flisiak R, Ferenci P, Jacobson I, et al. Once-daily simeprevir (TMC435) with pegylated interferon and ribavirin in treatment-naive genotype 1 hepatitis C: the randomized PIL-LAR study. *Hepatology.* 2013; 58: 1918–1929. doi: [10.1002/hep.26641](https://doi.org/10.1002/hep.26641) PMID: [23907700](https://pubmed.ncbi.nlm.nih.gov/23907700/)