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Commentary

Antiviral therapy of hepatitis C in 2014: Do we need resistance testing?

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ABSTRACT

The treatment of chronic hepatitis C has fundamentally changed since the approval of the first direct-acting antivirals (DAA) in 2011. In addition to telaprevir and boceprevir, in 2014 two new NS3 protease inhibitors (simeprevir and faldaprevir), one non-nucleoside polymerase inhibitor (sofosbuvir) and one NS5a replication complex inhibitor (daclatasvir) have expanded the treatment options for chronic hepatitis C. Resistance-associated variants (RAV) are naturally produced during the HCV life cycle. The frequency of RAVs within HCV quasispecies mainly depends on their replicational fitness. Variants conferring resistance to nucleos(t)ide analogues have not been detected, and the majority of NS3 protease-resistant variants are present at low frequencies (0.1–3%) before initiation of DAA-based therapies. However, the Q80K variant conferring resistance to simeprevir has been observed in 9–48% of untreated HCV genotype 1a-infected patients, leading to reduced SVR rates. Resistant variants are detectable in the majority of patients with treatment failure to NS3 protease inhibitor- or NS5a inhibitor-based antiviral therapy. Long-term follow-up studies by population-based sequence analysis have shown the disappearance of resistant variants in the majority of patients, with median times to loss of mutations of 4–64 weeks. For the nucleotide analogue sofosbuvir, the emergence of the S282T resistant variant has been observed only in single patients, with reversion to wild-type within several weeks. Data are sparse on retreatment of patients with the same DAA or the same class of DAAs. However, retreatment with a different class of DAAs after failure of NS3 protease inhibitor-based therapy has been successful in small studies. This article forms part of a symposium in *Antiviral Research* on "Hepatitis C: next steps toward global eradication."

1. Introduction

With the development of direct acting antivirals (DAA), a new era began in the treatment of chronic hepatitis C. These specifically targeted drugs combined with pegylated interferon-alfa and ribavirin have shown a distinct increase in sustained virologic response (SVR) rates. More recently, viral eradication has also been shown to be possible with interferon-free DAA combination therapies. In contrast to host-targeting agents (HTA), DAAs target different structures of the hepatitis C virus (HCV) genome, primarily the NS3/4A protease, the NS5A protein or the RNA-dependent NS5B polymerase.

Drug resistance plays a key role in patients with failure to DAA-containing therapies. HCV replication exhibits a rapid turnover with a daily production of 1012 virions (Neumann et al., 1998). The high genetic diversity, known as HCV quasispecies, is caused by the error-prone activity of the HCV polymerase. The selection of resistance-associated amino-acid variants (RAVs) from HCV quasispecies depends on drug-, host- and virus-related factors. The potency of a drug itself is primarily influenced by the viral susceptibility, the exposure to a drug and the genetic barrier to resistance. The ability of a RAV to persist and to induce treatment failure (relapse, non-response or viral breakthrough) is related to its fitness compared to the wildtype virus (Welsch and Zeuzem, 2012).

The detection of RAVs depends primarily on the method, which is used. For population sequencing, clonal sequencing and deep sequencing, variants below frequencies of approximately 25%, 5% and 0.5% respectively cannot be detected (Halfon and Sarrazin, 2012). This review will give an overview about the current issues of HCV-resistance and address the question of the potential need for resistance testing for future HCV-therapies.

2. Which treatment options will be available in 2014?

In HCV-genotype 1 infected patients (treatment naïve, relapser, partial non-responder and null-responder) triple therapy with

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pegylated interferon alfa (peg), ribavirin and the peptidomimetic linear ketoamide inhibitors of the NS3/4A protease (PI) telaprevir (TVR) or boceprevir (BOC) showed significantly improved SVR rates compared to peg/ribavirin alone (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011; Zeuzem et al., 2011). The treatment is complicated by the aggravated and expanded side effect profile, high frequency dosing together with a (fatty) meal and drug–drug-interactions. Since the approval in 2011, triple therapy has been the new standard of care in HCV genotype 1 infected patients.

Four new DAAs expand the HCV-treatment options for all genotypes in 2014:

- NS3 protease inhibitors simeprevir and faldaprevir together with peg/ribavirin for genotype 1 infected patients.
- Nucleotide NS5B polymerase inhibitor sofosbuvir together with peg/ribavirin for genotype 1–6 infected patients.
- Nucleotide NS5B polymerase inhibitor sofosbuvir together with ribavirin (interferon-free) for genotype 1–4 infected patients.
- Nucleotide NS5B polymerase inhibitor sofosbuvir together with simeprevir (study data available for genotype 1 patients (Jacobson et al., 2013c)).
- Nucleotide NS5B polymerase inhibitor sofosbuvir together with NS5a replication complex inhibitor daclatasvir (study data available for genotype 1–3 patients (Sulkowski et al., 2014).

The macrocyclic NS3/4A PI simeprevir (SMV) and the peptidomimetic linear ketoamide faldaprevir (FDV) in combination with peg/ribavirin recently demonstrated significantly improved SVR-rates (between 79% and 80%) in treatment-naïve genotype 1 patients in phase III studies (Ferenci et al., 2013; Jacobson et al., 2013b). FDV-based triple therapy was also investigated in peg/ribavirin treatment failures and showed improved SVR rates compared to peg/ribavirin alone between 33% and 70% (Jacobson et al., 2013a). Furthermore, SMV showed an increased response in patients with a relapse to prior peg/ribavirin treatment (Lawitz et al., 2013a). For the vast majority (approx. 80%) of treatment-naïve and relapse patients, shortening of treatment duration to 24 weeks is possible based on a response guided treatment approach with decline of HCV RNA concentrations to certain cut-offs at week 4 of treatment.

SMV has already been approved in the United States, Canada and Japan for combination therapy in HCV-genotypes 1 and 4. The approval of SMV in the European Union and the approval of FDV are expected in 2014.

In comparison to TVR/BOC the advantages of these second wave PIs are the lower frequency of side effects and probably also of drug–drug-interactions and a longer half-life, leading to a more user friendly dosing. Trials assessing the efficacy of SMV triple therapy in genotype 1 null- and partial responders as well as in patients infected with the HCV-genotype 4 are ongoing.

Sofosbuvir (SOF), a nucleotide inhibitor of the HCV-RNA-polymerase 5B (NI), has been approved for combination therapy of chronic hepatitis C infection with HCV-genotypes 1–6 in the United States, Canada and the European Union in late 2013 and early 2014, respectively. SOF demonstrated superior SVR rates in combination with peg/ribavirin in treatment-naïve genotype 1, 4, 5, 6 infected patients based on a flat 12-week treatment independent of HCV RNA viral kinetics. In genotype 2 and 3 infection, compared to peg/ribavirin, non-inferior SVR-rates for the interferon-free combination of SOF with ribavirin alone in previously untreated patients were achieved. However, SVR-rates were significantly lower for genotype 3 than for genotype 2 patients (Lawitz et al., 2013b). Another phase 3 study emphasized the improvement of efficacy in genotype 2/3 patients and prior peg/ribavirin treatment failure: Here, excellent results have been achieved in genotype 2 infected patients while for genotype 3 patients only moderate SVR rates especially in cirrhotics could be achieved (Jacobson et al., 2013d; Zeuzem et al., 2013a). Recently, SOF in combination with peg/ribavirin for 12 weeks in treatment experienced genotype 2/3 infected patients resulted in >80% SVR rates independent of the presence of cirrhosis (Lawitz et al., 2013c).

The combination of SOF and SMV with or without ribavirin was evaluated in a phase 2a study. Here, SVR-rates between 79% and 96% were achieved in prior peg/ribavirin null-responders (genotype 1). The study results suggest, that the addition of ribavirin as well as a treatment duration of 24 instead of 12 weeks do not improve treatment outcome (Jacobson et al., 2013c).

Another interferon-free treatment containing of SOF and the NS5a inhibitor daclatasvir (DCV) with or without ribavirin was investigated for previously untreated genotype 1–3 patients and genotype 1 patients with prior TVR or BOC triple therapy treatment failure. SVR rates were 98% in both, previously untreated and treatment-experienced genotype 1 patients (including TVR/BOC triple therapy failures) whereas SVR rates were 92% and 89% for genotype 2 and 3, respectively (Sulkowski et al., 2014).

3. Which patients will be treated?

- Treatment-naïve patients.
- Failure to peg/ribavirin.
- Failure to TVR/BOC triple therapy.

For all new drugs studies in treatment naïve and in peg/ribavirin failure patients have been performed. Pre-existing, naturally occurring resistance mutations for SOF (S282T) have not been described in these patients even by deep sequencing analysis (Svarovskaia et al., 2013b). For FDV and SMV the rate of naturally occurring NS3 resistance mutations is low (0.1–3%). However, for SMV one additional variant was observed to confer medium level resistance (Q80K) and this variant is observed in 9–48% of treatment naïve genotype 1a infected patients with the highest incidence in North America (Lenz et al., 2013a). Indeed patients with the Q80K variant achieved consistently lower SVR rates with SMV triple therapy (Forns et al., 2013; Jacobson et al., 2013b; Poordad et al., 2013b). Although relapse rates after treatment with SOF and DCV were low, all patients with relapse had Q80K at baseline (Jacobson et al., 2013c).

For TVR/BOC-experienced patients, in addition to naturally occurring variants, also resistant mutations selected during prior treatment might be of importance if re-treatment with another PI is considered. In contrast to HBV and HIV infection RAVs cannot be archived as DNA, so the possible impact of selected variants is a matter of debate. In the long-term follow-up of patients with treatment failure to TVR, 77% of patients had detectable RAVs at the time of treatment failure using population sequencing (Sullivan et al., 2013). After a median follow-up time of 29 months RAVs disappeared in 85% of patients (Sherman et al., 2011). Lower frequencies of detected RAVs are reported for patients with treatment failure to BOC, but also a clearance of RAVs and a dominance of wildtype virus were found in the majority of patients after 6 to 14 months using population sequencing (Barnard et al., 2013). For TVR a more rapid disappearance of RAVs in subtype 1b vs. 1a variants was observed (Sullivan et al., 2013). Because of a high level of cross-resistance between TVR and BOC a switch to the other PI in case of a treatment failure is not recommended (Ghany et al., 2011; Sarrazin and Zeuzem, 2010).

Studies with re-treatment of TVR/BOC treatment-failures with SMV-, FDV- or SOF-based triple therapies have not been performed. For SMV and FDV an at least partial overlapping resistance profile may impair virologic response to the protease inhibitor. For SOF no such cross-resistance is present. However, failure to TVR/

BOC triple therapy is typically associated with impaired responsiveness to peg/ribavirin (De Meyer et al., 2012; Howe et al., 2013b), which makes it unlikely that high SVR rates can be achieved by re-treatment with peg/ribavirin based triple therapies.

For the question of a potential persistence of NS3 resistant variants at low frequencies and re-selection after re-treatment with the same drug, only limited data are available. A short course of SMV monotherapy in 5 patients led to the occurrence of resistant mutants in all patients. Approximately 1.5 years later at baseline of retreatment with SMV + peg/ribavirin, no RAVs were found by deep sequencing analysis. Nevertheless, in 2 patients with treatment failure of triple therapy, RAVs recurred, emphasizing the potential impact of selected variants on virologic response (Lenz et al., 2012). A sequential short-term treatment with BOC led to the reselection of the same mutations in 3 of 9 patients, while 5 patients emerged different mutational patterns during subsequent BOC therapy (Vermehren et al., 2012).

While re-treatment with the same class of drugs as well as with peg/ribavirin based triple therapies and one DAA may have limited chances of efficacy, a combination of DAAs with no cross resistance may be effective. The NS5A inhibitors daclatasvir as well as ledipasvir in combination with SOF were assessed with or without ribavirin in small cohorts of TVR/BOC experienced patients and led to SVR-rates between 95% and 100%. The response was independent of the presence or absence of NS3/4A polymorphisms at baseline (Lawitz et al., 2013d; Sulkowski et al., 2013b).

4. Which factors influence resistance prior to treatment?

4.1. The role of natural occurring RAVs

The high turn over rate in HCV replication and a poor fidelity and high error rate of the RNA-dependent RNA polymerase lead to the continuous production of numerous variants known as HCV-quasispecies. In the natural course of HCV life cycle the wild-type virus is predominantly produced. Several isolates within the HCV-quasispecies can carry mutations which confer resistance to DAAs either by direct (binding site) or indirect effects (functional restoration of the protein). Natural occurring RAVs are selected early in monotherapy with TVR (Sarrazin et al., 2007) and BOC (Susser et al., 2009) and an occurrence in treatment-naïve patients could be confirmed (Kuntzen et al., 2008). Therefore selected variants are considered to be pre-existent mutations in natural HCV life cycle. The incidence of resistant variants is variable and depends on the binding domain, different populations and HCV- geno- and subtypes. Deep-sequencing analysis allows to detect viral variants with a sensitivity of approximately 0.5–1%. Using these techniques nearly all described RAVs in the NS3/4A gene can be detected (Verbinnen et al., 2010). To date, RAVs at very low frequencies have no impact on treatment response. Population-based sequencing showed cumulative frequencies of different protease inhibitor resistant mutations in 10.8% of patients (Paolucci et al., 2012). Table 1 shows the frequencies of the most relevant resistant mutations in genotype 1a and 1b DAA- naive patients treated in TVR phase 3 trials.

In this study treatment-naïve patients with TVR-resistant variants prior to treatment achieved similar SVR-rates compared to patients without RAVs (Bartels et al., 2013). Also SVR rates in a FDV-containing treatment of 6 patients were not influenced by pre-existing mutations at positions R155 and D168 (Sulkowski et al., 2013a). Further analysis of the TVR and BOC phase 3 studies emphasized, that treatment response is independent of the presence of pre-existing RAVs if there is a good responsiveness to the peg/ribavirin-backbone. On the other hand, patients with baseline RAVs who were also poor peg/ribavirin responders (<1log decrease in HCV-RNA during lead-in-phase) showed lower SVR rates compared to poor Peg/ribavirin responders without baseline RAVs (22% vs. 37%). Especially the presence of V36M, T54S, V55A or R155K at baseline combined with a poor interferon response led to a SVR in only 7% of BOC-treated patients (Howe et al., 2013b). Prior null-responders with the pre-existing variants T54S or R155K treated with TVR in the REALIZE-study always had on-treatment virologic failure whereas prior relapsers had SVR in most cases (De Meyer et al., 2012).

The mutational variant Q80K is a pre-existing RAV frequently found in HCV-genotype 1a (Prevalence: South America 9.1%, Europe 19.4%, North America 48.1%) and is rarely detected in genotype 1b (0.5%) (Lenz et al., 2013a). Resistance is associated with SMV only. As currently shown, SVR and RVR rates were distinctly reduced in prior relapsers with Q80K-mutation at baseline (SVR12 46.7% vs. 78.5%; RVR 43.3% vs. 75.9%) (Forns et al., 2013), which highlights the relevance of Q80K mutation also in good peg/ribavirin responders.

The NS5B-substitution S282T is the only resistant mutation associated with decreased susceptibility to SOF. At baseline of phase 3 studies in none of the 1292 patients S282T was detected. Also there was no correlation between other NS5B variants detected prior to treatment and treatment outcome (Svarovskaia et al., 2013a).

Viral breakthrough or relapse to DCV treatment is linked to the existence of the RAVs L31M/F and Y93H at baseline. In a Japanese study 58% of genotype 1b infected patients with these baseline mutations failed therapy (McPhee et al., 2013). The natural occurrence of Y93H is reported between 4% and 23% and is lower for genotype 1a than for subtype 1b (Karino et al., 2013; Kuiken et al., 2008). However, most cases of treatment failure have been observed in genotype 1a without baseline RAVs and also many patients with baseline RAVs exhibited SVR (Karino et al., 2013).

4.2. The influence of the HCV genotype/subtype

The phase 3 studies of TVR, BOC, FDV and SMV showed lower SVR rates for HCV-genotype 1a compared to genotype 1b (TVR 71% vs. 79%, BOC 59–62% vs. 66–73%, FDV 69–76% vs. 83–84%, SMV 71% vs. 90% for treatment-naïve patients) (Brass et al., 2011; Ferenci et al., 2013; Jacobson et al., 2013b, Jacobsen et al., 2011). Pre-existing dominant resistance mutations are more common in treatment-naïve patients infected with genotype 1a (cumulative incidence 8.6% vs. 1.4%) (Kuntzen et al., 2008). Compatible with the lower SVR-rates, RAVs were more frequently detected among genotype 1a in patients with PI-treatment failure (Barnard et al., 2013; Ferenci et al., 2013; Lenz et al., 2013a; Sullivan et al., 2013). The major resistance mutation for subtype 1a infected patients is associated with an amino acid exchange at position 155 of the NS3 protease. Most frequently arginine (R) is changed to lysine (K, mutation R155K). In subtype 1b typically two nucleotide changes are necessary to generate R155K whereas a subtype 1a strain requires only one change to encode for lysine (McCown et al., 2009).

Although the loss of RAVs was faster in genotype 1b after TVR and SMV treatment (median time 10.6 vs. 0.9 months and 36 vs. 24 months respectively) (Lenz et al., 2013a; Sullivan et al., 2013), the reversion rate to wildtype virus 3 years after the end of BOC-treatment was not significantly higher in genotype 1b (Howe et al., 2013a).

The linear PIs were primarily designed for the treatment of genotype 1 infection and have no or only moderate activity on other genotypes. TVR alone or with Peg/ribavirin reduced HCV RNA in patients with genotype 2 (monotherapy HCV-RNA decline 3,66 log10 after 15 days), but not genotype 3 infections (monotherapy HCV-RNA decline 0,54 log10 after 15 days) (Foster et al.,

Table 1
Prevalence of the most important natural occurring RAVs in DAA-naïve patients. *N* = 2111 for genotype 1a and 1336 for genotype 1b. n.o. = not observed prior treatment. Adapted from (Bartels et al., 2013).

Amino acid site	Resistance associated to:	Observed amino acid changes	Cumulative prevalence of naturally occurring RAVs (%)	
			1a	1b
V36	TVR, BOC	I, L, M	2.5	1.3
T54	TVR, BOC	A, S	3.1	1.9
V55	BOC	A, I	4.9	0.4
Q80	SMV	K, R	38.3	1.42
V107	BOC	I	0.2	0.6
R155	TVR, BOC, SMV, FDV	K	0.9	n.o.
A156	TVR, BOC.	F, N, S, T, V	n.o.	n.o.
D168	TVR, BOC, SMV, FDV	E	0.2	0.6
I/V170	BOC	A, T	0.1	0.2

2011). The RAVs detected when viral breakthrough or relapse occurred were equal and consistent to variants described for genotype 1, so no genotype 2/3-specific mutations were found (De Meyer et al., 2013). Also after SMV monotherapy in patients infected with genotypes 2–6 no additional mutations occurred. In summary, SMV showed activity across genotypes 4–6 and in the half of the genotype 2-infected patients whereas no antiviral activity was observed against genotype 3 (Lenz et al., 2013b). For BOC a moderate activity against genotype 2 and 3 is described (maximum decline during BOC monotherapy: 1.39–1.71 log10) (Silva et al., 2013). To date, analysis of resistance has not been published.

In vitro analysis of SOF indicates a pan-genotypic activity (Lam et al., 2010). However, SVR rates differed between sub- and genotypes in phase 3 trials:

- SVR-rates were consistently lower in all treatment groups for genotype 3 (30–62%) compared to genotype 2 (86–97%) (Jacobson et al., 2013d; Lawitz et al., 2013b).
- In treatment-naive patients SVR rates were significantly lower in subtype 1b than in subtype 1a (82% vs. 92%) (Lawitz et al., 2013b).

The reasons for these differences are unclear so far and larger studies have to be awaited to confirm different antiviral activities of SOF in HCV geno- and subtypes.

4.3. Influence of cirrhosis and IL28B genotype

Conventional triple therapies with peg/ribavirin backbone, but also interferon-free treatments have shown reduced antiviral activities/ SVR rates in patients with cirrhosis (Jacobson et al., 2013d; Vierling et al., 2013). This might also enhance the risk of virologic breakthrough due to resistance.

The influence of the IL28B polymorphism as a surrogate marker for interferon-sensitivity has been shown in many studies with DAAs and peg/ribavirin. Although the difference between IL28B-genotypes is decreasing when using potent direct antivirals, patients with CC-genotype exhibited consistently higher SVR-rates in phase 3 studies with TVR, BOC, SMV and FDV (Ferenci et al., 2013; Jacobson et al., 2013b; Manns et al., 2013; Pol et al., 2013; Poordad et al., 2012).

Using interferon-free treatment regimes the influence of the IL28B genotype is expected to decrease, but can still remain, depending on the antiviral activity and the barrier of resistance of the used substances. For example the PI ABT-450 combined with low-dose ritonavir in addition to the non-nucleoside NS5B polymerase inhibitor ABT-333 and ribavirin showed high and consistent SVR rates independent of IL28B genotype in treatment-naive patients (Poordad et al., 2013a). In contrast, the rate of SVR in an interferon-free treatment regimen containing FDV, deleobuvir (non-nucleoside polymerase inhibitor) and ribavirin was affected by the IL28B polymorphism (Zeuzem et al., 2013b).

5. What do we know about resistance for the different DAAs?

To date, 8 main amino acid positions within the HCV NS3/4A protease associated with resistance have been described (main variants: V36A/M, T54S/A, V55A/K, Q80R/K, R155K/T/Q, A156S/D/T/V, D168A/V/T/H, V170A/T). PIs, whether linear or macrocyclic, show different profiles of resistance, but exhibit a significant cross-resistance. In all PIs quasispecies at position R155 were selected. Second generation PIs like MK-5172 and ACH-1625 have a broader genotypic coverage and lower levels of resistance. During monotherapy with MK-5172, quasispecies containing resistant variants could be detected, but no virologic break-through occurred (Strizki et al., 2012), indicating a higher barrier of resistance. In contrast to other PIs, MK-5172 rather interacts with the catalytic triad than directly with amino acid sites that confer resistance (Romano et al., 2012).

5.1. Telaprevir

Early *in vitro* studies detected A156S as the major mutant conferring resistance to TVR (Lin et al., 2004). *In vivo*, further resistant mutations were detected during TVR monotherapy in phase 1 trials. Mutations that confer low-level resistance (V36A/M, T54A, R155K/T and A156S) showed a better fitness and were detected at higher levels than resistant variants with a higher barrier of resistance (A156V/T, 36+155, 36+156) (Sarrazin et al., 2007). In genotype 1a the most common variants are V36M and R155K whereas in genotype 1b mainly observed RAVs were V36A, T54A, A156S/T. The median time until loss of mutations after treatment failure were 10.6 and 0.9 months for genotype 1a and 1b, respectively (Sullivan et al., 2013). At baseline of retreatment with a TVR-containing triple-therapy, no resistant variants were detectable by deep sequencing 5.7 years after short term exposure to TVR in phase 1 studies. Patients who did not respond to triple therapy selected the same resistant variants as those previously detected after TVR monotherapy (Sarrazin et al., 2013). Different patterns of resistance are exhibited depending on the type of treatment failure: whereas in patients with on-treatment failure, RAVs with high-level resistance were predominantly detected, patients with relapse more likely emerged lower-level resistant variants or wildtype virus (De Meyer et al., 2012).

5.2. Boceprevir

Between TVR and BOC exists a significant cross-resistance. The RAVs T54A, V170A and A156S/T were detected *in vitro* (Tong et al., 2006). In addition, as BOC-resistant variants the amino acid

mutations V36M/A/G, Q41R, F43S/L, T54S, V55A R155K/T/L were observed *in vivo* (Susser et al., 2009; Vermehren et al., 2012). The mutations V36G, T54S, R155L conferred low-level resistance whereas medium-level resistance is observed for T54A, V55A, R155K, A156S and V170A. A156T is known as a high-level RAV to BOC (Susser et al., 2009). In patients with BOC treatment failure different RAVs were detected in genotype 1a (V36M (60%), R155K (67%) and T54S (19%)) compared to genotype 1b (54A (42%), T54S (37%), V55A (24%), A156S (26%) and V170A (32%)) (Barnard et al., 2013). The median time for all RAVs to become undetectable was 1.11 (1.05–1.2) years and was not significantly different between genotype 1a and 1b (Howe et al., 2013a).

5.3. Simeprevir

During *in vivo* studies mutations at 3 main amino acid sites were detected (Q80, R155, D168) whereas *in vitro* studies also showed emerging resistance at positions F43 and A156 (Lenz et al., 2010; Reesink et al., 2010). Mutations at position Q80 confer low-level-, at positions R155 and F43 moderate-level and at position D168 high-level resistance. At position A156 the susceptibility to SMV depends on the amino acid change: High-level resistance is observed for A156V and moderate-level resistance for A156G/T (Lenz et al., 2010). As mentioned above, the PROMISE study in relapsers as well as the QUEST trials in treatment-naïve subjects illustrated the impact of pre-existent Q80K in genotype 1 patients on treatment outcome. In QUEST-1 patients with pre-existing Q80K-variant did not show significantly superior SVR-rates compared to peg/ribavirin (Forns et al., 2013; Jacobson et al., 2013b). In the majority of patients (83.7%) in phase 2b and 3 studies with genotype 1a and baseline Q80K an emerging single R155K-variant at time of treatment failure was detectable, suggesting, that the presence of Q80K alone is not sufficient to explain a treatment failure. The median time until loss of mutation was 36 and 24 months for genotype 1a and 1b, respectively. Interestingly, the median time to loss of mutation for the R155K variant without baseline Q80K was 64 months compared to 32 months for patients who had emerging R155K and baseline Q80K (Lenz et al., 2013a).

5.4. Faldaprevir

In vitro studies found RAVs for FDV at positions R155, A156 and D168. Changes at V36, T54, F43 and Q80 did not confer resistance to FDV (Lagace et al., 2012). After FDV-treatment failure RAVs are predominantly found at positions R155 and D168 (Berger et al., 2013). Most of the RAVs confer moderate (R155Q, D168G) to high-level resistance (R155K, A156T/V, D168A/V). Although rates of virologic failure were consistently higher in genotype 1a vs. 1b, there was no influence of the Q80K mutation on SVR rates (Ferenci et al., 2013). Patients with treatment failure during phase 2 studies predominantly selected R155K variants in genotype 1a and D168V variants in genotype 1b (Sulkowski et al., 2013a). After treatment with FDV the time until loss of mutations was similar to TVR and BOC (median time 8–11 months) (Berger et al., 2012).

5.5. Sofosbuvir

S282T mutation is the primary SOF resistance mutation selected in genotype 1–6 *in vitro*. S282T confers a low to medium level resistance. Its replicative capacity compared to wildtype virus is significantly reduced (Rajyaguru et al., 2013). The S282T-substitution is a variant, which was already detected after treatment with other nucleos(t)ide polymerase inhibitors (Gane et al., 2012). To date S282T variant was only found in few patients after treatment: One patient (HCV-genotype 2b) relapsed 4 weeks after 12 weeks of SOF monotherapy (Svarovskaia et al., 2012). Another patient (treatment-naïve, genotype 1) had virologic relapse after 8 weeks of treatment with SOF and the NS5A inhibitor ledipasvir. Beside emergence of S282T, three RAVs associated with NS5A inhibitor resistance were detected. This patient was successfully retreated with SOF, ledipasvir and ribavirin over 24 weeks (Lawitz et al., 2013d).

In an analysis of all patients with treatment failure during phase 3 trials, the S282T substitution was not detected by deep sequencing in any of the 225 patients (Svarovskaia et al., 2013b). In general, SOF exhibits a high genetic barrier to resistance. Therefore, together with the low replicative fitness of the S282T variant, to date no viral break-through could be observed. For patients with virologic relapse after the end-of-treatment either survival of wildtype virus at low levels or selection of S282T variants with rapid reversion to wildtype before the time point of sequence analysis is a possible explanation for treatment failure (Vermehren and Sarrazin, 2012).

5.6. Daclatasvir

In general the barrier to resistance for DCV is low. After 14-day-monotherapy with DCV RAVs at positions M28, Q30, L31 and Y93 for subtype 1a and L31 and Y93 for subtype 1b were observed *in vivo* (Wang et al., 2013). In addition, *in vitro* studies found RAVs at position P32 for both subtypes as primary resistance mutations (Fridell et al., 2010). The main mutations Y93H and L31V confer medium-level resistance to DCV (Wang et al., 2013).

The antiviral activity of DCV is less potent for subtype 1a than subtype 1b and also less potent for genotype 3 than genotype 2 *in vitro* (Gao et al., 2010). In line with these preclinical data clinical studies demonstrated lower response rates for patients infected with subtype 1a (Lok et al., 2012) and genotype 3 (Dore et al., 2013).

In a small study with Japanese genotype 1b patients (prior null-responders and interferon-intolerant/ineligible) treated with DCV and the protease inhibitor asunaprevir all patients with viral breakthrough and 2 of 4 patients with relapse had Y93H mutation at baseline. In contrast, five other patients with Y93H at baseline achieved a SVR. Viral breakthrough and relapse was always associated with the emerging double mutation L31M and Y93H with or without other substitutions at position P58 or Q54. Interestingly, in all patients with viral breakthrough RAVs persisted in a 48-week follow-up period whereas RAVs became undetectable in three of four relapse patients (Karino et al., 2013). The possible impact of these long persisting and high-level resistant variants on a retreatment is currently unknown.

6. Do we need resistance testing in the future?

In summary, systematic trials evaluating the influence of pre-existing or persistent resistant mutations after prior treatment on the efficacy of DAA-containing therapies are lacking. Currently there is no indication for a general resistance testing before starting HCV-treatment. In some patient populations and when a certain treatment is considered, resistance testing can help to decide which DAA is the best treatment option for the individual patient:

6.1. Scenario 1: Treatment-naïve patients and patients after peg/ribavirin treatment failure

The frequency of pre-existing RAVs in this population is generally low. In fact, a successful treatment is independent of the pre-existence of RAVs to TVR, BOC and FDV. Given sufficient adherence to treatment, only in combination with other unfavourable factors, primarily unresponsiveness to the peg/ribavirin backbone,

treatment failure occurs. For these rare cases resistance testing is not justified. For SMV the relative high frequency of pre-existing Q80K variants in genotype 1a especially in European and North American populations has to be considered (19–48%) as this variant is associated with lower rates of SVR and RVR. Therefore, for genotype 1a patients in whom a treatment with SMV is considered, resistance testing for the detection of Q80K has to be discussed.

For SOF no pre-existing RAVs are known, therefore resistance testing is not indicated in this situation. Although a link between DCV-RAVs at baseline and treatment failure exists, combined with another potent antiviral like SOF the rates of breakthrough or virologic relapse are low (Sulkowski et al., 2014), so resistance testing is not indicated in this situation.

6.2. Scenario 2: Retreatment after failure of conventional TVR/BOC triple therapy

To date, no study data exists evaluating the significance of a retreatment with other PIs after TVR/BOC failure. In general, in these patients a poor interferon-responsiveness has to be expected and resistant variants can still be detectable especially for a short interval to re-treatment (<1 year). Thus, conventional PI-based triple therapy may not be an effective option for these patients. No cross-resistance exists between PIs and NS5A or NS5B inhibitors like SOF and DCV. Therefore resistance testing before SOF-containing interferon-free options together with an NS5a inhibitor as well as a conventional triple therapy is not required. However, while SOF in combination with DCV or another NS5A inhibitor like ledipasvir has been shown to be highly effective, no data exist on the efficacy of SOF-based peg/ribavirin triple therapy in these patients.

6.3. Scenario 3: Interferon-free treatment

In genotype 2 (and 3) the interferon-free therapy SOF and ribavirin has shown non-inferior SVR rates and is a new treatment option in these patients. As mentioned above, due to the lack of pre-existing variants, resistance testing prior to SOF treatment is not necessary.

A second potential treatment is the combination of SOF and a PI (especially SMV as clinical study data are available). Although high SVR rates have been observed in treatment-naïve and peg/ribavirin failure patients treated with SOF plus SMV combination therapy for 12 or 24 weeks (>90%), all patients with treatment failure had pre-existing Q80K variants at baseline (Jacobson et al., 2013c). Therefore, especially if also prior to PI treatment failure, patients are considered for SOF plus PI re-treatment, resistance testing may be useful to detect either Q80K and/or other NS3-variants.

In the recent study by Sulkowski at al. using SOF and DCV with or without ribavirin in genotype 1–3 patients, 16% of patients had baseline mutations associated with DCV resistance. Nevertheless, virologic relapse was detected in only one of these patients (Sulkowski et al., 2014). Thus, general resistance testing is not required.

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