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Review

Critical challenges and emerging opportunities in hepatitis C virus research in an era of potent antiviral therapy: Considerations for scientists and funding agencies

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ABSTRACT

The development and clinical implementation of direct-acting antivirals (DAAs) has revolutionized the treatment of chronic hepatitis C. Infection with any hepatitis C virus (HCV) genotype can now be eliminated in more than 95% of patients with short courses of all-oral, well-tolerated drugs, even in those with advanced liver disease and liver transplant recipients. DAAs have proven so successful that some now consider HCV amenable to eradication, and continued research on the virus of little remaining medical relevance. However, given 400,000 HCV-related deaths annually important challenges remain, including identifying those who are infected, providing access to treatment and reducing its costs. Moreover, HCV infection rarely induces sterilizing immunity, and those who have been cured with DAAs remain at risk for reinfection. Thus, it is very unlikely that global eradication and elimination of the cancer risk associated with HCV infection can be achieved without a vaccine, yet research in that direction receives little attention. Further, over the past two decades HCV research has spearheaded numerous fundamental discoveries in the fields of molecular and cell biology, immunology and microbiology. It will continue to do so, given the unique opportunities afforded by the reagents and knowledge base that have been generated in the development and clinical application of DAAs. Considering these critical challenges and new opportunities, we conclude that funding for HCV research must be sustained.

1. Introduction – the public health imperative

Infections with hepatitis C virus (HCV) are a major cause of acute and especially chronic liver disease. The World Health Organization (WHO) estimates that at least 71 million people are persistently infected with HCV and are at risk for serious liver diseases, including potentially fatal hepatic cirrhosis and hepatocellular carcinoma (HCC) (WHO, 2017). At least 400,000 people die from HCV infection annually, almost half of the one million deaths attributable to HIV/AIDS in 2016 (UN AIDS, 2016). In the U.S., HCV-related deaths have exceeded HIV-

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related deaths for well over a decade, and opioid addiction is now driving dramatic increases in new HCV infections – truly a 'syndemic'. HCV-related deaths are increasing worldwide, while HIV-related mortality is declining according to the WHO, yet there are staggering disparities in both public health and research investments aimed at controlling these viruses. One in four cases of liver cancer, the second most common cause of cancer death worldwide and accounting for about 800,000 deaths annually, results from HCV infection, making HCV one of only 7 viruses (and the only positive-strand RNA virus) known to be oncogenic in humans. Yet HCV-mediated oncogenesis has received relatively little attention from the United States National Cancer Institute and many other cancer research agencies.

The recent development of highly effective direct-acting antivirals (DAAs) that cure the vast majority of HCV infections after only 8 weeks of oral therapy represents an outstanding success of modern medicine. It started with the discovery of HCV in 1989 (Choo et al., 1989) and the generation and rapid implementation of HCV screening tests to protect the blood supply (Kuo et al., 1989), and culminated 25 years later with the approval of interferon-free therapies that eliminate the virus in > 95% of treated individuals (reviewed in (Pawlotsky et al., 2015)). These drugs are the results of sustained and collaborative efforts between industry, academia, and government funding agencies, a joint endeavor that has unfortunately fallen victim to its success. Some now consider mistakenly that HCV is a vanquished pathogen, capable of being controlled or even eradicated on a global scale solely by antiviral therapy. This general perception has found its way into some funding agencies where the imperative for support of HCV research has been lost, and applications for support are confronted with the argument that HCV is no longer clinically relevant and further research is unnecessary. Such a view is naïve and short-sighted, and overlooks several major obstacles to global control of HCV with antivirals (Fig. 1). First, a large proportion of persistent HCV infections are clinically silent, often undiagnosed, and will not be recognized by patients or practitioners until liver damage is advanced. Second, DAAs are expensive, and will likely remain out of the reach of a majority of infected persons worldwide for many years (Iyengar et al., 2016). Third, clinically-relevant antiviral resistance, now relatively uncommon, will likely increase with broader use of DAAs. Forth, protective immunity after viral clearance is most often insufficient and reinfection with HCV, in the absence of a vaccine, is all too easy following curative DAA therapy (Midgard et al., 2016). Another factor, poorly understood and discussed in greater detail below, is that eliminating HCV infection with DAAs does not eliminate the risk of developing liver cancer. Finally, in the history of mankind no infectious disease has been eradicated by antimicrobial therapy, whereas this has been proven possible by vaccination.

Apart from the continuing need to develop more effective approaches to control the spread of HCV worldwide, the two-decade search for effective DAAs has provided investigators with a unique molecular "toolbox" that offers unparalleled opportunities to make important fundamental discoveries in virology, cell biology and immunology. In addition to its real and compelling impact on human health, the continuing value of HCV as a model pathogen should not be overlooked.

In this opinion paper we summarize important challenges in public health, translational and basic science, review some key aspects of HCV and briefly discuss new research opportunities that have emerged in the HCV field. We conclude that the mission is far from over, and that reductions in support for HCV research will compromise an opportunity to eradicate HCV on a global scale, and to make important discoveries that reach far beyond hepatitis C.

2. Important public health research challenges

At a first glance the availability of highly effective antiviral drugs might make the development of novel therapies for chronic hepatitis C obsolete. However, the costs are too high for most high-prevalence countries, which are often resource limited. Although costs have been lowered due to competition in the HCV drug market or facilitated access to generic drugs in some high-prevalence countries, a global eradication will not be possible unless these drugs become widely available with no strings attached. In principle, the conditions for such a strategy are good because treatment with pan-genotypic activity, minor side effects and minimal contraindications have recently become available. This should allow provision of treatment outside of specialized primary care centers.

Another major public health challenge is to reduce under-diagnosis. In fact, it is estimated that in most countries the rate of diagnosis of HCV infection is below 50% or just unknown (Bruggmann et al., 2014; Gower et al., 2014). While this requires nation-wide hepatitis-specific action plans that have been implemented only in 18 countries according to WHO (Lazarus et al., 2013), costs for diagnosis especially to measure viremia, an important marker to monitor antiviral therapy, is a major hurdle, especially in resource-limited countries where costs for HCV RNA testing may surpass the costs for HCV treatment. Thus, cheaper diagnostic tests to measure HCV viremia or core antigen have to be implemented that are also easy to use and do not require expensive equipment or specialized training. Such tests, ideally combined with antiviral therapy would be one important step to move treatment out of

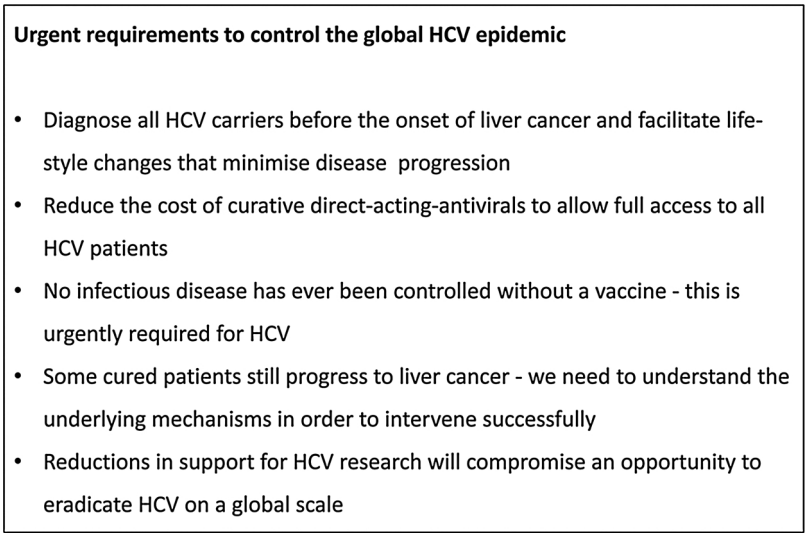

Fig. 1. Summary of the most urgent requirements to control the global HCV epidemic.

specialized clinical centers.

In parallel to intensified diagnosis of chronic hepatitis C, surveillance of incidence as well as therapy success will be required to monitor the disease burden and to make recommendations to governments and health agencies. These are required in order to set the budgetary frame to cover treatment costs, but also to monitor HCV prevalence in high-risk groups such as persons who inject drugs (PWIDs). In fact, in high- and many middle-income countries, injection drug use is a main cause of HCV transmission (Bruggmann et al., 2014). In those groups, prophylactic DAA treatment might in principle be considered, similar to a strategy pursued for HIV infection control (Cohen et al., 2011). However, such a strategy most likely will increase the risk for the selection of DAA-resistant HCV variants and will be unaffordable, at least under current circumstances.

3. Important clinical and translational research challenges

3.1. The case for a HCV vaccine

The greatest unmet medical need in the hepatitis C field is a prophylactic vaccine. In developed countries, most new HCV infections occur in PWIDs and even a partially effective vaccine would substantially lower the overall incidence of infection. Emerging data indicate that individuals who have been cured with DAAs remain susceptible to reinfection and viral persistence (Grebely et al., 2017; Midgard et al., 2016; Pineda et al., 2015; Simmons et al., 2016), a substantial risk for PWIDs that likely can be averted only by immunization. An HCV vaccine could also benefit other risk groups with high transmission rates including HCV/HIV co-infected men who have sex with men, infants born to HCV-positive mothers and healthcare workers with frequent exposure to blood and bodily fluids. Outside of North America and Western Europe, many countries experience a much higher prevalence and incidence of HCV infection where implementation of a vaccine would be expected to be highly beneficial (Strickland et al., 2008).

In spite of considerable scientific and clinical challenges (see below), effective immunization against HCV appears to be feasible. At least partial natural immunity has been demonstrated in the chimpanzee model (Bukh et al., 2008; Lanford et al., 2004; Major et al., 2002; Weiner et al., 2001) and in humans (Mehta et al., 2002) and immunity has been correlated with both virus-specific CD4+ and CD8+ T cell responses (Bowen and Walker, 2005) and neutralizing antibodies (Ball et al., 2014; Pestka et al., 2007). Direct evidence for the protective efficacy of neutralizing antibodies has been derived from the chimpanzee model (Bukh et al., 2015; Morin et al., 2012) and a chimeric mouse model populated with human liver cells (Meuleman et al., 2011). However, since the virus has an extensive glycan shield and is coated with various apolipoproteins, the development of neutralizing antibodies is delayed in infected individuals (Logvinoff et al., 2004); and when they appear they are less effective at neutralizing the infectivity of apolipoprotein-associated virus (Bankwitz et al., 2017) as compared to antibodies induced by infection with more accessible viruses like e.g., hepatitis A virus (HAV) or hepatitis B virus (HBV).

Despite these inherent challenges, a recombinant envelope glycoprotein prophylactic vaccine was shown to be sterilizing against experimental challenge with a homologous HCV strain (Choo et al., 1994) and was effective at significantly reducing the ensuing carrier state following experimental challenge with heterologous genotype 1a HCV, which is a common clade around the world (Houghton, 2011). This vaccine candidate was also shown to elicit cross-neutralizing antibodies in humans (Law et al., 2013), chimpanzees (Meunier et al., 2011) and goats (Wong et al., 2014). Other forms of recombinant envelope glycoproteins have also been shown to elicit cross-neutralizing antibodies in animals (Vietheer et al., 2017). Vaccination methods to elicit strong virus-specific cellular immune responses that have been shown to ameliorate the early acute phase of HCV infection have also been elucidated (Folgori et al., 2006; Houghton, 2011). Efforts to activate both arms of the adaptive immune response *via* vaccination are now underway. Such a vaccine eventually combined with the HBV vaccine, would be an important step towards global HCV eradication.

A major roadblock for vaccine development is the lack of an immune-competent small animal model (see below), because predictive *in vivo* models of vaccine efficacy are crucial to prioritize vaccine candidates before initiation of costly clinical development (Winer et al., 2016). This issue has only been compounded by recent changes in NIH policies that prevent further studies in chimpanzees, the only animal species other than humans that is fully permissive for HCV infection (Bukh, 2012). Therefore, further research is required to develop an immune competent and permissive small animal model that can be used for vaccine studies but also to study HCV-associated pathogenesis. The recent discovery of the Norway rat hepacivirus that can be propagated in lab strains of mice and that appears to share basic immunological features with HCV is an important step in this direction (Billerbeck et al., 2017; Trivedi et al., 2017).

The current lack of detailed knowledge concerning the correlates of immune protection is another major roadblock for devising vaccination strategies that overcome viral escape mechanisms. For instance, relatively little is known about how HCV continuously escapes antibodies. Clearly, one mechanism is the decoration of HCV particles with lipoproteins that facilitate cellular attachment and thus antibody escape (Bartenschlager et al., 2011). However, does this feature also reduce viral immunogenicity? Are there viral epitopes that are not protected by lipoproteins? How do rare broadly neutralizing antibodies evolve *in vivo*? What are productive B cell maturation pathways that govern their development? Which vaccination strategies elicit such responses? And which immune components in the cellular repertoire are important for successful recall immune responses and can the virus escape them? These are just a few important questions that merit future investigation. The recent determination of the 3D structure of the major target of neutralizing antibodies, the E2 envelope glycoprotein of HCV, at atomic resolution is an important step towards addressing these questions and should facilitate rational vaccine design (Khan et al., 2014a; Kong et al., 2013). Moreover, the large patient cohorts that were recruited during the licensing studies of DAAs and subjected to precise clinical monitoring as well as the associated biobanks provide a fantastic foundation to dissect key features of protective immunity in humans. Research taking advantage of these opportunities should guide and transform HCV vaccine development.

3.2. HCV-associated liver cancer

Liver cancer is the second leading cause of cancer-related death world-wide with increasing morbidity and mortality (Ryerson et al., 2016). Chronic hepatitis C is a major predisposing condition for liver cancer and owing to the high prevalence of chronic hepatitis C, the number of patients developing HCC will remain high (Chhatwal et al., 2016; Hoshida et al., 2014). Of note, HCC can occur even more than 10 years after successful HCV clearance with the annual post-therapy HCC incidence of ~1% being higher than cancer in other organs (Baumert et al., 2017). A reduction in HCC risk upon successful therapy has been reported in multiple retrospective studies of earlier interferon treatments. Although it is well possible that these data can be extrapolated to persons cured with DAAs, the risk of developing HCC persists especially in patients with advanced fibrosis or cirrhosis, genotype 3 infection or certain co-morbidities such as diabetes mellitus (El-Serag et al., 2016; Kanwal et al., 2017; van der Meer et al., 2012). Moreover, it is becoming clear that the decline of HCC incidence after successful therapy depends on how far liver disease had advanced at the time therapy was initiated. Indeed, patients suffering from decompensated liver disease have the highest risk to develop HCC in spite of successful HCV elimination. Future prospective studies will be needed to determine the extent with which DAA treatment reduces HCC incidence

and to define the "point-of-no-return" when persistent HCV infection has advanced to a stage where HCC will develop regardless of viral infection (Baumert et al., 2017).

It will also be essential to understand the molecular mechanisms driving HCV-related HCC. Since HCV does not integrate its genetic material into the host genome it most likely contributes to cancer development indirectly. For instance, chronic HCV infection has been shown to modulate hepatocyte gene expression associated with progression of liver disease, cancer and death (Bandiera et al., 2016). Moreover, viral proteins can disrupt signal transduction pathways that affect cell survival, proliferation, and transformation (Tu et al., 2017). Apart from these virus-dependent mechanisms, the genetic background of the host has also been shown to play a role in HCC pathogenesis, as demonstrated by the identification of specific mutations or polymorphisms that are associated with HCC development (Bandiera et al., 2016; Kumar et al., 2011). Since HCV and other HCC aetiologies share common pathways and produce similar genetic footprints (Fujimoto et al., 2016; Guichard et al., 2012; Schulze et al., 2015) chronic HCV infection may serve as model to understand hepatocarcinogenesis in general, which is of utmost importance given that tumor resection and liver transplantation are still the predominant therapeutic options. Moreover, the investigation of the molecular mechanisms of HCV-induced HCC should ultimately contribute to the development of urgently needed strategies for HCC prevention as well as treatment and identify biomarkers for predicting cancer risk.

3.3. DAA resistance

The proportion of patients with chronic hepatitis C failing DAA-based therapy is small but the absolute numbers are nevertheless important given the high prevalence of HCV infection. DAA failure is associated with the selection of viral variants harboring resistance-associated substitutions (RAS), especially in NS5A, which is a target of virtually all currently approved antiviral regimens (Pawlotsky, 2016; Sarrazin, 2016). Variants harboring these RAS are fit and persist for years. Second-generation DAA combinations can achieve high rates of sustained virological responses in this situation (Bourliere et al., 2017). However, these combinations are costly and not broadly available yet. In addition, regimens comprising protease inhibitors cannot be administered to patients with decompensated cirrhosis, limiting treatment options for patients with very advanced liver disease. Hence, DAA resistance represents a challenge; however, its dimensions are currently difficult to foresee and therefore, it will be critical to develop surveillance programs to detect and characterize resistance development against DAAs as their use will become more widespread, and for patient groups with a high risk of transmission. In fact, transmission of resistant variants has been demonstrated (Franco et al., 2014). In addition, it will be important to look at natural occurrence of RAS at a global scale (Smith et al., 2018) as it appears that some key RAS might be prevalent in specific geographical regions (Li et al., 2017). Moreover, it will be important to continue research with the aim to determine the potential of HCV for developing variants with resistance to the various DAA classes. For example, it has recently been demonstrated *in vitro* that HCV genotype 3 and 6 strains can readily develop highly fit variants with resistance to the nucleotide analog sofosbuvir, a key component of one of the two pangenotypic DAA regimens (Ramirez et al., 2016), arguing that RAS in one drug target severely compromises the effect of these regimens. These results and the experience with resistance development against HIV-specific antivirals collected over the past 25 years (van Zyl et al., 2018) should keep the HCV community alert, in spite of the high effectiveness of current drug regimens.

3.4. Risk of HBV reactivation under DAA-based therapy

An increased risk of reactivating HBV infection has been observed in HBV-HCV co-infected individuals undergoing treatment with DAAs (Holmes et al., 2017; Perrillo, 2017). At least in cell culture both viruses can co-replicate in the same cell without discernable differences between co- and mono-infected cells (Bellecave et al., 2009). This argues that the inverse relationship between HBV and HCV replication levels observed in co-infected patients might be linked to antiviral immune responses. Interestingly, interference with both HAV and HBV infection by prior HCV infection has been documented in experimentally infected chimpanzees (Bradley et al., 1983). The underlying mechanisms are unknown, but can now be elucidated by studying the immune reconstitution that occurs in patients undergoing DAA-based HCV elimination (see below).

4. New opportunities and impact on other fields

4.1. Understanding viral persistence by in vivo studies of DAA-treated patients

Considering its high rate of persistence it is obvious that HCV must employ highly effective strategies to escape innate and adaptive immune responses (Heim and Thimme, 2014; Thimme et al., 2012). Previous HCV research in this area has led to numerous important discoveries with implications far beyond the HCV field. For example, HCV served as an important tool to elucidate signaling pathways that induce interferon responses, and to identify and characterize MAVS (mitochondrial antiviral-signaling protein) that is cleaved by the viral NS3-4A protease (Cheng et al., 2006; Loo et al., 2006; Meylan et al., 2005). Moreover, HCV-related studies have spearheaded research on interferon lambda, an important antiviral cytokine discovered only more recently (Boisvert and Shoukry, 2016). Clinical studies revealed distinct polymorphisms in the interferon lambda gene that correlate with the ability to eliminate HCV during acute infection or by treatment with interferon-based regimens (reviewed in (Heim et al., 2016)). These studies laid the groundwork for dissecting the organization of the interferon-lambda gene locus and the identification of lambda interferon variants with different biological activities.

In terms of adaptive immunity, the role a of vigorous and multispecific CD4+ and CD8+ T cell responses for the control of HCV infection, the strong association between certain class I and class II HLA-alleles and spontaneous HCV elimination as well as the impact of CD4+ and CD8+ T cell depletion on the course of HCV infection *in vivo* have been described (Grakoui et al., 2003; Heim and Thimme, 2014; Park and Rehermann, 2014; Shoukry et al., 2003). However, in chronic hepatitis C T cell exhaustion predominates, driven most likely by ongoing antigen recognition in the absence of viral escape (reviewed in (Heim and Thimme, 2014)). Exhausted T cells are ineffective and unable to secrete antiviral cytokines or proliferate and kill infected hepatocytes. Moreover, they up-regulate several inhibitory receptors, such as PD-1 (Wherry and Kurachi, 2015), but PD-1 blockade alone does not restore the function of strongly inhibited HCV-specific CD8+ T cells in the liver and targeting of additional inhibitory receptors, such as CTLA-4 is required to increase T cell function (Nakamoto et al., 2008). While the underlying mechanisms are still poorly understood, the availability of highly efficient DAA-based therapies has made it possible to monitor (partial) immune reconstitution in patients as HCV antigen load declines and interferon-stimulated genes are downregulated (Meissner et al., 2014). For example, a normalization of NK cell phenotype and function (Serti et al., 2015) and a partial restoration of HCV-specific CD8+ T cell response has been observed (Martin et al., 2014; Wieland et al., 2017). Since HCV reinfection is possible it remains unclear whether $CD8^+$ T cells that persist after DAA-mediated HCV clearance are protective.

HCV has also evolved strategies to escape humoral immunity. Some of the underlying principles are similar to HIV such as high antigen variability and shielding targets for neutralizing antibodies within the viral envelope glycoprotein structures (reviewed in (Ball et al., 2014)). However, novel strategies have been discovered by studying HCV,

notably the protection of the virus particle through its association with components of the low density lipoprotein machinery (Bartenschlager et al., 2011). Other strategies are glycan shielding of the viral envelope proteins (Helle et al., 2007) and the use of the N-terminal region of E2 that 'protects' other functionally important, but less mutable epitopes (Bankwitz et al., 2010; Prentoe et al., 2016). It has also been suggested that HCV may evade neutralization by direct cell-to-cell spread (Timpe et al., 2008). While these properties are challenging hurdles for the development of an effective prophylactic vaccine, these examples illustrate the wealth of knowledge that has been gained from studies on HCV.

In this respect, the revolution in antiviral treatment of chronic hepatitis C has transformed HCV infection into an important and unique immunological model to study the impact of chronic inflammation, accompanied by persistent antigen stimulation, and its clearance on innate and adaptive immunity. Important questions can now be addressed. For example, what are the mechanisms governing the differential responsiveness of immune cell subsets? Is the partial restoration of the cellular immune response after antiviral therapy due to the normalization of cytokines such as interferon or the removal of viral antigens? Does the early induction of the innate response suppress HCV viremia to below the threshold required for efficient induction of adaptive immunity? Are vigorous T cell responses linked to the single nucleotide polymorphisms near the IL-28B gene encoding type III IFNs that predict spontaneous resolution of HCV infection? It is very likely that future discoveries made with HCV will continue to have profound impact on understanding of viral persistence in general.

4.2. Understanding the mode-of-action of effective antiviral drugs and resistance against them

Numerous highly effective antiviral drugs targeting HCV or host factors used by this virus have been developed and these antivirals provide unique opportunities to conduct drug-related mechanism-of-action studies as well as studies on the nature of antiviral resistance by using HCV as a model. For instance replicon-based screens identified a novel class of DAAs targeting a non-enzymatic factor of the HCV replicase, i.e. NS5A (Gao et al., 2010). This protein that lacks known enzymatic activity has been regarded as non-druggable, but turned out to be an extremely valid target. In fact, NS5A inhibitors have an unprecedented high potency and became a cornerstone in all recently approved combination therapies. Recent studies have shown that NS5A inhibitors block the HCV replication cycle at several steps (Berger et al., 2014; McGivern et al., 2014) most likely because NS5A can form multiple protein-protein and protein-RNA complexes with each of them exerting a different function (RNA replication, virion assembly, counteracting antiviral responses) (Bartenschlager et al., 2013). Since many viruses utilize multi-functional proteins the clinical success of HCV NS5A inhibitors should encourage future development of antivirals targeting such proteins without caring too much whether or not they are enzymes.

Proof of concept studies demonstrated the capacity of HCV to escape the most potent drugs, including DAAs (e.g. (Ramirez et al., 2016)), host-targeted agents (Badillo et al., 2017; Li et al., 2011) and human monoclonal antibodies (Keck et al., 2009; Velazquez-Moctezuma et al., 2017). Taking advantage of the large arsenal of drugs targeting pretty much every step of the HCV replication cycle, we are now in the position to dissect the molecular mechanisms of viral resistance by using HCV as tool. Such studies involve virology, evolution, chemistry, and structural biology. Research in this direction might provide much needed basic knowledge on resistance mechanisms and identify classes of molecules with high resistance barriers. For instance, the resolution of the crystal structures of NS5B ternary complexes in primed initiation and elongation states not only provided a model about the structural changes involved in HCV RNA replication (initiation and elongation), but also explained the extremely high resistance barrier to the nucleotidic inhibitor sofosbuvir (Appleby et al., 2015).

4.3. HCV as a tool to study fundamental principles in virology and cell biology

Studies of the HCV replication cycle and its interaction with the host cell have provided important insights into fundamental aspects of molecular and cellular biology. Since an exhaustive list of major contributions is beyond the scope of this opinion paper here we give a few arbitrarily selected examples. Readers who are more interested in this topic are referred to excellent recent reviews (Alvisi et al., 2011; Johnson et al., 2017; Lindenbach and Rice, 2013; Paul et al., 2014; Scheel and Rice, 2013).

Viral entry pathways: HCV entry is mediated by multiple host cell factors including CD81, claudin 1, occludin and scavenger receptor BI acting in a concerted manner (Zeisel et al., 2013). Interestingly, several kinases such as the epidermal growth factor receptor regulate cell entry by modulating host cell-receptor associations or trafficking. Deciphering the molecular mechanisms of cell entry paved the way for antiviral therapies targeting entry factors – an approach that is being pursued for other viruses such as HBV, hepatitis D virus (HDV), HIV or dengue virus (Zeisel et al., 2015) and shown for HCV to be not only prophylactic, but also having the potential to be curative (Mailly et al., 2015). HCV cell entry is also highly relevant for the pathogenesis of virus-induced liver disease and cancer since relevant signaling pathways become activated during the early steps of the viral replication cycle. Thus, the study of HCV - host cell interactions during cell entry such as virus-triggered signaling will yield important contributions to our understanding of the pathogenesis of liver disease and cancer in general.

Lipid metabolism: Like all positive-strand RNA viruses, HCV hijacks and expands cellular endomembrane systems to generate its membranous replication organelle. This requires multiple reprogramming steps of cellular lipid biosynthesis and transport pathways. By these mechanisms, HCV shapes the membrane composition of the infected cell in general and the membranes associated with viral replication in particular. This includes enhanced transcription of genes involved in lipid synthesis, resulting in steatohepatitis in a genotype-specific manner (Syed et al., 2010), activation of enzymatic activities of lipid generating enzymes (e.g. phosphatidylinositol 4-kinase IIIα, PI4KA) (Berger et al., 2011; Reiss et al., 2011) and exploitation of lipid transfer proteins such as OSBP (oxysterol-binding protein 1), FAPP2 (Golgi-associated four-phosphate adaptor protein 2), Sec14L2 and NPC1 (Niemann-Pick disease, type C1) (Khan et al., 2014b; Saeed et al., 2015; Stoeck et al., 2017; Wang et al., 2014) to generate a lipid environment conducive for viral replication.

By using HCV as tool, studies of virus - host lipid interaction not only have strongly increased our understanding of mechanisms governing HCV replication, but also have important implications for future research in virology, pathobiology of lipid-associated diseases, including liver cancer, and cell biology. The following examples shall illustrate that:

- First, HCV genotype-specific induction of liver steatosis in patients is well established and has been linked to the HCV core protein, thus providing an excellent model to study the fundamental mechanisms underlying steatohepatitis and its contribution to HCC.
- Second, studies of HCV replication in cell culture have largely relied on cell culture adapted variants, whereas propagation of patient isolates has been almost impossible. Recent studies have identified two independent mechanisms that allow replication of patient HCV isolates in cell culture, both linked to lipid metabolism (Harak et al., 2016; Saeed et al., 2015). While this will enable experiments using serum-derived virus and non-adapted primary isolates, e.g., to test for phenotypic drug resistance, the underlying mechanisms remain to be determined.

- Third, some HCV isolates are highly sensitive to lipid peroxidation while others are not, yet the molecular determinants have not been identified (Yamane et al., 2014). However, such virus pairs are excellent tools to understand the induction of lipid peroxidation by virus-induced oxidative stress, the deleterious effects of peroxidized lipids on protein function and the action of scavengers such as tocopherol (vitamin E) and its cellular transporters (e.g. Sec14L2) in counteracting lipid peroxidation.
- Forth, HCV dramatically re-shapes the lipid composition of ER membranes, by enhancing the enzymatic activity of PI4KA to generate lipid gradients that allow the recruitment and delivery of cholesterol and sphingolipids by lipid transfer proteins (Paul et al., 2014). In this regard, HCV is an attractive tool to study the cellular functions of these lipids as well as the contribution of alterations in lipid homeostasis to the pathogenesis of liver disease. In addition, a deeper understanding of the interactions of viral proteins with distinct lipids or lipid metabolizing enzymes will also provide valuable tools to engineer the lipid composition of intracellular membranes in living cells.

miRNAs in viral replication and pathogenesis: miRNAs are well established as negative regulators either blocking the translation of a targeted mRNA or inducing its degradation. However, studies conducted with HCV showed for the first time that miRNAs can act positively, i.e. enhance the function of an RNA, i.e. in this case the HCV genome (Jopling et al., 2005). The miRNA in question is miR-122, a tumor suppressor that is highly abundant in liver cells and regulates the expression of many lipogenic genes. It was shown that miR-122 binds to the HCV genome to stabilize the viral RNA, affect its translation and enhance its amplification (Masaki et al., 2015; Sarnow and Sagan, 2016). Moreover, in the infected cell HCV sequesters miR-122, thus causing a derepression of target genes that might contribute to liver cancer development (Luna et al., 2015). Of note, subsequent studies conducted for other viruses revealed an analogous stimulatory role for other miRNAs in viral replication (Scheel et al., 2016). Moreover, the discovery of miR-122 as a critical host dependency factor for HCV lead to the development of antagomirs sequestering miR-122 and making it unavailable for HCV. This approach turned out to be highly efficient in animal models and clinical studies revealing high tolerability of antagomirs with no induction of drug resistance in patients and high antiviral potency (Janssen et al., 2013; Sarnow and Sagan, 2016). This example nicely illustrates a new research direction seeded by studies on HCV that led to the discovery of fundamental new principles of miRNA biology, as well as the development of a new therapeutic paradigm.

4.4. RNA virus evolution and the origin of HCV

RNA viruses are characterized by high mutation rates allowing for rapid adaptation to the host and escape from antiviral drugs as well as antiviral immunity triggered by natural infection or vaccination. HCV is extremely heterogeneous and exists as at least 7 major genotypes with more than 85 subtypes. The frequent mutations create a population of variant RNA genomes that circulates as quasispecies in individual patients. The mutations are distributed throughout the genome, but frequently are linked on an individual virus genome and function in concert. This linkage can be important for efficient adaptation e.g. to drug treatment or antiviral immune responses, the latter being a major obstacle in vaccine development (Bukh, 2016; Simmonds et al., 2017). In this context, HCV samples collected longitudinally from infected individuals or from various HCV culture models offer unique opportunities to perform basic studies of virus evolution. Such studies profit a lot from novel sequencing techniques offering the opportunity to analyze entire HCV genomes and accurately link mutations. For example, a recent study elegantly evaluated how host and virus genomics are linked functionally (Ansari et al., 2017). Thus, HCV with its amazing heterogeneity offers an excellent opportunity to map in detail the multitude of mechanisms permitting or restricting the evolution of RNA viruses.

Until recently, HCV was considered the sole major member of the genus *Hepacivirus*. However, it has become clear that there are multiple related viruses circulating in numerous mammalian species, including horses, cattle, bats and rodents (Scheel et al., 2015). These related viruses provide new opportunities to dissect unique features of HCV and common strategies of the hepaciviral replication strategy. At the same time these comparisons raise important questions regarding the evolution of these viruses and their routes of transmission. For example, flaviviruses belong to the same family as HCV and are frequently transmitted *via* insect vectors, yet for hepaciviruses there is no evidence for this mode of transmission. Might the existence of HCV relatives in nature, predict a risk of future zoonoses? Is there cross-species transmission of hepaciviruses? What are the barriers that prevent this? Recent studies indicate that rodent hepaciviruses can be employed to help dissect mechanisms of liver tropic viral pathogenesis and immunity (Billerbeck et al., 2017; Trivedi et al., 2017). It will be of interest to determine whether they can also provide tractable models for exploring hepacivirus vaccine platforms. Clearly the discovery of novel HCV relatives in various species raises important basic scientific questions and opportunities with a high potential for translational impact.

4.5. Boosting research towards curative HBV therapy

The high rate of HCV elimination with DAAs has ignited hopes of achieving similar curative therapies also for the two other hepatotropic viruses causing chronic liver disease and cancer, HBV and HDV. While the molecular biology of these viruses and their replication cycles are completely different, they have in common an exquisite hepatotropism and liver disease biology. Research for curative HBV and HDV therapies is benefiting from knowledge and concepts obtained in HCV research on several levels. These include the establishment of robust and high-throughput cell culture and animal model systems to uncover and validate curative approaches – a key milestone for the development HCV-specific DAAs (Winer et al., 2016). Indeed, humanized liver chimeric mice are a common model for all three viruses and the humanization of mice by transgenic expression of respective human dependency factors, a concept pioneered for HCV (Dorner et al., 2013), is currently pursued for HBV and HDV. Other examples are the discovery of host dependency factors as targets for antiviral therapy by using functional genomics approaches. This is nicely illustrated by entry inhibitors such as antibodies targeting claudin 1 that turned out to eliminate HCV infection, at least in animal models (Mailly et al., 2015), and peptidic inhibitors of NTCP, the entry molecule utilized by HBV and HDV (Yan et al., 2012), that show great promise in ongoing clinical trials to treat persistent HDV infection (Blank et al., 2016).

Apart from that, infections with HBV and HCV share several key features of pathogenesis, such as the induction of an inflammatory milieu in the liver that is characterized, amongst others, by the production of cytokines such as lymphotoxin-alpha/beta (Haybaeck et al., 2009). Moreover, infections with both viruses are major risk factors for the development of liver cancer. While in both cases chronic inflammation induced by viral infection is regarded as a major driver of hepato-carcinogenesis, the more direct (viral) contributions appear to differ between these two viruses (Tu et al., 2017). Thus, comparative studies with both viruses should reveal important fundamental insights how viral infection directly or indirectly promotes tumor development.

5. Conclusion

Along with the prevention of post-transfusion HCV infection, the high level of HCV elimination achieved with all-oral interferon-free antiviral therapy in chronically infected patients is a remarkable example of the power and synergy of basic, translational and clinical research. Although this is rightfully regarded as a triumph of modern

medicine, a number of critical challenges remain that will substantially impede efforts to control this virus using DAA-based therapies only. Insufficient access to antiviral therapy, the urgent need to detect HCV carriers, the lack of immunity against reinfection, the uncertainty about the magnitude of viral resistance development and the continued risk for liver cancer, especially in elderly patients with advanced liver disease, regardless of HCV elimination, are prime challenges that need to be overcome. In addition, HCV-related research has played an important role in making groundbreaking discoveries in basic and translational science and will continue to do so if adequate funding is provided. It is surprising that support for basic HCV research is nowadays often considered of little relevance because of a perceived lack of medical need, yet studies with other model pathogens are well accepted. It is inevitable that priorities for HCV research need some readjustment and the availability of DAAs can be considered as starting point of new research directions. The need for cheaper drugs and therapies are obvious examples, the possibility to study the immune reconstitution in patients undergoing DAA-based therapy and eliminating the virus is just another one.

Clearly, history suggests that global eradication of a pathogen such as HCV will not be possible without a vaccine. Although scientifically challenging and currently met with little interest both by industry and academia, a vaccine for HCV is an unmet medical need that must be addressed and should be achievable. Even a partially protective vaccine able to prevent persistence, but not necessarily infection, would suffice. Given this need and the many scientific opportunities HCV research offers, it is disappointing that government and philanthropic funding agencies focus so little on the development of immunization strategies for HCV. Many of the scientific issues confronting efforts to develop an effective HCV vaccine mirror issues in HIV vaccine development, and a concerted effort to develop vaccines for both viruses could provide some much needed synergy. Cutting research funding by declaring “mission accomplished” would be a mistake, and mean missing many opportunities for new advances with impact far beyond HCV.

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