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Global evolutionary analysis of chronic hepatitis C patients revealed significant effect of baseline viral resistance, including novel non-target sites, for DAA-based treatment and retreatment outcome

Running Title: **DAA treatment failure of chronic hepatitis C and resistance**

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Abstract

Background and aims: Direct-acting antivirals (DAAs) have proven highly effective against chronic hepatitis C virus (HCV) infection. However, some patients experience treatment-failure, associated with resistance-associated substitutions (RASs). Our aim was to investigate the complete viral coding sequence in hepatitis C patients treated with DAAs to identify RASs and the effects of treatment on the viral population. We selected 22 HCV patients with sustained virologic response (SVR) to match 21 treatment-failure patients in relation to HCV genotype, DAA regimen, liver cirrhosis and previous treatment experience. Viral-titer data were compared between the two patient groups and HCV full-length open reading frame deep-sequencing was performed. The proportion of HCV NS5A-RASs at baseline was higher in treatment-failure (82%) than matched SVR-patients (25%) (p=0.0063). Also, treatment failure was associated with slower declines in viremia titers. Viral population diversity did not differ at baseline between SVR- and treatment-failure patients, but failure was associated with decreased diversity probably caused by selection for RAS. The NS5B-substitution 150V was associated with sofosbuvir treatment-failure in genotype 3a. Further, mutations identified in NS2, NS3-helicase and NS5A-domain-III were associated with DAA treatment-failure in genotype 1a patients. Six retreated HCV patients (35%) experienced 2nd treatment failure; RASs were present in 67% compared to 11% with SVR. In conclusion, baseline RASs to NS5A inhibitors, but not virus population diversity, and lower viral titer decline predicted HCV treatment-failure. Mutations outside of the DAA-targets can be associated with DAA treatment-failure. Successful DAA retreatment in patients with treatment-failure was hampered by previously selected RASs.

Keywords: Direct-Acting Antivirals; DAA; hepatitis C virus; HCV; Resistance-associated substitution; RAS; Next Generation Sequencing; NGS; Treatment-failure; GWAS.

Introduction

It has been estimated that 71 million people are chronically infected with hepatitis C virus (HCV) worldwide[1]. The introduction of direct-acting antivirals (DAAs) targeting essential functions of HCV have increased cure rates to greater than 90% in clinical trials and in real-life settings[2–5]. Nonetheless, with millions treated worldwide, treatment failures remain a significant problem[6]. The increasing number of approved DAAs have resulted in multiple resistance-associated substitutions (RASs) towards all DAAs with varying degrees of resistance[2,7–12]. The presence of RASs in the NS3 protease (NS3P), NS5A protein and the NS5B polymerase prior to treatment have been related to treatment failure and lower cure rates[6,13]. Some RASs inhibit multiple DAAs of the same class, especially those targeting the NS5A protein. Most studies have focused on the common RAS positions inside the three DAA targets[14]. However, we and others have previously shown that co-selected substitutions outside those positions also affect DAA treatment outcome[9,15]. Further, sequencing studies describing patients that experience treatment failure rarely obtain matched samples prior to and after treatment failure combined with complete patient information and viral load measurements during treatment. Finally, most studies omit viral sequencing data from matched SVR-patients for comparative analysis.

Here we present a study of HCV deep sequencing data from real-life DAA treated patients with chronic HCV infection experiencing treatment-failure matched with patients achieving cure in relation to HCV genotype (GT), liver cirrhosis, DAA regimen and previous treatment experience. We analyzed the entire HCV coding sequence, which permitted analysis of viral genome regions not targeted by DAA. Furthermore, sequencing allowed detailed analysis of resistance development and baseline resistance important for treatment and retreatment after treatment-failure. In addition, we measured viral load during treatment to investigate if treatment outcome and RASs reflected the DAAs' effect on viral load reduction.

Materials and methods

Matched patient groups

Twenty-one chronic hepatitis C (CHC) patients with treatment-failure were identified from The Danish Database for Hepatitis B and C (DANHEP), as described[16,17]. They initiated DAA treatment between 1st of January 2014 - 1st of May 2015, and were either treated outside clinical trials, fulfilling then applicable Danish treatment criteria, or participated in clinical trials with grazoprevir/elbasvir ± uprifosbuvir or ruzavir/grazoprevir/uprifosbuvir and ribavirin (RBV), including patients with different liver fibrosis status. A single failure patient who experienced a late viral relapse was previously described[18].

To evaluate any differences in the prevalence of baseline RASs between patients with treatment-failure versus patients with SVR, we selected 22 patients from the 407 patients with SVR in the inclusion period matched on HCV GT, DAA regimen, liver cirrhosis, and previous treatment. Matching were attempted 1:1. If this was not possible, matching was done prioritizing the parameters in the order described above.

Definition of Danish treatment criteria at the time was liver biopsy (Metavir score $\geq$F2)[19], transient elastography $\geq$10 kPa, clinical cirrhosis or, regardless of fibrosis status, extra-hepatic manifestations (see Supplemental Methods and Material)[19–21]. We previously published the demographic data for 22 patients with SVR, 17 patients with treatment-failure[16], and 13 patients with GT3[22].

Treatment-failure was defined as non-response (persistent HCV-RNA level >15 IU/ml during treatment), viral relapse (HCV-RNA >15 IU/ml within 12 weeks post-treatment), or viral breakthrough (HCV-RNA levels initially decreased to <15 IU/ml during treatment followed by a clinically relevant increase >15 IU/ml while on treatment). Cure was defined as SVR12 (sustained virologic response, HCV-RNA <15 IU/ml 12 weeks post-treatment). The Danish Data Protection Agency approved the study (2012-58-0004).

Statistical analysis

Categorical variables were reported as absolute numbers and relative frequencies. Continuous data were summarized as mean and standard error or median and interquartile ranges. SAS 9.4 software (SAS Institute) or GraphPad Prism for t-test and Fisher's exact test was used for all statistical analyses and p-values <0.05 (two-sided) were considered statistically significant.

Next generation sequencing (NGS)

All baseline samples from the 22 patients with SVR and from 20 of the 21 patients with treatment-failure, as well as 16 of the 21 post-treatment samples were sequenced using high coverage RT-PCR NGS. Sample preparation for RT-PCR NGS was performed as described[9,23]. In short, total RNA was extracted from 100 µl of serum samples. Except for the GT3h patient, full-length open reading frame (ORF) RT-PCR was performed for the different HCV subtypes using specific HCV primers (Table S1). The library prep was done by NEBnext Ultra II DNA library kit and all libraries were sequenced on Illumina Miseq with a 2 x 250 bp. v2 kit.

Four post-treatment samples (patient 1, 7, 15 and 16) were sequenced by RNA-seq with lower coverage. RNA was purified for RNA-seq from 0.5ml plasma using the NucliSens easyMAG System (BioMérieux). RNA-seq libraries were prepared with the NuGEN Ovation RNA-seq V2 kit followed by sequencing on the Ion Torrent Proton Platform (ThermoFisher).

The post-treatment sample from a single patient was originally Sanger sequenced to identify resistance, which did not leave any material for subsequent NGS. RNA was purified as described above for the RNA-seq, and amplicons for Sanger sequencing were amplified by an in-house RT-PCR assay using random hexamers, and published and in-house developed primers for specified regions of NS3[24], NS5A[25] and NS5B (NS5B primers in Table S1).

Sequence analysis

Data analysis was done using a customized data pipeline to detect major and minor variant RASs[26]. De novo assembly was performed by IVA, and Blast was used against NS3 of all subtypes to verify genotyping of the isolate. Subsequently, reads were aligned to reference subtype proteins NS3, NS5A and NS5B with BWA using the MEM algorithm. LoFreq was applied to detect the low frequency SNPs as described[9]. Translational effects were detected by VCF annotator (Broad Institute), and in-house scripts were applied to detect RASs by comparing to the Geno2Pheno database. We chose to record RASs with a 15% cutoff in both baseline and post-treatment failure samples[6]. Individual codons with more than one RAS were manually inspected to ensure the correct translation. Phylogenetic analysis of the baseline and post-treatment sequences was done as described[26] except that the phylogeny was built by the PhyML package[27]. Population heterogeneity was determined as described[9]. SNPgenie was applied with a 0.5% cutoff. Finally, we searched for novel resistance-associated mutations in genotype 1a sequences by using BMAGWA, which is a tool for genome-wide association studies (GWAS) (see Supplemental Methods and Material), using an equal number of patients with SVR and treatment failure[28].

Results

Patient characteristics

The complete study population is shown in Figure 1a, and the DAA regimens used are described in Tables 1 and 2. Among the 21 HCV patients with treatment-failure, 15(71%) were male, the median age was 62 years (43-72 years), and 11(52%) had GT1a infection, 3(14%) GT2b, 6(29%) GT3a and 1(5%) GT3h infections. Seventeen (81%) patients had liver cirrhosis (Table S2). Nine (43%) patients were treatment-naive, 9(43%) patients had previously been treated with pegylated-interferon (peg-IFN) and RBV, and 3(14%) patients had received peg-IFN and RBV treatment in combination with 1st. generation NS3P-inhibitors or daclatasvir.

Among the matched patients with SVR, 15(68%) were male, median age was 57 years (36 – 68 years), and 10(46%) had GT1a, 2(9%) GT1b, 4(18%) GT2b and 6(27%) GT3a infections. Fifteen (68%) SVR-patients had liver cirrhosis (Table S3). Four (18%) SVR-patients had previously been treated with peg-IFN and RBV.

In order to test how well the two patient groups were matched the following comparisons were made, and no difference was seen in relation to GT (p= 0.75), liver cirrhosis status (p= 0.05), gender (p= 1.0) and age (p= 0.39). The only difference found was that more patients in the treatment-failure group had been treated for CHC with peg-IFN based regimens and 1st. generation NS3P-inhibitors (p= 0.01).

Phylogenetic analysis of SVR-patients and patients with treatment-failure

Twenty baseline - and post-treatment samples from HCV patients with treatment-failure, with 19 patients having both (Table 1), and baseline treatment samples from 22 matched patients with SVR (Table 2) were sequenced by NGS covering the entire ORF, except for the GT3h patient that did not include the 3' part of NS5B. Figure 1b shows a phylogenetic tree based on this set of near full-length ORF sequences. The phylogeny illustrates that for patients with treatment-failure, virus present post-treatment is very closely related to the virus found at baseline, indicating that viral relapse/breakthrough and not reinfection had occurred.

Decrease in HCV-RNA titer was associated with DAA regimen and treatment outcome

One week after treatment initiation, the viral titer in patients with SVR had decreased significantly more than in patients with treatment failure (difference 0.66 $\log_{10}$ IU/ml; Figure 2a). In addition, we could detect the viral RNA significantly longer in the treatment failure group (Figure 2b). Non-synchronized sampling could affect these results, but there was a clear tendency that patients with treatment-failure had detectable HCV-RNA in plasma for a longer period (4 weeks) compared to

the SVR-patients (3 weeks) (Figure 2b). NS5A inhibitors as part of the DAA regimen significantly reduced the duration of detectable viremia within the treatment-failure group, but did not result in a larger drop in viral titer at week 1 (Figure 2c and d). However, this tendency could not be observed within the SVR group where patients cleared the virus between week 3-6 on average and had a large titer decline at week 1 regardless of DAA regimen composition.

Resistance associated substitutions in patient groups with SVR or treatment failure

Among patients with treatment failure, 11 out of 20 (56%) were found to have at least one RAS in a drug-target relevant to treatment at baseline (Table 1). For comparison, RASs towards the used DAA regimen was found in 5 out of 22 (23%) of the patients with SVR (Table 2).

Examining the patients with treatment failure more closely we found that for 9 of 11 patients (82%) treated with an NS5A inhibitor, NS5A RASs were present at baseline (Figure 3a). Baseline RASs towards NS3P-inhibitors were detected in 3 (30%) of 10 patients, and to sofosbuvir in only one (6%) of 16 treated patients.

For patients with SVR we found that in 16 patients, treated with a NS5A inhibitor, only 4 patients (25%) had RASs in the NS5A region at baseline (Figure 3a). In 6 patients treated with a NS3P-inhibitor, substitutions conferring low-level of resistance against the used DAA were found in 2 patients (33%). No RASs towards sofosbuvir were detected (Table 2).

The RASs associated with resistance to at least one of the drugs from the used DAA regimen containing either a NS3P-inhibitor and/or an NS5A inhibitor were found post-treatment in 15 (94%) of 16 patients. For 6 (67%) of 11 analyzed patients treated with an NS3P-inhibitor, high-level NS3P-inhibitor-RASs were found post-treatment. In addition, for 10 (91%) of 11 patients treated with an NS5A inhibitor high-level NS5A-RASs were detected post-treatment. In contrast, only 2 (13%) of 16 patients treated with sofosbuvir had detectable NS5B-RASs post-treatment (Table 1).

Sofosbuvir and ribavirin treatment failure

Only 2 among 16 patients (13%) treated with sofosbuvir, with or without RBV, had detectable NS5B RASs after the end of treatment. However, we observed the 150V NS5B substitution at baseline in 4 out of 5 (80%) and 1 out of 5 (20%) of the GT3a failure and SVR patients treated with sofosbuvir, respectively. In addition, when correlating to NS5A RASs relevant to treatment regimen, HCV isolated from 4 out of 5 (80%) treatment-failure patients, and 0 out of 5 (0%) SVR patients had both the 150V NS5B substitution and relevant NS5A RASs (Table 1 and 2). In addition, three of the failure patients with 150V also received RBV as part of their treatment. The

substitution 150V was also found in virus isolated at baseline from one of the two GT2b failure patients treated with sofosbuvir, while none of the three SVR GT2b patients treated with sofosbuvir had the mutation. This indicated that 150V might play a role in treatment outcome in GT3a and GT2b patients. None of the GT1a patients had the 150V substitution and sequence comparison before and after treatment did not reveal any specific amino acid change in the treatment-failure group compared to the SVR group that could explain the different treatment outcome to sofosbuvir with or without RBV treatment.

Resistance persistence and off-target putative compensatory NS3-helicase mutations in patients with treatment failure

All NS5A RASs, once fixed in the virus population, persisted long-term with no sign of reversion. Consistent with these observations, no clear mutation development outside the NS5A domain I was observed. Similarly, persistence was observed for NS3P RAS 80K, 155K and 168/H/E/V. However, for the one patient with the 168V RAS, relapse from a non-NS3P regimen led to almost complete reversion going from fixation at baseline to 3% post 2nd treatment. For patients treated with the NS3P-inhibitors simeprevir or paritaprevir, we observed mutations in 4 out of 6 patients in the NS3 helicase at conserved aa positions S1368P, E1383D/A and V1516M.

Treatment effects on virus population structure

It has been suggested that a heterogeneous viral population may be more fit than a homogeneous population and may more easily adapt to changes such as drug treatment. In order to test whether heterogeneity influenced treatment outcome, diversity was quantified as the mean pairwise sequence difference in each sample across all coding sites in the genome (Figure 3b). No significant difference was found between diversity in the treatment-failure and SVR groups at baseline. This suggests that heterogeneity per se was not associated to treatment outcome in this analysis, but as seen above the specific nature of the sequence variants present at baseline did have an impact.

Although a substantial spread in diversity was observed within each group, a large and significant drop was observed in patients with treatment-failure post-treatment indicating that treatment induced a pronounced selective pressure. Further, analysis of the treatment failure group showed that this drop in the diversity was caused mostly by patients in whom RASs evolved during treatment, while patients who already had fixed RASs at 100% at baseline, and no additional evolution of RAS, had no loss of heterogeneity (Figure 3c). This indicates that in patients with pre-existing fixed RAS there is no or very little further selection, and the previously

accumulated sequence diversity thus remains. In patients that do not have fixed RAS at the outset, the few, newly evolved or pre-existing RAS-containing viruses are rapidly selected, thus leading to a strong decrease in heterogeneity.

Retreatment, resistance and outcome in patients with treatment failure

Retreatment with DAAs in experienced patients can be difficult due to development of multi DAA target resistance. Seventeen (81%) of 21 patients with treatment failure were retreated, while 3 (14%) patients declined retreatment, and one (5%) patient was diagnosed with terminal hepatocellular carcinoma, and therefore not retreated. In total, 9 (53%) out of 17 patients achieved SVR, one patient had SVR at week 8, but deceased before week 12, and one patient deceased at treatment week 7 during retreatment before SVR. Six retreated patients (35%) experienced retreatment failure (Figure 1a).

For all retreated patients, DAA regimens containing a NS3P-inhibitor and/or NS5A inhibitors were used in combination with an NS5B inhibitor ± RBV (Table 3). Four (67%) out of 6 patients with 2nd treatment failure had RASs against the NS5A or the NS3P part of the treatment regimen at baseline of 2nd treatment (Figure 3d). Only one (11%) out of 9 patients who were retreated and achieved SVR had RASs against the NS5A and the NS3P part of the treatment regimen (Figure 3d). In addition, the retreated patient with SVR (Pt. nr. 5) treated with grazoprevir had 155K and 80R in NS3P that has been shown to only give minor resistance in vitro[8]. Interestingly, 3 (50%) out of 6 patients with 2nd treatment failure were retreated with the same regimen composition regarding the drug targets, while only 1 (11%) of the retreatment SVR patient received the same regimen composition directed at the same drug target as original treatment (Figure 3e). This observation correlated well with the observed RASs pattern described above.

We found RASs in the NS5A region post 2nd treatment in 4 patients who failed a NS5A containing regimen. In the NS3P-inhibitor treated patient (Pt. nr. 1) with relapse, RAS 155K conferring with high resistance against the NS3P-inhibitor simeprevir was already present before retreatment.

Search for novel resistance-associated mutations

We used a GWAS approach to search for novel resistance-associated mutations in all the sequenced GT1a patients at baseline, including 7 out of 10 with treatment failure and 8 out of 10 with SVR treated with sofosbuvir (Table 1 and 2). The idea was to find variants besides RAS in the nonstructural proteins that were predictive of treatment-failure. Figure 4a shows a summary of

the results in the form of a Manhattan plot. The analysis revealed "high confidence" amino acid sites that are shown in dark blue on the plot. The indicated sites include several residues in NS2 (mostly in the protease domain), NS3 (both protease and helicase domains), and one site near the C-terminus of NS5A (Figure 4b).

Discussion

Although DAA treatment of CHC patients has shown high SVR rates, some patients fail treatment and the ability to understand and predict treatment failure is important for future treatment and prevention of global resistance development[29]. Our results demonstrated that RASs present at baseline could be linked to treatment outcome and decline of viral titer in two matched groups of patients experiencing either SVR or treatment-failure. Often in real-life studies, the number of patients with DAA treatment failure and available samples for analysis are limited, and therefore we considered it a strength of the present study that the entire virus polyprotein data, including RASs, from baseline and at treatment-failure for 20 patients, as well as frequent blood sampling during treatment for viral titer determination for patients with both SVR and failure, were available[30,31].

Most of the included patients had advanced liver disease and the majority of the DAA regimens used are now considered obsolete, which means that the SVR rate might be higher, and the treatment-failure rate lower, in patients with less advanced liver disease or with treatment including more superior DAA regimens. However, since we had a matched SVR group with these parameters, liver cirrhosis, HCV genotype and DAA regimen was unlikely to be the sole cause of treatment failure. In addition, the strength of these groups being matched allowed that differences in viral loads, resistance development and virus evolution could be linked directly to treatment failure. Although some of the DAAs in this study were not the ones currently used, cross-resistance to the novel DAAs within a given drug target is well recognized[9,11] and therefore the resistance patterns are still relevant to treatment with the newest DAA regimens.

The treatment-failure group had a smaller reduction in viral load at week 1 and a longer period of detectable HCV RNA compared to the SVR group. This was probably due to the RASs already present or developing in the treatment failure group as observed in other studies[31,32]. The use of NS5A inhibitors reduced the period with detectable HCV RNA in the treatment group. This is in accordance with other studies that has observed increased reduction of viral load with NS5A inhibitors[33–36]. However, titer drop at week 1 did not differ from the non-NS5A containing regimens probably since most patients in the treatment failure group already had NS5A RASs at baseline.

We detected baseline RASs causing high levels of resistance towards the used NS5A inhibitor in 82% of the patients with treatment-failure compared to 25% in the matched patients, who achieved SVR. The presence of viral variants resistant to NS5A inhibitors at baseline has

been associated with lower SVR rates especially in patients with HCV GT1a and 3, liver cirrhosis and/or prior non-response to peg-IFN based regimens[6,37]. The selected NS5A RASs often remain in the virus population for years, and could influence future treatment outcome, as we observed in the patients with 2nd treatment failure. Since all currently used DAA regimens contain NS5A inhibitors and cross resistance is well documented[11], NS5A RASs may pose a threat to treatment efficacy and especially retreatment options.

In patients with SVR, we only detected RASs giving a low level of resistance to the given NS3P-inhibitor grazoprevir, whereas 25% and 58% of the patients with treatment-failure had RASs conferring a high-level of resistance at baseline and post-treatment, respectively. This supports the hypothesis that the presence of RASs in the NS3 region at positions other than 155, 156 and 168, do not affect clinical outcome. No RASs were detected for the patients treated with grazoprevir post-treatment. The reason could be that the mutations at position 156 conferring high resistance to grazoprevir also come with a high fitness cost and therefore quickly revert after the end of treatment[9]. It is relevant that the patients were sequenced late after end of treatment, thus giving the virus more time to revert. However, rapid NS5A escape could be enough to make treatment fail without the development of the 156 RAS because of the high fitness cost. This could also explain why no NS3 helicase mutations were observed in these patients in contrast to the patients treated with paritaprevir or simeprevir.

RASs towards sofosbuvir are rarely found in patients with treatment failure[6], which might be due to the large reduction in replication capacity caused by these RASs. We detected low level RASs towards sofosbuvir in two patients (13%) post-treatment, which is in accordance with what has been seen in clinical trials [38]. We also found substitution A150V in the NS5B polymerase associated with treatment failure in GT3a patients as seen in a previous study[39] and indications that this mutation could also affect GT2b. The high prevalence of 150V in GT3a ranging from 19-44%[40] at baseline could affect treatment with sofosbuvir/velpatasvir (Epclusa®) and sofosbuvir/velpatasvir/ voxilaprevir (Vosevi®), especially if NS5A RASs are also present.

The observed differences across the whole coding sequence that was detected post-treatment compared to baseline was in accordance with a previous study[31]. One of the main observations we made regarding evolutionary dynamics of HCV in this study, is that there are two distinct subsets in the treatment failure group: In one group of patients the viral population already has RAS present at high frequency (or fixed) at the outset. For these patients, viral diversity is found to be more or less constant from baseline to post-treatment, indicating a lack of purifying

selection, presumably because the virus is already resistant. In the other group of treatment-failure patients, RAS are present at low frequencies at baseline, and for this group we find a marked decrease in viral diversity over time. This seems to suggest that there is strong selection for the pre-existing or newly evolving RAS, thus only the subset of resistant viruses survive.

The high confidence positions related to failure for GT1a detected by GWAS included sites in NS2, NS3, and NS5A (Figure 4). We note that two of the sites (amino acid 210 in NS2 and amino acid 441 in NS5A) both are located 8 amino acids N-terminally to protease cleavage sites that could be related to a switch from replication to assembly as observed in cell-culture[41,42]. None of the aa residues are associated with resistance in HCV databases Geno2Pheno or HCV-GLUE[43,44]. It will require additional investigations in cell-culture systems to determine the effect of these mutations to DAA treatment, in particular to sofosbuvir treatment.

It was recommended that patients who fail 2nd generation DAA treatment should be retreated with a different DAA combination, and that the addition of RBV and/or extension of the treatment period should be considered[2]. This recommendation was followed in the majority of patients who were retreated and lead to an SVR rate of 53% (n=9/17) in patients who could be evaluated. However, a clear correlation between RASs and previous DAA combination could be seen in the 2nd treatment failure patients. In addition, 2nd treatment failure in some cases resulted in RASs against both NS3 and NS5A inhibitors, which could have a crucial impact on further retreatment. A similar finding has been reported, where patients were retreated after initial DAA failure. In one study, two patients required four DAA treatments in total to obtain SVR and a single patient still relapsed after the 4th treatment[45]. At present, systematic resistance testing before first-line therapy is not recommended, since it will limit the access to care due to no standardized assay available. It should, however, in relation to our data be considered in difficult to treat patients with advanced liver disease to identify possible high-level RASs in the NS5A region if appropriate expertise is available.

Our data highlights baseline resistance to NS5A inhibitors and lower viral titer decline was associated with treatment failure. In addition, successful DAA retreatment in patients with treatment failure was hampered by previously selected RASs. We found substitutions in NS5B associated with failure to sofosbuvir treatment. Finally, we found novel mutations outside the target sequence associated with failure to DAA-treatment.

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Figure Legends

Figure 1. Patient group description and phylogenetic analysis of HCV from patients with confirmed treatment failure and from a matched control group with sustained virological response (SVR) after DAA-based therapy. **a)** Flowchart of the matched patient groups with chronic hepatitis C who initiated DAA treatment. The patients with SVR were selected to match the patients with treatment-failure based on HCV genotype, DAA regimen, liver cirrhosis status and previous treatment experience. Patients retreated are indicated with outcome when available. **b)** Phylogenetic tree based on near full-length ORF consensus sequences (nucleotides 342 to 9283 on HCV reference strain H77) from both treatment failure and SVR patients. The treatment failure patient samples are coded in red, while the SVR samples are coded in blue as in a. For the treatment-failure patients, baseline and post-failure samples are marked by the suffix B and P, respectively. Note how viruses from baseline and post treatment samples for a specific patient are always each other's closest relatives, indicating that failure is caused by relapse or breakthrough, and not by reinfection. For treatment failure patient 6 no baseline sample was available for sequencing. The patient 9 post sample had only Sanger sequencing of NS3, NS5A and NS5B performed and could not be included in the phylogenetic analysis. For four out of six patients with 2nd treatment failure, baseline and post failure samples were sequenced and are marked 2nd_B and 2nd_P, respectively. The genotypes and subtypes are indicated to the right of the node names. The assembled consensus sequences were aligned with reference subtypes (coded in black) using MAFFT. Subsequently, the phylogenetic tree was constructed using PhyML. The bar indicates genetic distance measured as nucleotide substitutions per site.

Figure 2. Viral kinetics during DAA treatment was associated with treatment outcome in patients with chronic hepatitis C. a: Decrease in virus titer is larger for the SVR group: decrease of HCV RNA titer was measured in serum (mean IU/ml ± SEM of log_{10} decrease) at week 1 post treatment initiation compared to baseline for SVR (n=21) and treatment-failure (n=13) patients. A t-test was applied to determine significance of differences between SVR and treatment-failure patients ($p = 0.025$) with * indicating significant values. b: Virus is detectable longer in the treatment failure group. This plot shows the time of last HCV RNA positive sample for SVR (n=22) and treatment-failure (n=21) patients measured in medians with interquartile range of weeks from baseline during treatment. A Mann Whitney test was applied to determine significance of differences between SVR and treatment failure patients ($p = 0.013$) with * indicating significant values. c and d: Viral kinetics compared to regimen composition. The

patients were divided into 3 groups of regimen composition; NS5A/(NS3P)/NS5B: regimen always containing NS5A and NS5B inhibitors and sometimes NS3P; NS3P/NS5B: regimen always containing NS3P and NS5B inhibitors; and NS5B: regimens containing NS5B inhibitors. c: decrease of HCV RNA titer (means ± SEM of $\log_{10}$ decrease) at week 1 compared to baseline for SVR (NS5A/(NS3P)/NS5B n=14, NS3P/NS5B n=1, NS5B n=5) and treatment failure patients (NS5A/(NS3P)/NS5B n=7, NS3P/NS5B n=5, NS5B n=2) measured in serum divided into regimens of treatment. d: time of last positive HCV RNA sample for SVR and treatment failure patients measured in medians with interquartile range of weeks from baseline during treatment divided into regimens of treatment.

Figure 3. Baseline NS5A RASs are correlated with HCV treatment outcome in NS5A containing DAA regimens and viral population heterogeneity does not predict treatment outcome. a: Fraction of SVR and treatment-failure patients having pre-existing NS5A RASs relevant to treatment. Fischer's exact test was applied to determine significance of differences between the SVR and the treatment failure group ($p = 0.0063^*$) with * indicating significant value b: pair-wise genetic distance within each sample for SVR (n=22) and treatment-failure patients (n=20) at baseline and for the treatment failure patients, post-treatment (means ± SEM). c: pair-wise genetic distance for two subgroups of treatment-failure patients. Fixed RASs (n=6): The patient's viral population all had pre-existing RAS at baseline and no further RASs development during treatment. Evolving RASs (n =12): presence of low-frequency RAS only at baseline, or development of RASs during treatment. A t-test was applied to determine significance of differences between baseline and post samples in Fixed RASs and Evolving RASs ($p = 0.5206$, $p = 0.0028^*$), respectively with * indicating significant values. d: Fraction of patients with pre-existing NS5A and NS3P RASs relevant to retreatment after 2nd treatment and 2nd treatment failure. Fischer's exact test was applied to test if differences were significant ($p = 0.0889$) e: similarity between retreatment regimen composition and 1st treatment for 2nd treatment-SVR and 2nd treatment failure patients; higher values indicate that the same regimen composition had been used twice.

Figure 4: GWAS analysis of GT1a baseline sequences. a: Manhattan plot showing the strength of association between HCV genomic variants and drug resistance. Points represent genomic variants, with the location in the genome shown on the x-axis and the y-axis showing how much support there is for this site having an impact on drug resistance. For the strongest signals, the location of the site in each protein is indicated on a label next to the dark blue point. Analysis was based on nonstructural protein amino acid sequences for HCV genotype-1a from 20 patients – 10

with SVR and 10 with treatment failure at baseline. An alignment of the nonstructural protein sequences was then analyzed using a Bayesian method for GWAS (BMAGWA), with SVR/treatment-failure as phenotype and the amino acid alignment recoded into pseudo-genotypes. The strength of association is here expressed as Bayes factors, which essentially quantify the degree to which the sequence data supports the site having an impact on drug resistance. To distinguish between real and spurious signals, we performed the analysis on 1000 data sets where the phenotype had been randomly shuffled. Sites having Bayes factors larger than 95% of these random values are labeled as high confidence. The indicated sites include several in NS2, NS3 (both protease and helicase domains), and one site near the C-terminus of NS5A. b: Table depicting aa residue composition in SVR and failure groups at high-confidence sites as number of sequences represented by each residue. Protein numbers shown for both individual mature protein and polyprotein according to the genotype 1a H77 reference sequence.

Table 1. Resistance associated substitutions for 21 hepatitis C patients with treatment failure

Patient No.	GT	DAA treatment (wks)	Previous treatment	RAS BL NS3 protease (%)	RAS BL NS5A (%)	RAS BL NS5B polymerase (%)	RAS Post NS3 protease (%)	RAS Post NS5A (%)	RAS Post NS5B polymerase (%)	Post sequenced weeks after EOT
1	1a	SOF/LED/RBV (12)	PR	155K (96.5)	None	None	155K (100)	30R (99)	None	31
2	1a	SOF/SIM/RBV (12)	BOC/PR	None	None	None	155K (99.3)	None	None	12
3	1a	SOF/SIM/RBV (18)	BOC/PR	54S (51.6)	None	None	168V (96.2)	None	None	19
4	1a	ELB/GRZ/UPR (16)	PR	None	28M (48.1), 28V (51.9), 31M (36.9)	None	None	30R (92.3), 31M (99.9)	None	16
5	1a	SOF/SIM/RBV (24)	TVR/PR and DAC $/PR	54S (14.1)¶, 155K (21.8)	28T (18.5), 30E (37.9), 30H (19.1)	None	54S (99.7), 80R (99.8), 155K (99.8)	30E (99.9)	None	12
6	1a	SOF/SIM (12)	TN	NA	NA	NA	None	None	None	33
7	1a	PAR/OMB/DAS/Rit/RBV (12)	PR	80K (99.9)	58D (48.1)	None	80K (100)	58D (100)	None	4
8	1a	SOF/SIM/RBV (12)	PR	80K (99.9)	None	None	80K (99.3), 155K (53.8), 168E (43.6)	None	None	28
9	1a	SOF/SIM/RBV (24)	PR	None	None	None	D168H (Sanger)	None	None	(Sanger)
10	1a	ELB/GRZ (12)	TN	None	None	None	None	None	None	14
11	1a	SOF/LED/RBV (12)	PR	None	30E (54.7)	None	None	30E (99.8), 31M (99.8)	None	14
12	2b	SOF/RBV (24)	PR	None	None	None♯	None	None	None	15
13	2b	ELB/GRZ/UPR (8)	TN	None	31M (56.2)	None	None	31M (99.9)	None	27
14	2b	SOF/RBV (16)	TN	None	None	None	None	None	None	38
15	3a	SOF/LED (12)	TN	None	30K (100)	None♯	None	30K (100)	None♯	21
16	3a	SOF/RBV (16)	PR	None	30K (99.9)	None♯	None	30K (99.8)	None♯	4
17	3a	SOF/LED/RBV (12)	TN	None	30K (99.9)	None♯	None	30K (91.8)	None♯	4
18	3a	ELB/GRZ/UPR (8)	TN	None	93H (99.9)	None	None	93H (99.9)	None	24
19	3a	SOF/DAC/RBV (12)	TN	None	93H(31.9)	None	None	93H (100)	None	59
20	3a	SOF/RBV (24)	TN	None	None	None♯	None	None	159F (100)♯	26
21	3h	DAC/SOF (24)	PR	None	28A (100)	289L (99)	None	28A (100)	289L (98), 321A (2.8)¶	3

DAA: direct acting antivirals; RAS: resistance associated substitution; ¶ RASs below 15% cut off level. $: DAC = 20 mg.

GT: genotype; BL: baseline; TN: treatment naïve; EOT: End of treatment; Wk: Week. NA: not available.

TVR: Telaprevir; BOC: Boceprevir; PR: Pegylated-interferon/Ribavirin; Rit: Ritonavir; GRZ: Grazoprevir; ELB: Elbasvir; UPR: Uprifosbuvir; SOF: Sofosbuvir; SIM: Simeprevir; LED: Ledipasvir; RBV: Ribavirin; DAC: Daclatasvir; PAR: Paritaprevir; DAS: Dasabuvir (RAS were only recorded for DAS when it was relevant for treatment); OMB: Ombitasvir. ♯: NS5B substitution 150V was detected in these patients

Table 2. Baseline Resistance Associated Substitutions for 22 patients with chronic hepatitis C who achieved Sustanied Virological Response.

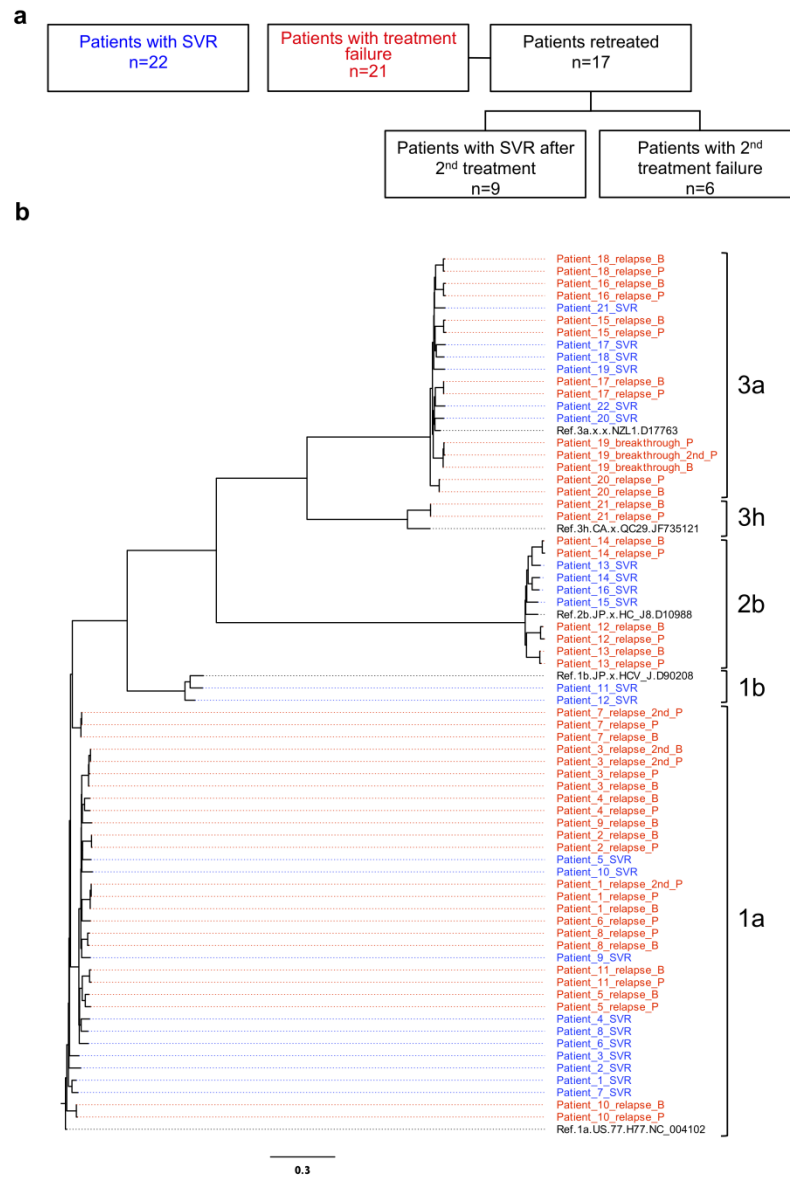| Patient No.	GT	DAA treatment (Wks)	Previous treatment	RAS BL NS3 protease (%)	RAS BL NS5A (%)	RAS BL NS5B polymerase (%)
1	1a	SOF/DAC (24)	TN	None	None	None
2	1a	ELB/GRZ (12)	TN	80K (100)	None	None
3	1a	ELB/GRZ (12)	TN	80R (36)	None	None
4	1a	SOF/LED (12)	TN	None	None	None
5	1a	SOF/LED (12)	TN	None	None	None
6	1a	SOF/LED/RBV (13)	TN	None	None	None
7	1a	SOF/LED (12)	TN	None	None	None
8	1a	SOF/LED (12)	TN	None	28M (100), 31M (96)	None
9	1a	SOF/SIM (12)	PR	None	None	None
10	1a	SOF/LED (13)	PR	55A (100)	None	None
11	1b	SOF/LED (12)	TN	None	30R (84)	None
12	1b	PAR/OMB/DAS/Rit (12)	TN	None	30R (100)	None
13	2b	ELB/GRZ/UPR (9)	TN	None	30K(100)	None
14	2b	SOF/RBV (16)	TN	None	30K(100)	None
15	2b	SOF/RBV (12)	TN	None	30K (100)	None
16	2b	SOF/RBV (24)	TN	None	30K (100)	None
17	3a	SOF/DAC (12)	TN	None	None	None †
18	3a	SOF/DAC (12)	TN	None	None	None
19	3a	ELB/GRZ/UPR (8)	TN	None	None	None †
20	3a	SOF/RBV (24)	TN	None	None	None
21	3a	SOF/RBV (24)	PR	None	None	None
22	3a	SOF/DAC/RBV (24)	PR	None	None	None

DAA: direct acting antivirals; GT: genotype; BL: baseline; PR: Pegylated-interferon/Ribavirin; TN: treatment naïve; Wk: Week.

Rit: Ritonavir; GRZ: Grazoprevir; ELB: Elbasvir; SOF: Sofosbuvir; SIM: Simeprevir; LED: Ledipasvir; RBV: Ribavirin; DAC: Daclatasvir; PAR: Paritaprevir; DAS: Dasabuvir

(RAS were only recorded for DAS when it was relevant for treatment); OMB: Ombitasvir. † NS5B substitution 150V was detected in these patients
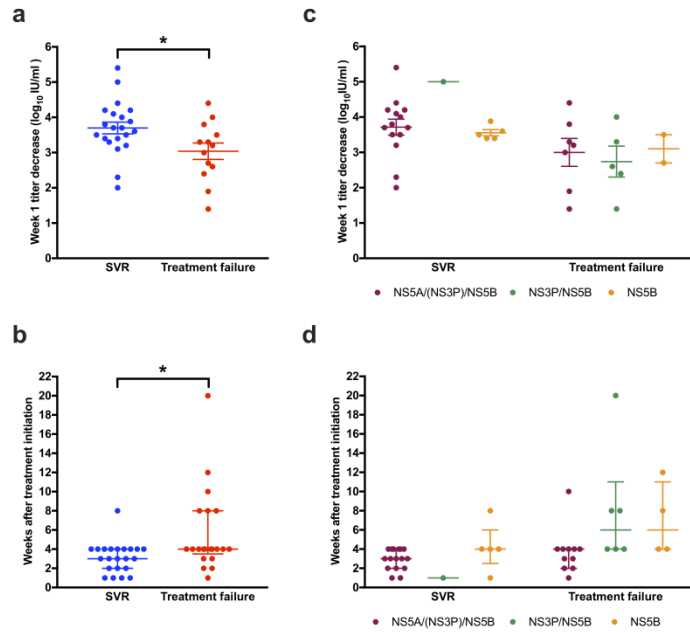
Table 3. Re-treatment regimen and resistance associated substitutions for 21 hepatitis C patients with treatment failure

Patient No.	GT	DAA treatment (wks)	Re-treatment (wks)	Outcome	RAS BL NS3 protease (%)	RAS BL NS5A (%)	RAS BL NS5B polymerase (%)	RAS Post NS3 protease (%)	RAS Post NS5A (%)	RAS Post NS5B polymerase (%)
1	1a	SOF/LED/RBV (12)	SOF/SIM/RBV (16)	Relapse and HCC	155K (100)	30R (99)	None	155K (100)	30R (99)	None
2	1a	SOF/SIM/RBV (12)	SOF/LED (24)	SVR	155K (99.3)	None	None	NA	NA	NA
3	1a	SOF/SIM/RBV (18)	SOF/LED/RBV (24)	Relapse and HCC	168V (99.7)	None	None	168V (3.3)¶	30R (99.9)	None
4	1a	ELB/GRZ/UPR (16)	PAR/OMB/DAS/Rit/RBV (24)	SVR	None	30R (92.3), 31M (99.9)	None	NA	NA	NA
5	1a	SOF/SIM/RBV (24)	SOF/GRZ/ELB/RBV (12)	SVR	54S (99.7), 80R (99.8), 155K (99.8)	30E (99.9)	None	NA	NA	NA
6	1a	SOF/SIM (12)	SOF/LED/RBV (12)	Relapse	None	None	None	None	28T (26.4) 93H (100)	None
7	1a	PAR/OMB/DAS/Rit/RBV (12)	SOF/LED (12)	Relapse	80K (100)	58D (100)	None	80K (100)	58D (99.8)	None
8	1a	SOF/SIM/RBV (12)	None due to HCC	NA	NA	NA	NA	NA	NA	NA
9	1a	SOF/SIM/RBV (24)	SOF/DAC/RBV (24)	SVR	D168H (100)	None	None	NA	NA	NA
10	1a	ELB/GRZ (12)	None	NA	NA	NA	NA	NA	NA	NA
11	1a	SOF/LED/RBV (12)	SOF/SIM/RBV (24)	SVR	None	30E (99.8), 31M (99.8)	None	NA	NA	NA
12	2b	SOF/RBV (24)	SOF/LED/RBV (12)	SVR	None	None	None	NA	NA	NA
13	2b	ELB/GRZ/UPR (8)	None	NA	NA	NA	NA	NA	NA	NA
14	2b	SOF/RBV (16)	None	NA	NA	NA	NA	NA	NA	NA
15	3a	SOF/LED (12)	SOF/DAC/RBV (24)	SVR8, died before SVR12	None	30K (100)	None♯	NA	NA	NA
16	3a	SOF/RBV (16)	SOF/DAC/RBV (12)	SVR	None	30K (99.8)	None♯	NA	NA	NA
17	3a	SOF/LED/RBV (12)	SOF/DAC/RBV (24)	Relapse	None	30K (100)	(A150V)	No data	No data	No data
18	3a	ELB/GRZ/UPR (8)	GRZ/RUZ/UPR/RBV (16)	SVR	None	93H (99.9)	None	NA	NA	NA
19	3a	SOF/DAC/RBV (12)	VEL/SOF (12)	Relapse	None	93H (100)	None	None	93H (100)	None
20	3a	SOF/RBV (24)	PIB/GLE (12)	SVR	None	None	159F (100)♯	NA	NA	NA
21	3h	DAC/SOF (24)	SOF/DAC/RBV (24)	Died during	None	28A (100)	289L (98), 321A	NA	NA	NA

treatment wk 7		(2.8)¶

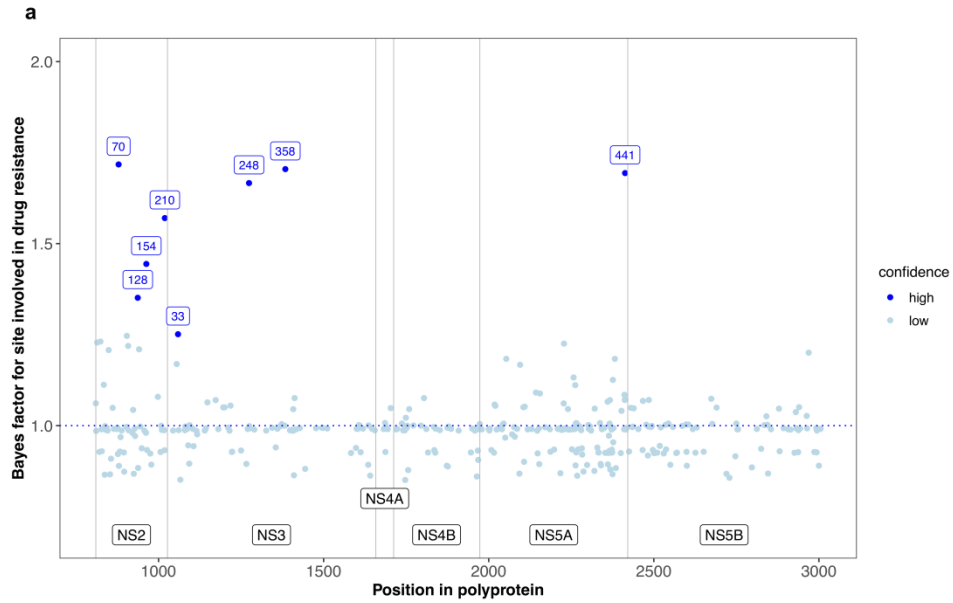jvh_13430_f1.tiff

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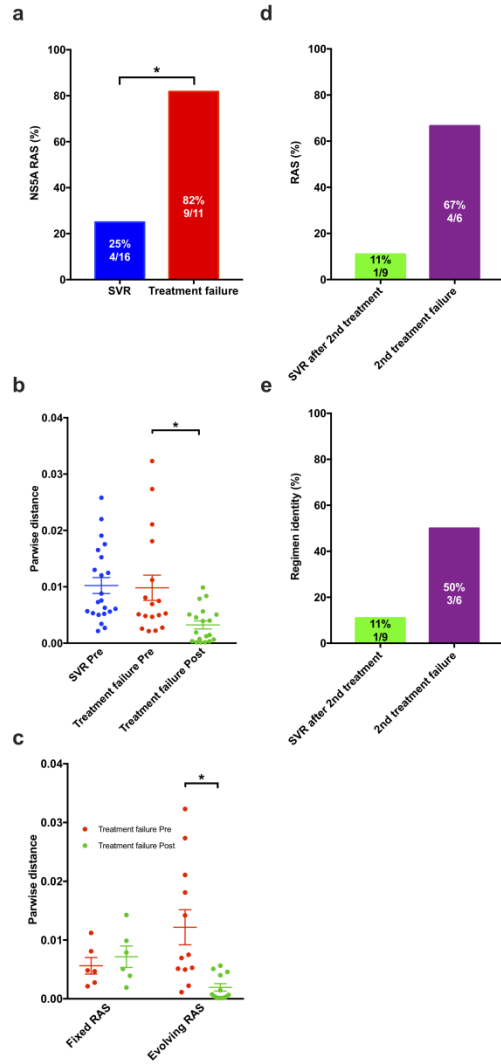
b

Protein	Position in protein	Position in polyprotein	SVR (aa)	Relapse (aa)
NS2	70	879	A:5 V:4 I:1	A:1 V:9
	128	937	A:9 V:1	A:5 V:4 I:1
	154	963	G:6 S:4	G:10
	210	1019	V:7 A:3	V:2 A:3 D:3 T:2
NS3	33	1059	V:10	V:7 I:3
	248	1274	I:7 V:3	I:2 V:8
	358	1384	V:7 A:3	V:2 A:8
NS5A	441	2413	G:7 D:3	G:2 D:8

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