Hepatology Publish Ahead of Print
DOI:10.1097/HEP.0000000000000379

Virological characterization of treatment failures and retreatment outcomes in patients infected with "unusual" HCV genotype 1 subtypes

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Keywords: hepatitis C virus; antiviral resistance; direct-acting antiviral drugs; unusual subtypes; retreatment.

FOOTNOTE PAGE

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List of abbreviations: HCV: hepatitis C virus; DAA: direct-acting antiviral; GT: genotype; SVR: sustained virological response; EASL: European Association for the Study of the Liver; RAS: resistance-associated substitution; IQR: interquartile range; EC50: effective concentration 50%.

Funding: National surveillance of HCV treatment failures and resistance to DAAs is funded by Santé publique France through the National Reference Center for Viral Hepatitis B, C and D. The EPIRES-C study has been funded by the Agence Nationale de Recherche sur le SIDA et les Hépatites Virales/Maladies Infectieuses Émergentes (ANRS/MIE).

Conflicts of interest disclosures:

Christophe Rodriguez consults, advises and is on the speakers' bureau for Illumina. Stéphane Chevaliez advises and/or is on the speakers' bureau for Abbott, Abbvie, Cepheid, Gilead, and Roche Diagnostics. Vincent Leroy consults, advises and/or is on the speakers' bureau for AbbVie and Gilead. He advises and/or is on the speakers' bureau for Alexion, Beker, Intercept, Mayoly, Pfizer, and Tillots. Slim Fourati consults for, advises, and/or is on the speakers' bureau for GSK, Cepheid, and AstraZeneca. Jean-Michel Pawlotsky advises and is on the speakers' bureau for AbbVie and Gilead. He advises Abbott, Arbutus, Assembly Biosciences, and GSK. The remaining authors have nothing to disclose.

Authors contributions: E.V.Q., J.-M.P., S.F., V.L.: conception of the study; E.V.Q.: data collection; E.V.Q., J.-M.P., S.F., V.L, M.N.: data analysis and interpretation; E.V.Q., A.S., M.N., C.R., S.C., V.L., S.F., J.-M.P.: drafting and editing of the manuscript. The authors declare that they have seen and approved the final version of the manuscript.

Graphical abstract

Abstract

Background & Aims: Suboptimal rates of sustained virological response (SVR) have been reported in patients infected with an "unusual", non-1a/1b hepatitis C virus (HCV) genotype 1 subtype. The objectives of this study were to assess the proportion of non-1a/1b genotype 1 subtypes in a population of HCV-infected patients who failed to achieve SVR after first-line direct-acting antiviral treatment, to virologically characterize their failures, and to assess their outcomes on retreatment.

Approach and Results: Samples addressed between January 2015 and December 2021 to the French National Reference Center for Viral Hepatitis B, C and D were prospectively analyzed by means of Sanger and deep sequencing. Among 640 failures, 47 (7.3%) occurred in patients infected with an "unusual" genotype 1 subtype. Samples were available in 43 of them; 92.5% of these patients were born in Africa. Our results show the presence at baseline and at treatment failure of NS3 protease and/or NS5A polymorphisms conferring inherent reduced susceptibility to DAAs in these patients, together with the presence at failure of additional RASs not naturally present as dominant species, but jointly selected by first-line therapy.

Conclusions: Patients infected with "unusual" HCV genotype 1 subtypes are overrepresented among DAA treatment failures. Most of them were born and likely infected in sub-Saharan Africa. "Unusual" HCV GT-1 subtypes naturally carry polymorphisms that confer reduced susceptibility to the drugs currently used to cure hepatitis C, in particular the NS5A inhibitors. Retreatment with sofosbuvir plus an NS3 protease and an NS5A inhibitor is generally efficacious.

Introduction

Hepatitis C virus (HCV) infection remains a major public health threat, with 58 million people chronically infected worldwide, i.e. approximately 0.7% of the world population, and approximately 1.5 million new infections each year (1). Chronic HCV infection is responsible for 19% of hepatocellular carcinoma and 21% of cirrhosis cases worldwide, making it a leading cause of liver-related mortality (2,3). The global prevalence of chronic HCV infections has decreased by approximately 7 million since 2015, as a result of the approval of combinations of direct-acting antiviral (DAA) agents and broadening access to these therapies (1). Numerous clinical trials and real-world studies, performed in high-income countries, have demonstrated the safety, tolerability and high efficacy of pangenotypic DAA regimens (4–8). Virological failures (most often post-treatment relapses) are rare with these therapies. In this context, the World Health Organization set the goal to eliminate hepatitis C as a major public health threat by 2030, i.e. to reduce the incidence of new infections by 90%, treat 80% of diagnosed patients and reduce HCV-related mortality by 65% (9).

Eight HCV genotypes and nearly 100 subtypes have been identified thus far. HCV genotype 1 (GT-1) predominates, accounting for 44% of all infections. GT-1 most frequent subtypes, GT-1a and GT-1b, are highly prevalent in Europe, the Americas and Asia (10). Numerous studies have confirmed the efficacy of HCV DAAs in patients infected with GT-1a and GT-1b (6,11). In contrast, little information is available on the response to DAA therapy in patients infected with one of the 12 known non-1a/1b subtypes (GT-1c to 1n) or with an undetermined subtype of GT-1. Such information would be particularly useful, because GT-1 non-1a/1b subtypes, which are considered “unusual” in Europe or North America, are common in regions of West Africa where they represent 2% to 82% of all GT-1 infections, as well as in patients born in these regions who now live in high-income areas (10,12).

Suboptimal rates of sustained virological response (SVR) have been recently reported in patients of African origin living in London who were infected with an “unusual”, non-1a/1b

HCV GT-1 subtype (13). Indeed, only 75% of them achieved SVR, contrasting with a 100% SVR rate in patients infected with GT-1a or GT-1b. Very few clinical trials with new DAAs have been conducted in Africa, and data are lacking as to treatment responses and patterns of treatment failure and viral resistance in subjects infected with non-1a/1b GT-1 subtypes. The objectives of this study were: (i) to assess the proportion of non-1a/1b GT-1 subtypes in a population of DAA-treated HCV-infected patients living in France who failed to achieve SVR; (ii) to virologically characterize their treatment failures; and (iii) to assess their outcomes on retreatment. In this report, we will use the term "unusual GT-1 subtype" to characterize non-1a/1b subtypes of GT-1, as was done in a previous study (13).

Patients and Methods

Patients

The French National Reference Center for Viral Hepatitis B, C and D at Henri Mondor University Hospital receives samples from patients with HCV infection who failed to achieve SVR from a large number of laboratories across the country for genotype/subtype determination, genotypic resistance testing and advice on retreatment. We retrospectively analyzed data from all patients who failed an NS5A inhibitor-based treatment regimen prospectively included in the National Reference Center cohort between January 2015 and December 2021 and selected those who were infected with an "unusual" GT-1 subtype for the present study. Demographic and clinical data were collected. The choice of the hepatitis C retreatment was guided by the Final Update of the European Association for the Study of the Liver (EASL) Recommendations on Treatment of Hepatitis C (14) and retreatment outcomes were collected when available.

Study design

When received at the National Reference Center, the samples were prospectively analyzed by means of Sanger sequencing for: (i) HCV genotype/subtype determination; (ii) assessment of the presence of HCV resistance-associated substitutions (RAS) in the 3 target regions of currently approved HCV DAAs, including the NS3 protease, the NS5A protein domain I and the NS5B polymerase regions. Sanger sequencing was performed in all cases at treatment failure and, when available, at treatment baseline. Whenever possible, the samples were also analyzed by means of deep sequencing using shotgun metagenomics generating full-length genome sequences. RASs were interpreted according to the 2020 EASL Recommendations on Treatment of Hepatitis C, at the following positions: NS3 protease positions 56, 80, 155, 156, and 168; NS5A protein domain I positions 24, 28, 30, 31, 58, 92, and 93; NS5B polymerase positions 159, 282, 316, 320, and 321 (14). Reference GT-1a sequence NC004102 was used for comparisons. Then, a reference sequence for each different GT-1 subtype was used for comparison within each subtype group.

HCV genotype/subtype determination

The HCV genotype and subtype were determined by means of our in-house Sanger sequencing technique targeting the NS5B gene followed by phylogenetic analysis, the reference method for HCV genotype determination (15). Phylogenetic analysis was carried out by means of the Phylogeny Inference Package (PHYLIP; version 3.695), using genotype 1-8 subtype reference sequences available in GenBank. Nucleotide sequences (positions 724-1,009 according to the H77-1a prototype strain) obtained by Sanger sequencing were aligned

with reference sequences using CLUSTAL W (16). Phylogenetic relationships were deduced by means of DNADIST-NEIGHBOR from PHYLIP. For neighbor-joining analysis, a Kimura two-parameter distance matrix with a transition/transversion ratio of 2.0 was used (17). Phylogenetic trees were plotted with FigTree v1.4 (18). Their robustness was assessed by bootstrap analysis of 1,000 replicates by means of the SEQBOOT program from PHYLIP.

Sanger sequencing of the 3 target regions

Briefly, HCV RNA was extracted with the QIASymphony DSP Virus/Pathogen kit in a QIASymphony device (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Complementary DNA synthesis was performed with the OneStep RT-PCR kit (Qiagen) with sets of primers adapted to the viral regions targeted (19). Nested PCR was then performed with pangenotypic primers specific for each region amplified and subsequently sequenced (Supplementary Table 1 http://links.lww.com/HEP/F20). PCR products were purified with Amicon Ultra-0.5 mL Centrifugal Filters (EMD Millipore, Darmstadt, Germany) and sequenced with the BigDye Terminator Cycle Sequencing Kit v3.1 on an ABI Prism 3730 DNA Analyzer (Applied Biosystems, Foster City, California). Nucleotide sequences were manually corrected and aligned with genotype 1a or 1b reference sequences, respectively, and amino acid changes were deduced from the nucleotide sequence.

Deep sequencing of full-length genomes by means of shotgun metagenomics

An original in-house method based on shotgun metagenomics using deep sequencing and MetaMIC software was used to characterize full-length HCV genome sequences, as already described (20). Briefly, after pretreatment with a combination of mechanical, enzymatic and

chemical fragmentations, RNA was extracted by means of the DNA midi kit in a QiaSymphony device (Qiagen). The quality and amount of extracted nucleic acids were verified (Tape Station, Agilent, Santa Clara, California, and Quant-it, ThermoFisher Scientific, Waltham, Massachusetts, respectively). Sequencing used the TruSeq Total RNA kit (Illumina, San Diego, California) after automated library preparation in a MicroLab StarLet device (Hamilton Robotics, Reno, Nevada). Twelve-sample multiplexing was performed to allow for minor variant detection. Sequencing was run on a NextSeq500 device with sequencing kit High Output 2*150 (Illumina). Sequences were analyzed by means of our in-house software MetaMIC, developed for bioanalysis of microbiological big data generated by metagenomics. MetaMIC uses different modules for demultiplexing, quality check, dusting, pair-end long sequence identification using several different databases, genotyping and mapping of viral sequences with the best reference sequence, variant calling, and estimation of the rates of nucleotide or amino acid changes. The cutoffs for detection of minority variants were set at 1% and also at 15% (equivalent to Sanger sequencing), as is usually the case for deep sequencing studies of viral quasispecies. All sequences are accessible at GenBank under accession number BankIt2638897.

Ethical aspects

The study was conducted in accord with the Declaration of Helsinki and French law for biomedical research and was approved by our institutional review board (Registration number: Mondor IRB082018).

Results

Characteristics of the patients infected with an "unusual" GT-1 subtype who failed to achieve SVR after receiving an NS5A inhibitor-containing treatment

Between January 2015 and December 2021, samples taken at the time of relapse in 640 patients who failed to achieve SVR after receiving an NS5A inhibitor-containing regimen were analyzed at the French National Reference Center for Viral Hepatitis B, C and D at Henri Mondor University Hospital. Among these 640 patients, 285 (44.5%) were infected with GT-1, including 142/640 (22.2%) with subtype 1a and 96/640 (15.0%) with subtype 1b. The remaining 47 patients (7.3%) were infected with an "unusual" GT-1 subtype, including: 1d (n=8), 1e (n=13), 1f (n=1), 1g (n=2), 1i (n=2), 1k (n=1), 1l (n=18) and 1-undetermined (n=2) (Figure 1A). Biological samples were not available for 4 patients, including 2 infected with GT-1e and 2 with GT-1l. The remaining 43 patients were studied.

The characteristics of these 43 patients, infected with an "unusual" GT-1 subtype and who failed to achieve SVR after receiving an NS5A inhibitor-containing treatment, are shown in Table 1. Thirty-seven of the 40 patients (92.5%) for which the country of birth was known were born in Africa, including 17/40 (42.5%) in Cameroon. The distribution of the "unusual" GT-1 subtypes according to the patients' countries of birth is shown in Figure 1B. The median age of the patients was 61 years (interquartile range (IQR): 54-65) and the male/female ratio was 0.51. Two of the 43 patients (4.7%) were coinfected with HIV and 9/43 (20.9%) had cirrhosis (Table 1). All patients relapsed after receiving a full course of an NS5A inhibitor-containing DAA regimen. The treatments received are shown in Table 1.

Sequencing results

A sample taken at treatment failure was available for all of the 43 patients infected with an "unusual" GT-1 subtype. An additional sample taken at baseline of initial treatment was available in 6 patients. Sequence determination was based on Sanger sequencing, deep sequencing, or both. Polymorphisms at known RAS positions are shown for each patient in Table 2. Results generated by Sanger and deep sequencing matched at a deep sequencing cutoff of 15%, while deep sequencing with a 1% cutoff was more sensitive than Sanger for the detection of polymorphisms and RASs (Table 2). Supplementary Table 2 http://links.lww.com/HEP/F20 shows the same information as Table 2 according to the DAA regimen received.

Table 3 shows the most frequent amino acids present at known RAS positions in reference sequences from "unusual" GT-1 subtypes represented in the study. No reference sequences spanning the three DAA-targeted regions were found in international databases for GT-1f. Data in Table 2 are presented accordingly, with identification of: (i) polymorphisms found at treatment baseline or failure in our patients that should be considered as potential RASs relative to the reference GT-1a sequence, and (ii) polymorphisms likely conferring inherent DAA resistance to the corresponding GT-1 subtype.

HCV RNA sequences and RASs at baseline of initial treatment in patients with "unusual" GT-1 subtypes (n=6)

At baseline, Q80L in the NS3 protease region was found in 4 of the 6 patients infected with an "unusual" GT-1 subtype who subsequently failed to achieve SVR (Table 2). None of them had been previously exposed to an NS3 inhibitor. Three of these 4 patients were infected with GT-1l, for which an L at position 80 conferring inherent reduced susceptibility to NS3 protease inhibitors is usual (Table 3). The remaining patient was infected with an

undetermined GT-1 subtype, for which the frequency of an L at this position cannot be established.

In the NS5A region, 2 to 4 polymorphisms known to be associated with reduced susceptibility to NS5A inhibitors were present as dominant species (>99%) at baseline in 5 of the 6 patients by deep sequencing, including 3 infected with GT-1l, 2 with GT-1e and 1 with GT-1-undetermined (Table 2). The NS5A polymorphisms present were K24R (n=3), Q30R (n=1), L31M (n=3), H58P (n=5) and A92T (n=2). As shown in Table 3, polymorphisms that confer reduced susceptibility to NS5A inhibitors are naturally present in GT-1e (L31M and A92T) and in GT-1l (L31M and H58P).

No polymorphisms possibly conferring reduced susceptibility to NS5B polymerase inhibitors were detected in the 6 patients at baseline.

HCV RNA sequences and RASs at failure according to the initial treatment regimen in patients with "unusual" GT-1 subtypes (n=43)

Eight of the 43 patients had been treated with both an NS3 protease and an NS5A inhibitor (plus the allosteric NS5B inhibitor dasabuvir in one case, plus the nucleotide analogue NS5B inhibitor sofosbuvir in one case), including 6 infected with GT-1e, 1 with GT-1g and 1 with GT-1k (Tables 1 and 2). These patients harbored a combination of 1 to 4 dominant substitutions at NS5A RAS positions at treatment failure, including K24R (n=2), M28T (n=1), Q30R/D (n=3), L31M (n=2), H58P (n=3), A92T (n=5) and Y93H/C (n=2). L31M and A92T are naturally present in GT-1e, Q30R and Y93F in GT-1g, and M28A and H58P in GT-1k (Table 3). The NS3 protease RAS Q80K was present at failure in one case with GT-1k infection (Table 2).

Thirty-five patients had been treated with the nucleotide analogue NS5B polymerase inhibitor sofosbuvir in combination with an NS5A inhibitor (ledipasvir, daclatasvir, velpatasvir or elbasvir) (Tables 1 and 2). These patients were all infected at failure with viruses highly resistant to NS5A inhibitors. Indeed, using the GT-1a sequence as a reference (14), 34/35 (97.1%), 39/35 (82.9%), 16/35 (45.7%) and 4/35 (11.4%) patients harbored 1, 2, 3 or 4 NS5A RASs at treatment failure, respectively. The median numbers of RASs at treatment failure did not differ according to the "unusual" GT-1 subtype: 3 for GT-1d (IQR: 2-3); 4 for GT-1e (IQR: 3-4); 2 for GT-1f (IQR: 2-2); 3 for GT-1g (IQR: 3-3); 2 for GT-1i (IQR: 1-2); 2 for GT-1l (IQR: 2-3); and 2 for GT-1-undetermined (IQR: 2-2) (Figure 2A). Figure 2B shows the frequency of the different NS5A RAS patterns according to the "unusual" GT-1 subtype. Most patients harbored NS5A RASs at positions 31, 58 and 93 at treatment failure, irrespective of the GT-1 subtype. The GT-1g sequence harbored NS5A RASs at positions K24, H58 and Y93, while the two GT-1-undetermined ones harbored NS5A RASs at positions L31 and H58. The GT-1f sequence harbored NS5A RASs at positions 31 and 93 at failure. In the 16 GT-1l-infected patients, NS5A RASs were mainly at positions 31 and 58. Table 3 shows the polymorphisms at RAS positions naturally present in the different GT-1 subtypes represented in this group: Q30R, L31M and H58P for GT-1d; L31M and A92T for GT-1e; Q30R and Y93F for GT-1g; K24Q, Q30R and H58P for GT-1i; M28A and H58P for GT-1k; and L31M and H58P for GT-1l. Of note, no relevant reference sequence was found for GT-1f in GenBank.

Nineteen of the 43 patients (44.1%) had at least one RAS in the NS3 protease region at treatment failure, at position 80 in 16/43 (37.2%) cases and D168 in 3/43 (7.0%) cases. As shown in Table 3, Q80K is naturally present in GT-1d and Q80L in GT-1l, whereas D168 is conserved across the different GT-1 subtypes.

One NS5B polymerase RAS (C316Y) was present at failure in a GT-1e-infected patient who received dasabuvir.

Sequence changes between baseline and failure in 5 patients with samples available at both time points characterized by deep sequencing

Figure 3 shows the proportion of RASs detected by deep sequencing in both the NS3 protease and NS5A regions in 5 patients at baseline of initial treatment and at the time of failure. In 3 patients (Figures 3A, 3B and 3C), NS3 protease and NS5A RAS profiles did not change between baseline and treatment failure. In the remaining 2 patients (Figures 3D and 3E), an NS5A RAS not present at baseline (Y93F) was selected by therapy, as a minority variant (20%) in one case, as a majority variant (>99%) in the other case.

Retreatment

As shown in Table 2, 3/43 (7.0%) patients were not retreated due to comorbidities, while 26/43 (60.5%) completed DAA retreatment and 12-week post-treatment follow-up. All of them achieved SVR, except one who had been suboptimally retreated with a combination of sofosbuvir and daclatasvir. All patients treated with a triple DAA combination or with the combination of glecaprevir and pibrentasvir achieved SVR.

Discussion

We report here a large cohort of patients infected with “unusual” GT-1 subtypes who failed to achieve SVR to DAA therapy in the real-world, tertiary referral setting of the French National Reference Center for Viral Hepatitis B, C and D. Most of these patients were born

in Africa and most likely infected in their country of origin. More than 85% of them were infected with GT-1 subtypes known to naturally carry NS3 and/or NS5A polymorphisms at RAS positions that confer reduced susceptibility to NS3 protease or NS5A inhibitors, respectively. Overall, most patients infected with "unusual" GT-1 subtypes in our study harbored viruses carrying at least 2 NS3 and/or NS5A RASs at treatment failure. Most of these NS3 and/or NS5A RASs were preexistent as subtype-specific polymorphisms when baseline samples were studied in the same patients. Our results confirm the presence at baseline and at treatment failure of NS3 protease and/or NS5A polymorphisms conferring inherent reduced susceptibility to DAAs in patients infected with a variety of "unusual" GT-1 subtypes, together with the presence at failure of additional RASs not naturally present as dominant species, but jointly selected by first-line therapy.

The proportion of "unusual", non-1a/non-1b subtypes among GT-1-infected patients who failed to achieve SVR after an NS5A inhibitor-based therapy was substantially higher in this study than their prevalence in the French population of HCV-infected patients in a recent nationwide multicenter study (7.3% *vs* 0.8%, respectively) (21). This indicates that patients infected with these rare-in-Europe subtypes are more likely to fail DAA therapy than patients infected with the most frequent GT-1 subtypes, i.e. subtypes 1a and 1b.

Information on the worldwide epidemiology of "unusual" GT-1 subtypes is limited. In a recent study performed in a referral center in London, United Kingdom, "unusual" (GT-1e, -1g, -1h, -1l, -1p) or undetermined GT-1 subtypes were found in 63% of patients born in Africa, *vs* 3% of non-African patients, while SVR to sofosbuvir plus an NS5A inhibitor was observed in only 75% of them (13). In a Dutch Nationwide Cohort Study, the majority of patients with non-usual HCV subtypes was infected with "unusual" GT-2 (41%) or GT-4 (31%) subtypes, whereas "unusual" GT-1 subtypes (GT-1c, -1d, -1g) represented only 12% of cases (22). SVR was achieved in all patients infected with an "unusual" GT-1 subtype in

this study. Discrepancies in the epidemiology of "unusual" GT-1 subtypes likely reflect differences in the countries and regions of origin of migrant populations in different European countries (22).

The diversity of GT-1 subtypes that circulate in Western and Central Africa is far greater than that in patients infected in Western Europe or North America. This is not surprising, because Africa is the region where these subtypes initially emerged, diversified and spread. GT-1 subtypes 1a and 1b have been more recently exported from Africa to Europe and North America where they widely spread and became dominant (23). For instance, in Cameroon, subtypes 1e, 1h and 1l have been reported to account for more than 80 % of HCV infection cases (24). Because approximately 14% of HCV-infected patients are believed to live in the WHO Africa Region, the actual prevalence and incidence of infections with "unusual" GT-1 subtypes is probably largely underestimated.

In the context of sustained immigration from sub-Saharan Africa to Western Europe and other industrialized destinations, the prevalence of HCV infections with "unusual" GT-1 subtypes is likely to increase, in parallel to the progressive decrease in proportion of genotypes and subtypes thus far endemic in these regions, as a result of HCV elimination initiatives. Currently, most of the newly diagnosed HCV cases in our center are in patients born outside of France, essentially in sub-Saharan Africa. Thus, treatment failures remain likely to occur in patients infected with "unusual" GT-1 subtypes inherently resistant to NS5A inhibitors over the coming years, despite the overall decrease of the HCV infection prevalence.

The majority of patients in our study, like in previous reports (13,22,25), had been treated with older generations of HCV DAAs than those currently used as first-line therapy (i.e. sofosbuvir/velpatasvir or glecaprevir/pibrentasvir). Indeed, sofosbuvir/ledipasvir is known to be less efficacious against "unusual" HCV subtypes than more recent pangenotypic

combinations. This is due to the pre-existence, in several GT-1 and GT-4 subtypes (for example GT-4r), of NS5A polymorphisms at RAS positions that confer substantially reduced susceptibility to ledipasvir (5,6,20,26). Importantly, 5 patients in our study failed to achieve SVR despite the first-line use of a pangenotypic regimen: 2 patients who received sofosbuvir/velpatasvir (one with GT-1f and one with GT-1l) and 3 who received glecaprevir/pibrentasvir (two with GT-1e and one with GT-1k). Table 4 summarizes phenotypic data available in the literature describing, when available, the fold-shifts in effective concentrations 50% (EC50) and the alterations in replicative fitness conferred, in a GT-1a backbone, by the RASs or combinations of RASs observed in these 5 patients at treatment failure (27–29).

Because simplification of therapy is essential to achieve the worldwide goal to eliminate hepatitis C as a major public health threat, first-line treatment with pangenotypic regimens should be initiated without knowledge of the HCV genotype and subtype in most instances (14). Nevertheless, as indicated in the EASL Recommendations on Treatment of Hepatitis C, in settings where HCV genotype and subtype determination are available and affordable and would not limit access to care, [accurate genotype/subtyping by means of sequencing] remains useful to optimize the virological results of HCV therapy". This is the case in tertiary referral centers like ours for patients born in Africa or Asia found to be infected with GT-1l, GT-4r, GT-3b, GT-3g, GT-6u, GT-6v or any other subtype naturally harboring one or several NS5A RAS(s), who can be treated first-line with the triple combination of sofosbuvir/velpatasvir/voxilaprevir in order to optimize the likelihood to achieve an SVR, as recommended by EASL (14).

Only one patient in our study (Patient 23) failed to achieve SVR after retreatment with 12 weeks of sofosbuvir and daclatasvir. The reason for failure in this noncirrhotic patient already exposed to simeprevir and daclatasvir was most likely the inadequacy of the

retreatment strategy, with re-exposure to the same suboptimal NS5A inhibitor that initially failed. To our knowledge, no data have been published on the efficacy of retreatment with sofosbuvir/velpatasvir/voxilaprevir or glecaprevir/pibrentasvir with or without sofosbuvir in patients with "unusual" GT-1 subtype infection who failed first-line DAA therapy. High SVR rates have been reported in patients infected with "usual" HCV genotypes/subtypes in several studies, with no or modest impact of the presence of RASs at retreatment start on virological response (30–33). RASs Y93H and C316N have been suggested to be associated with sofosbuvir/velpatasvir/voxilaprevir failures (34), but this was not the case in our study. Overall, our results suggest that retreatment with sofosbuvir/velpatasvir/voxilaprevir or with glecaprevir/pibrentasvir with or without sofosbuvir is efficacious in the vast majority of patients infected with "unusual" GT-1 subtypes who failed first-line therapy.

The utility of resistance testing by means of sequencing of the drug target region(s) prior to retreatment in patients who failed first-line therapy remains debated. The EASL Recommendations on Treatment of Hepatitis C indicate that "HCV resistance testing prior to retreatment in patients who failed after any of the DAA-containing treatment regimens is useful to guide retreatment by probabilities of response, according to the resistance profile observed"(14). Our results suggest that resistance testing is dispensable in most retreatment cases, based on the high probability of SVR with any pangenotypic triple combination. Nevertheless, our tertiary referral team routinely uses resistance testing prior to retreatment to guide decisions in difficult-to-retreat patients with complex RAS profiles (preference for glecaprevir-based regimens, addition of ribavirin, and/or prolongation of retreatment duration up to 16 or 24 weeks).

The limitations of our study are the size of the cohort, as DAA failures have become rare in general and "unusual" GT-1 subtypes have a low prevalence in the general population of HCV-infected individuals in our area. The incidence of treatment failure in patients infected

with “unusual” GT-1 subtypes could have been overestimated, because most patients in our study had been treated with older generations of DAAs than currently used pangenotypic combinations (in particular, many patients received sofosbuvir/ledipasvir). Baseline samples were available in only a small number of patients because the selection process was through post-treatment failure sequencing. We could not determine whether RASs persisted in the absence of long-term follow-up in our patients. Nevertheless, a recent real-world study performed in Germany indicated that low- to medium-level RASs generally persist after relapse, whereas high-level RASs tend to disappear over time (35). Finally, our study brings no information on the global SVR rate in patients infected with “unusual” GT-1 subtypes failing first-line therapy. Importantly, retreatment with either a triple combination of an NS3 protease inhibitor, an NS5A inhibitor and an NS5B polymerase inhibitor or glecaprevir/pibrentasvir was successful in 100% of cases. It is therefore likely that treatment failures will be less frequent in such patients with the most recent generations of pangenotypic drugs used first-line, but this remains to be demonstrated.

In summary, patients infected with “unusual” HCV GT-1 subtypes are overrepresented among DAA treatment failures relative to their actual prevalence in the infected population in the Northern hemisphere. Most of them were born and probably infected in sub-Saharan Africa. “Unusual” HCV GT-1 subtypes naturally carry polymorphisms that confer reduced susceptibility to the drugs currently used to cure hepatitis C, in particular the NS5A inhibitors. Our results emphasize the need to identify these subtypes after treatment failure and propose a retreatment strategy adapted to the subtype and RAS profile, as indicated in the 2020 EASL Recommendations on Treatment of Hepatitis C.

Acknowledgments

We are grateful to Christine Silvain (Centre Hospitalier Universitaire, Poitiers, France), Aurore Baron (Centre Hospitalier Sud-Francilien, Corbeil-Essonnes, France), Jean-Michel Molina and Caroline Lascoux-Combe (Hôpital Saint-Louis, Paris, France), Guy Bellaiche (Centre Hospitalier Intercommunal Robert Ballanger, Aulnay-sous-Bois, France), Sophie Hillaire (Hôpital Foch, Suresnes, France), Frédéric Mechai (Hôpital Avicenne, AP-HP, Bobigny, France), Suna Bakan and Pierre Leroy (Hôpital Lariboisière, AP-HP, Paris, France), Hélène Labadie (Centre Hospitalier, Saint-Denis, France), Martine Escure (Centre Hospitalier, Roubaix, France), Pierre-François Sandrine (Centre Hospitalier Universitaire, Fort-de-France, Martinique, France), and Anty Rodolphe (Centre Hospitalier Universitaire, Nice, France) for their help in collecting missing clinical information.

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Figure 1. **(A)** Distribution of "unusual" GT-1 subtypes among the 47 patients infected with HCV non-1a/1b GT-1 who failed to achieve SVR after a full course of an NS5A inhibitor-containing regimen between January 2015 and December 2021

. **(B)** Countries of origin of the 43 patients infected with an "unusual" GT-1 subtype who failed to achieve SVR after a full course of an NS5A inhibitor-containing regimen with blood samples available.

Figure 1A

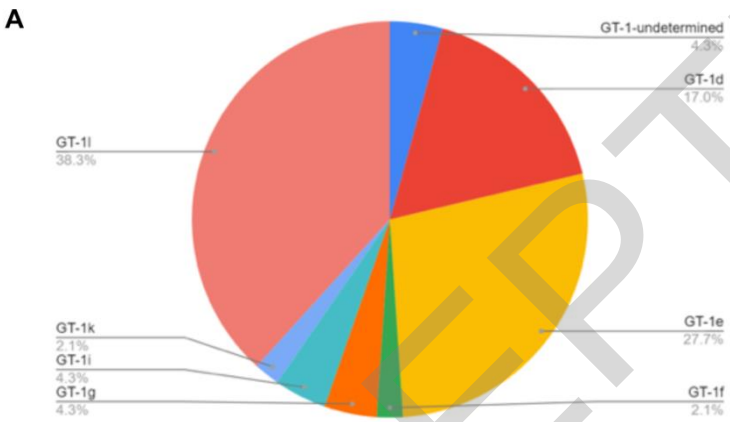

Figure 1B

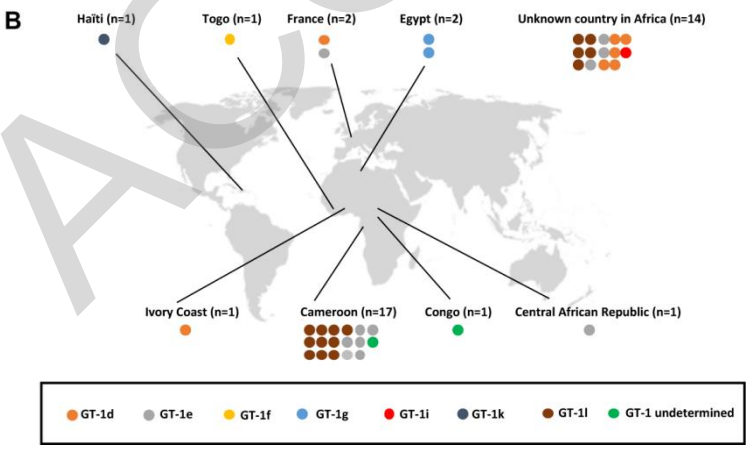

Figure 2. (A) Number of NS5A RASs at treatment failure according to the "unusual" HCV GT-1 subtype in the 35 patients exposed to a combination of the nucleotide analogue NS5B polymerase inhibitor sofosbuvir and an NS5A inhibitor. **(B)** Frequency of the different NS5A RAS patterns observed at treatment failure according to the "unusual" HCV GT-1 subtype in the 35 patients exposed to a combination of the nucleotide analogue NS5B polymerase inhibitor sofosbuvir and an NS5A inhibitor.

Figure 2A

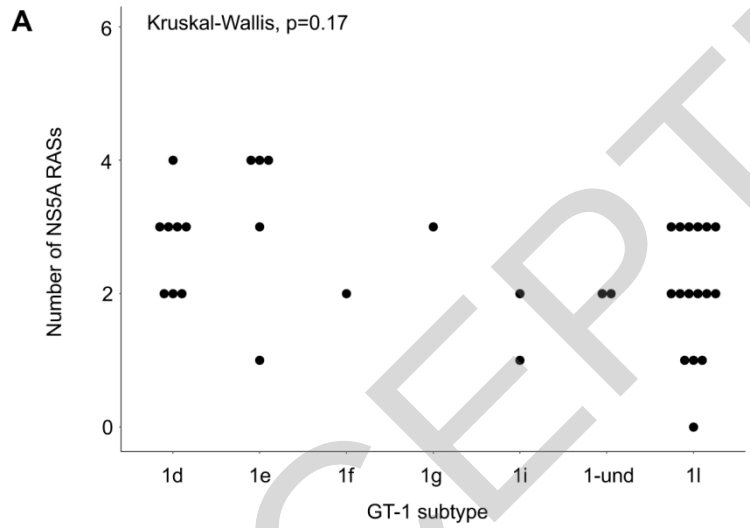

Figure 2B

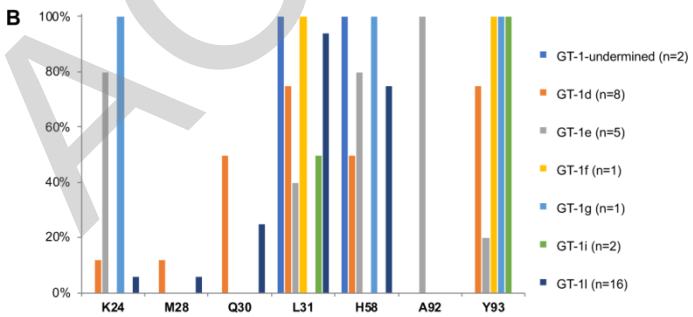

Figure 3. Full-length HCV genome sequence analysis by means of deep sequencing of baseline and treatment failure samples in patients with both time points available. The NS3 protease and NS5A regions are shown using a 1% deep sequencing cut-off for polymorphism/RAS detection at both baseline and treatment failure. **(A)** Patient 40, GT-1l treated with sofosbuvir/velpatasvir for 12 weeks. **(B)** Patient 41, GT-1e treated with glecaprevir/pibrentasvir for 4 weeks. **(C)** Patient 42, GT-1l treated with sofosbuvir/ledipasvir for 24 weeks. **(D)** Patient 39, GT-undetermined treated with sofosbuvir/ledipasvir for 12 weeks. **(E)** Patient 38, GT-1e treated with grazoprevir/elbasvir for 8 weeks. The NS5B region is not shown because no polymorphisms or amino acid changes were observed. Amino acid positions without polymorphisms in NS3 and/or NS5A are not shown. Polymorphism/RAS positions are indicated by vertical black bars. The proportion of RAS is indicated in the circles when present.

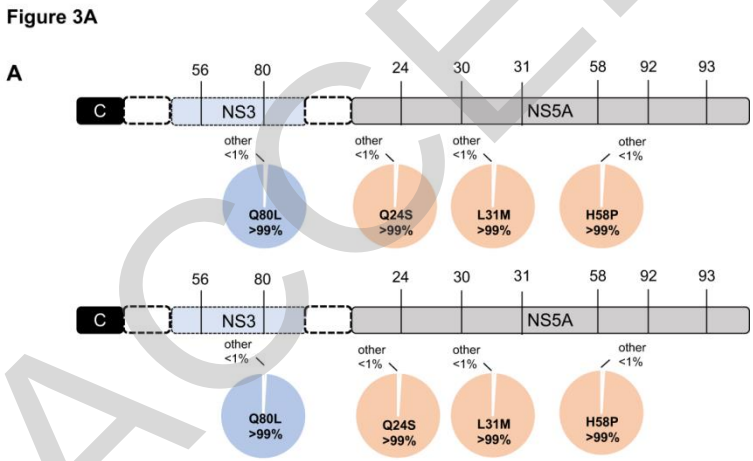

Figure 3B

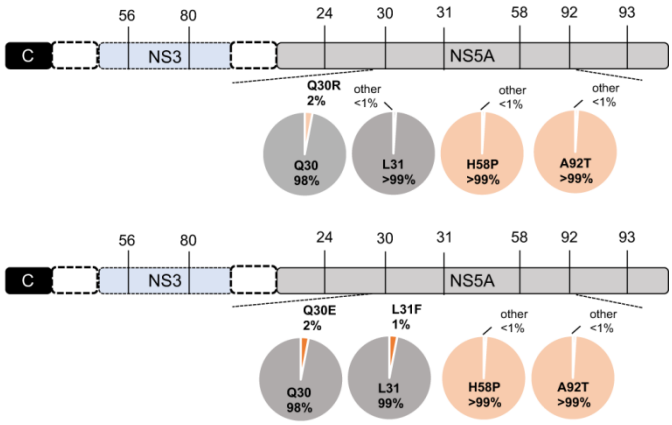

Figure 3C

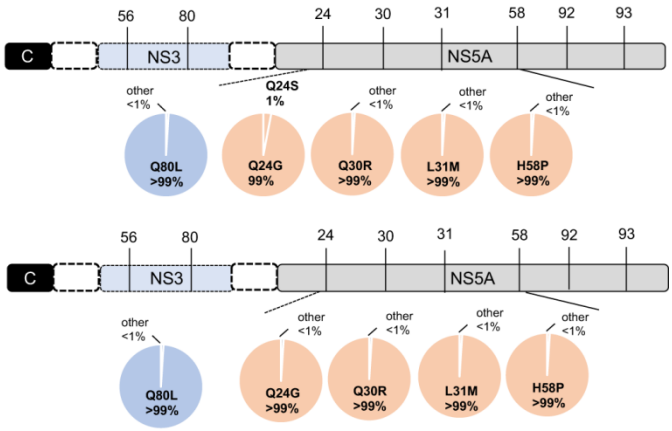

Figure 3D

D

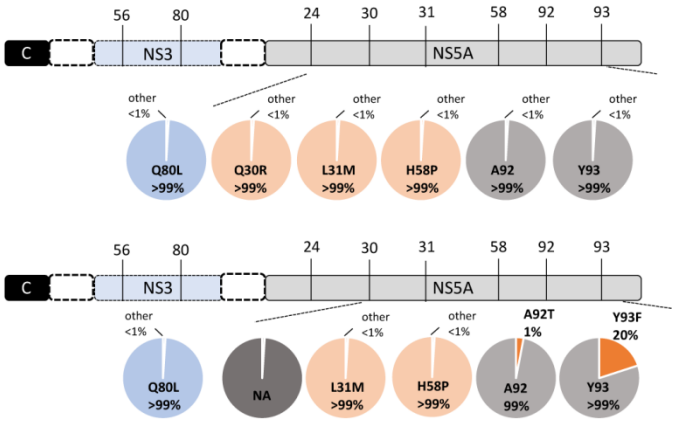

Figure 3E

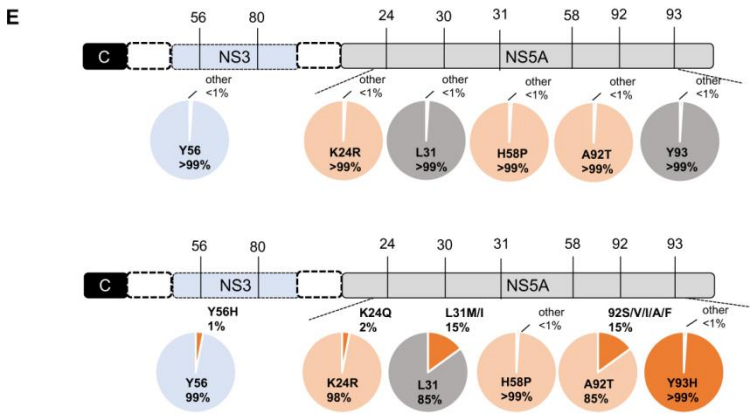

Table 1. Characteristics of the 43 patients infected with an "unusual" GT-1 subtype who failed to achieve SVR after receiving a full course of an NS5A inhibitor-containing treatment regimen.

Parameters	**N=43**
Age, median (IQR)	61 (54-65)
Male sex, n/N (%)	22/43 (51.2%)
African birth, n/N (%)	37/40 (92.5%)
HIV-positive, n/N (%)	2/43 (4.7%)
Cirrhosis, n/N (%)	9/43 (20.9%)
Failed DAA regimen received, n/N (%)	
- NS5B inhibitor + NS5A inhibitor	35/43 (81.4%)
Sofosbuvir/ledipasvir ± ribavirin	32/43 (74.4%)
Sofosbuvir/velpatasvir	2/43 (4.7%)
Sofosbuvir + daclatasvir	1/43 (2.3%)
- NS3 protease inhibitor + NS5A inhibitor	6/43 (14.0%)
Ombitasvir/paritaprevir/ritonavir + ribavirin	1/43 (2.3%)
Glecaprevir/pibrentasvir	3/43 (7.0%)
Grazoprevir/elbasvir	1/43 (2.3%)
Simeprevir + daclatasvir	1/43 (2.3%)
- NS3 protease inhibitor + NS5A inhibitor + NS5B inhibitor	2/43 (4.7%)
Ombitasvir/paritaprevir/ritonavir + dasabuvir + ribavirin Sofosbuvir + grazoprevir/elbasvir	1/43 (2.3%) 1/43 (2.3%)

Table 2. Amino acid changes relative to reference GT-1a sequence NC004102 at known RAS positions at baseline of initial treatment (when available) and at treatment failure, and retreatment outcomes in the 43 patients infected with an "unusual" GT-1 subtype. Samples were analyzed by means of Sanger sequencing and/or deep sequencing, as indicated. Polymorphisms known to confer reduced susceptibility to the corresponding drug class are shown in bold. RASs naturally present at baseline in the "unusual" GT-1 subtype reference sequence conferring inherent resistance of the subtype to the corresponding drug class (see Table 3) are underlined. NA: not available; DS: deep sequencing. DCV: daclatasvir; DSV: dasabuvir; EBV: elbasvir; GLE: glecaprevir; GZV: grazoprevir; LDV: ledipasvir; OBV: ombitasvir; PTV: paritaprevir; PIB: pibrentasvir RBV: ribavirin; r: ritonavir; SIM: simeprevir; SOF: sofosbuvir; VEL: velpatasvir; VOX: voxilaprevir.

Patient number	**Subtype**	**Initial treatment**	**Polymorphisms at treatment baseline**						**RASs at treatment failure**						**Retreatment regimen**	**Retreatment SVR**
			NS3 (DS)	**NS3 (Sanger)**	**NS5A (DS)**	**NS5A (Sanger)**	**NS5B (DS)**	**NS5B (Sanger)**	**NS3 (DS)**	**NS3 (Sanger)**	**NS5A (DS)**	**NS5A (Sanger)**	**NS5B (DS)**	**NS5B (Sanger)**		
	1. Patients with a sample at treatment failure only (n=37)															
	A. Samples analyzed by both deep sequencing and Sanger sequencing (n=18)															
P1	GT-1l	SOF/LDV 12 wk	-	-	-	-	-	-	**Q80L**	NA	**Q30R, L31M, H58P**	NA	None	None	Not treated	-
P2	GT-1d	SOF/LDV 8 wk	-	-	-	-	-	-	None	**Q80K**	**L31M, H58P, Y93H**	**L31M, Y93H**	None	None	SOF/VEL/VOX 12 wk	Yes
P3	GT-1e	SOF/LDV 12 wk	-	-	-	-	-	-	None	None	**K24R, L31M, H58P, A92T**	NA	None	None	SOF + DCV + RBV 12 wk	Yes
P4	GT-1l	SOF/LDV 24 wk	-	-	-	-	-	-	**Q8**	**Q80**	**M28V, L31M,**	**L31M,**	No	None	GLE/PIB +	Yes

									0L	**L**	**H58P**	**H58P**	ne	e	SOF 12 wk	
P5	GT-1l	SOF/LDV 12 wk	-	-	-	-	-	-	None	None	**Q30R, L31M, H58P**	**L31M**	None	None	NA	-
P6	GT-1e	SOF/LDV 12 wk	-	-	-	-	-	-	None	None	**K24R, H58P, A92T, Y93H**	**A92T, Y93H**	None	None	NA	-
P7	GT-1l	SOF/LDV 12 wk	-	-	-	-	-	-	**Q80L**	NA	**L31M, H58P**	NA	None	None	SOF + SIM + RBV 12 wk	Yes
P8	GT-1d	SOF + DCV 12 wk	-	-	-	-	-	-	**D168F**	**Q80K**	**L31M, H58P**	NA	None	NA	NA	-
P9	GT-1d	SOF/LDV 12 wk	-	-	-	-	-	-	**D168F**	NA	**L31M, H58P, Y93H**	**L31M, Y93H**	None	NA	GZR/EBR + SOF + RBV 12 wk	Yes
P10	GT-1-undetermined	SOF/LDV 12 wk	-	-	-	-	-	-	**Q80K**	NA	**L31M, H58P**	**L31M**	None	None	GLE/PIB + SOF 12 wk	Yes
P11	GT-1e	SOF/LDV 8 wk	-	-	-	-	-	-	None	NA	**K24R, L31M, H58P, A92T**	**K24R, L31M, A92T**	None	None	Not treated	-
P12	GT-1d	SOF/LDV 24 wk	-	-	-	-	-	-	None	NA	NA	**Q30R, Y93H**	None	None	NA	-
P13	GT-1d	SOF/LDV 12 wk	-	-	-	-	-	-	None	None	**M28V, Y93N**	**M28V, Y93N**	None	None	NA	-
P14	GT-1d	SOF/LDV 12 wk	-	-	-	-	-	-	**Q80K**	**Q80K**	**Q30G, L31M, H58P**	**Q30G, L31M**	None	None	SOF/VEL/VOX 12 wk	Yes
P15	GT-1e	SOF/LDV 8 wk	-	-	-	-	-	-	No	Non	**K24R, H58P,**	None	No	NA	NA	-

									ne	e	**A92T**		ne				
P16	GT-1l	SOF/LDV 12 wk	-	-	-	-	-	-	**Q80L**	NA	**L31M, H58P**	**L31M**	None	None	GLE/PIB 12 wk	Yes
P17	GT-1l	SOF/LDV 12 wk	-	-	-	-	-	-	**Q80L**	None	**L31M, H58P**	**L31M**	None	None	GLE/PIB 12 wk	Yes
P18	GT-1l	SOF/LDV 8 wk	-	-	-	-	-	-	**Q80L**	None	**Q30K, L31M, H58P**	**Q30K, L31M**	None	None	NA	-
	B. Samples analyzed by deep sequencing only (n=3)																
P19	GT-1l	SOF/LDV 12 wk	-	-	-	-	-	-	**Q80L**	-	**L31M, H58P**	-	None	-	Not treated	-
P20	GT-1l	SOF/LDV 12 wk	-	-	-	-	-	-	**Q80L**	-	**L31M, H58P**	-	None	-	NA	-
P21	GT-1l	SOF/LDV + RBV 12 wk	-	-	-	-	-	-	**Q80L**	-	**L31M, H58P**	-	None	-	SOF/VEL/VOX 12 wk	Yes
	C. Samples analyzed by Sanger sequencing only (n=16)																
P22	GT-1k	GLE/PIB 4 wk	-	-	-	-	-	-	-	**Q80K**	-	**M28T, Q30D**	-	None	SOF/VEL/VOX 12 wk	Yes
P23	GT-1e	SIM + DCV 24 wk	-	-	-	-	-	-	-	NA	-	**L31M, A92T**	-	None	SOF + DCV 12 wk	No
P24	GT-1l	SOF/LDV 12 wk	-	-	-	-	-	-	-	NA	-	**L31M**	-	None	NA	-
P25	GT-1g	OBV/PTV/r + RBV 12 wk	-	-	-	-	-	-	-	NA	-	**K24R**	-	None	SOF/VEL/VOX 12 wk	Yes
P26	GT-1l	SOF/LDV + RBV 12 wk	-	-	-	-	-	-	-	NA	-	**L31M**	-	None	NA	-
P27	GT-1d	SOF/LDV 12 wk	-	-	-	-	-	-	-	Non	-	**Q30R,**	-	Non	NA	-

										e		**L31M, Y93H**		e		
P28	GT-1d	SOF/LDV 12 wk	-	-	-	-	-	-	-	None	-	**K24R, L31M, Y93S**	-	None	GZR/EBR + SOF 12 wk	Yes
P29	GT-1f	SOF/VEL 12 wk	-	-	-	-	-	-	-	None	-	**L31V, Y93H**	-	None	SOF/VEL/VOX 12 wk	Yes
P30	GT-1i	SOF/LDV 12 wk	-	-	-	-	-	-	-	NA	-	**L31V, Y93N**	-	None	NA	-
P31	GT-1g	SOF/LDV 12 wk	-	-	-	-	-	-	-	None	-	**K24R, H58P, Y93F**	-	None	NA	-
P32	GT-1e	GLE/PIB 8 wk	-	-	-	-	-	-	-	None	-	**Q30R, A92T**	-	None	SOF/VEL/VOX 12 wk	Yes
P33	GT-1i	SOF/LDV 12 wk	-	-	-	-	-	-	-	None	-	**Y93H**	-	None	NA	-
P34	GT-1e	OBV/PTV/r + DSV + RBV 12 wk	-	-	-	-	-	-	-	**D168V**	-	**H58P, A92T, Y93C**	-	**C316Y**	SOF/VEL/VOX 12 wk	Yes
P35	GT-1e	SOF + GZR/EBR 8 wk	-	-	-	-	-	-	-	None	-	**Q30R, L31M**	-	None	SOF/VEL/VOX 12 wk	Yes
P36	GT-1e	SOF/LDV 8 wk	-	-	-	-	-	-	-	NA	-	**A92T**	-	None	SOF/VEL/VOX 12 wk	Yes
P37	GT-1l	SOF/LDV + RBV 12 wk	-	-	-	-	-	-	-	NA	-	None	-	None	SOF + SIM + RBV 12 wk	Yes
	2. Patient with samples at both baseline and treatment failure (n=6)															
	A. Samples analyzed by both deep sequencing and Sanger sequencing (n=1)															
P38	GT	GZR/EB	N	N	**K24**	**A**	N	N	N	N	**K24R,**	**A92**	N	N	GLE/PI	Yes

	-1e	R 8 wk	one	one	**R, H58P, A92T**	**92T**	one	one	one	one	**H58P, A92T, Y93H**	**T, Y93H**	one	one	B 12 wk	
	B. Samples analyzed by deep sequencing only (n=4)															
P39	GT-1-undetermined	SOF/LDV 12 wk	**Q80L**	-	**Q30R, L31M, H58P**	-	None	-	**Q80L**	-	**L31M, H58P**	-	None	-	SOF/VEL/VOX 12 wk	Yes
P40	GT-1l	SOF/VEL 12 wk	**Q80L**	-	**L31M, H58P**	-	None	-	**Q80L**	-	**L31M, H58P**	-	None	-	SOF/VEL/VOX 12 wk	Yes
P41	GT-1e	GLE/PIB 4 wk	None	-	**H58P, A92T**	-	None	-	None	-	**H58P, A92T**	-	None	-	GLE/PIB 12 wk	Yes
P42	GT-1l	SOF/LDV 24 wk	**Q80L**	-	**Q30R, L31M, H58P**	-	None	-	**Q80L**	-	**Q30R, L31M, H58P**	-	None	-	GLE/PIB 12 wk	Yes
	C. Samples analyzed by Sanger sequencing only (n=1)															
P43	GT-1l	SOF/LDV 24 wk	-	**Q80L**	-	**L31M**	-	None	-	NA	-	**L31M**	-	None	SOF/VEL/VOX 12 wk	Yes

Table 3. Amino acids at known RAS positions in reference sequences from all "unusual" GT-1 subtypes represented in the study. Amino acids conferring reduced susceptibility to the corresponding DAAs are shown in bold underlined.

GT-1 subtype reference sequence	Most frequent amino acids at baseline at known RAS positions																
	NS3 positions					NS5A positions							NS5B positions				
	56	80	155	156	168	24	28	30	31	58	92	93	159	282	316	320	321
GT-1a 1a_NC004102	Y	Q	R	A	D	K	M	Q	L	H	A	Y	L	S	C	L	V
GT-1b 1b_AB154189	Y	Q	R	A	D	**Q**	L	**R**	L	**P**	A	Y	L	S	C	L	V
GT-1d A1d_KJ439768	Y	**K**	R	A	D	K	L	**R**	**M**	**P**	A	Y	L	S	C	L	V
GT-1e 1e_KC248194	Y	Q	R	A	D	K	M	Q	**M**	S	**T**	Y	L	S	C	L	V
GT-1f*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GT-1g 1g_AM910652	Y	Q	R	A	D	S	L	**R**	L	P	A	**F**	L	S	C	L	V
GT-1h 1h_KC248198	Y	Q	R	A	D	**Q**	L	**R**	L	**P**	A	Y	L	S	C	L	V
GT-1i 1i_KJ439772	Y	Q	R	A	D	**Q**	L	**R**	L	**P**	A	Y	L	S	C	L	V
GT-1j 1j_KJ439773	Y	Q	R	A	D	K	M	Q	L	**P**	A	Y	L	S	C	L	V
GT-1k 1k_KJ439774	Y	Q	R	A	D	K	**A**	Q	L	**P**	A	Y	L	S	C	L	I
GT-1l 1l_KC248197	Y	**L**	R	A	D	S	M	Q	**M**	**P**	A	Y	L	S	C	L	V

*No reference sequence available for GT-1f in international databases: NA = not applicable.

Table 4. Phenotypic characteristics of RASs observed at failure in 5 patients infected with an "unusual" GT-1 subtype who failed to achieve SVR to one of the currently used pangenotypic DAA regimen , including sofosbuvir/velpatasvir (n=2) or glecaprevir/pibrentasvir (n=3). Data retrieved from the literature, generated with the corresponding NS5A inhibitor (i.e. velpatasvir or glecaprevir, respectively) (27–29). RASs are shown using GT-1a sequence 1a_NC004102 as a reference. EC50 : effective concentration 50% ; wt : wild-type ; NA: not available; GLE: glecaprevir; PIB: pibrentasvir; SOF: sofosbuvir; VEL: velpatasvir.

Patient	**GT-1 subtype**	**Initial treatment**	**NS5A RASs found at treatment failure**	**Fold-shift in EC50 vs wt**	**Replicative Fitness (% of wt)**
P29	GT-1f	SOF/VEL 12 wk	L31V + Y93H	>100x	NA
P40	GT-1l	SOF/VEL 12 wk	L31M + H58P L31M H58P	NA 16x <1x	NA 67% NA
P22	GT-1k	GLE/PIB 4 wk	M28T + Q30D M28T Q30D	NA 2.1x 94x	NA 89% 50%
P32	GT-1e	GLE/PIB 8 wk	Q30R + A92T Q30R A92T	NA 1.7x 0.4x	NA 86% 4.1%
P41	GT-1e	GLE/PIB 4 wk	H58P + A92T H58P A92T	NA 0.6x 0.4x	NA 129% 4.1%