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Overview of hepatitis C infection, molecular biology, and new treatment

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ABSTRACT

The World Health Organization estimates that 71 million people worldwide have chronic hepatitis C viral infection. A major challenge is overall lack of public awareness of hepatitis C, particularly among infected people of their infection status. Chronic hepatitis C infection is associated with advanced liver disease, is the main cause of hepatocellular carcinoma and causes many extra-hepatic manifestations. The existence of seven viral genotypes complicates targeting of treatment. Recent years have seen the approval of many direct acting antivirals targeted at hepatitis C virus non-structural proteins. These have revolutionized therapy as they allow achievement of extremely high sustained virologic responses. Of great significance is the development of pan-genotypic drug combinations, including the NS3/4A-NS5A inhibitor combinations sofosbuvir–velpatasvir and glecaprevir–pibrentasvir. However, resistance-associated mutations can result in failure of these treatments in a small number of patients. This, combined with the high costs of treatment, highlights the importance of continued research into effective anti-hepatitis C therapies, for example aimed at viral entry. Recent developments include identification of the potential of low-cost anti-histamines for repurposing as inhibitors of hepatitis C viral entry. In this review we focus on molecular biology of hepatitis C virus, and the new developments in hepatitis C treatment.

Introduction

Hepatitis C is caused by infection with the hepatitis C virus (HCV). 60–80% of patients with acute hepatitis C develop the chronic form when the virus overcomes host innate and adaptive immune defenses [1–4]. HCV can be classified into seven confirmed genotypes and 67 subtypes [5]. These genotypes lead to differential prognosis of hepatitis C disease and influence antiviral therapy selection [6,7].

Public awareness of HCV is low, despite World Health Organization (WHO) estimates of 71 million people worldwide having chronic HCV infection [8,9]. Many people with chronic HCV are unaware of their infection, as they are often asymptomatic for a prolonged period and have not been tested for HCV [8,10,11]. In the National Health and Nutrition Examination Survey (NHANES) in the United States between 2001 and 2008, 393 of 30,140 participants were currently or previously infected with HCV. Of the 170 who could be subsequently followed up, only 49.7% were previously aware of their HCV infection status [12]. Estimates in Europe suggest that only between 10% and 40% of HCV-positive individuals are aware of their infection status [13,14].

HCV infection is one of the leading causes of chronic liver disease, is the main cause of hepatocellular carcinoma (HCC) and is the major indicator for liver transplantation in Western countries [7,13,15,16]. Additionally, chronic HCV infection is associated with many extrahepatic manifestations (EHMs) [17–21]. A predominantly Th1 immune response occurs in chronic HCV infection and

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EHMs, with CXCL9, -10 and -11 mediated recruitment of inflammatory infiltrates also impacting on liver damage and liver cirrhosis [22,23].

The American Association for the Study of Liver Diseases (AASDL) provides comprehensive and regularly updated guidance on risk factors, testing, evaluating, and monitoring HCV infection, and on new developments in treatment [7]. HCV testing is variable between countries, particularly in low- and middle-income countries (LMICs) [24]. The aim of the WHO Global Health Sector Strategy is testing of 90% and treatment of 80% of people with HBV and HCV by 2030 [25].

In this review, we focus on molecular biology and up-to-date developments in HCV therapy, including a consideration of resistant HCV variants.

Prevalence of hepatitis C virus genotypes: worldwide, Middle East, and Saudi Arabia

According to the most recent WHO and Polaris Observatory updates, there were 1.75 million new cases of HCV infection in 2015 [26,27]. The highest chronic infection prevalence (2.3%) was in the WHO Eastern Mediterranean region, including the Middle East, followed by the European region (1.5%) and the African region (1%) [26]. The lowest prevalence was in the South-east Asia region (0.5%) [26].

There are seven known HCV genotypes, GT1, GT2, GT3, GT4, GT5, GT6 and GT7, and 67 confirmed subtypes [5,28]. A recent systematic review of data from 138 countries suggests that GT1 is the most prevalent genotype globally at 49.1%, followed by GT 3 at 17.9%, GT4 at 16.8%, GT2 at 11.0%, and GT5 and 6 at <5% between them [29]. However, relative prevalence varies (Table 1). For example, while GT1 predominates in North America, Latin America and Europe, GT4 predominates in Africa and the Middle East [29] (Table 1). GT7 has only been reported once, in four immigrants from the Democratic Republic of Congo in Canada [30]. In the Middle East, the HCV GT4 is more prevalent in Egypt, Iraq, Saudi Arabia, and Syria, while GT1a and 1b are predominant in Turkey, Israel, Cyprus, and Iran [31].

Infection by all genotypes depends on the action of a variety of structural and non-structural viral proteins interacting with host proteins. Genotype dictates responsiveness to therapy and variability in genotype frequency complicates the task of designing effective HCV vaccines with potential universal use.

Molecular biology of the hepatitis C virus

The HCV is an enveloped, single-stranded RNA virus of the genus *Hepacivirus*, and the family *Flaviviridae*. The central 9.6 kb genome encodes a large polyprotein precursor subject to proteolytic cleavage by viral and host proteases to generate both structural (core, E1 and E2) and non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. The single-stranded RNA genome is encapsulated in an icosahedral protein coat, within a lipid envelope in which the highly glycosylated E1 and E2 glycoproteins are embedded [32,33].

E1 and E2 are implicated in viral entry. Entry involves a series of steps mediated by entry factors including scavenger receptor BI (SR-BI), tetraspanin CD81, and the tight junction proteins claudin-1 (CLDN1) and occludin (OCLN). E2 interacts with both SR-B1 and CD81 in viral entry and is the main target of many neutralizing antibodies [34–39]. E1 is likely to be involved in the viral fusion phase of entry, modulation of E2 CD81 receptor binding, selection of CLDN1 as the viral entry factor and in cross-talk with viral genomic RNA during morphogenesis [40–42].

NS3 to NS5B are sufficient *in vitro* for viral replication, while NS3, NS4B, and NS5A are also involved in particle production and secretion [43,44]. Both NS2 and NS3 have serine protease activity which cleaves precursor NS proteins. NS3 also has NTPase/helicase activity which unwinds the viral RNA. Cleavage at the NS2/NS3 junction, catalyzed by NS2 autoprotease, releases the NS3 protease activity [45]. After NS2/NS3 cleavage, the conserved part of the NS3 serine protease domain becomes available to promote NS5A hyperphosphorylation [45]. NS4A is an NS3 cofactor, and together they are responsible for cleavage of the remaining NS proteins. NS4B associates with the membrane of the host cell endoplasmic reticulum (ER) and interacts with host cellular proteins to induce the formation of a membranous web HCV replication compartment containing the replicase proteins NS3, NS4A, NS4B, NS5A and NS5B, and viral RNA [46]. NS4B interacts with multiple host proteins including the Ras superfamily Rab proteins Rab5 and Rab7, [47], the calcium- and phospholipid-binding protein annexin A2 [48], the ER lipid droplet protein and putative methyltransferase AAM-B [49], and the prolactin regulatory element binding (PREB), a vesicle budding regulator [46]. The second amphipathic N-terminal helix (AH2) of NS4B is important in the lipid bilayer remodeling that accompanies the membranous web structure formation [50]. Activity of NS5A in interaction with core protein and production of infectious virus is dependent on its phosphorylation/hyperphosphorylation by kinases such as casein kinase I-α (CKI-α) [51] and promoted by free NS3 following cleavage from N2 [45]. NS5A-core protein is transported along with the replication complex to lipid droplets via microtubules. The NS5A-core protein is then transported to the plasma membrane to be incorporated into the core of low-density virus particles [52]. NS5A interacts *in vitro* with apolipoprotein E (ApoE) in a process mediated by the Golgi protein 73 (GP73) to facilitate HCV particle secretion [53] and with cyclophilin A (CypA) for promotion of viral RNA replication [54]. It also interacts *in vitro* with annexin A2 in a ternary complex with viral RNA, which may be key to HCV infection progression, DAA treatment effectiveness, and development of HCC [55]. NS5B is an RNA-dependent RNA polymerase (RdRp) [56]. Recent *in vitro* studies suggest that NS5B may mediate viral replication and HCV-induced cancer progression by inhibition of the NORE1A (RASSF5) tumour suppressor [57]. Yeast two-hybrid assay and cell culture studies also suggest that the host CYP4F12 protein, a member of the cytochrome P450 superfamily, may also interact directly with NS5B to promote HCV replication [58]. On the other hand, the tumor suppressor Fbw7, a component of E3 ubiquitin ligase, can target NS5B for ubiquitination via interaction with a Cdc4 phosphodegron (CPD) site on NS5B, decreasing NS5B expression and reducing viral replication [59]. Treatment of HCV has improved in recent years due to development of several direct-acting antivirals (DAAs) which mainly target NS proteins and hence viral replication [60].

Recent developments in HCV DAA treatment

The main aim of HCV therapy is to achieve SVR, which is defined as undetectable levels of HCV RNA twelve (SVR12) or 24 weeks (SVR24) after treatment ends [61]. The relatively modest efficacy, resistance of some genotypes to treatment, and side-effects of pegIFN/RBV prompted the search for new HCV drugs and the development of direct-acting antiviral (DAA) agents [62–66].

Table 2 shows a summary of the currently FDA-approved DAAs, along with their NS targets and genotype effectiveness, and the clinical trial series on which approval was based. The two NS3/4A protease inhibitors, boceprevir, and telaprevir, have recently been withdrawn in the United States, superseded by newer, more effective drugs [7,61,67–69].

Sofosbuvir (SOF) (NS5B polymerase inhibitor) and simeprevir (SMV) (viral NS3/4A protease inhibitor) were approved in 2013 [70,71]. However, SOF-SMV is not currently recommended except

Table 1
Geographical distribution of HCV genotypes.

HCV genotypes relative prevalence	GT1	GT2	GT3	GT4	GT5	GT6
North America	74.5 %	10.2%	10.6%	1.7%	0.1%	–
Europe	64.4%	5.5%	25.5%	37.0%	0.1%	0.1%
Asia	46.6%	18.6%	22.4%	1.0%	0.1%	7.0%
Africa	26.3%	23.7%	6.3%	28.2%	12.2%	–
Middle East/North Africa	27.3%	0.8%	6.3%	65.3%	0.3%	–
Australasia	55.0%	6.5%	36.0%	1.2%	–	1.3%

Data taken from Petruzziello et al. [29].

for GT1 treatment in areas where no other IFN-free treatment is available, as it gives inferior SVR12 results compared to newer treatments [61]. SOF is included on the WHO List of Essential Medicines and is part of other FDA-approved combination therapies. These include with ledipasvir (LDV), an NS5A inhibitor. SOF–LDV was approved by the FDA under the brand name Harvoni in 2014 for GT1 HCV treatment [72]. SOF–LDV treatment in the presence or absence of RBV has been shown to be effective in patients infected with GT1, GT3 or GT6 HCV, including previous therapy non-responders in the SIRIUS trial, but its effectiveness can be compromised by resistance-associated variants (RAVs) associated with NS5A gene mutations [73,74].

A major breakthrough in DAA therapy for chronic HCV infection has been the recent development of pan-genotypic drug combinations. SOF in combination with the NS5A inhibitor velpatasvir (VEL) is a pan-genotypic front-line treatment for HCV [28,61]. SOF–VEL was approved by the FDA under the brand name Epclusa in 2016 and is the current standard of care, with or without RBV, for chronically infected HCV patients of any genotype without cirrhosis or with compensated cirrhosis, or for patients with decompensated cirrhosis in combination with RBV [7,28,61,75]. Approval was granted on the basis of the results of the ASTRAL-1-4 phase III clinical trials (Table 3) [76–78]. The SOF–VEL ASTRAL-5 trial is ongoing, on treatment-naïve and treatment-experienced patients co-infected with HIV. Initial results from 106 patients indicate SVR12 achievement in 95% of patients [79].

In 2017, SOF–VEL in combination with the NS3/4A protease inhibitor voxilaprevir (VOX) was also approved by the FDA under the brand name Vosevi, for pan-genotypic treatment in situations where the patient had been previously treated with an NS5A inhibitor-containing regimen, and for GT1a and GT3 if the patient had been previously treated with a SOF-containing regimen in the absence of NS5A inhibitor [80,81]. Approval was granted following the POLARIS-1-4 phase III clinical trials (Table 3) [82,83].

SOF is also a component of a treatment for chronic GT3 HCV infection, in combination with daclatasvir (DCV), an NS5A inhibitor which was initially approved by the FDA in 2015 under the brand name Daklinza for GT3 therapy. The combination of SOF–DCV was approved in 2016 for GT3 and GT1 treatment on the basis of the phase 3 ALLY-3 trial, in which 101 treatment-naive and 51 treatment-experienced GT3 patients were treated with SOF-DCV for twelve weeks and achieved 90% and 86% SVR12 respectively [84,85]. RBV was added to the treatment regime in the ALLY-3+ trial in which 50 GT3 patients with cirrhosis (n = 36) or advanced fibrosis (n = 14) were randomised to twelve or to sixteen weeks of SOF-DCV-RBV; SVR12 was achieved in 90% and 92% of patients respectively [84,86].

Another recently approved pan-genotypic treatment is the combination of NS3/4A protease inhibitor (Glecaprevir; GLE) and NS5A inhibitor (Pibrentasvir; PIB), approved by the FDA in 2017 under the brand name Mavyret, and Maviret by the European Commission [87,88]. A major advantage of GLE–PIB is its suitability for use in patients with renal impairment, and it has also been shown to be effective in cases where previous DAAs have failed [89,90]. The drug was approved on the basis of numerous phase II and III trials [89,91–97]. The results of a pooled analysis of the findings of these trials are shown in Table 4, for the optimised GLE–PIB dose of 300 mg and 120 mg respectively [98]. Other GLE–PIB trials include MAGELLAN-2, which showed SVR12 of 98% in 100 patients with chronic HCV types GT1-6 who have received a liver or kidney transplant [99]. The CERTAIN-1 and -2 trials established that GLE–PIB for eight weeks was as efficacious as twelve weeks of ombitasvir/paritaprevir/ritonavir (OBV/PTV/r) in GT1 patients with or without the NS5A Y93H RAV variant, and as efficacious as twelve weeks of SOF–RBV in GT2 patients [100,101]. Trials of GLE–PIB are ongoing, including for paediatric patients with chronic HCV infection, adult GT1 patients previously treated with SOF plus NS5A inhibitor, adult GT5 and GT6 patients, and treatment-naïve GT1, GT2, and GT4-6 patients with compensated cirrhosis [91]. The currently active MAGELLAN-3 trial is designed to evaluate efficacy and safety of GLE–PIB in combination with SOF–RBV in HCV patients who did not respond to treatment in a previous AbbVie GLE–PIB clinical study (ClinicalTrials.gov Identifier: NCT02939989). EXPEDITION-5 is designed to evaluate the efficacy and safety of GLE–PIB in chronic GT1-6 HCV adult patients with renal impairment (ClinicalTrials.gov Identifier: NCT03069365) [102,103].

Other more recently approved treatments are targeted at specific genotypes. These include the combination of ombitasvir (OBV) (NS5A inhibitor)/paritaprevir (PTV) (NS3/4A inhibitor)/ritonavir (r) (CYP3A4 enzyme inhibitor). This was approved by the FDA in 2014, packaged with dasabuvir (NS5B inhibitor), under the brand name Viekira Pak (Incivo in Europe) for chronic HCV GT1 treatment. [104]. OBV/PTV/r was further approved in 2015 under the brand name Technivie, without dasabuvir, for treatment of chronic HCV GT4 infection, which is responsible for 13% of global HCV infections and for which IFN-based treatments have poor success rates and tolerability [105]. Approvals were based on the basis of the PEARL-1 and AGATE-1 trials, which included 255 patients chronically infected with HCV GT4, both cirrhosis-free (PEARL-1) or with compensated cirrhosis (AGATE-1), both treatment-naive and IFN or pegIFN/RBV treatment-experienced [105–107]. Another phase III trial, AGATE-2, was carried out in Egypt on 160 chronic HCV GT4 patients; results indicated that a shorter twelve-week treatment regimen for OBV/PTV/r with RBV is as effective as longer treatment periods [108].

Another recently approved GT1/GT4-targeting treatment is Elbasvir (EBR; NS5A inhibitor)/grazoprevir (GZR; NS3/4a inhibitor). EBR/GZR was approved by the FDA in 2016 under the brand name Zepatier based on multiple clinical trials. These confirmed the efficacy of EBR/GZR for GT1, GT4 and GT6, including patients previously treated with pegIFN/RBV plus DAA (boceprevir, telaprevir or simprevir), but not GT2 or GT5 patients (Table 2) [109–116]. Efficacy of EBR/GZR has been shown in patients co-infected with HIV (C-EDGE-COINFECTION phase III study) [111]; and in GT1 patients with stage 4 or 5 CKD (C-SURFER study) [116]. For the C-SALVAGE trial, 79 patients with chronic HCV GT1 infection who had not previously achieved SVR12 with were assigned to EBR/GZR + RBV for twelve weeks, with 96.2% SVR12 overall [114]. Importantly, this included 75% of patients with baseline NS5A RAVs (n = 8) and 66.7% of patients with baseline NS3 and NS5A RAVs (n = 6); in a 24-week

Table 2
Summary of FDA-approved HCV direct-acting antivirals.

DAA	FDA approval	Target NS	HCV gentoype	Additional information
Generic name: Boceprevir	2011	NS3 inhibitor (used in combination with peg-IFNa/RBV)	GT1	Voluntarily withdrawn January 2015, due to superiority of newer DAAs, such as ledipasvir/sofosbuvir.
Brand name: Victrelis				Approval based on SPRINT-2 clinical trial
Company: Merck Sharp & Dohme Corp. [7,61,67–69]				
Generic name: Telaprevir	2011	NS3/4A serine protease inhibitor (used in combination with peg-IFNa/RBV)	GT1	Discontinuation of production announced in August 2014 due to falling demand for the drug caused by competition from newer hepatitis C treatments.
Brand name: Incivek				Approval based on ADVANCE, ILLUMINATE and REALIZE trials
Company: Vertex Pharmaceuticals [7,61,67–69]				
Generic name: Simeprevir	2013	NS3/4A serine protease inhibitor (used in combination with peg-IFNa/RBV)	GT1	Approval based on QUEST 1, QUEST 2, PROMISE trials.
Brand name: Olysio			(GT4 indicated off-label)	
Company: Janssen Pharmaceuticals[70,71]				
Generic name: Sofosbuvir	2013	Nucleotide analog NS5B inhibitor	GT1, GT2, GT3, GT4, GT5, GT6 (in combination with other drugs)	On the WHO List of Essential Medicines.
Brand name: Sovaldi				Recommended in combination with velpatasvir for all genotypes — cure rate > 90%.
Company: Gilead Sciences [70,71]				Approval based on NEUTRINO, FISSION, POSITRON, VALENCE trials.
Generic name: Sofosbuvir and Ledipasvir	2014	NS5B inhibitor (Sofosbuvir) and NS5A inhibitor (Ledipasvir)	GT1	Approval based on ION-1, ION-2, ION-3 trials.
Brand name: Harvoni				
Company: Gilead Sciences [72–74]				
Generic name: Ombitasvir, Paritaprevir and Ritonavir tablets co-packaged with Dasabuvir tablets Brand name: Viekira Pak (Incivo- Europe)	2014	NS5A inhibitor (Ombitasvir); NS3/4A protease inhibitor (Paritaprevir), a CYP3A inhibitor (Ritonavir) NS5B inhibitor (Dasabuvir)	GT1	Approval based on SAPPHIRE-I and II, PEARL-II, III and IV, TURQUOISE II trials.
Company: AbbVie Inc. [104–108]				
Generic name: Ombitasvir, Paritaprevir and Ritonavir	2015	NS5A inhibitor (Ombitasvir); NS3/4A protease inhibitor (Paritaprevir), a CYP3A inhibitor (Ritonavir) (used in combination with RBV)	GT4	Indicated for GT4 chronic HCV infection without cirrhosis.
Brand name: Technivie				Approval based on PEARL-I, AGATE-I.
Company: AbbVie Inc. [104–108]				Warning issued for risk of HBV reactivation in co-infected patients and hepatic decompensation and or failure in cirrhosis patients.
Generic name: Daclatasvir	2015/2016	NS5A replication complex inhibitor	GT3	On the WHO List of Essential Medicines. Other medications used in combination include sofosbuvir, RBV, and IFN, depending on the virus type and presence/absence of cirrhosis.
Brand name: Daklinza			GT1 (when used in combination with sofosbuvir ± RBV)	Approval in combination with sofosbuvir based on ALLY-3 and ALLY-3+ trials.
Company: Bristol-Myers Squibb Company [84–86]				
Generic name: Elbasvir and Grazoprevir	2016	NS5A inhibitor (Elbasvir), and NS3/4A protease inhibitor (Grazoprevir) (used with or without RBV)	GT1, GT4	Approval based on C-EDGE TN, C-EDGE COINFECTION, C-SCAPE, C-EDGE TE, C-SALVAGE, C-SURFER trials.
Brand name: Zepatier				Warning issued for risk of HBV reactivation in co-infected patients.
Company: Merck Sharp Dohme [109–116]				
Generic name: Sofosbuvir and Velpatasvir	2016	NS5B inhibitor (Sofosbuvir) and NS5A inhibitor (Velpatasvir)	GT1, GT2, GT3, GT4, GT5, GT6	Indicated in patients without cirrhosis or with compensated cirrhosis, or with decompensated cirrhosis in combination with RBV.
Brand name: Epclusa				Approval based on phase 3 ASTRAL-1,-2, -3 and -4 trials.

Table 2 (*Continued*)

DAA	FDA approval	Target NS	HCV gentoype	Additional information
Company: Gilead [76–78]				
Generic name: Sofosbuvir and Velpatasvir and Voxilaprevir.	2017	NS5B inhibitor (Sofosbuvir) and NS5A inhibitor (Velpatasvir) and NS3/4A protease inhibitor (Voxilaprevir)	GT1, GT2, GT3, GT4, GT5, GT6 (patients previously treated with NS5A inhibitor-containing regimen)	Approval based on phase 3 POLARIS-1, -2, -3 and -4 studies.
Brand name: Vosevi			GT1a, GT3 (patients previously treated with a sofosbuvir-containing regimen without NS5A inhibitor)	Warning issued for risk of HBV reactivation in co-infected patients.
Company: Gilead Sciences [80–83]				
Generic name: Glecaprevir and Pibrentasvir	2017	NS3/4A protease inhibitor (Glecaprevir) and NS5A inhibitor (Pibrentasvir)	GT1, GT2, GT3, GT4, GT5, GT6.	Warning issued for risk of HBV reactivation in co-infected patients.
Brand name: Mavyret			GT1 (patients previously treated with a regimen containing NS5A inhibitor or NS3/4A protease inhibitor, but not both)	Indicated for patients without cirrhosis or with compensated cirrhosis.
Company: AbbieVie [87–97]				Approval based on ENDURANCE-1, -2, -3, -4, SURVEYOR-1, -2, EXPEDITION-1, 4 and MAGELLAN-1 trials.

Table 3
Overview of SVR12 rates in ASTRAL and POLARIS clinical trials of sofosbuvir–velpatasvir–voxilaprevir.

Trial	Treatment	SVR12 (% of patients)						
		Genotype						
		1a	1b	2	3	4	5	6
ASTRAL-1 (with or w/o compensated cirrhosis) [76–78]	SOF-VEL (n = 624)	98%	99%	100%	–	100%	97%	99%
	Placebo (n = 116)							
ASTRAL-2 and -3 (with or w/o compensated cirrhosis) [76–78]	SOF-VEL (n=411)	–	–	99%	95%	–	–	–
	SOF + RBV (n = 407)			94%	80%			
ASTRAL-4 (with decompensated cirrhosis) [76–78]	SOF–VEL (12 weeks) (n = 89)	88%		100%	50%	100%	100%	100%
	SOF–VEL + RBV (12 weeks) (n = 89)	96%		100%	85%	100%	100%	100%
	SOF–VEL (24 weeks) (n = 89)	92%		100%[a]	50%	100%	100%	100%
POLARIS-1 (with or w/o compensated cirrhosis) (Prior NS5A inhibitor treatment) [82,83]	SOF–VEL–VOX (n = 264)	96%		100%	100%	95%	91%	100%
	Placebo (n = 150)							
POLARIS -2 and -3 (with or w/o compensated cirrhosis; NOT GT3 with cirrhosis) (No prior DAA) [82,83]	SOF–VEL–VOX (8 weeks) (n = 611)	92%		97%	97%	96–99%	94%	94%
	SOF–VEL (12 weeks) (n = 549)	99%		97%	100%	97%	98%	
POLARIS -4 (with or w/o compensated cirrhosis) (Prior DAA treatment EXCLUDING NS5A inhibitor treatment) (No prior PEG, RBV +PI) [82,83]	SOF–VEL–VOX (12 weeks) (n = 182)	98%		96%	100%	96%	100%	–
	SOF–VEL (12 weeks) (n = 151)	89%		95%	97%	85%		

SOF–VEL: sofosbuvir–velpatasvir; SOF–VEL–VOX: sofosbuvir–velpatasvir–voxilaprevir; DAA: direct-acting antiviral.
[a] One exception; patient who died of advanced liver failure. -: genotype not included in trial.

Table 4
Pooled analysis of SVR12 rates in SURVEYOR-I, -II, EXPEDITION-4 and ENDURANCE-1-4 clinical trials of glecaprevir–pibrentasvir.

Duration	SVR12					
GLE–PIB [89,91–97]	Genotype					
	GT1	GT2	GT3	GT4	GT5	GT6
8 weeks	99% (383/387)	98% (193/197)	95%[a] (177/186)	93% (43/46)	1.0% (2/2)	90% (9/10)
12 weeks	1.0% (400/401)	99% (232/234)	95% (302/319)	99% (111/112)	1.0% (28/28)	1.0% (31/31)

GLE–PIB: glecaprevir–pibrentasvir.
[a] No treatment-experienced patients.

follow-up of C-SALVAGE, SVR24 was achieved in 76 of 79 patients (96.2%) (1115). For the three patients who experienced virologic failure, baseline NS3 and/or NS5A RAVs reappeared at relapse, with NS5A RAVs persisting throughout the entire 24-week follow-up period [115].

DAA development has revolutionized HCV treatment worldwide. For example, in Egypt the high prevalence of HCV infection, almost entirely associated with GT4, has necessitated the implementation of the world's largest HCV national treatment program, spearheaded by the Egyptian National Committee for the Control of Viral Hepatitis (NCCVH) [117–120]. The NCCVH was founded in 2006 and they established the first specialized centres for treatment of viral hepatitis in 2007, since when more than 54 centres have been established [117]. Treatment was initially reliant on pegIFN/RBV, however following the introduction of SOF in 2014 and the development of further DAAs, the NCCVH negotiated deals with several pharmaceutical companies which ensured availability of DAAs to Egyptian HCV patients at a greatly reduced cost [117–120]. Local production of generic versions of DAAs also contributed to a major increase in program uptake. While SOF–RBV therapy has achieved an SVR12 rate of 82.7%, success for other therapies has been much higher at between 94% and 98% [118,120]. The

Table 5
Summary of viral resistance mutations.

	Gene		
Therapy	NS3	NS5B	NS5A
Simeprevir (SMV) (viral NS3/4A protease inhibitor)	R155K D168V Q80 K Genotype GT1a and 1b [128]		
Sofosbuvir (SOF) (NS5B polymerase inhibitor)		S282T L159F (15% SOF-treated patients with virologic failure; GT1) L320F V321A (5% SOF-treated patients with virologic failure; GT1) [129–131]	
Pibrentasvir (PIB) (NS5A inhibitor)			Hotspot mutation site 93: low resistance (cell culture) [132] Amino acid 32 deletion + hotspot mutations (aminoacids 28, 30, 31, 93): high resistance (cell culture) [132] L31V GT6: resistant (and emergence of L28S in NS5A) (cell culture) [137] GT3: A30K+L31M or A30K+Y93M- resistance (BOSON clinical trial) [139]
Velpatasvir (VEL) (NS5A inhibitor)			Hotspot mutation sites 28, 93: relatively high resistance [132,136] GT6a: NS5A RAS L31V (cell culture) GT4: SOF–VEL effective regardless of NS5A RAS [138] GT3: A30K+L31M or A30K+Y93M- resistance (BOSON clinical trial) [139]
Ledipasvir (LDV) (NS5A inhibitor)			Hotspot mutations (aminoacids 28, 30, 31, 93): high resistance (cell culture) [132] Y93H/N/C, Q30R/H and M28T/A: ≥1000-fold L31M (100–1000 fold) [134,135]
Daclatasvir (DCV) (NS5A inhibitor)			Hotspot mutations (aminoacids 28, 30, 31, 93): high resistance (cell culture) [132] Hotspot mutation site 93 reduces sensitivity in vitro [133]
Ombitasvir (NS5A inhibitor), Paritaprevir (NS3/4A inhibitor) and Ritonavir (CYP3A4 enzyme inhibitor) (OBV/PTV/r)	D168A/V (PTV); GT1b [145]		Y93H (OBV) GT1b [145]
Elbasvir (NS5A inhibitor) and Grazoprevir (NS3/4A protease inhibitor (EBR/GZR)			Hotspot mutations (aminoacids 28, 30, 31, 93): high resistance in small number GT1a patients USA [142] Q30H/R and Y93C/H/N (single mutations) and Q30H+Y93H and Q30R+Y93H double mutations (Spain) [143]

main limitation facing the program is the low rate of post-treatment follow-up [120].

The impact of RAVs is a major challenge in DAA treatment effectiveness for a small proportion of patients, particularly for those who fail NS5A inhibitor-based treatments.

RAVs and viral resistance to new HCV treatment protocols

The low fidelity of the HCV polymerase and the high rate of viral replication trigger a high volume of mutations in the HCV genome, resulting in novel HCV strains or quasispecies which are DAA-resistant *in vitro* and *in vivo* due to mutations in the NS3, NS5A and NS5B genes [121–123]. Important resistance-associated substitutions (RAS) vary with genotype.

Mutations associated with resistance to DAAs are summarized in Table 5. Boceprevir and telaprevir resistance was associated with several mutations in the NS3 and NS5B genes [124]. Development of resistant viral strains was also observed for treatment with SOF, SMV, DCV, and OBV/PTV/r [125–128]. The S282T, L159F, L320F and V321A mutations in the NS5B polymerase are common in SOF-resistant HCV strains [129–131]. L159F and V321A are most relevant in terms of emergence during treatment, including in 15% and 5% respectively of patients with virologic failure (Table 5) [129–131]. However, addition of LDV reduced both L159F and V321A to 2% of patients with virologic failure [130,131]. L159F at baseline in GT1 patients does not appear to have any impact on SOF/LDV treatment outcomes [131].

With the recent rise of combination therapies which include NS5A inhibitors, including LDV, VEL, DCV, PIB, OBV and EBR, the impact of NS5A mutation-associated RAVs has become increasingly prominent. NS5A resistance-associated substitution (RAS) hotspots have been identified at amino acid positions 28, 30, 31, and 93 in domain 1 of the 447 amino acid NS5A, which contains a zinc-binding domain (Table 5) [132,133]. A recent *in vitro* study showed that when GT 1-7 HCV recombinants containing either wild-type or RAS NS5A were expressed in cell culture, PIB had uniformly high anti-HCV activity against all genotypes [132], with the exception of low resistance conferred by RAS at position 93 and significant resistance due to some RAS combinations along with amino acid 32 deletion [132]. RAS at hotspot positions 28 and 93 resulted in high resistance levels to the other NS5A inhibitors, although VEL was more effective against position 30 and 31 RAS variants than other NS5A inhibitors.

In vitro studies suggest that LDV and DCV exert their inhibitory effect via direct NS5A binding and that mutation in domain 1, for example at the RAS hotspot 93, reduces binding affinity [133]. In the phase II and III clinical trials for LDV–SOF, the rare cases of virologic failure were mainly due to RASs in NS5A that resulted in increased resistance to LDV, mainly in GT1 subtype patients, especially GT1a [134,135]. Y93H/N/C, Q30R/H and M28T/A mutations caused ≥1000-fold reduced LDV susceptibility (Table 5) [134].

Decreased LDV susceptibility due to NS5A mutation at RAS hotspots can often be overcome by extension of SOF/LDV treatment duration [134,135].

In an overview of the ASTRAL-1-3, ASTRAL-5 and POLARIS-2-3 studies on the pangenotypic SOF-VEL treatment, 28% of 1778 had NS5A RASs at baseline [136]. Of the twenty patients who experienced virologic failure, seventeen had single NS5A class resistance (VEL) but not SOF resistance [136].

Cell culture-based propagation of HCV GT6a strains from patients has shown that compared to GT1A, GT6a recombinants are equally sensitive to the NS5A inhibitors DCL, EBR, VEL, PIB, and SOF but less sensitive to LDV, OBV, and DCV [137]. Long-term SOF–VEL treatment of HCV GT6a-infected cells cleared the infection but if single inhibitor treatments were used, the infection escaped due to emergence of the NS5A RAS L31V (VEL-resistant), or S282T in NS5B (SOF-resistant). Cells infected with a recombinant HCV GT6a containing RAS NS5A-L31V also allowed resistance to PIB, with further emergence of the L28S NS5A RAS [137].

Some GT subtypes are linked with more frequent occurrence of NS5A RASs. With respect to GT4, SOF–VEL and LDV–SOF are both highly efficacious for all subtypes regardless of NS5A RASs particularly for the common 4a/d subtypes, and the 4c/f/k/l/m/n/o/p/r/t subtypes [138]. In one study on 454 patients with chronic HCV GT4 infection, the rare GT4b resulted in a higher resistance to LDV, associated with presence of 2-4 NS5A RASs, some including Y93H, while two patients with the GT4r subtype who experienced virologic relapse had rare triple RASs [138]. For GT3, a study on 496 GT3 patients enrolled in the BOSON clinical trial showed that there is a high level of RASs in HCV GT3, with the hotspot RASs A30K and Y93H most common [139]. Notably, paired hotspot RASs, namely A30K + L31M and A30K + Y93H, were present in eighteen patients (nine each), with the A30K + L31M pair being present in all GT3b and GT3g subtype samples; these paired RASs resulted in resistance to VEL, as well as to DCV, EBR and PIB (Table 5) [139].

PIB was found to be active against the hotspot RASs associated with resistance to other NS5A inhibitors in a recent study on in HCV chimeric replicons containing GT 1-6 NS5As, including at positions 28, 30, 31, and 93, consistent with the results of cell culture studies [132,140]. Consistent with this robustness of PIB in the presence of NS5A RASs, an overview of the results of the CERTAIN-1 and -2 trials on GLE–PIB treatment showed that common NS3 or NS5A baseline polymorphisms had no effect on treatment outcomes in GT1- and GT2-infected patients [141]. However, the resistance to PIB and other NS5A inhibitors induced by paired NS5A RASs, particularly in some GT3 subtypes, should not be discounted [139].

Impact of RASs may be more significant for EBR/GZR treatment. A recent overview of resistance data from selected phase II/III clinical trials submitted to the FDA showed that EBR/GZR for twelve weeks failed in a small percentage of GT1a patients, associated with baseline hotspot RASs at NS5A positions M28, Q30, L31, or Y93 (Table 5) [142]. Most patients in whom treatment failed also acquired RASs in NS3 and NS5A during treatment [142]. Increasing the treatment duration to sixteen weeks with addition of RBV may increase efficacy in the GT1a patients with NS5A polymorphisms. By contrast, for 26 patients chronically infected with GT4a or 4d HCV who had NS5A polymorphisms, all achieved SVR12 on twelve-week EBR/GZR treatment [142]. This suggests that RAS testing may be beneficial in GT1a patients for whom EBR/GZR treatment is considered. However, prevalence may vary between countries. A recent study from Spain on 617 patients infected by HCV GT1 showed that NS5A RASs potentially conferring EBR resistance, most commonly Y93C/H/N and Q30E/H/R, were only present in 6.2% of patients [143]. Of these, increased EBR resistance arose only due to Q30 H/R (n = 7) and Y93C/H/N (n = 8) as single mutations and Q30H + Y93H (n = 4) and Q30R + Y93H (n = 2) as double mutations [143]. Thus, there was a lower prevalence of RASs conferring EBR resistance in Spanish patients compared to the USA. Meta-analysis suggests that NS3 RASs do not have any impact on efficacy of EBR/GZR treatment in HCV GT1 patients [144].

For OBV/PTV/r treatment, studies on HCV GT1b Japanese patients showed a virologic failure rate of 3% (13/436) across the M12-536 and GIFT-I studies [145]. The most common NS5A baseline mutation associated with OBV resistance was Y93H (Table 5) [145]. For patients with virologic failure, NS3 D168A/V and NS5A Y93H, alone or in combination with other variants, were the most common RASs. As treatment progressed, levels of NS3 RAVs reduced while NS5A RAVs persisted. For 204 GT2-infected Japanese patients in the M12-536 phase 2 study (OBV/PTV/r twelve weeks) and phase 3 GIFT II study (OBV/PTV/r and RBV 16 weeks), baseline OBV-resistance-conferring NS5A polymorphisms were not observed to have any significant effect on treatment outcome [146]. Meanwhile an analysis of HCV NS3/4A, NS5A, and NS5B nucleotide sequences in 132 patients from different countries with the GT4 subtypes 4a, 4b, 4c, 4d, 4f, 4g/4k, and 4o suggested that baseline RASs also had no significant effect on treatment outcome [147].

In terms of impact on retreatment, a recent review showed that baseline NS5A RASs may have an inhibitory effect on SVR percentages for retreatment with LDV-SOF but not for EBR/GZR + SOF + RBV, OBV/PTV/r + DSV + SOF, SOF/VEL + RBV, SOF/VEL/VOX or GLE/PIB [148]. Given the rapid pace of HCV DAA development, forthcoming clinical guidelines for chronic HCV treatment may suggest that resistance testing for retreatment has less clinical utility than formerly as there are so many retreatment options that appear to be unaffected by the presence of RASs [148].

Targeting viral entry

While DAAs targeting viral replication have obviously revolutionized chronic HCV treatment, emergence of RAVs can result in resistance. Cost of DAAs may also be prohibitive, especially in LMICs [149,150]. Research is ongoing on other possible therapeutic options for HCV, most notably targeting of viral entry.

The developments for inhibiting viral entry as a novel antiviral strategy against HCV were comprehensively reviewed in 2016, including inhibitors, antagonists, peptides and antibodies targeting both host and viral proteins including E2-CD81 and E2-SR-B1 interactions, ApoE, E1CD81-CLDN1 co-receptor complex formation, viral trafficking and internalization, or viral fusion via interference with E1 or targeting of lipids and membrane fluidity [151]. These types of therapeutic agents may be of particular relevance for prevention of reinfection of grafts in liver transplant patients with HCV infection [152]. For example, the polyclonal anti-HCV immunoglobulin Civacir®, which targets E1/E2, has recently completed a phase III trial (NCT01804829) to test its safety and efficacy in preventing HCV recurrence in 80 liver transplant patients. Civacir® has been shown in HCV pseudoparticles and cell culture-derived models to be broadly neutralizing of HCV variants of all the main genotypes isolated from patients before and after liver transplantation [153]. SR-B1 is the target of another drug, ITX5061, which has completed a phase I clinical trial (NCT01292824) in 23 liver transplant patients with HCV infection [154]. In GT1-infected patients, treatment with ITX5061 before and after transplant and daily for one week thereafter induced a sustained reduction in HCV RNA levels when compared control patients [154]. The epidermal growth factor receptor (EGFR) inhibitor Erlotinib (Tarceva™), which is already an approved cancer drug with a well characterized safety profile in humans, is in phase I/II proof of concept trials (NCT01835938); it targets both EGFR, which is a HCV entry host co-factor, and also blocks viral entry mediated by SR-BI-dependent high-density lipoprotein (HDL) [155]. In cell culture models, inhibition by ertolinib can be overcome by mutations of the E2 envelope

protein tryptophan residue 420 (W420), which is essential for E2 binding to both SR-B1 and CD81 [155].

Recent developments in identification of promising viral entry inhibitors include studies on plant-based agents. Both rutin, a flavonoid derived from *Prunus domestica* (plum), and the dehydrorotenoid boeravinone H derived from the herb *Boerhavia diffusa*, have been shown to inhibit HCV and HCV-like particles (HCV-LPs) binding and entry to hepatoma cells *in vitro* and *ex vivo* [156,157]. Meanwhile (-)-epigallocatechin-3-gallate (EGCG), a polyphenol found in green tea, both reduces HCV viral entry and induces innate immune responses in human hepatocytes in cell culture [158]. The challenge of developing affordable novel HCV inhibitors was addressed in a study in which natural product compound libraries were screened and micrococcin P1, a macrocyclic peptide antibiotic, was identified as a pan-genotypic HCV entry and spread inhibitor with activity against SOF-resistant strains and a synergistic effect with selected DAAs [159]. The potential for repurposing clinically approved ion channel inhibitors and related anti-histamines in HCV therapy has been recently comprehensively reviewed and again presents a possible means of addressing affordability [160]. Both first- and second-generation anti-histamines have been identified as potential anti-HCV entry therapies, while Ca^{2+} ion channels inhibitors of the phenothiazine, diphenyl-piperazine, diphenyl-piperidine, and thioxanthene chemical scaffold types are already in clinical use as neuroleptics in clinics to treat migraines or psychiatric diseases [160]. Proposed mechanisms of action of these drugs in HCV inhibition include direct effects on lipids and cholesterol and hence viral membrane fusion and/or direct binding to viral or cellular transmembrane domains involved in fusion [160–162].The diphenylmethylpiperazine flunarizine, a licensed migraine treatment, inhibits HCV *in vitro* entry into human primary liver cells, as well as *in vivo* into humanized mouse hepatocytes in a HCV cell entry reporter mouse model, with preference for the GT2 genotype [161]. Resistance to flunarizine mapped to both E1 and E2 envelope proteins. The over-the-counter anti-histamine chlorcyclizine HCl (CCZ) has been shown *in vitro* to inhibit HCV infection of human hepatoma cells and primary human hepatocytes via viral entry inhibition and to act synergistically with DAAs including telaprevir, boceprevir, sofosbuvir and daclatasvir [163]. *In vivo*, in chimeric mice engrafted with primary human hepatocytes, CCZ significantly reduced infection with GT1b and 2a, most potently against GT2 [163]. CCZ derivatives have been assessed *in vitro* [164]. These lead compounds are achiral, in contrast to CCZ, and are therefore easier to synthesize, and also have lower anti-histamine activity, reducing the possibility of off-target effects [164]. Pharmokinetic profiling of six lead compounds led to compound 3, an alkyl analogue at the piperazine nitrogen, for further *in vivo* analysis in chimeric mice engrafted with primary human hepatocytes and infected with HCV GT1b. Compound 3 time-dependently reduced HCV viral load over four weeks, with no apparent toxicity, in three out of four mice tested [164]. These CCZ derivatives are promising candidates for development as HCV therapies, for example in combination with existing DAA therapies, with potential benefits for affordability and efficacy of treatment over reduced treatment durations [164].

Conclusions

Overall lack of public awareness of HCV is a major challenge, given the association of chronic HCV infection with advanced liver disease, HCC and many extra-hepatic manifestations. The emergence of DAAs, and the recent approvals of a variety of combination treatments capable of targeting all HCV genotypes, has revolutionized treatment of HCV and made very high SVRs achievable. A cohort of patients remain in whom resistance to these treatments is an issue, for example due to pre-existing RAVs or emergence of RASs during treatment. Often, this can be overcome due to retreatment options that appear to be unaffected by the presence of RASs [148]. However, development of alternative or complementary therapies, such as viral entry inhibitors, is an important line of research, especially given the cost barriers associated with prolonged DAA treatment and retreatment, for example in LMICs and in some at-risk groups [156–160]. These types of therapies may be of particular importance for the treatment of liver transplant patients and for development of potential HCV vaccines. The potential of repurposing of existing drugs such as some anti-histamines is of particular note in the context of cost.

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Informed consent

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