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REVIEW

Clinical Laboratory Testing in the Era of Directly Acting Antiviral Therapies for Hepatitis C

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SUMMARY Directly acting antiviral (DAA) combination therapies for chronic hepatitis C virus (HCV) infection are highly effective, but treatment decisions remain complex. Laboratory testing is important to evaluate a range of viral, host, and pharmacological factors when considering HCV treatment, and patients must be monitored during and after therapy for safety and to assess the viral response. In this review, we discuss the laboratory tests relevant for the treatment of HCV infection in the era of DAA therapy, grouped according to viral and host factors.

KEYWORDS directly acting antiviral therapy, hepatitis C virus, viral resistance

INTRODUCTION

The treatment of chronic hepatitis C virus (HCV) infection has changed dramatically over the past 5 years. In the past, the duration and continuation of treatment with interferon (IFN)- and ribavirin (RBV)-based therapies were guided by laboratory testing and demographic factors. Those individuals with so-called "unfavorable treatment characteristics," including infection by particular HCV genotypes, male sex, distinct genetic polymorphisms, advanced hepatic fibrosis (including cirrhosis), and coinfection

with human immunodeficiency virus (HIV), were less likely to achieve a sustained virologic response (SVR), defined as the absence of detectable hepatitis C virus 12 weeks after the completion of therapy, now synonymous with cure (1–3). The first approved directly acting antiviral (DAA) agents, which specifically inhibit HCV serine protease, were introduced in 2011. However, the use of these initial agents was complicated by coadministration with pegylated IFN (pegIFN) and RBV, which resulted in severe side effects and required frequent laboratory monitoring throughout protracted treatment courses. With the development of newer combination DAA regimens, first approved by the U.S. Food and Drug Administration (FDA) in late 2014, patients have safe and highly effective all-oral, interferon-free HCV treatment options, with cure rates exceeding 90% for most HCV genotypes and stages of fibrosis (4–8). The advent of DAAs has changed the paradigms not only for treatment of HCV but also for the relevant associated laboratory testing as well.

The DAA agents currently approved for IFN-free combination therapy of HCV infection are shown in Table 1. These agents target three different HCV proteins via four different pathways. The nucleotide HCV nonstructural protein 5B (NS5B) polymerase inhibitor sofosbuvir (SOF) (Gilead Sciences, Inc.) (generics are also available in certain countries) is the only approved member of its class; a uridine analog chain terminator, it is approved for combination therapy with other DAAs or with pegIFN-RBV (9). There is also an approved nonnucleoside NS5B inhibitor, dasabuvir (DSV; AbbVie, Inc.), that inhibits the polymerase separately from the active site (10). The second drug target, the HCV NS5A protein, is thought to help stabilize infectious HCV replication complex formation on the surface of the endoplasmic reticulum within hepatocytes (11). Approved NS5A inhibitors include ledipasvir (LDV) and velpatasvir (VEL) (both from Gilead Sciences, Inc.) (12, 13), daclatasvir (DCV; Bristol-Myers Squibb) (14), ombitasvir (OMV; AbbVie, Inc.) (15), and elbasvir (EBR; Merck & Co., Inc.) (16). The final class of approved second-generation DAAs is the NS3/4A protease inhibitors, which include simeprevir (SMV; Janssen Pharmaceuticals) (17), grazoprevir (GZR; Merck) (18), paritaprevir (PTV; AbbVie) (19), and asunaprevir (ASV; Bristol-Myers Squibb) (currently approved in Japan and Russia) (20). There are also several coformulations of these medications that greatly simplify their administration: LDV and SOF are coformulated as Harvoni (Gilead), GZR and EBR are coformulated as Zepetier (Merck), and OMB and PTV are coformulated with ritonavir (inactive against HCV but included to potentiate PTV to facilitate once-daily dosing) as Technivie and copackaged with DSV under the name Viekira Pak (both from AbbVie). The American Association for the Study of Liver Diseases (AASLD) and the Infectious Diseases Society of America (IDSA) have together created dynamic guidelines (available online at http://www.hcvguidelines.org/) that are regularly updated in response to new data and drug approvals with recommendations and expert opinion regarding the combination of these medications for the treatment of chronic HCV infection (21).

Despite the high efficacy and tolerability of these regimens, treatment decisions remain complex. After a patient is found to be seropositive, having detectable anti-HCV antibody, a positive molecular test for HCV RNA is required for a diagnosis of chronic HCV infection (22). Depending on the immunological status, between 20 and 45% of persons exposed to HCV may naturally clear the infection, usually in the first 6 months after exposure (23). Clinicians must then use laboratory testing to evaluate a range of viral, host, and pharmacological factors when considering initiating treatment to ensure that patients realize the full benefit of new HCV therapies. Patients must be monitored during and after therapy for safety and to assess the viral response (the testing timeline is shown in Fig. 1). In this review, we discuss the laboratory tests relevant for the treatment of HCV infection in the era of DAA therapy, grouped according to viral and host factors.

VIRAL FACTORS

Prior to the start of DAA therapy for HCV, viral testing is required for two reasons: first, to confirm chronic HCV infection (while it is rare, even patients with documented

TABLE 1 FDA-approved formulations for the treatment of chronic hepatitis C[a]

Generic name(s) (concn [mg])	Abbreviation	U.S. brand name	Dosing	Indication(s)
Daclatasvir (60)	DCV	Daklinza	One tablet taken once daily	Approved for treatment of GT1 or GT3 infection when used with sofosbuvir, +/− ribavirin, for 12 wk
Elbasvir (50)-grazoprevir (100)	EBR-GZR	Zepatier	One tablet taken once daily	Approved for treatment of GT1 or GT4 infection, +/− ribavirin, for 12-wk duration or for 16-wk duration for GT1a infection, treatment-experienced patients with NS5A resistance-associated variants, and GT4-infection treatment-experienced patients
Ledipasvir (90)-sofosbuvir (400)	LDV-SOF	Harvoni	One tablet taken once daily	Approved for treatment of GT1, GT4, GT5, and GT6 infection, +/− ribavirin, for 12 wk, or for 24 wk for patients with GT1a infection with compensated cirrhosis
Ombitasvir (12.5)-paritaprevir (75)	PrO	Technivie	Two ombitasvir-paritaprevir-ritonavir tablets taken once daily (morning)	Approved for treatment of GT4, +/− ribavirin, for 12 wk, including patients with compensated cirrhosis
Ombitasvir (12.5)-paritaprevir (75)-ritonavir (50) + dasabuvir (250)	PrOD	Viekira Pak	Two ombitasvir-paritaprevir-ritonavir tablets taken once daily (morning), one dasabuvir tablet taken twice daily	Approved for treatment of GT1b infection, and GT1a infection when used with ribavirin, for 12 wk; for patients with GT1a infection and compensated cirrhosis, treatment should be extended to 24 wk
Ombitasvir (8.33)-paritaprevir (50)-dasabuvir (200)-ritonavir (33.33)	PrOD	Viekira XR	Three fixed-dose combination tablets once daily	Approved for treatment of GT1b infection, and GT1a infection when used with ribavirin, for 12 wk; for patients with GT1a infection and compensated cirrhosis, treatment should be extended to 24 wk
Simeprevir (150)	SMV	Olysio	One capsule taken once daily	Approved for treatment of GT1 infection when used with sofosbuvir for 12-wk duration
Sofosbuvir (400)	SOF	Sovaldi	One tablet taken once daily	Approved for treatment of GT1, GT2, GT3, or GT4 infection when combined with other antiviral medications.
Sofosbuvir (400)-velpatasvir (100)	SOF-VEL	Epclusa	One tablet taken once daily	Approved for treatment of GT1, GT2, GT3, GT4, GT5, or GT6 infection, with or without cirrhosis (compensated), for 12 wk; for those with advanced cirrhosis (decompensated), approved for use with ribavirin for 12 wk

[a]Shown are IFN-free treatment regimens for chronic hepatitis C currently approved by the FDA as of July 2016. GT, genotype.

infection for more than a decade can occasionally clear HCV on their own); second, to select the treatment regimen and determine the optimal duration of treatment. Therapeutic regimen selection depends upon the HCV genotype and subgenotype, and for some regimens, resistance to DAAs and the baseline HCV load must also be considered. In this section, we discuss the tests used to assess these factors and the data supporting these recommendations.

HCV Genotype

There are seven genotypes of HCV, numbered in the order in which they were discovered, and these distinct genotypes may differ in their genetic sequences by >30% (24, 25). Each genotype has many subtypes, identified by a letter, also in the order of discovery. People can be infected with more than one HCV genotype,

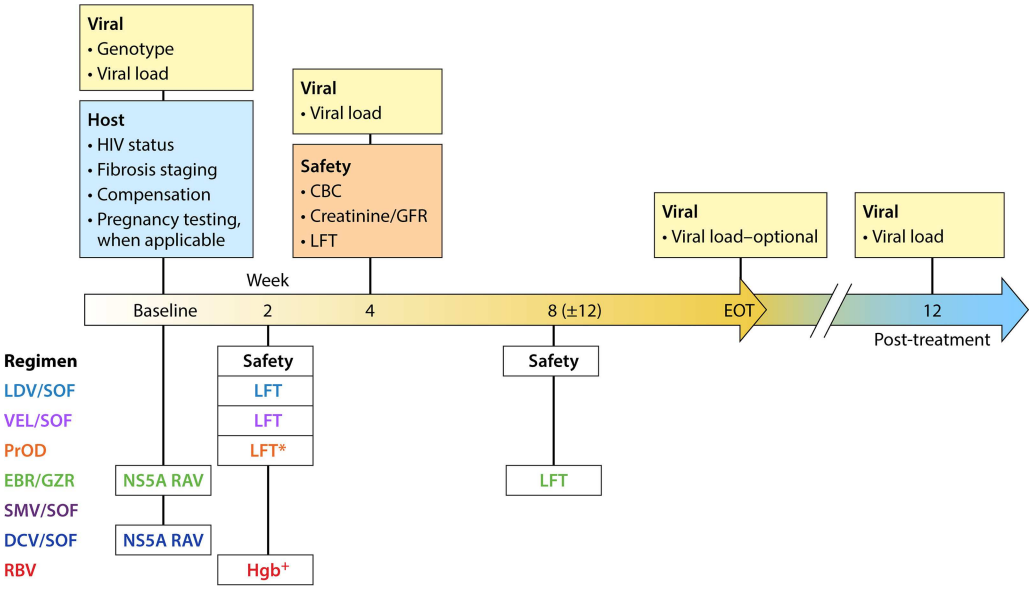

FIG 1 Timing of laboratory testing for treatment of hepatitis C. The schematic shows the timing of host, virus, and safety laboratory testing prior to, during, and following combination DAA therapy for the treatment of chronic hepatitis C. Regimen-specific testing is color-coded according to regimen. Abbreviations: EOT, end of therapy; HIV, human immunodeficiency virus; RAV, resistance-associated variant; CBC, complete blood count; GFR, glomerular filtration rate; LFT, liver function testing; Hgb, hemoglobin. *, indicated if patient is cirrhotic; +, repeat as clinically indicated.

known as mixed infection, which occurs in up to 25% of those persons infected via blood transfusions or intravenous drug use, exposures which carry the highest risk (24, 26). Patients may also be infected with recombinant infections, most commonly subgenotype 2k/1b infections in Georgia and subgenotype 2b/1a infections in the United States (25).

HCV genotype may predict disease progression, with genotype 3 infection being associated with accelerated fibrosis (27, 28) and, in the era of IFN-based therapy, treatment response as well. Dual therapy with pegIFN-RBV cured only 20 to 50% of patients with genotype 1 or 4 infection, compared to 75 to 90% of those infected with genotypes 2 and 3 (29–31). DAA-based regimens have become the standard of care for the vast majority of patients; even so, knowledge of genotype, and in some cases subgenotype, remains an integral part of selecting the most appropriate DAA regimen and duration of treatment. Whether this will change with the approval of the first pangenotypic combination DAA regimen, SOF-VEL, remains to be seen.

HCV genotype and subtype testing is available commercially, although these tests differ in their approach. The Versant HCV Genotype INNO-LiPA 2.0 assay (Siemens Healthcare Diagnostics) relies upon a reverse hybridization line probe. The Trugene HCV 5′NC (Visible Genetics, Inc.) and M2000 RealTime HCV Genotype 2.0 (Abbott Laboratories) (FDA approved for HCV genotyping) assays use direct sequencing to differentiate genotypes. All three tests tend to be reproducible and have high degrees of concordance (32), but in some cases, including cases of mixed infections, non-genotype 1 subtypes, and recombinant infections, further discrimination with additional tests can be required (33–36). Most clinical trials, if they report the testing method, have used the Siemens INNO-LiPA 2.0 assay for genotype determinations, but either method is reliable for distinguishing genotypes and subgenotypes in the majority of patients.

Throughout the world, the distribution of HCV genotypes and risk factors for exposure vary. HCV genotype 1 is the predominant genotype in the Americas, Asia, and Europe (37), with most people being infected with one of two subgenotypes: subgenotypes 1a and 1b. All FDA-approved DAA therapies are active against HCV genotype 1. In initial studies of LDV-SOF (5) and in some studies of EBR-GZR-based (38) and

DCV-based (39) regimens, the authors reported high response rates (>95%) regardless of the subgenotype. Studies of SOF (40), DCV (41), ombitasvir-paritaprevir-dasabuvir-ritonavir (PrOD) (6, 42), and EBR-GZR (43), however, have reported differences in activity against subgenotypes 1a and 1b, with the majority of studies reporting lower response rates in patients infected with genotype 1a (6, 41–43). It is important to identify patients with HCV subgenotype 1a infection, as treatment outcomes may be improved by increasing the duration of therapy and/or adding RBV (PrOD [6] and EBR-GZR [44]), neither of which is required for treatment of patients with genotype 1b infection (42, 45). Patients with genotype 1b infection, in contrast, tend to do less well than genotype 1a-infected patients when treated with only SOF-RBV (40, 46). This combination is no longer recommended for the treatment of any genotype 1-infected patients where other, more effective DAA regimens are available. In some developing countries, however, dual therapy with SOF-RBV remains the first-line regimen for pegIFN-ineligible patients for infection with all genotypes, especially where generic sofosbuvir is available (47).

Genotypes 2 and 3, as discussed above, were historically easier to treat with pegIFN-RBV. Many DAA agents, however, lack *in vitro* activity against these genotypes, with reduced clinical efficacy even when combined with a pangenotypic backbone, such as SOF. For infection with genotype 2, the combination of SOF and RBV (46, 48, 49) and/or DCV (50, 51) is highly effective, with cure rates of ~90 to 100% (48). For patients infected with HCV genotype 3, recommended treatment regimens are similar to those for genotype 2 infection, with evidence to support treatment with SOF combined with RBV (49), DCV (52), and even pegIFN-RBV (53). Limited data have also suggested that while the 50% effective concentration (EC_{50}) of LDV is greatly increased in genotype 3 infection, LDV-SOF with RBV may also treat HCV in those with genotype 3 infection (54), with the advantage of decreased duration and adverse effects, but the findings of this one small study have yet to be replicated, and this regimen is not recommended by guidelines of any major professional society, although it is occasionally recommended based on formulary or availability in selected institutions. Many DAA regimens have demonstrated efficacy in genotype 4 infection, including LDV-SOF (55), ombitasvir-paritaprevir (PrO) with or without RBV (56), and the combination of SOF-RBV (57, 58). For genotype 5 and 6 infections, LDV-SOF has shown high efficacy in small clinical trials (54, 59), but these data are limited. Table 1 summarizes the currently approved regimens in the United States and Europe and their spectrum of genotype coverage.

Viral Load

Baseline HCV RNA load. HCV RNA testing is required prior to the initiation of treatment to confirm chronic HCV infection and, over the course of treatment, to assess treatment response. There are several approved tests for HCV RNA load quantification. In clinical trials, the preferred test has been either the Cobas TaqMan HCV, version 2.0, test (CTM2; Roche Molecular Systems), with a lower limit of quantification (LLOQ) of 25 IU/ml, or the Abbott RealTime HCV assay (ART), with a LLOQ of 12 IU/ml, both of which are FDA approved. Some comparative analyses have shown that these tests were highly correlative and have comparable linearity for HCV RNA quantification across all genotypes (60, 61). However, recent testing has raised questions about the comparability of the results of the various tests used in clinical practice, including CTM2, ART, and the new Aptima HCV Quant Dx assay (Hologic, Inc.), available in Europe but not currently FDA approved for confirmation of HCV infection, with measurements between tests varying widely, from 1.3- to 1.8-fold for genotype 1 samples (62). Nucleic acid tests may use different methodologies (i.e., PCR-based assays, like ART and CTM2, versus signal amplification-based branched-DNA-based assays, like the FDA-approved Versant HCV 3.0 assay [Siemens Healthcare Diagnostics]), and therefore, patients should be monitored by using the same test over the course of therapy. Even when patients are monitored by using the same HCV RNA assay, the HCV set point remains relatively stable although less so than the HIV load set point. One analysis showed that 15% of

those with chronic HCV infection not receiving antiviral therapy had HCV RNA levels that varied by a log or more in consecutive measurements over time (compared with only 4% of those with untreated HIV infection), and 44% of HCV-infected patients had an HCV RNA load that varied by at least 0.5 logs (63).

Many studies have looked at treatment responses to DAAs stratified by pretreatment HCV RNA measurements, as this had been shown to predict treatment responses to IFN-based therapies (64), but the exact HCV RNA cutoff varies. In a *post hoc* analysis of the ION-3 trial restricted to patients with an HCV RNA load of $<$6,000,000 IU/ml, treatment response rates after 8 or 12 weeks with LDV-SOF were similar (65), and the LDV-SOF prescribing information recommends that 8 weeks of therapy can be considered for treatment-naive patients without cirrhosis and with an HCV RNA load of $<$6,000,000 IU/ml (66). A separate analysis of publically available data (coauthored by one of the authors of this review) found no evidence to support a cutoff of 6,000,000 IU/ml (67). While this specific recommendation remains in dispute, other studies have also suggested that the baseline viral load impacts DAA therapy for HCV infection. A lower proposed HCV RNA load cutoff of $\leq$800,000 IU/ml has been shown to predict SVR rates following 24 weeks of SOF-RBV therapy (40) and 12 weeks of EBR-GZR therapy (43), and an HCV RNA level of $\leq$2,000,000 IU/ml was shown to predict a favorable response in one study of patients coinfected with HCV and HIV who were treated with DCV-SOF for 8 weeks (50).

On-treatment monitoring of HCV RNA load. Current AASLD-IDSA guidelines recommend a repeat HCV RNA measurement after 4 weeks of combination DAA therapy (68), as a measure of adherence only. Of patients without advanced cirrhosis, most have undetectable HCV RNA by week 4 of therapy, while patients with cirrhosis may experience a slower viral decline. The guidelines go on to state explicitly that there are no data to support cessation of therapy if a patient has detectable HCV RNA at week 4, unless it represents a $>$10-fold increase from the baseline measurement (based on expert opinion) (68). In a large study of patients treated through the Veterans Affairs Healthcare System, Backus et al. reported that across SOF-based regimens (including SOF-RBV, SOF-SMV, and SOF-SMV-RBV), patients who achieved an HCV RNA load lower than the LLOQ by week 4 of therapy were more likely to go on to achieve SVR, but importantly, this analysis included patients who had discontinued therapy prior to week 4 for a variety of reasons (69). Some advocate for HCV RNA testing at the end of therapy, in order to differentiate viral breakthrough from relapse or reinfection, but an intensive analysis of patients receiving a variety of combination DAA therapies of various durations demonstrated that patients who went on to develop SVR occasionally had residual viremia detected up until the end of therapy (70), further reinforcing the limitations of HCV RNA monitoring in predicting therapeutic response and in guiding treatment decisions.

Measurement of the HCV RNA load at least 12 weeks after the completion of therapy, or SVR12, is used as a surrogate endpoint for cure of HCV infection. Previously, during the era of IFN-based therapies, SVR was assessed 24 weeks following the completion of therapy (71), but relapse after 12 weeks following the completion of combination DAA-based therapy is rare, and the majority of clinical trials now use SVR12 as the primary endpoint (72). Monitoring of HCV RNA levels more than 12 weeks after the completion of therapy is indicated only if there is concern that a patient may have been reinfected; current guidelines do not recommend routine monitoring for relapse after SVR12 is achieved. In contrast, the HCV antibody test often remains reactive after successful treatment but these antibodies are not protective against reinfection.

Resistance Testing

With high levels of viral replication and an error-prone polymerase, HCV exhibits broad genetic diversity in chronic infection (73), and some amino acid substitutions exhibit reduced susceptibility to DAAs *in vitro*. The presence of resistance-associated variants (RAVs), also known as resistance-associated polymorphisms or substitutions,

NS5A Inhibitor	Amino Acid Position and Substitutions													
	Genotype 1a											Genotype 1b		
	M28		Q30			L31		H58	Y93			L31		Y93
	T	V	E	H	R	M	V	D	C	H	N	M	V	H
Daclatasvir (DCV) (77, 78)	205	–	7,500	435	365	105	1,000	–	555	1,600	14,100	3	15	12
Elbasvir (EBR) (79)	15	1	56	–	16	10	61	6	–	220	929	–	–	–
Ledipasvir (LDV) (77, 78)	61	–	952-5,458	183	632	554	–	1,127	1,602	1,677-3,309	14,706	–	–	1,319
Ombitasvir (OMV) (77, 80)	8,965	58	–	3	800	2	–	243	1,675	41,383	66,740	1	8	77
Velpatasvir (VEL) (87)	8	–	18	2	2	16	68	7	4	609	2,758	2	3	3

No data <5 fold 5–100 fold >100 fold

FIG 2 NS5A inhibitors and NS5A resistance-associated variants (77–80, 87). Numbers denote fold changes in reduced susceptibility to the NS5A inhibitor for the indicated amino acid substitution, rounded to the nearest integer.

has been shown to predict treatment failure of some DAA-containing regimens (74); AASLD-IDSA guidelines and the FDA recommend that RAVs should be assessed prior to therapy in some treatment situations when using selected agents (particularly EBR and, if desired, DCV) or in those who have previously failed DAA-based therapies. While the potency of DAAs may depend upon the genetic barrier to resistance and viral susceptibility, the role of resistance testing in predicting treatment outcomes is far from clear.

There are different methods of testing for RAVs. Some studies and the majority of clinical laboratories have used population-based sequencing, which detects only polymorphisms comprising more than 15 to 25% of the patient's viral population. In contrast, clonal sequencing and deep sequencing can detect variants that are present in as little as 5% and <1% of the population, respectively, depending on the volume of the sample (73). It remains unclear whether the proportion of RAVs in the overall viral population is important for treatment outcomes. Qualitative tests for the presence of RAVs, rather than a quantitative measure of the proportion of the patient's viral population comprised of individual RAVs, are available from a variety of clinical laboratories, including Monogram Biosciences and Quest Diagnostics.

One RAV associated with an adverse effect on the treatment response is the Q80K polymorphism in NS3, present in up to half of those individuals with genotype 1a infection at baseline (75), which predicts higher rates of virologic failure in those receiving SMV in combination with IFN-RBV. However, it appears this reduced susceptibility may be overcome by using combination DAA therapy for a sufficient duration: in patients receiving standard 12-week, rather than 8-week, courses of combination therapy with SMV-SOF, the presence of the Q80K polymorphism at baseline did not alter outcomes (76).

Polymorphisms in the NS5A region of HCV also confer reduced susceptibility to NS5A inhibitors *in vitro* (77–80). Selected NS5A RAVs and the corresponding fold changes for NS5A inhibitors are shown in Fig. 2. A pooled analysis of data from LDV-SOF phase 3 clinical trials found baseline NS5A RAVs in ~16% of all patients, but reduced rates of SVR (by ~30%) were observed only in those patients with prior HCV treatment

experience and NS5A RAVs conferring a >100-fold reduction in susceptibility (81). A subanalysis of data from EBR-GZR phase 2 and 3 clinical trials showed reduced efficacy (by ~10%) in patients with preexisting NS5A RAVs conferring 5-fold-decreased susceptibility to EBR when detected by population-based sequencing (38, 82). A subsequent analysis, this time in patients undergoing retreatment after failing initial combination DAA therapy with LDV-SOF, found that those individuals with NS5A RAVs identified by deep sequencing, a more sensitive technique than population-based sequencing, were less likely to respond to longer courses of LDV-SOF plus RBV (83), with only 60% achieving SVR, as opposed to 100% of those without NS5A RAVs detected. Our group has shown that patients with baseline high-level NS5A RAVs (>25-fold-reduced susceptibility to LDV), detected by deep sequencing, were less likely to respond to short-duration combination DAA-based therapies (4 weeks of therapy with three or four DAAs) (84). In contrast, we found that similar patients treated with combination DAA therapy containing LDV-SOF for at least 6 weeks (85) or retreated after failing previous short-duration therapy with LDV-SOF for at least 12 weeks (86) achieved SVR at the same frequency as patients without NS5A resistance. Another study of SOF-based therapy, this time SOF-VEL, showed that reduced efficacy was genotype dependent, with marginally lower rates of viral decline in patients with genotype 1a and 3 infections and RAVs, while no such reduction was noted despite the presence of RAVs in patients with genotype 1b, 2, and 4 infections (87). Other studies also suggest that increasing the duration of therapy, and/or adding RBV, can overcome the presence of baseline NS5A RAVs (81, 88).

While some amino acid substitutions within the NS5B gene associated with reduced susceptibility to SOF and DSV have been reported, their clinical significance remains unknown. Patients who have failed SOF-based regimens have been reported to have S282T and V321I substitutions (86, 89), but *in vitro* data suggest that these substitutions confer only slightly reduced (<5-fold) susceptibility to SOF (77) and that the S282T substitution may be present only transiently, possibly because this variant exhibits reduced viral fitness and is rapidly replaced with the wild-type virus (89).

Interestingly, NS5A RAVs have been shown to be remarkably stable, persisting for months to years in the absence of selective pressure (90), suggesting that the substitutions that confer reduced susceptibility to NS5A inhibitors replicate and persist with a fitness similar to those of wild-type viruses. In contrast, the prevalence of NS3 RAVs following therapy with an NS3 inhibitor declined over time (86, 90), as the viral variants carrying resistance-associated substitutions are outcompeted by variants with wild-type NS3 sequences. Current guidelines recommend pretreatment evaluation for the presence of NS5A RAVs in a patient with genotype 1a HCV infection if treatment with EBR or DCV (if the patient is cirrhotic) is being considered. If NS5A RAVs conferring >5-fold-reduced susceptibility to EBR are identified (in particular at position M28, Q30, L31, or Y93), current recommendations are that the duration of EBR-GZR treatment should be extended to 16 weeks and that RBV should be added (91). Any other RAV testing, including testing for substitutions within NS5B, has yet to be supported by clinical studies.

HOST FACTORS

As with virus testing, it is important to evaluate several host factors prior to the start of DAA therapy for HCV infection. Some comorbid conditions, like renal dysfunction or anemia, or infections may complicate HCV treatment and influence the selection of a therapeutic regimen. Other factors, including host genotype and fibrosis staging, can affect treatment outcomes. In this section, we discuss the tests used to assess these factors and the data supporting these recommendations.

IL28B Genotype

Polymorphisms within or near the IL28B gene have been strongly associated with prediction of treatment responses to IFN-based regimens (92–94), likely mediated by the levels of intrahepatic expression of IFN-stimulated genes (ISGs) (95).

The extent to which IL28B polymorphisms remain relevant in the era of DAA-based therapies is unclear. A report by Backus et al. on a large cohort of veterans treated for HCV infection found that patients with the favorable IL28B (rs12979860) genotype CC were more likely to respond to SOF plus pegIFN-RBV; no such difference was shown for combination DAA treatment with SMV-SOF (69). In a small study of IFN-free therapy with investigational agents, a favorable CC IL28B genotype was associated with more rapid hepatitis C virus decline (96), but this early response did not translate into higher SVR rates. The IL28B genotype of study participants has been reported in multiple trials of combination DAA therapy, including LDV-SOF (4, 5, 8, 65), PrOD (7, 42, 45), DCV (50, 51), and EBR-GZR (38, 43, 97), without a significant impact on SVR. Commercial sequence-based testing is available, and because of the effect of the IL28B genotype on the response to IFN-based therapies, the IL28B genotype should be evaluated for all patients receiving IFN-based therapies. This evaluation is not currently recommended for those receiving combination DAA-based IFN-free therapies; IL28B genotypes may play a role in the response to short-course (6 weeks or less) combination DAA-based therapies, but this would require further investigation.

IFNL4 Genotype

The IFN lambda-4 gene (IFNL4-ΔG) is an exonic deletion that is closely associated and in close linkage disequilibrium with the IL28B genotype. Our group has identified a possible association with reduced spontaneous clearance of HCV (98), a reduced response to IFN-based regimens (99), and a slower early HCV decay in response to SOF-RBV therapy (100). This polymorphism has been rarely reported in clinical trials of combination DAA-based, IFN-free therapy, and when reported, it has not been associated with different rates of viral clearance (85). As such, testing for the presence of IFNL4-ΔG is not routinely recommended in clinical practice and is not commercially available at this time. Allele-specific probes are available commercially from Applied Biosystems and can be used with PCR-based sequencing systems, but these probes are not covered under U.S. Clinical Laboratory Improvement Amendments (CLIA) regulations.

Viral Coinfection

Because of shared routes of transmission, chronic HCV infection is common in patients with HIV infection, and patients with HCV infection are routinely screened for HIV (101). Individuals coinfected with HIV and HCV have been shown to have worse outcomes than persons with HCV infection alone, with more rapid and more frequent development of cirrhosis and hepatocellular carcinoma (HCC) (102). While patients coinfected with HIV and HCV are less likely to respond to immune-based HCV therapies, DAA-based therapies appear to maintain high SVR rates similar to those observed for HIV-negative HCV-infected patients (103). While the selection of a treatment regimen for HCV infection requires thoughtful consideration of the potential interactions with antiretroviral therapy, HCV treatment outcomes do not appear to depend upon the patient's HIV status, regardless of the selected DAA regimen (50, 97, 104).

Similarly, hepatitis B virus (HBV) and HCV also share common routes of transmission, and patients chronically coinfected with HBV and HCV have accelerated liver fibrosis and are at increased risk for hepatic decompensation and HCC (105). IFN-based HCV therapies also have activity against HBV, and patients with inactive or resolved HBV infection were at risk for HBV reactivation with IFN-based regimens; this has also been reported, albeit infrequently, in patients treated with DAA-only regimens (106). Currently, AASLD-IDSA guidelines and other professional organizations recommend screening of patients for HBV prior to DAA-based therapy with a hepatitis B virus surface antigen test (22), but there is no consensus on the best way to monitor patients for HBV reactivation during or after treatment.

Fibrosis Staging

Although DAAs have demonstrated nearly universal efficacy regardless of most baseline demographic characteristics, fibrosis staging remains an important part of pretreatment evaluation, as treatment outcomes continue to be impacted by the degree of liver fibrosis. This may be due in part to the impact of hepatic fibrosis on HCV clearance and drug delivery. Individuals with advanced hepatic fibrosis may experience reduced drug delivery due to venous shunting, limited drug uptake secondary to fibrotic changes, decreased drug metabolism from reduced liver function, and impaired immune signaling pathways (107). However, given the direct antiviral activity of DAAs, the significance of the host immune response in achieving SVR remains unclear. Most clinical trials have divided patients into two categories: no cirrhosis (stage 0 to 3 fibrosis) and compensated cirrhosis (4, 5, 108). Due to disparities in SVRs between these groups, the presence of cirrhosis may change the recommended duration of treatment or warrant the addition of RBV (109). In addition, from a clinical perspective, it is important to be aware if a patient has cirrhosis or advanced fibrosis (stage 3 fibrosis) because surveillance for hepatocellular carcinoma and esophageal varices are recommended, regardless of treatment outcome. It is also important to distinguish between patients with compensated and those with decompensated cirrhosis, as this may change the recommended treatment. While patients with advanced fibrosis have a clear benefit from achieving SVR, it remains unknown whether outcomes vary for those treated at early, as opposed to moderate, stages of fibrosis. At this point, the main reason for distinguishing between early and moderate stages of fibrosis is to identify which patients meet insurance standards for treatment, as many insurance companies in the United States restrict access to DAAs, reserving them for patients with moderate or advanced fibrosis (110). The evaluations most commonly used for the evaluation of hepatic fibrosis are shown in Table 2.

Liver biopsy. The "gold standard" for staging of liver fibrosis is liver biopsy. Patients undergo a percutaneous, transvenous, or surgical/laparoscopic procedure to obtain a needle core biopsy specimen. Optimal outcomes have been identified when the needle gauge is 16, the core length is 3 cm after fixation, and three separate cores are taken to reduce sampling errors (111). The specimens are fixed and paraffin embedded and undergo staining with hematoxylin and eosin (H&E) to determine the degree and extent of hepatic inflammation and the presence of disease-specific abnormal cells. Specimens are also stained with Masson's trichrome in order to determine the extent and nature of fibrosis. Scoring systems have been developed to help standardize the classification of the degree of hepatic inflammation and fibrosis.

The Ishak (modified Knodell) scoring system was developed in 1995 in an attempt to grade the intensity of hepatic necroinflammatory activity and stage hepatic fibrosis and architectural alteration. For grading, scores are given for pathological assessment of the degree of periportal or periseptal interface hepatitis (piecemeal necrosis), confluent necrosis, focal (spotty) lytic necrosis, apoptosis and focal inflammation, as well as portal inflammation. For staging, architectural changes, fibrosis, and cirrhosis are taken into account by assessing fibrous expansion within portal areas, the presence and extent of septation, and bridging. The stage of fibrosis ranges from 0 to 6, with stage 5 or 6 indicating cirrhosis (112).

The Metavir scoring system was specifically designed and validated for individuals with chronic HCV infection. By using this system, pathologists assess the degree of histological activity through assessment of the number of necroinflammatory foci per lobule (focal lobular necrosis) and alteration of the periportal plate in portal tracts (piecemeal necrosis). Histological activity is scored as A0 to A3 (where A0 is no activity and A3 is severe activity). A fibrosis score is determined based on the extent of portal fibrosis and the degree of septation and is reported as a score of F0 to F4 (where F0 is no fibrosis and F4 is cirrhosis) (113).

Although liver biopsy is considered the gold standard, this status is being called into question, as this methodology has many limitations and challenges. Because

TABLE 2 Tests for evaluation of hepatic fibrosis[a]

Test	Availability	Formula	Advantage(s)	Disadvantage(s)
Liver biopsy	Hospital/surgical centers; limited availability in resource-limited settings	Pathological tissue evaluation	Gold standard	High cost, invasive/risk of complications, painful/patient disinterest, sampling error due to heterogeneous tissue, requires expert proceduralist/pathologist
APRI	Anywhere where basic laboratory tests are done	[(AST/ULN)/Plt] × 100	Cheap, not proprietary, helpful for ruling in F3–F4	Low scores do not exclude advanced fibrosis, suboptimal for patients with CD4 counts of <250
FIB-4	Anywhere where basic laboratory tests are done	(Age × AST)/(Plt × $\sqrt{}$ALT)	Cheap, not proprietary, low values have high NPV for F3–F4, high values have high specificity for F3–F4, validated for patients with CD4 counts of <250	Difficult to classify patients with values in the mid-range
FibroTest/FibroSure	Laboratory send-out; FibroTest in the European Union and FibroSure in the US	Formula is proprietary; components include α-2-macroglobulin, haptoglobin, gamma globulin, apolipoprotein A1, GGT, total bilirubin, age, sex	Useful for distinguishing between significant fibrosis and mild fibrosis	More expensive than APRI and FIB-4, proprietary formula
HepaScore	Laboratory send-out	Formula is proprietary; components include α-2-macroglobulin, GGT, bilirubin, age, sex		
Transient elastography	Only where machines are purchased; limited access currently	Measures shear-wave velocity; a 50-MHz wave is passed into the liver from a small transducer and is then converted into a liver stiffness score associated with stage of fibrosis	Noninvasive, immediate results, low values have high NPV for F3–F4, high values effectively rule in F3–F4, higher values are associated with increased risk of complications	Requires an expensive machine; not possible for patients with ascites or narrow intercostal space; difficult for patients with morbid obesity; confounded by elevated ALT levels, inflammation, cholestasis, or recent food or alcohol intake; can be operator dependent

[a]Plt, platelets.

liver biopsy is an invasive procedure, it is more costly and requires the presence of specialists to perform the procedure as well as experienced pathologists to review and score the sample. In addition, 1 to 5% of patients experience significant complications, and 1/1,000 to 1/10,000 procedures result in death (114). Because of the risks and discomforts associated with this invasive procedure, patients are often reluctant to consent.

In addition, there are many limitations to the accuracy of liver biopsy. Due to the heterogeneity of the liver, there is potential for sampling error: one study demonstrated that a single percutaneous liver biopsy missed the diagnosis of cirrhosis in 10 to 30% of cases. Even when biopsies were done on both sides of the liver, 33% had a difference of at least one stage (114). Increasing the number and size of biopsy specimens can reduce inaccuracies; however, this may also increase complication rates. Given the risks of biopsy and potential inaccuracies of this staging methodology, newer, noninvasive

staging methodologies have become available, decreasing the necessity for liver biopsy for the management of HCV infection.

Laboratory staging. Laboratory-based staging is often the most accessible and affordable method of staging. This allows staging to be integrated into the initial workup without requiring access to experienced proceduralists or tertiary care centers. This is crucial as general practitioners become increasingly involved in the management and treatment of HCV infection.

(i) APRI. The AST-to-platelet ratio index (APRI) score incorporates the aspartate aminotransferase level (AST) and the number of platelets using the following formula: {[AST/upper limit of normal (ULN)]/platelets} $\times$ 100. It was initially developed in 2003 after evaluation of HCV-infected individuals who underwent laboratory testing and subsequent liver biopsy. For evaluation of significant fibrosis, the APRI was found to have an area under the receiver operating characteristic curve (AUROC) of 0.88, and for cirrhosis, the APRI was found to have an AUROC of 0.94. Thus, the APRI is a basic test that can be very helpful in identifying patients with advanced fibrosis; however, lower scores do not sufficiently exclude the possibility of advanced fibrosis or cirrhosis (115). The APRI has been validated in patients with HIV-HCV coinfection. While the accuracy of the APRI for HIV-HCV-coinfected people with CD4 counts of $>$250 was similar to that for individuals with HCV monoinfection, the APRI was found to be suboptimal in individuals with CD4 counts of $<$250 (116). The authors of that study postulated that HIV-associated thrombocytopenia, and mechanisms of fibrosis progression associated with HIV but not reflected in these markers, may account for the poor predictive value of this test in HIV-infected individuals with low CD4 counts. Suboptimal APRI performance in individuals with low CD4 counts was not found to be associated with alcohol use or antiretroviral use (116).

(ii) FIB-4. The Fibrosis-4 (FIB-4) test was developed in an attempt to create a model using routine tests that would predict liver fibrosis in HIV-HCV-coinfected individuals. By using multivariate logistic regression, those researchers identified platelet counts, AST levels, and the international normalized ratio (INR) as the factors most significantly associated with fibrosis while not finding any associations for CD8, HIV RNA load, HCV RNA load, genotype, and highly active antiretroviral therapy (HAART) use. Thus, the FIB-4 test was developed to incorporate age, platelet counts, AST levels, and alanine aminotransferase (ALT) levels in the following formula: (age $\times$ AST)/(platelets $\times$ $\sqrt{}$ALT) (117). It was found that by using cutoffs of $<$1.45 for minimal fibrosis and $>$3.25 for advanced fibrosis, staging could correctly classify 87% of patients (117). Subsequent validation in HCV-monoinfected patients again demonstrated a strong negative predictive value (NPV) (94.7%) for FIB-4 values of $<$1.45 and 98.2% specificity for values of $>$3.25. Unlike the APRI, the accuracy of the FIB-4 test was similar regardless of CD4 counts (116).

FIB-4 is a useful test for accurately ruling in advanced fibrosis for patients with values of $>$3.25 and ruling out advanced fibrosis for patients with values of $<$1.45. However, patients with FIB-4 values of between 1.45 and 3.25 remain difficult to classify and will likely require further testing to discriminate the stage of fibrosis.

(iii) FibroTest/FibroSure and HepaScore. FibroTest (Europe)/FibroSure (United States) and HepaScore are commercially available, proprietary tests that calculate fibrosis based on indirect serum markers that reflect alterations in hepatic function. FibroTest/FibroSure incorporates α-2-macroglobulin, haptoglobin, gamma globulin, apolipoprotein A1, gamma-glutamyl transferase (GGT), and total bilirubin levels as well as age and sex. HepaScore combines α-2-macroglobulin, GGT, and bilirubin levels as well as age and sex. The score ranges between 0 and 0.99 and is correlated with a stage of fibrosis. Both FibroTest/FibroSure and HepaScore have been validated against liver biopsy specimens (118, 119). FibroTest was found to be concordant with FIB-4 results for values outside a range of 1.45 to 3.25. In most clinical trials, a FibroTest score of $>$0.75, in addition to an FIB-4 value of $>$3.25, is considered sufficient to diagnose cirrhosis.

Transient elastography. Transient elastography (TE) is a noninvasive procedure that requires the use of a TE machine (often called FibroScan) and takes only a few minutes

to perform. TE measures shear-wave velocity: a 50-MHz wave is passed into the liver from a small transducer and is then converted into a liver stiffness score associated with fibrosis staging (120). Scores are reported in kilopascals and generally range between 0 and 70 kPa. In a meta-analysis of 50 studies evaluating the efficacy of TE, F4 fibrosis (score of >12.5 kPa), synonymous with cirrhosis, was diagnosed with an AUROC of 0.94 (121). In addition, scores of <7.3 kPa have been found to have excellent NPVs, effectively ruling out the presence of significant fibrosis (122). Overall, this technique is best at discerning individuals with F0 to F1, indicating early-stage fibrosis, from those with F4 fibrosis. In addition to identifying the stage of fibrosis, an increased TE score has been associated with the occurrence of severe complications, including bleeding, primary liver cancer, and hepatic insufficiency (123).

TE measures a volume of liver stiffness that is 100× greater than the size of a liver biopsy specimen and therefore is felt to be less impacted by heterogeneous liver tissue (114). However, TE cannot be performed in patients with ascites or narrow intercostal spaces, and measurements can be difficult in individuals with morbid obesity. Furthermore, values can be confounded by elevated ALT levels that are >2 times the ULN, inflammation, cholestasis, or recent food intake or alcohol use (124).

Decompensated Cirrhosis

The most reliable diagnosis of decompensated cirrhosis is made by clinical identification of jaundice, upper gastrointestinal bleeding, abdominal ascites, or hepatic encephalopathy. However, laboratory values can also help estimate the severity of cirrhosis.

The Child-Turcotte classification was developed in 1964 as a means of predicting risk in patients undergoing shunt surgery for portal hypertension and became a tool for assessing the severity of cirrhosis and the prognosis for cirrhotic patients. In 1972, the score was modified as the Child-Turcotte-Pugh (CTP) score, which assigns points for encephalopathy, ascites, bilirubin, albumin, and either prothrombin time or INR in order to calculate a score ranging from 5 to 15 (113). In clinical trials of DAA treatment in cases of compensated cirrhosis, inclusion was limited to individuals with a CTP score of 5 to 6 (consistent with class A cirrhosis) (4, 5), while a score of 7 to 9 indicates CTP class B (moderate hepatic impairment), and a score of 10 to 15 indicates CTP class C (severe hepatic impairment). In clinical trials of DAAs and according to AASLD-IDSA guidelines, a score of 7 or above is considered to be indicative of decompensated cirrhosis (21, 125). These individuals should be treated with regimens specified for those with decompensated cirrhosis and should be referred to specialty care, ideally at a liver transplant facility. AASLD-IDSA guidelines specifically recommend that patients with decompensated cirrhosis should not receive treatment with regimens containing NS3 protease inhibitors (PTV, SMV, or GZR), which are often metabolized hepatically, due to increased drug exposure and a lack of safety data (68).

Recommendations

Overall, we recommend that all individuals with HCV infection receive baseline staging within a year of initiation of treatment. Optimal staging would include concordance between two different modalities, such as transient elastography and FibroSure/HepaScore or FIB-4 (126, 127). For individuals with early-stage fibrosis who achieve SVR, we do not recommend ongoing restaging after HCV cure, unless the individual has other risk factors for progression of liver fibrosis. However, individuals with advanced fibrosis should continue to receive routine follow-up care for annual laboratory monitoring for decompensation, imaging (either ultrasound or magnetic resonance imaging [MRI]) for HCC surveillance every 6 months, and variceal screening with upper endoscopy as needed. Studies are ongoing to evaluate if SVR after DAA treatment results in regression of fibrosis and a decreased risk of complications; however, at present, we must err on the side of caution and continue recommended screening for all patients who were ever diagnosed with cirrhosis.

Safety Monitoring

During treatment with combination DAA regimens, patients should be monitored, both clinically and by laboratory testing, for patient safety. For all patients on DAA-based therapies, it is currently recommended that laboratory testing, including complete blood count (CBC), creatinine level and calculated glomerular filtration rate, and hepatic function panel, be conducted at week 4 of therapy and repeated as clinically indicated (68). Patients who have a >10-fold elevation in the ALT level over the baseline or any elevation of the ALT level in association with clinical symptoms, including nausea, vomiting, jaundice, or weakness, or increased bilirubin levels, alkaline phosphatase levels, or INRs should stop therapy immediately, whereas asymptomatic increases of ALT levels of <10-fold should be monitored closely at 2-week intervals, and therapy should be discontinued if levels remain elevated. Patients with compensated cirrhosis receiving PrOD should also have additional liver function testing at week 2 of therapy, and therapy should be discontinued if patients develop clinical or laboratory signs of decompensation (128). During clinical trials with EBR-GZR, with and without RBV, ~1% of study subjects experienced elevations of ALT levels of at least 5-fold, generally after week 8 of therapy. Because of this, the FDA recommends that patients receiving EBR-GZR have an assessment of liver function at week 8, as well as at week 12 for those receiving 16 weeks of therapy (91).

When RBV is used in combination with DAAs, CBCs should be monitored after 2 weeks of therapy, and the RBV dose should be adjusted if hemoglobin levels fall more than 2 g/dl, or RBV should be discontinued entirely if hemoglobin level falls below 8.5 g/dl (129).

CONCLUSION

DAA therapies have been greatly successful in the treatment and cure of chronic HCV infection. While all patients benefit from these highly effective and well-tolerated regimens, patients with HIV coinfection and/or advanced liver disease, at risk for rapid progression and hepatic decompensation, are likely to have the most immediate benefit. At present, laboratory testing, particularly HCV genotyping and fibrosis staging, remains vital for the selection of the most appropriate antiviral regimen and treatment duration for each patient. The high cost of new combination DAA HCV drugs has been widely discussed, as has the cost-effectiveness of all-oral HCV therapies (130); the costs of the laboratory testing required for the selection of therapy and therapeutic monitoring are not insignificant, but some tests have the potential to lower the costs of therapy by streamlining the number of medications, duration of therapy, and required therapeutic monitoring. Furthermore, the complexity of interpreting the results of host and viral genetic testing may discourage nonspecialists from engaging in the treatment of hepatitis C. This is of concern, as the current number of patients with chronic hepatitis C exceeds the capacity of trained providers. We anticipate that pangenotypic DAA regimens, including the first such regimen, SOF-VEL, which was recently approved by the FDA, combined with future innovations to simplify the clinical laboratory data required for pre- and on-treatment evaluation will lead to expanded access and treatment options for patients with chronic HCV infection. The tools currently available will remain important for stratification of risk and staging of fibrosis, but also, as more patients are treated for and cured of HCV infection, questions remain about how they should be monitored following SVR, especially those patients at risk for complications of advanced fibrosis and the development of hepatocellular carcinoma. Prospective measurements of hepatic function and fibrosis will enable providers to offer patients more information about the long-term benefits, in terms of prognosis and outcomes, of eradicating this chronic viral infection.

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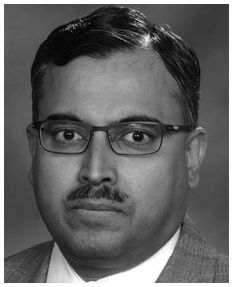

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