Research article

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Rare HCV subtypes and retreatment outcomes in a cohort of European DAA-experienced patients

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Background and Aims: Data on the prevalence and characteristics of so-called rare HCV genotypes (GTs) in larger cohorts is limited. This study investigates the frequency of rare GT and resistance-associated substitutions and the efficacy of retreatment in a European cohort.
Methods: A total of 129 patients with rare GT1-6 were included from the European resistance database. NS3, NS5A, and NS5B were sequenced and clinical parameters and retreatment efficacies were collected retrospectively.
Results: Overall 1.5% (69/4,656) of direct-acting antiviral (DAA)-naive and 4.4% (60/1,376) of DAA-failure patients were infected with rare GT. Although rare GTs were almost equally distributed throughout GT1-6 in DAA-naive patients, we detected mainly rare GT4 (47%, 28/60 GT4; of these n = 17, subtype 4r) and GT3 (25%, 15/60 GT3, of these n = 8, subtype 3b) among DAA-failures. A total of 62% (37/60) of DAA failures had not responded to first-generation regimes and the majority was infected with rare GT4 (57%, 21/37). In contrast, among patients with failure to pangenotypic DAA regimens (38%, 23/60), infections with rare GT3 were overrepresented (57%, 13/23). Although NS5A RASs were uncommon in rare GT2, GT5a, and GT6, we observed combined RASs in rare GT1, GT3, and GT4 at positions 28, 30, 31, which can be considered as inherent. DAA failures with completed follow-up of retreatment, achieved a high SVR rate (94%, 45/48 modified intention-to-treat analysis; 92%, 45/49 intention-to-treat). Three patients with GT4f, 4r, or 3b, respectively, had virological treatment failure.
Conclusions: In this European cohort, rare HCV GT were uncommon. Accumulation of specific rare GT in DAA-failure patients suggests reduced antiviral activities of DAA regimens. The limited global availability of pangenotypic regimens for first line therapy as well as multiple targeted regimens for retreatment could result in HCV elimination targets being delayed.
Impact and implications: Data on the prevalence and characteristics of rare HCV genotypes (GT) in larger cohorts are still scarce. This study found low rates of rare HCV GTs among European HCV-infected patients. In direct-acting antiviral (DAA)-failure patients, rare GT3 subtypes accumulated after pangenotypic DAA treatment and rare GT4 after first generation DAA failure and viral resistance was detected at NS5A positions 28, 30, and 31. The limited global availability of pangenotypic DAA regimens for first line therapy as well as multiple targeted regimens for retreatment could result in HCV elimination targets being delayed.

Keywords: Direct-acting antivirals; Hepatitis C Virus; rare HCV genotypes; resistance-associated substitutions; treatment response.
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Introduction

Chronic Hepatitis C virus (HCV) infection remains a major global cause leading to chronic liver disease, cirrhosis, and hepatocellular carcinoma (HCC).[1] The WHO has set the goal of eliminating HCV as a public health threat by 2030. Although the global HCV prevalence has recently declined to 50 million infections, only a few countries are currently on track to meet elimination targets.[1] The availability of second generation, pangenotypic direct-acting antiviral (DAA) combination therapies with sustained virologic response (SVR) rates of over 95% across all HCV genotypes (GT) has revolutionised HCV therapy and enables HCV elimination.[2–4] There are various reasons for DAA treatment failure, such as the presence of cirrhosis/HCC, resistance-associated substitutions (RASs) in the HCV non-structural protein 3 (NS3), non-structural protein 5A (NS5A), and non-structural protein 5B (NS5B) genes or drug–drug interactions.[4] In addition, several studies detected viral resistance outside the DAA target genes, for example in NS2, the NS3 helicase or NS5B, which were associated with DAA treatment failure.[5,6] Reduced SVR rates have also been observed in certain HCV GTs and subtypes, such as GT3a (in combination with the presence of cirrhosis) or also in patients infected with 'rare' HCV GTs or subtypes.[4] Rare or unusual HCV GTs have a low prevalence in industrialised countries and were therefore underrepresented in clinical trials for initial approval of DAAs and cell culture studies investigating DAA efficacy.[7,8] However, rare GT are more common in Africa and Asia and some of them, such as, 1l, 3b and 4r had lower virologic response rates to different DAA regimens.[7] This was particularly the case when therapy was conducted using first-generation DAAs.[9–11] HCV is currently divided into eight GTs and 90 subtypes, and its genetic diversity, which depends on the geographic region, has not been fully characterised, and the probability of DAA resistance in many HCV subtypes has not been fully investigated.[7] In addition, as a result of migration, the prevalence of HCV GT4, GT5, and GT6 is increasing in industrialised countries.[7,12] Especially in resource-limited settings, the prevalence of rare GT is higher and the limited availability of second generation DAAs could lead to reduced SVR rates. Furthermore, there is a lack of data on the prevalence of RASs in patients with rare GTs in larger cohorts as well as on the efficacy of retreatment. The aim of the current study was therefore, to investigate the frequency of rare HCV GTs in a cohort of European patients with DAA failure compared with DAA-naive patients and to evaluate the efficacy of retreatment.

Patients and methods

Patients

The serum samples from patients with chronic hepatitis C infection were collected at different study sites in Germany, Belgium, and Switzerland and were part of the European DAA resistance database at the University Hospital Frankfurt, Germany, which was established based on a non-interventional study described elsewhere.[13] In this retrospective observational study, patients were treated as part of the clinical routine at the European gastroenterology centres and only residual blood sample volumes were used for HCV resistance analysis and limited clinical data (such as the HCV geno-/subtype, the pre-treatment status, the presence of cirrhosis, the DAA treatment regimen, the treatment duration, virologic response and the country of origin) was collected in a retrospective manner. Therefore, no safety aspects had to be taken into account.

In this study, we searched the European DAA resistance database for rare HCV geno-/subtypes. Based on other studies rare HCV geno-/subtypes or non-epidemic subtypes[5,9] were defined as follows: Patients with HCV non-GT1a/1b, non GT2a/b/c, non-GT3a, non-GT4a/4d, GT5a, and non-GT6a infection or unassigned HCV GT. In total, samples from 69 DAA-naive and 60 patients with failure to DAA-based treatment of at least 4 weeks' duration and who were treatment adherent were included in the study, collected between 2014 and 2022. Patients with HCV reinfection after successful DAA treatment were not included in the analysis.

The efficiency of DAA retreatment of patients with a previous DAA failure was also assessed retrospectively. In accordance with our previous study,[14] we defined as intention-to-treat (ITT) population all patients who initiated retreatment and as modified ITT (mITT) the analysis of all patients who completed retreatment and follow-up visits at week 12.

Ethics statement

This study was conducted in accordance with the Declaration of Helsinki. The use of patients' blood samples and the retrospective collection of limited pseudonymised patient data was approved (ethics vote number 16/15) by the ethics committee of the University Hospital Frankfurt, Germany.

NS3, NS5A, and NS5B amplification and sequencing analyses

For HCV resistance analysis, HCV RNA was extracted from serum and subsequently cDNA synthesis, and NS3, NS5B, and NS5B nested PCR amplification and population-based sequencing on an Abi Prism analyser were performed as described previously.[13] Proofread sequences of an NS5B fragment amplified with universal primers were used to determine HCV genotypes and subtypes, as previously described[15,16] (Table S2). The HCV genotypes/subtypes were determined using the HCV genotyping tool from the Los Alamos sequence database (https://hcv.lanl.gov). To verify the subtype and for RAS analyses, the sequences were manually edited and compared with published reference sequences (Table S3) in BioEdit version 7.2.5 (T. Hall, Ibis Therapeutics, Carlsbad, Germany).

RASs were defined as substitutions that conferred a greater than twofold changed DAA susceptibility in *in vitro* replicon assays or as substitutions that were associated with virologic *failure in vivo* and were considered clinically relevant, as previously described.[13,17] Part of this study, the prevalence of RASs in patients with rare GT4 infection, was previously published.[18]

Results

A total of 7,987 samples from patients with chronic HCV infection collected within the European resistance database were included. Of these patients, 57% (4,656/7,987) were DAA-naive and 17% (1,376/7,987) of patients had failed DAA-based treatment. A further 17% of patients (1,299/7,987) did not have sufficient treatment information available or the treatment took place within a clinical trial. A total of 9% (656/7,987) of patients were treated with a combination of DAAs with pegylated-interferon (PEG-IFN). HCV sequencing revealed that 1.5% (69/4,656) of DAA-naive patients and 4.4% (60/1,376) of DAA failure patients were infected with rare HCV GTs or subtypes (rare GTs) (Fig. 1).

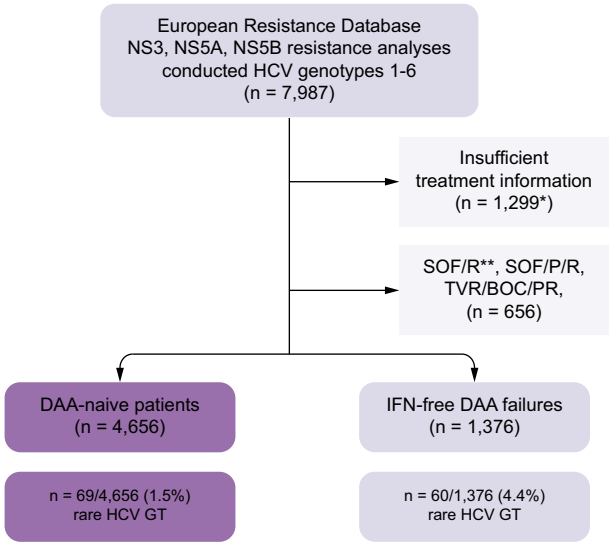

Fig. 1. Flow chart of the study cohort. BOC, boceprevir; GT, genotype; IFN, interferon; P/R, pegylated interferon/ribavirin; SOF, sofosbuvir; RBV, ribavirin; TVR, telaprevir. *also includes patients who were treated other DAAs from clinical studies, **only in patients with GT1.

Frequencies of rare HCV GTs in DAA-experienced *vs.* DAA-naive patients

Many different rare HCV subtypes have been detected in DAA-naive patients. These included almost equal proportions of the various rare HCV subtypes (rare GT1, 17%, 12/69, rare GT2, 16%, 11/69, rare GT3, 15%, 10/69, GT5a, 15%, 10/69, and rare GT6, 12%, 8/69), whereas rare GT4 (26%, 18/69) was slightly overrepresented (Fig. 2A).

The pattern of rare HCV GT subtypes was different in DAA-failure patients, where we observed higher rates of patients with rare GT3 and GT4 subtypes. Almost half of the patients were infected with rare GT4 (47%, 28/60) and a quarter (25%, 15/60) with rare GT3, while other GTs were rarely detected (Fig. 2B). Among patients with rare GT4, subtype 4r dominated (n = 17) and among rare GT3, subtype 3b (n = 8) was most frequent.

Of note, we observed interesting differences in the frequencies of rare GT3 subtypes. Although only subtypes 3b and 3h were detected in DAA-naive patients, larger numbers of different subtypes were present in DAA-failure patients, such as subtypes 3b, 3i, 3h, 3k, and one unassigned subtype.

Clinical characteristics and countries of origin of DAA failures with rare HCV GTs

The mean age of DAA failure patients infected with rare GTs was 55 years, 41% (20/49 with data available) had received prior PEG-IFN/ribavirin (P/R) treatment, 33% (19/57 with data available) had cirrhosis, and 5% (3/57) had a HCC at the time of study inclusion (Table 1).

All samples from patients with rare HCV GT were collected in European centres and the country of origin was documented retrospectively. Patients with rare GT1 and GT2 originated from West Africa, but also from Europe. Patients with rare GT3 mainly originated from South-Asia (India, 33%, 5/15; Bangladesh, 20%, 3/15; or Pakistan, 13%, 2/15). However, there were differences concerning rare GT4 subtypes: patients with subtypes 4n and 4o were of Egyptian descent (14%, 4/28), whereas patients with a subtype 4b or 4r infection were mainly from sub-Saharan Africa (DR Congo, 18%, 5/28; Eritrea, 18%, 5/28) as well as single patients from Angola, Burundi, and Nigeria). Rare GT6 infections were detected mainly in patients of Southeast-Asian origin (Vietnam, 29%, 2/7, and Thailand 29%, 2/7). However, for all rare GTs, we also identified patients who originally came from Central Europe (Germany, France, Belgium, Switzerland) (Fig. 3 and Table S1).

Rare HCV GTs in DAA-experienced compared with DAA-naive patients

To directly compare DAA-naive *vs.* DAA-experienced patients, patients were stratified into those who had not responded to first-generation DAA regimens (paritaprevir/ritonavir/ombitasvir with dasabuvir [PrOD]; grazoprevir/elbasvir [GZR/EBR]; ledipasvir/sofosbuvir [LDV/SOF]; sofosbuvir/ribavirin [SOF/R]) and those who had failed to second-generation, pangenotypic regimens (daclatasvir/sofosbuvir [DCV/SOF]; velpatasvir/sofosbuvir [VEL/SOF]; glecaprevir/pibrentasvir [G/P]). Overall, rare GTs were detected more frequently in patients in whom first generation

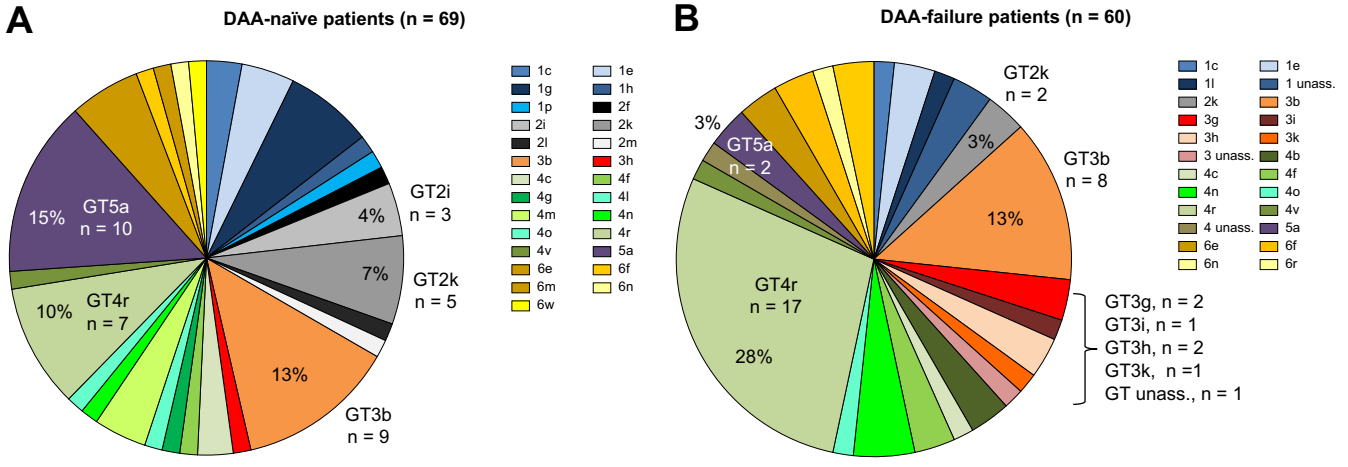

Fig. 2. Distribution of rare HCV GTs and subtypes. (A) Frequencies of rare HCV GT subtypes among DAA-naive patients and (B) rare HCV GT subtypes detected in DAA-failure patients. DAA, direct-acting antiviral; GT, genotype.

Table 1. Patient characteristics of DAA failure patients with rare GT (n = 60) at study inclusion.

	Rare GT1 n = 6	Rare GT2 n = 2	Rare GT3 n = 15	Rare GT4 n = 28	GT5a n = 2	Rare GT6 n = 7
Mean age, years	55.3	54.7	46.1	58.2	63.6	58.3
Male sex, n (%)	4 (67)	1 (50)	14 (93)	23 (93%)	1 (50)	7 (100)
Cirrhosis, n (%)	1 (17)	0 (0)	7 (47)	10 (40) n = 25	0 (0)	1 (14)
HCC, n (%)	0 (0)	0 (0)	0 (0)	2 (8) n = 25	0 (0)	1 (14)
Prior IFN-experience, n (%)	3 (60) n = 5	1 (50)	5 (46) n = 11	11 (48) n = 23	0 (0)	0 (0) n = 6
DAA treatment history, n (%)						
2D/3D PrOD (PTV/r/OBV ± DSV)	1 (17)	–	–	6 (21)	–	2 (29)
GZR/EBR	1 (17)	–	1 (7%)	2 (7)	–	1 (13)
LD/VSOF	4 (66)	–	1 (7%)	12 (43)	–	2 (29)
SOF/RBV	–	2 (100)	–	1 (4)	1 (50)	–
DCV/SOF	–	–	6 (40)	4 (14)	–	–
VEL/SOF	–	–	4 (26)	2 (7)	1 (50)	2 (29)
G/P	–	–	3 (20)	1 (4)	–	–

DCV/SOF, daclatasvir/sofosbuvir; EBR/GZR, elbasvir/grazoprevir; LDV/SOF, ledipasvir/sofosbuvir; G/P, glecaprevir/pibrentasvir; (pegylated); HCC, hepatocellular cellular carcinoma; IFN, interferon; 2D/3D, paritaprevir/r/ombitasvir ± dasabuvir; RBV, ribavirin; VEL/SOF, velpatasvir/sofosbuvir.

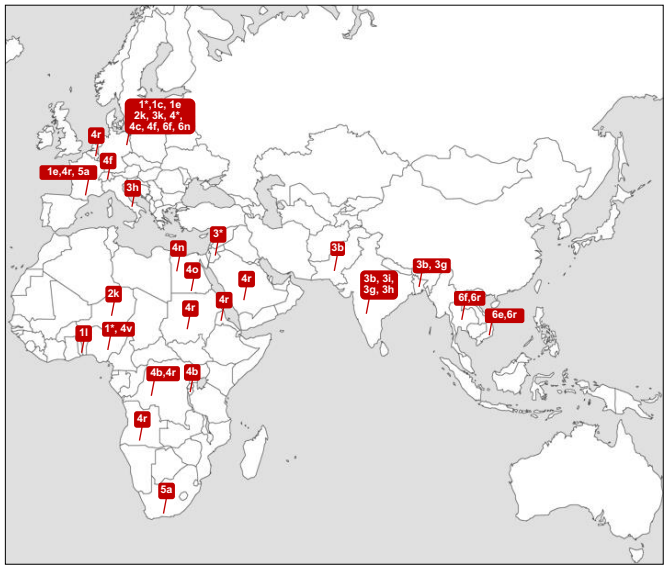

Fig. 3. Countries of origin of DAA-failure patients with rare GT subtypes. DAA, direct-acting antiviral; GT, genotype. *Subtype unassigned. (background picture: power point, creative commons, https://www.dahmen-quilt.com).

DAAs had failed (62%, 37/60) compared with patients who had failed to respond to second generation DAAs (38%, 23/60). Interestingly, more than half of the patients who failed first-generation DAA regimens were infected with rare GT4 subtypes (57%, 21/37), while the majority of patients without SVR after second-generation, pangenotypic, DAA treatment, were infected with rare GT3 subtypes (57%, 13/23) (Fig. 4A).

Strikingly, 62% (37/60) of the DAA failures with rare GTs had been treated with first-generation DAAs, whereas only 38% had received second-generation DAAs (Fig. 4B). This suggest a reduced activity of first generation DAAs towards rare GT.

Interestingly, subtype 4r was overrepresented in patients after PrOD (56%, 5/9) and LDV/SOF (42%, 8/19) failure. We have also observed higher rates of patients with rare GT3 subtypes (mainly subtype 3b) after failure to second generation DAAs such as DCV/SOF (60%, 6/10), VEL/SOF (45%, 4/9), and G/P (75%, 3/4). Of note, two additional VEL/SOF failures were infected with subtype 4r (Fig. 4B).

RASs in patients with rare vs. *common HCV GT*

The prevalence of NS3 RASs after protease inhibitor failure was low and limited to individual patients. No patients with rare GT2 and NS5A inhibitor (NS5Ai) failure could be included and the number of patients with GT5a was small.

However, regarding other HCV GTs, we identified interesting NS5A RAS patterns in patients with rare GT1, GT3, and GT4 and combined NS5A RASs were common after NS5Ai failure. Even though the number of patients with rare GT1 was smaller, we observed a trend towards combined NS5A RASs (M28V, Q30R, and L31M). In patients with rare GT3, combinations of A30K plus L31M/V were frequent and 92% harboured A30K and 85% L31M/V. The double mutation A30K + L31M/V was found in 73% (11/15) of patients (n = 10 with A30K + L31M), including subtypes 3b (n = 8), 3g (n = 2; n = 1 with A30K + L31V) and 3k (n = 1) (Fig. 5A). In patients with rare GT4, the results have already been partially published.[18] Here, combined RASs L28M/V + L30R/S + M31L/V were predominant. L28M/V occurred in 64%, L30R/S in 96% and M31L/V in 44% of patients, respectively, with triple RAS combinations being particularly characteristic for patients with subtype 4r (Fig. 5A). The NS5A signature RASs that we detected in rare GTs are listed in Table 2. Comparison with published data[15] shows that combined RASs are less common in patients with GT1a, 1b, or 3a. In common GT, Y93H was frequent and additional RASs were found at the following positions: In GT1a at positions 28 and 30, in GT1b at position 31 and in GT3a at position 30 (Fig. 5A).

In DAA-naive patients with rare GT1, GT3, or GT4, NS5A RASs at positions 28, 30, and 31 were also common (Fig. 5B). Overall, it can be considered that these RASs are inherent and already existed before DAA treatment.

There were also interesting differences in the frequency of NS5A Y93H, which confers high-level resistance to first-generation NS5Ai. Whereas in common HCV GTs, such as GT1b or GT3a, Y93H could be detected in 72–84% of patients after NS5Ai failure across all regimens, this was only the case in 15–20% of patients with rare GTs (Fig. 5C). In contrast, the opposite was observed concerning the prevalence of S282T in NS5B. The frequency of S282T in common GTs was between 1% and 5% only, whereas this variant was more frequent in rare GT3, GT4, and GT6 with frequencies of 10–22% (Fig. 5D). Of the six patients with S282T (n = 1, GT3g; n = 1 GT4b; n = 1, GT4o; n = 2, GT4r; n = 1, GT6r), we detected S282C as an additional variant to S282T in one patient with GT4r.

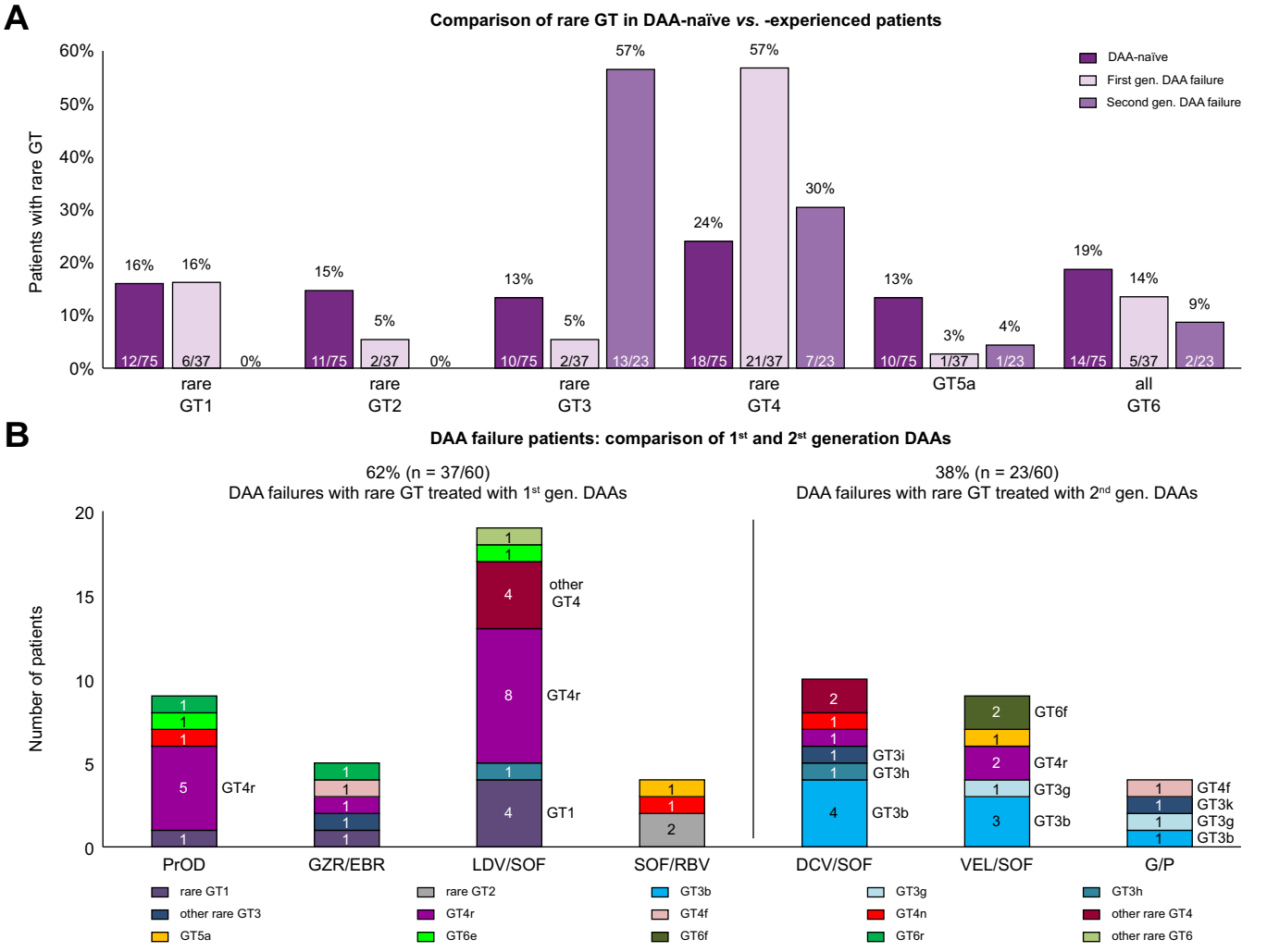

Fig. 4. Rare HCV GT subtypes in DAA-naive *vs.* -experienced patients. (A) Overall frequencies of rare GT subtypes in patients after first and second generation DAA failure compared to DAA-naive patients. (B) Distribution of rare HCV subtypes among DAA-failure patients, stratified for first and second generation DAAs. DAA, direct-acting antiviral; GT, genotype. Patients with rare GT6 subtypes and PrOD, GZR/EBR, LDV/SOF failure were partially misclassified as GT1 in commercial genotyping assays.

Overall, no relevant RASs were detected in patients with rare GT6.

Retreatment of patients with rare HCV GTs

Overall, 82% (49/60) of DAA-failure patients started retreatment, of whom 98% (48/49) had follow-up data available. The overall mITT SVR rate across all regimens and genotypes was 94% (45/48) (Fig. 6). One further patient had started voxilaprevir VOX/VEL/SOF retreatment and was lost during the on-treatment visits. Thus, the overall ITT SVR rate was 92% (45/49). A total of three patients had virological treatment failure. One patient with GT4f and cirrhosis, who was pretreated with GZR/EBR, had failed to subsequent G/P retreatment. Unfortunately, no sample was available for RAS testing before the start of retreatment. One patient who was GT4r-infected with F3/F4 fibrosis harboured NS5A L30R + Y93S and NS5B S282T after LDV/SOF failure. Directly before retreatment initiation, we detected again L30R + Y93S in NS5A, whereas S282T was undetectable. This patient failed again to achieve SVR after a repetition of NS5Ai treatment with VEL/SOF + RBV and the RAS profile was identical to that before retreatment initiation. Of note, this patient achieved SVR12 after a third rescue treatment with VOX/VEL/SOF. The last patient with subtype 3b and cirrhosis and NS5A RASs A30K + L13M (and no NS3 RASs) detectable after G/P treatment failed to achieve SVR after VOX/VEL/SOF treatment. Unfortunately, no sample was available after retreatment failure from this patient. Both patients with subtype 4f and 3b have not yet been retreated again.

Overall, the SVR rate after VOX/VEL/SOF retreatment was high with 96%. However, retreatment according to the concept of a DAA drug class switch (using DAAs, which were not used in first line treatment) was also successful in the majority of patients.

Discussion

Data on the prevalence of rare HCV GT, their RASs and (re)-treatment efficacies are limited and are based on individual studies in individual Western countries.[8,10,11,18–20] Therefore, in this real-world study, we analysed patient samples from different European countries for the presence of rare HCV GT 1–6

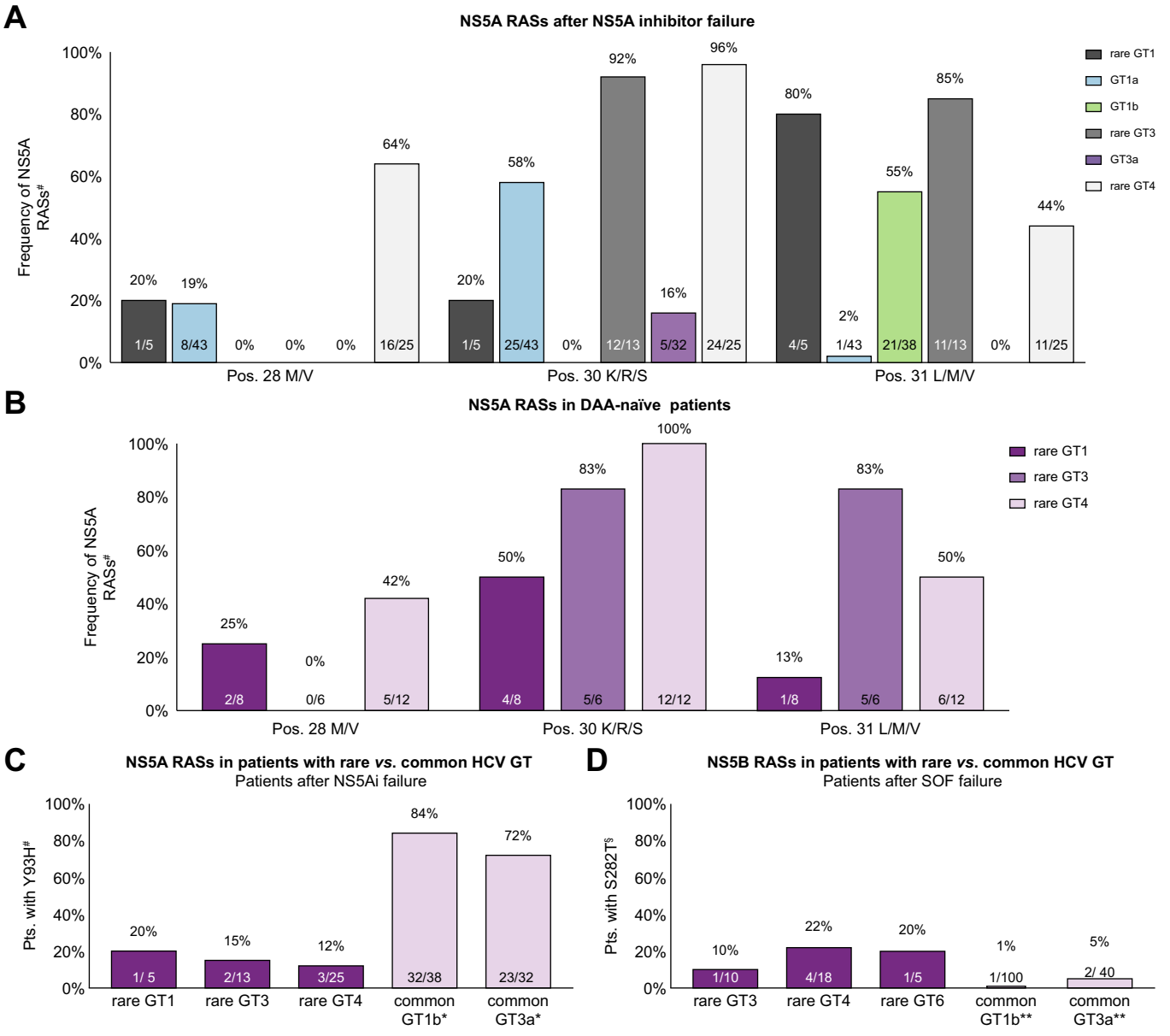

Fig. 5. RASs in patients with rare *vs.* common HCV GTs. (A) NS5A RASs detected in patients after NS5Ai failure. Pos. 28 not resistant in GT3a, Pos. 30 not resistant in GT1b. The data for GT1a, GT1b and GT3a are taken from Dietz *et al. J. Hepatol.*, 2023. (B) NS5A RASs in DAA-naive patients. (C) Frequency of Y93H after NS5Ai failure in patients with rare GTs compared with common subtypes. (D) Prevalence of S282T after sofosbuvir treatment failure in patients with rare GT in comparison to patients with common subtypes. DAA, direct-acting antiviral; GT, genotype; NS5Ai, NS5A inhibitor; RASs, resistance-associated substitutions. #In patients with NS5A sequencing data available; §in patients with NS5B sequencing data available; *data from Dietz *et al. J. Hepatol.*, 2023; **data from Dietz *et al. Gastroenterology*, 2018.

and RASs and evaluated the retreatment efficacies in DAA-failure patients.

In our study, many different rare GT subtypes were found in DAA-naive patients, without specific accumulation of certain GTs. In contrast, higher rates of rare GT3 and GT4 subtypes were detected after DAA failure. A direct comparison with French and British studies regarding the prevalence of rare GTs is difficult. In England, rare GT1 subtypes were overrepresented in DAA-naive patients,[10] and in France, rare GT4 and GT1 were mainly detected after DAA failure.[11,19] The overrepresentation of rare GT1 and GT4 subtypes in these countries is probably attributable to the fact that immigrants in these countries are mainly of African origin. Similarly, also in our study, most patients with rare GT1 and GT4 subtypes originated from Africa. However, we were also able to include many patients with rare GT3 or GT6 subtypes from South(East) Asia for whom data has been sparse. This reflects the diverse composition of the population at the treating centres.

The data are limited for rare GT2 and GT5a, as they are endemic only in certain geographical regions, and respond well to NS5Ai-based treatment. The individual patients in our study had mainly failed to SOF/RBV and RASs were not detected.

Several studies have investigated the prevalence of RASs in patients with rare GT1, GT3, and GT4 subtypes. In a British study,

Table 2. NS5A signature RASs detected in DAA-failure patients with rare GT.

Rare GT	Pos. 24	Pos. 28	Pos. 30	Pos. 31	Pos. 93
GT1c/e/l/*	K24G/R	M28V (n = 1 pt.)	Q30R (n = 1 pt.)	L31M	Y93H (n = 1 pt.)
GT2k	No. pts. with NS5A inhibitor pretreatment				
GT3b/g/h/i//*	–	–	A30K	L31M	Y93H (n = 1 pt.)
GT4b/c/f/n/o/r/v*	–	L28M/V	L30R	M31L	Y93H (single pts.)
GT5a	No RASs				
GT6e/f/n/r	–	F28M (n = 1 pt.)	–	–	Y93S (n = 1 pt.)

DAA, direct-acting antiviral; GT, genotype; NS5A, non-structural protein 5A, pt., patient; RASs, resistance-associated substitutions.
* Subtype unassigned.

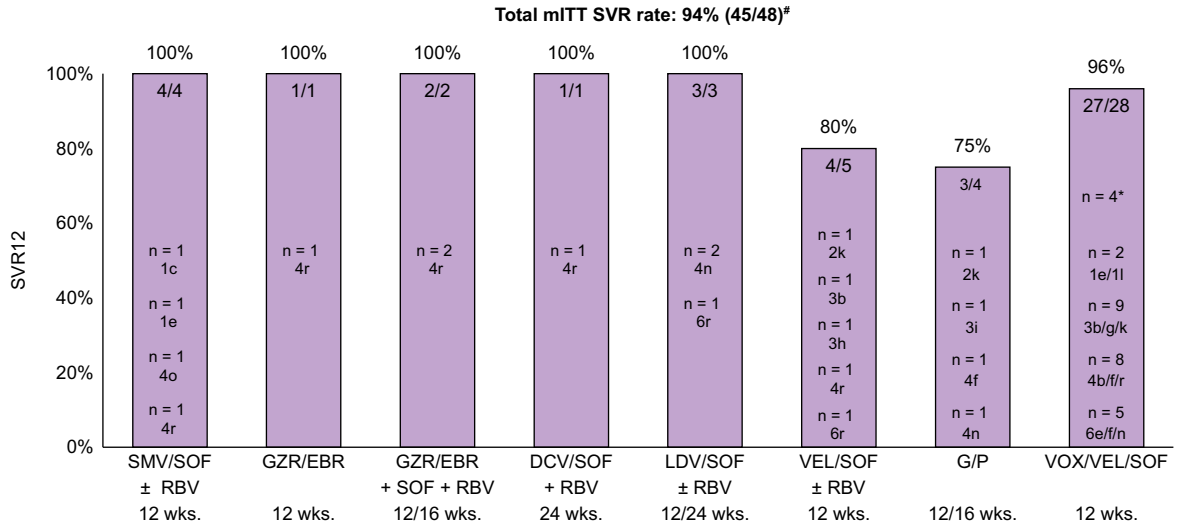

Fig. 6. Retreatment efficacies (mITT) in DAA-failure patients with rare GTs. DAA, direct-acting antiviral; GT, genotype. mITT (modified intention-to-treat): retreated patients with completed follow-up visits. #One further patient started retreatment with VOX/VEL/SOF for 12 weeks and was lost during the on-treatment visits. Thus, the overall TT SVR rate was 92% (45/49) and the VOX/VEL/SOF ITT SVR rate was 93% (27/29). Further 11 patients were not retreated, of whom 8 patients were lost and further 3 patients died due to HCC. *n = 4 patients with HCV GT1/GT4 subtypes unassigned.

39% of DAA-naive African patients were infected with rare GT1 and combined NS5A RASs K24G/R, Q30L/R, and L31M were frequent. After LDV/SOF-based treatment, only 75% of patients achieved SVR and post-treatment RASs largely overlapped with those at baseline.[10] A French study also considered NS5A RASs as inherent in rare GT1 subtypes.[11] We detected similar RASs in the limited number of DAA failures in our study. Overall, subtypes 1e, 1d, and 1l seem to be the most susceptible to LDV/SOF failure,[10,21] and also our study identified subtypes 1e and 1l in 3/6 DAA-failure patients.

In patients with rare GT3 subtypes, the present study found that 73% harboured NS5A A30K + L31M/V before initiation of antiviral therapy, particularly subtypes 3b and 3g. This is in accordance with another large study that examined DAA-naive patients with GT3 and showed that all patients with subtypes 3b and 3g harboured A30K + L31M double variant, whereas this variant was undetectable in subtype 3a.[22] As in our study, Y93H was undetectable in GT3b and GT3g isolates. *In vitro* experiments revealed that A30K + L31M confers high level DCV and VEL resistance and subtype 3b can be considered as inherently resistant.[22] Also, in an infectious GT3a cell culture system A30K + L31M conferred 12-fold resistance to pibrentasvir (PIB) and 9,420-fold resistance to VEL. However, in long-term combination treatments with gelcaprevir (GLE)/PIB or VEL/SOF or VOX/VEL/SOF, the treatment response was comparable with that of the wild type.[23] Clinical studies have assessed VEL/SOF and G/P efficacies in Asian patients with GT1-6. The SVR rate was >95% across all GT, but was reduced in GT3b: 75% SVR12 after VEL/SOF and 70% after G/P, respectively.[24,25] The reduced VEL/SOF response can be explained by the high-level VEL resistance of A30K + L31M compared with the wild type.[22,23] A30K + L31M also confers 10,000-fold DCV resistance,[22] explaining our observation of high rates of patients with GT3b infection after DCV/SOF failure and that rare GT3 subtypes accumulated among pangenotypic treatment failures. However, it remains unclear why the response to G/P was reduced, even though A30K + L31M confers only low level PIB resistance.[22,23,26] Table 3 shows the different published *in vitro* resistance levels in rare GTs. The NS3 baseline polymorphism A166S was associated with reduced clinical efficacies in GT3[26] and could explain the lower SVR rates. In our study, three patients with rare GT3 had G/P failure (n = 1, 3b; n = 1, 3g; n = 1, 3k) and one patient had post-treatment A166S.

We detected high frequencies of combined NS5A RASs in rare GT4 subtypes, especially L28M/V, L30R, and M31L. Other

Table 3. Published *in vitro* resistance levels of RASs in rare GT1, GT3, and GT4 subtypes.

Rare GT	Tested isolate	DCV EC_{50} fold change*	VEL EC_{50} fold-change*	PIB EC_{50} fold change*	Reference
Subtype 1l	Q30R + L31M	~3	~2	~30	Nguyen *et al.*
Subtype 4r	L28M + L30R + L31M	~40	~1	~0.8	Nguyen *et al.*
Subtype 4r	L28M + L30R + Y93H	~700	~8	~7	Nguyen *et al.*
Subtype 3b/3g†	A30K + L31M	~10,000	~40,000	~60	Smith *et al.*
Subtype 3b/3g†	A30K + L31M + Y93H	~40,000	~40,000	~60	Smith *et al.*

* Compared with wild-type replicon *in vitro*.
† Substitutions tested in subtype 3a replicon. DCV, daclatasvir; GT, genotype; PIB, pibrentasvir; RASs, resistance-associated substitutions; VEL, velpatasvir.

researchers have also described similar RAS patterns, particularly in subtype 4r, in DAA-naive and -experienced patients. Therefore, as suggested in previous studies, these NS5A RASs can be considered as inherent.[18,19,27] A large study on common HCV genotypes showed a high diversity of RASs and high rates of combined L31M + Y93H RASs in patients with GT1b, particularly after LDV/SOF failure.[28] Interestingly, in our study, Y93H was rare and combined NS5A RASs were observed in rare GT at baseline and after DAA treatment failure with all NS5Ai regimens.

In addition, clinical studies also demonstrated lower SVR rates in patients with subtype 4r (and subtype 4b) after LDV/SOF treatment.[9,29,30] *In vitro* data demonstrated that LDV susceptibility decreased with increasing numbers of NS5A RASs in subtype 4r, whereas subtype 4r was sensitive to VEL.[29] In clinical trials, SVR rates after VEL/SOF treatment were high in GT4-infected patients, but only few patients with subtype 4r were included.[29] In our study, two VEL/SOF failures were infected with subtype 4r. Precise antiviral activity of VEL/SOF in subtype 4r in clinical practice therefore remains to be determined.

The increased occurrence of NS5B S282T in rare GT4 in the present study as well as the reduced frequency of Y93H in rare GT3 and GT4 were also confirmed in other studies.[19,22] The different prevalence of these RASs could be because of a different codon usage or a different fitness of viral variants. In common GT, S282T showed short persistence after the end of treatment as its replicative fitness is relatively low.[15,31,32] However, it has been postulated that the replicative fitness of S282T was higher in subtype 4r, which may explain the higher detection rates.[14] Several cell culture studies also demonstrated that S282T is more frequently selected under SOF treatment in GT3a, GT4, and GT6a and persists after end of treatment, presumably because the resistance barrier of S282T to SOF is lower in these GTs.[33–35] The fitness of Y93H was also reduced and requires compensatory mutations at positions 30 or 31 in common GTs.[15] The different RAS patterns in rare GTs *vs.* common GTs could be explained by replicative differences in different backbones.

In patients with GT6 infection, Asian clinical studies demonstrated high SVR rates of >95% after VEL/SOF or G/P treatment, including mainly patients with GT6a/6b infection.[24,25] GT6 is considered the most diverse HCV lineage and characteristic NS5A RASs detected at baseline were F28M/V and T93S.[36,37] In our study, NS5A RASs were rare after DAA failure and most of the patients had not responded to first generation DAAs, which have been prescribed in the assumption of a GT1 infection. It is known that the GT can be misclassified in commercial genotyping assays, for example GT6 was misclassified as GT1.[7,38,39] Two patients with subtype 6f infection had failed to pangenotypic VEL/SOF treatment; however, the clinical relevance remains unclear as data on GT6f-infected patients are sparse.[24,36]

Overall, we observed different effects of NS5Ai-based treatments across all rare GT with hotspots for RASs at positions 28, 30, and 31. This was similarly shown in a large cell culture study for all common GTs, with VEL and PIB showing the highest antiviral activity against all GTs.[40]

In addition, there are few data concerning the retreatment of DAA failures with rare GTs, particularly for rare GT3 subtypes. For patients with rare GT4 subtypes, two studies demonstrated SVR rates of >95% across all regimens.[18,19] Also, in patients with rare GT1 subtypes, SVR rates of 96% were achieved after retreatment, and all patients retreated with VOX/VEL/SOF or G/P achieved SVR.[11] In our study, 94% of patients achieved SVR12 after retreatment. Only three patients had virologic treatment failure. In addition to the two patients who had not responded to suboptimal retreatment with VEL/SOF or G/P, one patient with GT3b with cirrhosis and NS5A RASs had virologic treatment failure after VOX/VEL/SOF retreatment. Overall, VOX/VEL/SOF was very effective against all GTs in clinical and real-world studies, despite the presence of baseline RASs and only few patients with GT3, cirrhosis, HCC, and NS5A RAS at pretreatment showed reduced treatment response.[4,16,41–44] However, the majority of these studies did not include patients with rare GT or subtyping was not conducted. Systematic studies in consecutively collected patients would be needed to directly compare possible factors for (re)treatment response in patients with rare GT *vs.* common GT. The present study demonstrates that VOX/VEL/SOF is very effective in patients with rare GT, including patients with rare GT3 subtypes and cirrhosis.[4] Overall, in resource-limited settings without availability of VOX/VEL/SOF, a switch of the DAA drug class should be considered for retreatment, since this concept was also successful in most cases in our study. Prolongation of the treatment duration and additional administration of ribavirin may also be considered.[14,39,45,46]

A limitation of the study is that it does not describe the true prevalence of rare HCV GT subtypes in Western countries. Furthermore, only small numbers of patients with GT2 and GT5a could be included. Unfortunately, it was also not possible to conduct next generation sequencing (NGS), this would have been interesting to identify minor RASs with a frequency of <25% in the HCV quasispecies, as these RASs could also have an impact on salvage therapy after DAA failure as reported in a study.[28] NGS would also have been helpful to identify additional minor combined NS5A RASs that were not detected by Sanger sequencing, but could have an impact on treatment response. Furthermore, it would have been informative to analyse the complete HCV genome to identify possible viral variants in non-DAA target regions, which have been shown to also influence the DAA treatment response.[5,6]

In summary, this study shows that rare GT were more common in DAA failures than in DAA-naive patients and rare GT3 and GT4 subtypes dominated. Concerning RASs, combined NS5A RASs were detected in DAA-naive and -experienced patients with rare GT1, GT3 and 4 subtypes, which can be regarded as inherent resistance. Although mainly rare GT4 subtypes were detected among patients with failure to first generation DAAs, rare GT3 subtypes accumulated after pangenotypic, second generation DAA treatment. As overall rare GTs occurred more frequently after first generation DAA failure, effective regimens such as VEL/SOF or G/P should be used for first-line treatment in countries with a high prevalence of rare GT, but DCV/SOF as pangenotypic regimen is also feasible. An important finding of this study is that rare GT3 subtypes were detected after treatment with all currently approved regimens, confirming suboptimal SVR rates observed in clinical studies. Retreatment was effective with high SVR rates across all rare GT subtypes and regimens.

In conclusion this study suggests that the different rare HCV subtypes may require different treatment regimens. Therefore, HCV subtyping in DAA failures remains critical[28] and should be conducted in countries with a high prevalence of rare GT, at least at a population level, to obtain more data concerning the prevalence of rare HCV subtypes.[7] Overall, first generation DAAs seem to be too inefficient in regions with high frequencies of rare GT1, GT3, and GT4 subtypes and in regions with high proportions of rare GT3 subtypes, DCV/SOF, VEL/SOF and G/P may also be suboptimal for first line treatment, which may lead to the global HCV elimination goals not being met by 2030.

Abbreviations

DAA, direct-acting antiviral; DCV, daclatasvir; EBR, elbasvir; GLE, glecaprevir; G/P, glecaprevir/pibrentasvir; GT, genotype; GZR, grazoprevir; HCC, hepatocellular carcinoma; ITT, intention-to-treat; LDV, ledipasvir; mITT, modified intention-to-treat; NGS, next generation sequencing; NS3, non-structural protein 3; NS5A, non-structural protein 5A; NS5Ai, NS5A inhibitor; NS5B, non-structural protein 5B; PEG-IFN, pegylated interferon; PIB, pibrentasvir; P/R, PEG-IFN/ribavirin; PrOD, paritaprevir/ritonavir/ombitasvir with dasabuvir; RASs, resistance-associated substitutions; RBV, ribavirin; SOF, sofosbuvir; SVR, sustained virologic response; VEL, velpatasvir; VOX, voxilaprevir.

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Conflicts of interest

JD: research support from Gilead. CG: speaking and/or consulting fees from AbbVie and travel support from AbbVie and Gilead. CPB: speaking and/or consulting fees: AbbVie, BMS, Gilead, Merck/MSD. KP: no conflicts to disclose. KD: speaking and/or consulting fees: Gilead, AbbVie, Alnylam. PB: speaking and/or consulting fees: AbbVie, BMS, Falk, Gilead, Janssen, Merz Pharma, Merck/MSD. K-HP: no conflicts to disclose. JV: speaking and/or consulting fees from Abbott, AbbVie, Bristol-Myers, Squibb, Gilead, Medtronic, Merck/MSD and Roche. GD: speaking and/or consulting fees from Abbvie, Gilead. AG: advisor and steering committee member for AbbVie, Advanz, Albireo, Alexion, Astra Zeneca, Bayer, BMS, CSL Behring, Eisai, Gilead, Heel, Intercept, Ipsen, Merz, MSD, Novartis, Pfizer, Roche, Sanofi-Aventis and as speaker for AbbVie, Advanz, Alexion, BMS, Burgerstein, CSL Behring, Falk, Gilead, Intercept, Merz, MSD, Novartis, NovoNordisk, Roche; research support from Intercept and Falk (NAFLD CSG), Novartis. FPR: honoraria for lectures, consulting activities and travel support from the Falk Foundation, AbbVie, Gilead, Ipsen, Astra Zeneca, Roche and Novartis. TB: speaking and/or consulting fees or travel support from Abbvie, Gilead, SOBI, CSL Behring, Merck, Gore, Advanz. JMS: consultant: Akero, Alentis Therapeutics, Astra Zeneca, Apollo Endosurgery, 89Bio, Boehringer Ingelheim, GSK, Ipsen, Inventiva Pharma, Madrigal, MSD, Northsea Therapeutics, Novartis, Novo Nordisk, Pfizer, Roche, Sanofi, Siemens Healthineers. Research Funding: Boehringer Ingelheim, Siemens Healthcare GmbH. Stock Options: AGED diagnostics, Hepta Bio. Speaker Honorarium: Gilead Sciences, Advanz, Echosens, MedPublico GmbH. ED: speaking fees: Abbvie, Gilead. TG: advisory board for GoLiver Therapeutics. CM: speaking and/or consulting fees from Abbvie, Gilead, MSD, Intercept. Research support: Abbvie, Gilead, MSD, Intercept. JT: speaking and/or consulting fees from Abbvie, Gilead, Viiv. TD: Speaking and/or consulting fees: Abbvie, BMS, Gilead, MSD, Roche. JF: no conflicts to disclose. TB: Speaking and/or consulting fees: AbbVie, Alexion, Bayer, Boehringer Ingelheim, BMS, Gilead, GSK, Intercept, Janssen, MSD/Merck, Merz, Novartis, Sequana Medical and Roche. Research support: AbbVie, Roche, BMS, Gilead, Novartis, Merck/MSD, Intercept, Janssen, Novartis, Sequana Medical, and Pfizer. AEK: speaking and/or consulting fees: Abbvie, Advanz, Alentis, AlphaSigma, AOP Orphan, AstraZenca, Avior, Bayer, BMS, CMS, CymaBay, Eisai, Escient, Falk, Gilead, GSK, Guidepoint, Intercept, Ipsen, Lilly, Medscape, Mirum, MSD, Myr, Novartis, Roche, Takeda, Viofor, Zambon. Research support: Intercept, Gilead. BM: speaking and/or consulting fees: Merck/MSD, AbbVie, Intercept, Astra, Bayer, BMS, Gilead. Research support: Gilead. SZ: consultancy and/or speaker's bureau: Abbvie, BioMarin, Boehringer Ingelheim, Gilead, GSK, Ipsen, Madrigal, MSD/Merck, NovoNordisk, and SoBi. CS: speaking and/or consulting fees: AbbVie, Gilead, Merck/MSD. Research support: AbbVie, Gilead.

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Contributed data or analysis tools: CPG, KP, KD, PB, KHP, JV, GD, AG, FPR, TB, JMS, ED, TG, CM, JT, TD, JF, TB, AEK, BM
Designed the research study and wrote the paper: JD, CS, SZ
Reviewed the results and approved the final version of the manuscript: all authors

Data availability statement

The authors confirm that the data supporting the findings of this study within the article and/or supplementary materials are available upon request.

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Supplementary data

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Appendix A

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Supplemental information

Rare HCV subtypes and retreatment outcomes in a cohort of European DAA-experienced patients

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Rare HCV subtypes and retreatment outcomes in a cohort of European DAA-experienced patients

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Table S1: Countries of origin of DAA-failure patients with rare GT

Patient	**Rare GT**	
Country	**Rare GT1 (n=6)**	**Number of patients n (%)**
Germany	1*, 1c, 1e	n=3
France	1e	n=1
Togo	1l	n=1
Nigeria	1*	n=1
	Rare GT2 (n=2)	
Germany	2k	n=1
Niger	2k	n=1
	Rare GT3 (n=15)	
Germany	3k	n=1
Italy	3h	n=1
India	3b, 3g, 3h, 3i	n=5
Bangladesh	3b, 3g	n=3
Pakistan	3b	n=2
Syria	3*	n=1
n.d.	3b	n=2
	Rare GT4 (n=28)	
Germany	4*,4c, 4f	n=3
Belgium	4r	n=1
France	4r	n=1
Switzerland	4f	n=1
Egypt	4n, 4o	n=4
Angola	4r	n=1
DR Congo	4r	n=5
Burundi	4b	n=1
Eritrea	4r	n=5
Nigeria	4v	n=1
Saudi-Arabia	4r	n=2
n.d.	4r	n=3
	GT5a (n=2)	
France	5a	n=1
South Africa	5a	n=1
	Rare GT6 (n=7)	
Germany	6f, 6n	n=2
Vietnam	6e, 6r	n=2
Thailand	6f, 6r	n=2
n.d.	6e	n=1

*subtype unassigned
n.d., not determined

Table S2: Primer and PCR conditions for NS5B amplification

Primer Name	Sequence	PCR*
RZ_1b_NS5B_out_fw	CTC CGT GTG GRA GGA CTT G	Outer PCR
RZ_1a_NS5_out_fw	CCG TGT GGA ARG ACC TTC TG	
P3_8713_out_rev	GAV RCR TTG GAG GAG CAN GAT GT	
NS5B_7998_out_F	CCA ATH SMY ACH ACC ATC ATG GC	Inner PCR
P3_6816_rev**	GGC GGA ATT CCT GGT CAT AGC CTC CGT GAA	
P6_8611_rev**	AAT TCC TGG TCA TAG CCT CCG TGA AGA CTC	

*outer and inner PCRs were each performed with 40 cycles at 54°C. Sequencing of PCR products was conducted using P6_8611_rev. Further details can be found in [1]
**Primer sequence adapted from [2]

Table S3: Published reference sequences

Author	Title	Citation
R. Xu	A panel of 16 full-length HCV genomes was characterized in China belonging to genotypes 1–6 including subtype 2f and two novel genotype 6 variants	[3]
C. Li	Characterization of full-length hepatitis C virus sequences for subtypes 1e, 1h and 1l, and a novel variant revealed Cameroon as an area in origin for genotype 1	[4]
L. Lu	Full-length genomes of 16 hepatitis C virus genotype 1 isolates representing subtypes 1c, 1d, 1e, 1g, 1h, 1i, 1j and 1k, and two new subtypes 1m and 1n, and four unclassified variants reveal ancestral relationships among subtypes	[5]
L. Lu	Full-length genome sequences of five hepatitis C virus isolates representing subtypes 3g, 3h, 3i and 3k, and a unique genotype 3 variant	[6]
C. Li	Complete genomic sequences for hepatitis C virus subtypes 4b, 4c, 4d, 4g, 4k, 4l, 4m, 4n, 4o, 4p, 4q, 4r and 4t	[7]
C. Li	An expanded taxonomy of hepatitis C virus genotype 6: Characterization of 22 new full-length viral genomes	[8]

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