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Pre-existing, treatment-specific resistance-associated substitutions in hepatitis C virus genotype 1 and 3 and viral RNA titers during treatment with direct-acting antivirals

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The introduction of direct-acting antiviral (DAA) treatment of hepatitis C virus (HCV) infected patients has greatly increased treatment success rates. However, viral response kinetics to DAA treatment may depend on pre-existing resistance-associated substitutions (RASs) in HCV. The aim of this study was to describe how pre-existing RASs affect DAA treatment-induced reduction in HCV RNA titers in HCV genotypes 1- and 3-infected individuals. Patients with HCV genotype 1 infection (N = 31) treated with either sofosbuvir/ledipasvir/ribavirin or paritaprevir/ombitasvir/ritonavir/dasabuvir/ribavirin and HCV genotype 3-infected patients (N = 16) treated with either sofosbuvir/daclatasvir/ribavirin or sofosbuvir/ribavirin were analyzed. HCV RNA levels were determined at baseline and frequently during treatment, and RAS profiles were obtained by deep sequencing at baseline. In total, 33/47 (70.2%) of the patients had baseline RASs. However, treatment-specific RASs were detected at baseline only in 12.9% and 18.8% of HCV genotypes 1- and 3-infected patients, respectively. In genotype 1-infected individuals, reduction in HCV RNA titer during the first week of treatment was not affected by evidence of either treatment-specific RASs or cirrhosis or treatment regimen. In genotype 3-infected individuals receiving sofosbuvir/daclatasvir/ribavirin, the presence of daclatasvir-specific NS5A RASs at baseline correlated with a reduced decline of HCV RNA in the first treatment week. For both genotypes 1- and 3-infected individuals, cirrhosis but not treatment-specific RAS were associated with the time of clearance of HCV RNA. It is, however, important to note that this study involves DAA regimens that were used only during the original introduction of interferon-free DAA-based treatments.

Key words: Hepatitis C virus; direct-acting antiviral therapy; sofosbuvir; viral kinetics; clearance; resistance-associated substitutions.

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Treatment of hepatitis C virus (HCV) infection with direct-acting antiviral (DAA) drugs targets key replicative viral nonstructural (NS) proteins NS3, NS5A, and NS5B, and is highly successful with cure rates (defined as sustained virologic response (SVR): HCV-RNA negative 12 weeks after the end of treatment) above 95% [1–3]. Treatment duration is generally 8–12 weeks [4], but shorter treatment periods have been considered in relation to patient compliance and reduced treatment cost [5–7].

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Treatment failure may depend on multiple host factors such as advanced liver disease, treatment intensity, duration, and adherence [8], as well as the presence of resistance-associated substitutions (RASs) within the drug target regions of the HCV genome [9, 10], and in non-targeted regions such as NS2 [11, 12]. Current available DAA treatment includes three sofosbuvir-containing regimens [ledipasvir/sofosbuvir (Harvoni, Gilead, San Dimas, CA, USA), velpatasvir/sofosbuvir (Epclusa, Gilead, San Dimas, CA, USA), and voxilaprevir/velpatasvir/sofosbuvir (Vosevi, Gilead, San Dimas, CA, USA] and two regimens containing only protease and NS5A inhibitors [grazoprevir/elbasvir (Zepatier, MSD, Rahway, NJ, USA) and glecaprevir/pibrentasvir (Maviret, abbVie, North Chicago, IL, USA) [4]. Sofosbuvir has a potent antiviral activity across a diverse range of NS5B variants in different HCV genotypes and is considered the backbone in the sofosbuvir-containing regimens [13]. No new DAA treatment options are in the pipeline and the cost of DAA treatment is still to be considered in relation to achieving the goal of eliminating HCV as a public health threat by 2030 [14].

Shortened treatment durations from 3 to 6 weeks have been tested on small cohorts of genotype (GT) 1-infected patients obtaining rates of SVR between 20% and 100%, as reviewed previously [6]. Retrospective modeling of viral kinetics during DAA treatment estimated that 80% of the treated study cohort could have had a reduced treatment period [15]. Romani et al. [16] have associated specific $CD8^+$ T cell frequencies at baseline and end of treatment with patients achieving SVR with 4 weeks of therapy. In this context, immunological markers including $CD3^+$ and $CD8^+$ T cells have been found to be predictors of fast (<4 weeks) or slow (>4 weeks) viral clearance during DAA treatment. In contrast, patient age, HCV genotype, and fibrosis score were not associated [17]. The effect of pre-existing RASs on treatment failure can be difficult to demonstrate in clinical studies due to the low number of failures. Reduction in HCV RNA titers after initiation of treatment thus merited studies as a potential surrogate parameter for treatment efficacy.

Resistance-associated substitutions that are important for therapy outcomes are selected during DAA treatment and may be present as only minority genome populations prior to treatment. We have developed an Illumina-based sequencing assay of almost full-length HCV open-reading frame amplicons capable of detecting low-frequency RASs [18]. We have applied this method to baseline samples obtained from a published clinical study evaluating adverse events to DAA treatment [19]. In this study, HCV GT1-infected patients were randomized to either paritaprevir/ombitasvir/ritonavir/dasabuvir/ribavirin (PrODR) or sofosbuvir/ledipasvir/ribavirin (SLR) and HCV GT3 patients to either sofosbuvir/daclatasvir/ribavirin (SDR) or sofosbuvir/ribavirin (SR). HCV RNA was measured at baseline and weekly intervals. Using baseline samples from the above-mentioned study, we determined the presence of pre-existing RASs and investigated if the presence of RASs in the allocated treatment influenced the reduction of HCV RNA titers. The overall objective was to access whether pre-existing RASs could affect DAA treatment-induced reduction in HCV RNA in HCV GT1- and GT3-infected individuals.

MATERIALS AND METHODS

Patient samples

Forty-seven patients with HCV GT1 or GT3 infection, previously described in detail [19], were initially included from a single center that routinely performs Next Generation Sequencing (NGS) for HCV GT and DAA resistance determination. All patients had a baseline sample taken on the day of initiation of DAA treatment and consecutive samples taken during and after treatment at weeks 1, 2, 3, 4, 8, 12, 16, 24, and 36. In short, patients were DAA treatment-naïve but could have had prior treatment with pegylated-interferon and/or ribavirin. Cirrhosis was previously confirmed in 22 patients and was according to national treatment guidelines defined as the presence of one of the following: a liver biopsy with a Metavir score of F4 and/or median elasticity at transient elastography (TE) of ≥17 kPa [19]. All samples were stored at −80 °C until usage. Patients were treated according to national guidelines at the time of treatment [19, 20] with different DAA regimens including ribavirin. Combinations were 12 weeks of SLR or PrODR for GT1-infected patients, SR for 24 weeks, or SDR for 12 weeks for GT3-infected patients (Table S1). HCV RNA in plasma samples was quantified using the Aptima HCV Quant Dx Assay (Hologic Inc, San Diego, CA, USA) with a lower limit of quantification at 10 IU/mL, as previously described [21].

Near full-length genome amplification and sequencing

Near full-length genome HCV sequences and RASs profiles were obtained from baseline samples using the method previously described [18]. Briefly, the near full-length HCV genome was amplified in a long-range RT-PCR. Library preparation and

sequencing of the PCR product were conducted using a NEBNext Ultra II DNA Library Prep Kit and an Illumina MiSeq Reagent Kit v2 (300 cycles). An in-house pipeline [18] processed the reads, and all RASs detected above a 2% frequency threshold were included. Treatment-specific RASs were further confirmed with HCV-GLUE project version 0.1.44 [22]. Genome-wide population heterogeneity analysis was calculated with SNPGenie [23], as previously described [24], with a 1% threshold.

Statistical analysis

The effect of virus genotype (GT1/GT3) and the presence of cirrhosis on HCV RNA at baseline was evaluated using univariate analysis of variance in a general linear model. The effect of the presence of treatment-specific RASs, HCV subtype, cirrhosis, and DAA regimen on the reduction in HCV RNA in the first week of treatment was evaluated using a univariate analysis of variance in a general linear model. In the latter analysis, GT1- and GT3-infected patients were analyzed separately. All calculations were done using IBM SPSS Statistics version 25. All statistical analyses were two-tailed and the tests were considered statistically significant if the p value was ≤ 0.05.

Ethics

The study was conducted in compliance with the Declaration of Helsinki and written informed consent was provided by all patients. The Regional Ethical Committee (H-15007265) and the Danish Data Protection Agency (2012-58-0004) approved the study and patient plasma samples were obtained from the Danish Database for Hepatitis B and C (DANHEP) biobank [25].

RESULTS

Patient characteristics

We analyzed 47 HCV-infected individuals initiating antiviral treatment; 31 were infected with GT1 and 16 with GT3. In the GT1-infected patients, of whom 14/31 (45.2%) had evidence of cirrhosis, 16/31 (51.6%) received treatment with SLR while 15/31 (48.4%) received treatment with PrODR. In the GT3-infected patients, of whom 8/16 (50%) had evidence of cirrhosis, 8/16 (50%) received treatment with SR, and 8/16 (50%) received SDR. Only two patients (Id. #1 and 43) failed treatment; one experienced a late virus relapse and the other turned non-compliant after 10 weeks of treatment.

Virus baseline characteristics

Hepatitis C virus viral load at baseline before initiation of antiviral treatment was available for all 47 patients and did not differ significantly between cirrhotic and non-cirrhotic patients (HCV RNA 6.06 Log IU/mL (95% CI: 5.80–6.32) and 6.06 Log IU/mL (95% CI: 5.81–6.31), respectively) or between GT1- and GT3-infected patients (HCV RNA 6.10 Log IU/mL (95% CI: 5.89–6.31) and 6.02 Log IU/mL (95% CI: 5.73–6.31), respectively). An unexpected and unexplained significant interaction between GT and cirrhosis and baseline HCV RNA was observed ($p = 0.019$; univariate ANOVA), in which baseline HCV RNA was higher in GT1-infected patients with cirrhosis than in patients without cirrhosis, and the opposite was the case for GT3-infected patients. This was not explored further.

Detection of baseline RASs

In total, 33/47 (70.2%) of the patients had baseline RASs but the majority were not relevant in relation to the used DAA treatment regimen or viral GT (Table S1). Genotype-relevant baseline RASs towards the used DAA treatment regimen were detected in only 7/47 (14.9%) of the sequenced patient samples.

In 4/31 (12.9%) GT1-infected individuals, treatment-relevant RASs were detected. The RASs intra-population frequencies ranged from 4.5% to 99%. In 4 patients (Id. # 10, 14, 27, 33) RASs at position 30R in the NS5A region and at position 159F, 316N, 321I, and 556G in the NS5B region were detected. We did not detect treatment-specific RASs in the NS3 region.

In 3/16 (18.8%) HCV GT3-infected patients (Id. # 11, 43, 47), treatment-relevant RASs were detected. RASs in the NS5A region at positions 30K and 93H with intra-population frequencies between 48% and 99% were found while no treatment-relevant RASs were detected in the NS5B region.

Viral population heterogeneity

The occurrence of RASs towards NS5A inhibitor daclatasvir, the first NS5A inhibitor developed [26], was hypothesized to be due to an overall higher heterogeneity in the samples. Samples from Id. # 1, 8, 11, 43, 47, and 48 with RASs towards daclatasvir were compared to the other GT3a samples. The analysis did not show an increased population heterogeneity compared to the other samples in NS5A (with RASs $\pi = 0.01244$ (95% CI: 0.001989–

0.02289); without RASs $\pi = 0.008296$ (95% CI: 0.0009754–0.01562); p = 0.49), or across the ORF (with RASs $\pi = 0.01093$ (95% CI: 0.003009–0.01885); without RASs $\pi = 0.008072$ (95% CI: 0.001536–0.01461); p = 0.49). Visual investigation of the heterogeneity across the genomes did not either indicate any differences (Fig. S1).

Viral load response to treatment

Hepatitis C virus RNA viral load 1 week after initiation of antiviral treatment was available for 44/47 (93.6%) of patients for whom the reduction in HCV RNA during the first week of treatment was used as a measure of treatment effect. The effects of cirrhosis, treatment regimen, and the presence of treatment-specific RASs were investigated in a univariate model analyzing GT1- and GT3-infected patients separately.

For GT1-infected individuals, a reduction in HCV RNA during the first week of treatment did not differ significantly between patients with GT1a or 1b infection, with or without evidence of cirrhosis, by treatment regimen, or by the presence of RASs (Table 1). Individuals treated with PrODR without treatment-specific RASs (N = 13) had an average reduction in HCV RNA during the first treatment week of 3.80 Log IU/mL (95% CI: 3.37–4.24), while the individual with treatment-specific RASs showed a reduction of HCV RNA on 3.94 Log IU/mL. Similarly, individuals treated with SLR without treatment-specific RASs (N = 13) had an average reduction in HCV RNA on 3.85 Log IU/mL (95% CI: 3.49–4.21) during the first week of treatment, while the three individuals with treatment-specific RASs displayed an average reduction of 4.48 Log IU/mL (95% CI: 3.57–5.38). The presence of cirrhosis did apparently not affect the reduction of HCV RNA during the first week of treatment, as seen in Table 1.

For GT3-infected individuals, reduction of HCV RNA during the first week of treatment was associated with treatment regimen [SDR 3.79 Log IU/mL (95% CI 3.42–4.17) vs SR 3.39 Log IU/mL (95% CI: 3.03–3.73)] (Table 2). The presence of treatment-specific RASs in NS5A at baseline in SDR-treated individuals reduced the decline of HCV RNA compared to SDR-treated individuals without RASs [RASs present 3.40 Log IU/mL (95% CI; 2.79–4.00) vs RASs absent 3.79 Log IU/mL (95% CI: 3.51–4.07)] (Fig. 1). Evidence of cirrhosis was not associated with reduction of HCV RNA during the first week of treatment [with cirrhosis 3.43 Log IU/mL (95% CI: 3.06–3.80); without cirrhosis 3.86 Log IU/mL (95% CI: 3.47–4.30)].

Clearance of HCV RNA

Hepatitis C virus RNA was measured during treatment and the first week of undetectable HCV RNA was registered. The week of clearance of detectable HCV RNA was not influenced by the presence or absence of treatment-specific RASs, the median week of clearance was 4 [IQR: 4–8 (N = 7)] and 4 [IQR: 3–8 (N = 40)], respectively.

The week of clearance of detectable HCV RNA appeared to be influenced by the presence of cirrhosis. In cirrhotic and non-cirrhotic patients, the median week of clearance was 8 [IQR: 4–8

Table 1. Univariate model of reduction of HCV RNA in genotype 1-infected individuals during the first week of treatment

	N	ΔHCV RNA (week 1) (Log IU/mL)	95% CI	p-value
Subtype				
1a	20	4.11	3.74–4.49	0.065
1b	8	3.79	3.26–4.31	
Cirrhosis				
Yes	11	3.90	3.39–4.41	0.807
No	17	4.04	3.69–4.41	
Treatment				
PrODR	13	3.80	3.37–4.24	0.821
SLR	15	4.10	3.71–4.52	
RASs				
Yes	4	4.25	3.68–4.89	0.474
No	24	3.81	3.50–4.51	

Treatment combinations were sofosbuvir/ledipasvir/ribavirin (SLR) and paritaprevir/ombitasvir/ritonavir/dasabuvir/ribavirin (PrODR). Subtype, cirrhosis, treatment, and resistance-associated substitutions (RASs) did not have a significant effect on viral load reduction during the first week of treatment.

Table 2. Univariate model of reduction of HCV RNA in genotype 3-infected individuals during the first week of treatment

	N	ΔHCV RNA (week 1) (Log IU/mL)	95% CI	p-value
Cirrhosis				
Yes	8	3.89	3.47–4.30	0.086
No	8	3.43	3.06–3.80	
Treatment				
SDR	8	3.79	3.42–4.17	0.010
SR	8	3.39	3.04–3.73	
RASs				
Yes	3	3.40	2.79–4.00	0.039
No	13	3.80	3.51–4.07	

Treatment combinations were sofosbuvir/ribavirin (SR) and sofosbuvir/daclatasvir/ribavirin (SDR). Treatment and resistance-associated substitutions (RASs) had a significant effect on viral load reduction during the first week of treatment.

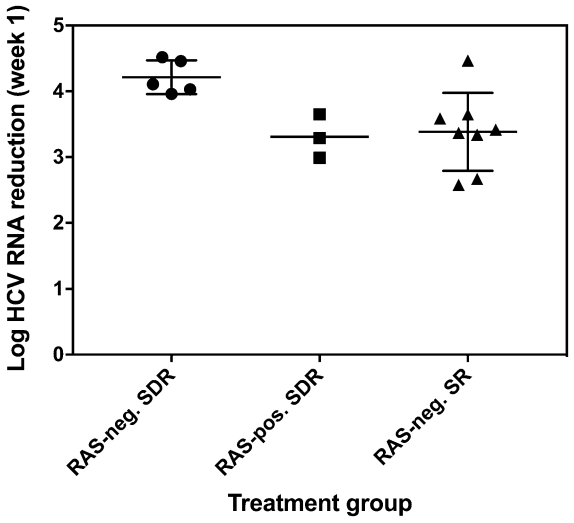

Fig. 1. Genotype 3-infected patients constituted three groups: (1) sofosbuvir/daclatasvir/ribavirin (SDR) treated individuals without treatment-specific RASs (N = 5), (2) SDR-treated individuals with treatment-specific RASs (N = 3), and (3) sofosbuvir/ribavirin (SR) individuals without treatment-specific resistance-associated substitutions (RASs) (N = 8). When these three groups were directly compared (1) SDR-treated individuals without RASs had a significantly better treatment response (mean 4.22 Log IU/mL; 95% CI: 3.90–4.54) than both (2) SDR-treated individuals with RASs (mean 3.31 Log IU/mL; 95% CI: 2.49–4.13) (p = 0.039; one-way ANOVA) and (3) SR treated individuals without RASs (mean 3.39 Log IU/mL; 95% CI: 2.89–3.88) (p = 0.017; one-way ANOVA). Error bar indicates SD.

(N = 22)] and 3 [IQR: 3–4 (N = 25)], respectively (p = 0.0021; Mann–Whitney U test). This difference was significant among GT1-infected patients [8 (IQR: 4–8 (N = 14)) vs 3 (IQR: 2.5–4 (N = 17)) (p = 0.045) in the presence and absence of cirrhosis, respectively]. A similar trend, though not statistically significant, was observed for GT3-infected patients [6 (IQR: 3–8 (N = 8)) and 3 (IQR: 3–4 (N = 8)) (p = 0.226) for cirrhotic and non-cirrhotic patients, respectively].

DISCUSSION

In this study, pre-existing treatment-specific RASs did not influence the kinetics of treatment-induced virus reduction in GT1 patients, which has also been observed in another study [27]. We observed that viral load reduction after the first week of treatment was increased for GT3-infected patients if daclatasvir was added to treatment with sofosbuvir and ribavirin. However, if treatment-specific RASs against daclatasvir were present, the viral load reduction did not differ between patients receiving sofosbuvir and ribavirin compared to those receiving sofosbuvir, daclatasvir, and ribavirin. The observed increase in viral load reduction at week 1 by adding daclatasvir to sofosbuvir compared to treatment with only sofosbuvir has also been observed in other studies with different NS5A inhibitors [28, 29].

Three GT1-infected patients had pre-existing RASs within NS5B, and one had pre-existing RASs within NS5A. The NS5B RAS 321I has to our knowledge not been tested in *in vitro* experiments with subtype 1b, but has been observed at treatment failure [30]. The observed RASs NS5B 159F and 316N only convey a 1.6-fold increase in EC_{50} to sofosbuvir when combined [31, 32]. NS5B RAS 556G results in an 11-fold increase in EC_{50} towards dasabuvir [33]. This limited loss of potency is apparently not reflected in the reduction of HCV RNA under treatment. The remaining GT1-infected patient had NS5A 30R, which confers a 632 to >1000-fold increase in EC_{50} to ledipasvir used in treatment [34, 35]. Despite this considerable loss in potency, the patient responded well to treatment with SLR and experienced a rapid reduction of HCV RNA.

All three GT3-infected patients with pre-existing RASs in the NS5A region had RASs that conferred significant resistance to daclatasvir. NS5A 30K and NS5A 93H increase EC_{50} of daclatasvir by 29.6–44 fold [36, 37] and 1000–2154 fold [35–37], respectively, explaining the decreased efficacy of daclatasvir in these patients. A relation between a high viral population heterogeneity in NS5A or the entire ORF and the presence of RASs against daclatasvir in the GT3 samples was not present. The newer NS5A inhibitors used for GT3, pibrentasvir, and velpatasvir, however, are less inhibited by RASs presence [35], and current HCV treatment guidelines [4] do not recommend that baseline testing for RASs is done prior to DAA treatment initiation as it can hamper access to treatment. However, as these RASs only have a low cost of fitness they have been observed to be sustained a long time after treatment discontinuation [38] and might influence retreatment, which is why resistance-guided retreatment after DAA failure is useful [4] and have shown good results in relation to successful retreatment outcome [39, 40].

Viral load information is no longer used to guide treatment decisions as in the interferon treatment era [41] and patients are treated for fixed treatment durations regardless of HCV RNA results during treatment. New DAA treatment is shorter, highly effective, and generally well tolerated, but shorter treatment duration may be beneficial in patients

with cirrhosis where ribavirin is added to treatment in some cases and where side effects may influence treatment, and in patients with severe comorbidities where drug–drug interactions can be an issue. Treatment costs can also advocate for more individual approaches using viral kinetics modeling. Prior studies have indicated that viral kinetics can have an impact on outcomes. Paolucci et al. [42] detected significantly higher viral loads during treatment at weeks 1, 4, and 8 in patients failing DAA treatment compared to patients with SVR. Furthermore, Maasoumy et al. [43] found that week 2 viral load can predict viral relapse in GT3-infected patients when treated solely with sofosbuvir and ribavirin but not with other DAAs.

The treatment week for clearance of detectable HCV RNA was not influenced by pre-existing RASs but instead by the presence of cirrhosis. Our result is in accordance with the study by Gambato et al. [44] including patients with compensated and decompensated cirrhosis, where a slower viral clearance rate was seen in patients with a transient elastography >21 kPa. Persistence of detectable HCV RNA may rather reflect the presence of less rapidly clearing compartments than the reduced potency of treatment.

Our study presents some limitations. First, the low number of patients in each treatment arm makes it difficult to compare RASs and their influence among the different DAA regimens. Additionally, only patients with compensated cirrhosis at baseline were included which excludes patients considered most difficult to treat. Lastly, the majority of the patients treated received DAA regimens that are currently considered sub-optimal, and we cannot exclude different results when using next-generation pan-genotypic DAA regimens. Larger clinical studies are warranted to validate if viral kinetics can be used as a surrogate marker in relation to response-guided therapy, and whether baseline RAS may hamper viral kinetics. As different factors such as HCV genotype, liver fibrosis status, baseline HCV RNA load, presence of baseline RAS, and DAA regimen may influence viral load reductions these factors should be considered when designing future studies, as larger homogeneous patient groups would provide more conclusive data.

CONCLUSION

We found that pre-existing RASs in the NS5A - and NS5B regions did not influence viral kinetics in patients with GT1. In patients with GT3 treated with SDR pre-existing RASs in the NS5A region reduced the decline of HCV RNA in the first treatment week but was not related to the time of clearance of HCV RNA. The presence of cirrhosis was related to the time of clearance of HCV RNA for both GT1 and GT3 patients. Further studies including a larger number of patients who are treated with DAA regimens including 2nd and 3rd generation NS5A inhibitors with regular viral load testing during treatment are needed to understand the interaction between RASs and viral kinetics during DAA treatment.

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CONFLICT OF INTEREST

NW has participated as a clinical investigator for Abbvie and Merck. The other authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Visual investigation of the heterogeneity across the HCV genomes.
Table S1. Patient, treatment, and resistance-associated substitution information.