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The role of human lipoproteins for hepatitis C virus persistence

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Hepatitis C virus (HCV) is a hepatotropic virus that establishes a chronic infection in most individuals. Effective treatments are available; however, many patients are not aware of their infection. Consequently, they do not receive treatment and HCV transmission remains high, particularly among groups at high risk of exposure such as people who inject intravenous drugs. A prophylactic vaccine may reduce HCV transmission, but is currently not available. HCV has evolved immune evasion strategies, which facilitate persistence and complicate development of a protective vaccine. The peculiar association of HCV particles with human lipoproteins is thought to facilitate evasion from humoral immune response and viral homing to liver cells. A better understanding of these aspects provides the basis for development of protective vaccination strategies. Here, we review key information about the composition of HCV particles, the mechanisms mediating lipoprotein incorporation, and the functional consequences of this interaction.

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Abbreviations: HCV, hepatitis C virus; TAGs, triacylglycerides; CEs, cholesterol esters; LDLR, low-density lipoprotein receptor; SR-BI, scavenger receptor class B type I; RO, replication organelle; ER, endoplasmic reticulum; LDs, lipid droplets

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Introduction

Virus particles are direct offspring of a previously infected host cell. Therefore, they may incorporate not only viral but also cellular components, which together define their structure, composition, and, importantly, function. This is particularly obvious for enveloped viruses, such as HCV, as they use a portion of a cellular lipid bilayer for their envelopment. Consequently, their particles pick up lipids and proteins resident in the lipid membrane environment used for assembly and release. To date, a range of studies has explored the lipid and protein composition of enveloped viruses [1]. Owing to the identification of specific lipid and protein signatures in released virus particles, this work has provided important information about the cellular sites of virus production and the cellular processes shaping viral progeny. However, we have a relatively limited understanding about the processes that coordinate selective uptake of cellular components into nascent virus particles. Likewise, in most cases, we do not know if cellular proteins coincorporated into virus particles are simply bystanders randomly associating with these particles or if they play specific functional roles relevant for virus transmission. Given the critical function of virus particles for carrying the viral genetic information to new cells and host organisms, the functional traits of virus particles are under evolutionary pressure. This pressure may also entail the selection of viral assembly processes that favor incorporation of 'useful' cellular factors. Such host-derived factors may facilitate selection and infection of novel and susceptible host cells. Furthermore, they may facilitate evasion from host-derived immune effector mechanisms. Conceivably, both of these roles may enhance virus propagation, transmission, and consequently evolutionary success. In case of HCV, more than thirty years ago, Thomssen et al. reported the peculiar observation that HCV particles can be effectively purified from human sera by using antibodies specific for human lipoproteins [2,3]. This finding suggested that HCV particles directly associate with — or incorporate — human lipoproteins (Box 1). However, why and how HCV engages in this interaction remained elusive for a long time and continues to be investigated.

In this concise review, we summarize the most important findings related to the peculiar composition of HCV particles. We also discuss how HCV incorporates

Box 1 Lipoproteins, apolipoproteins, and lipoprotein receptors

Lipoproteins transport lipids in the circulation and distribute them between specific cells and tissues. They are composed of a core of neutral lipids (TAGs and CEs) packaged by a lipid monolayer composed of phospholipids, free cholesterol, and a range of proteins called apolipoproteins (Apos, e.g. ApoA, B, C, etc). Apolipoproteins play a structural role by initiating the lipoprotein biogenesis and lipid incorporation and by solubilizing the lipids in the plasma. They regulate enzymes responsible for the lipoprotein metabolism and allow selective cellular uptake via binding to specific lipoprotein receptors. One of these receptors is the SR-BI. SR-BI is also an entry factor of HCV. It binds HCV E2 and apolipoproteins such as ApoA-I, ApoC-III, and ApoE. The LDLR has particular affinity for ApoB and ApoE — it is also implicated in facilitating HCV infection. The liver is the primary source for apolipoprotein production. Of note, most apolipoproteins freely exchange between lipoprotein species (e.g. ApoA-I, ApoC-I to -III, and ApoE), while the large ApoB (around 540 kDa) is nonexchangeable.

Lipoproteins are very diverse and categorized depending on their size, density, and their content in lipids and apolipoproteins. The three main lipoprotein classes relevant for HCV infection are HDL, low-density lipoprotein (LDL), and VLDL. ApoA-I is incorporated in multiple copies on the surface of HDL particles and accounts for 70% of their protein content. It is secreted by the liver and the intestine forming a HDL precursor that gets enriched in CEs by absorbing cholesterol effluxed from peripheral tissues. These CEs are then drawn from the HDL particles and taken up by interaction of ApoA-I with SR-BI in particular in the liver, without internalization of the lipoprotein. Thus, HDL ensures reverse cholesterol transport from the peripheral tissues to the liver. VLDL are triglyceride-rich lipoproteins produced by the hepatocytes and incorporating a single copy of ApoB as the main apolipoprotein. Hydrolysis of the core triglycerides results in cholesterol enrichment and in the conversion to smaller lipoproteins, such as the LDL, which are the main carriers of ApoB and cholesterol in the circulation. LDL particles are taken up by endocytosis mediated by the LDLR that recognizes ApoB and ApoE. LDLR is mostly abundant in the liver and its expression is regulated by the hepatocyte cholesterol level. More details on the lipoprotein metabolism have been comprehensively reviewed by K. Feingold [4].

lipids and apolipoproteins. Finally, we summarize current information about the function(s) of these host components within virions and we speculate how this may influence chronic persistence of HCV.

The composition of HCV lipo-viro-particles

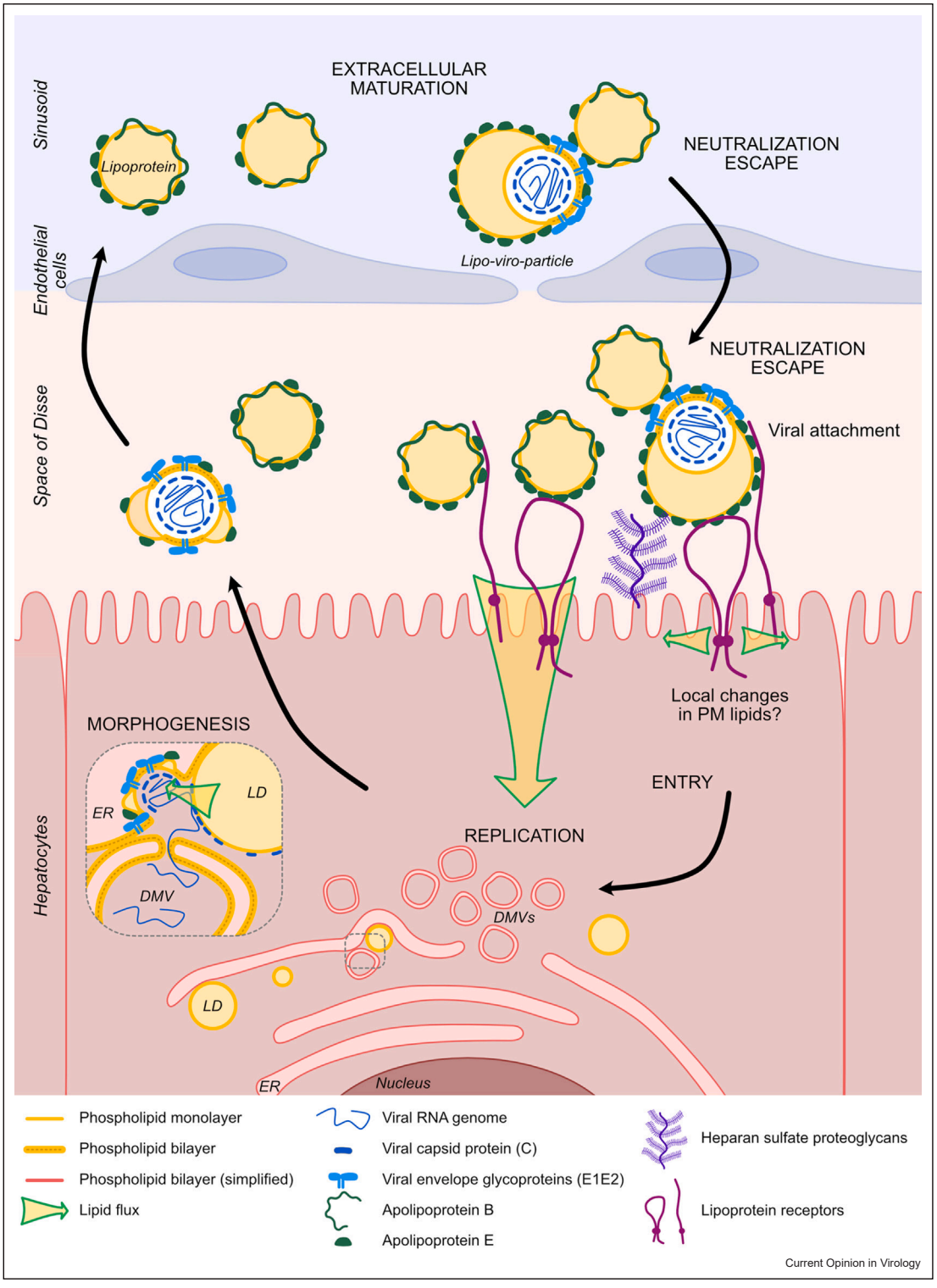
Twenty years ago already, André et al. proposed the term of 'lipo-viro-particle' to describe the nature of HCV virions circulating in patient plasma and its hybrid properties, partly virus, partly lipoprotein [5]. Additional studies carefully assessed the composition of HCV particles derived from patients. It was noted that HCV also interacts with immunoglobulins [3,6]. Moreover, analysis of HCV particles in density gradients revealed that virions exhibit a surprisingly variable and in part unusually low buoyant density. This range of densities is due to the particle interactions with different proteins and the uptake of large amounts of lipids. Hijikata and Bradley found out that the low-density particles are highly infectious, whereas particles with intermediate and high density, likely due to association with immunoglobulins, are less infectious [6,7]. Felmlee reported that consumption of a high-fat meal influences the density of circulating virions in patients [8]. These transiently exhibit a low density, suggesting that availability of lipid-rich lipoproteins after a rich meal may influence the composition and properties of the viral particles. Finally, immunoprecipitation analyses of patient serum-derived HCV suggested that ApoB and -E are present within HCV particles [9].

The development of fully permissive tissue culture models of HCV opened doors for dissecting the mechanisms and functional relevance of the HCV lipoprotein interaction [10–12]. Lindenbach et al. reported that cell culture-derived hepatitis C virus (HCVcc) has a lower specific infectivity and a lower buoyant density than the same virus that had been passaged through human liver chimeric mice or chimpanzees [13], suggesting that this model does not fully recapitulate the lipoprotein association of the virion. Nevertheless, even in cell culture, a range of apolipoproteins, including ApoA-II, ApoB, ApoC-II, ApoC-III, ApoE, and more recently ApoM, were identified in purified HCV preparations or coprecipitated with HCV genome [14–17]. Moreover, the lack of a symmetrical particle appearance in electron microscopy [18,19], and its protein and lipid content [15,16] set HCV aside from the canonical virions observed in the *Flavivirus* genus. Today, the precise architecture of the mixed HCV viral particle remains unclear. Contrary to the host-derived quasi envelope recently described for several naked viruses, in particular the hepatitis-A and -E viruses [20], HCV lipo-viro-particle incorporates both host apolipoproteins and the viral envelope glycoproteins at its surface. Electron microscopy suggests a one-particle model rather than a peripheral association between HCV and lipoproteins [18,19]. However, lipoproteins are limited by a phospholipid hemilayer, which is likely incompatible with the incorporation of HCV E1 and E2 envelope glycoproteins — even though their transmembrane domain can transiently adopt such a configuration in the context of the immature HCV polyprotein precursor [21,22]. Therefore, rather than harboring a surface monolayer such as a lipoprotein, it is possible that HCV envelope consists of a typical bilayer and accommodates its neutral lipids locally as lenses between the two membrane leaflets, reminiscent of the lipid lenses forming in the endoplasmic reticulum (ER) during lipid droplet (LD) and lipoprotein biogenesis (Figure 1).

Role of lipoproteins in HCV morphogenesis

This lipoprotein imprint on the HCV virion suggested that the lipoprotein production pathway is involved in HCV morphogenesis (Figure 1). In this regard, the in vitro systems proved very useful to pinpoint the cellular

Figure 1


EXTRACELLULAR MATURATION
Sinusoid
Lipoprotein
NEUTRALIZATION ESCAPE
Endothelial cells
Lipo-viro-particle
NEUTRALIZATION ESCAPE
Space of Disse
Viral attachment
Local changes in PM lipids?
MORPHOGENESIS
ENTRY
LD
ER
REPLICATION
DMV
DMVs
Hepatocytes
LD
ER
Nucleus
Phospholipid monolayer
Phospholipid bilayer
Phospholipid bilayer (simplified)
Lipid flux
Viral RNA genome
Viral capsid protein (C)
Viral envelope glycoproteins (E1E2)
Apolipoprotein B
Apolipoprotein E
Heparan sulfate proteoglycans
Lipoprotein receptors
Current Opinion in Virology

Putative roles of human lipoproteins for HCV replication cycle and persistence. The role played by host lipoproteins during viral entry has been well-described. Apolipoproteins incorporated in the lipo-viro-particles enhance HCV attachment to the host hepatocytes by engaging heparan sulfate proteoglycans and lipoprotein receptors. Furthermore, lipoprotein receptors might mediate local lipid transfers from the lipo-viro-particle to the target host cell plasma membrane, which could facilitate subsequent steps of the entry process, for instance, the recruitment of more specific HCV receptors toward the virus entry site, the virus trafficking at the cell surface, or its fusion with the endosomal membrane. Overall, virus entry is accelerated and fusion facilitated, which decreases HCV sensitivity to neutralizing antibodies. Lipid transfer from circulating lipoproteins might also provide essential building blocks for the biogenesis of virus-induced double-membrane vesicles (DMVs), which form the viral RO. HCV morphogenesis takes place at the ER membrane close to the DMVs and cytosolic LDs. Not only E1 and E2 glycoproteins but also host apolipoproteins are essential for the assembly of the viral particle and get incorporated in the virion. HCV morphogenesis also depends on LD lipolysis, suggesting that lipid flux between cytosolic LDs and budding lipoproteins might also fuel the production of infectious HCV progeny. Released viral particles are likely to further mature in the bloodstream by exchanging apolipoproteins, acquiring further neutral lipids and possibly associating with circulating lipoproteins. Whether lipoprotein association affects HCV immunogenicity and hides neutralizing epitopes is still not clearly proven.

requirements for the association of HCV with lipoproteins. Initial work had suggested that an elaborate machinery of host proteins, which is usually engaged with production of ApoB-containing very-low-density lipoprotein (VLDL) particles, is needed for virus production [23,24]. However, more recent complementation studies in nonhepatic human cell lines, such as 293T or HeLa, both of which lack the machinery for production of lipoproteins, established that ApoE is both necessary and sufficient for infectious HCV production [25,26]. In fact, this requirement was further narrowed down to the amphipathic α-helices of ApoE [27,28]. Curiously, this structural determinant can be provided by a range of other apolipoproteins [27,28] or by ectopically expressing unrelated secretory proteins, including the *Flaviviridae* proteins nonstructural protein 1 (NS1) and E^{rns} [29,30]. Mechanistically, all ApoE mimicks are secreted proteins and most can carry a lipid cargo [1], suggesting that ApoE and substitutes might vehicle the HCV lipo-viro-particle on the way out of the cell. From the virology point of view, ApoE or its mimicks are essential for the intracellular assembly of infectious virions but not for their envelopment [26,28,31]. E^{rns} and NS1 from pesti- and flaviviruses do not have any homolog in the HCV polyprotein. The fact that these proteins can complement HCV assembly in nonhepatic cell lines [30] suggests that HCV might have 'learned' to use abundant hepatic host factors, especially the apolipoproteins ApoE and -B, to fill in a gap in its assembly machinery. Interestingly, ApoE is also used as assembly cofactor and incorporated in hepatitis-B virion, another enveloped but unrelated liver-tropic virus [32], suggesting that the lipoprotein production is a coveted exit route for hepatic viruses.

Apart from incorporating apolipoproteins, HCV also draws neutral lipids from its host cells. The role played by the lipolytic machinery, with the cytosolic, calcium-dependent, group-IVA phospholipase A2 [33], the adipocyte triglyceride lipase and its cofactor α/β-hydrolase domain-containing protein 5 (ABHD5/CGI-58), in HCV assembly and release [34,35], suggests that at least the triacylglycerides (TAGs) flow from the LDs to the virion — involving their lipolysis and re-esterification — in a manner similar to lipoprotein lipidation. However, physical evidence for this lipid flux is still lacking, because inhibiting lipolysis already hinders HCV assembly and secretion [34]. Also, the role of TAG re-esterification enzymes in HCV assembly or the process for cholesterol ester (CE) incorporation in the virion has not been reported yet.

A further specificity of HCV and commonality with lipoproteins is its capacity to mature after secretion from the host cells. On one hand, the transfer of exchangeable apolipoproteins and in particular ApoE between lipoproteins and the virion has been well-documented [36–38], although it has to our knowledge not been studied whether this process also relies on the structural determinants of ApoE amphipathic α-helices. On the other hand, extracellular lipidation of the viral particles was observed in the serum of patients fed with a high-fat diet [8] but also in vitro by incubating HCVcc with human serum [39]. In this last study, the decrease in virion density was also concomitant with ApoB association [39]. Since ApoB is not an exchangeable apolipoprotein, this suggests that HCV particles might also mature by associating peripherally with circulating lipoproteins (Figure 1), as previously proposed by Lindenbach [40]. In both cases, virion maturation correlated with increased infectivity [8,36–39]. This plasticity of the viral particle likely contributes to the broad distribution of serum particles in density gradients.

Altogether, a lot is known about the host factors participating in HCV assembly and the composition of the lipo-viro-particle, even though most results were obtained in hepatoma cell lines that only partially recapitulate the hepatic lipid metabolism and lipoprotein secretion. In this direction, progress has been made to establish more physiological stem-cell-derived hepatocyte-like cells, which were shown to be permissive for HCV production [41,42]. Furthermore, human fetal and adult primary hepatocyte models are being used for analysis of HCV in a more authentic environment [43–45]. Finally, modifying the culture conditions of the standard hepatoma cell lines can increase their differentiation and favor HCV lipo-viro-particle production [46–48].

Like for the lipoproteins, gray areas subsist in particular to comprehend the architecture of the virion and the discrete steps of its morphogenesis. High-resolution imaging of the morphogenesis process and of the viral particle could help addressing these shortcomings. Although this would have sounded unrealistic a decade ago, tremendous progress in cryo-EM was achieved enabling the imaging and reconstruction of 3D structures for membrane-associated proteins as well as heterogeneous material [49,50]. The reconstitution of multiple 3D structures, including several biological states of the proteasome from size-fractionated cell lysates, is a showcase demonstrating how cryo-EM approaches hold great promise into determining the structure of proteins or particles with ever-increasing heterogeneity and complexity [51]. However, the extreme heterogeneity of the HCV particle, its possible complete lack of symmetry, and the very weak efficiency of viral particle production in cell culture systems remain major hurdles to overcome. Thus, even though the site of HCV assembly is now well-defined and a spectrum of mutants or host factor modulations arresting assembly at discrete stages is available, and despite the power of correlative light and electron microscopy, HCV budding or intracellular HCV particles still resist imaging [52]. Efforts to further increase HCV assembly efficiency in cell culture and to artificially restrain the virion heterogeneity might help getting first structural insights into these and better understanding the structural role played by lipoproteins in HCV morphogenesis.

Functional roles of lipoproteins and potential relevance for viral persistence

Work from different laboratories supports at least three roles of the lipoprotein components within HCV particles: these are (1) attachment to liver cells, (2) support for membrane fusion, and (3) facilitation of evasion from antibodies. Regarding the first, HCV attachment seems to be particularly supported by ApoE resident in the lipo-viro-particles. ApoE is incorporated during HCV assembly within infected cells and can also be recruited after budding by interaction with circulating ApoE-containing lipoproteins. Jiang showed that ApoE-specific antibodies blocked HCV infection and also limited attachment of HCV to Huh-7.5 cells and primary human hepatocytes [53]. By complementing ApoE-deficient Huh-7.5 cells with mutant ApoE proteins, they provided evidence that ApoE variants with mutated receptor-binding site poorly rescued HCV infection. They also showed that the capacity of ApoE to bind cellular heparan sulfate proteoglycans was important for HCV attachment and they confirmed this observation with patient-derived HCV [54]. Beyond this, also direct binding between ApoE and the low-density lipoprotein receptor (LDLR) and the scavenger receptor class-B type I (SR-BI) may facilitate HCV infection [55–58]. In fact, HCVcc of various densities engages different lipoprotein receptors, ranging from LDLR usage by low-density HCVcc to SR-BI preference of high-density HCVcc [59]. In case of the latter, it is well- documented that not only binding but also the lipid transfer function of SR-BI aids in HCV infection, because SR-BI mutants lacking this property do not support, and inhibitors of SR-BI lipid transfer, impede HCV infection [60,61]. Furthermore, efficient HCV RNA replication depends on LDLR function. In fact, blocking the receptor with an antibody after virus entry or after genome transfection decreased viral genome accumulation and correlated with changes in the cellular neutral and membrane lipid content [62]. These observations suggest that not only direct binding, but also postbinding steps are influenced by lipoproteins, which may directly deliver fatty acids and cholesterol at the site of cell attachment but also to internal membranes that form the viral replication organelle (RO). This effect of the lipid transfer mediated by LDLR and SR-BI on HCV entry versus replication also coincides with the divergent lipid uptake mechanisms used by the two lipoprotein receptors. Thus, LDLR internalizes the whole lipoprotein via clathrin-mediated endocytosis and cholesterol is delivered to the cell internal compartment after lysosomal degradation of the lipoprotein [4]. In contrast, SR-BI uses selective lipid uptake: it remains sequestered by oligomerization in patches at the plasma membrane [63] and draws the CEs from high-density lipoproteins (HDL) particles through an intramolecular tunnel [64] without internalizing the lipoproteins, thereby directly transferring the CEs to the plasma membrane. It is not clear if the lipid transfer function of SR-BI, which facilitates infection, is fueled directly by lipids derived from the HCV lipo-viro-particle or if this occurs in *trans*. While these options are not mutually exclusive, it is well-established that addition of lipoproteins such as, for instance, HDL, can enhance HCV infection in *trans*. Considering the recent observation that released HCV particles accept additional ApoE and the role of ApoE in virus attachment, this presumed 'trans-effect' may actually be mediated by incorporation of ApoE directly into the virions. In addition, or alternatively, it may be caused by stimulation of the lipid transfer function of SR-BI.

Regarding the second functional role of lipoproteins, Dreux et al. reported that ApoC-I, another apoliprotein, which associates primarily with HDL, has the capacity to enhance HCV membrane fusion [65]. Using mutant HCV pseudoparticles and in vitro membrane fusion assays, they showed that this function may depend on the hypervariable region 1 of HCV E2. Thus, collectively, virus-resident apolipoproteins enhance attachment and membrane fusion. In addition to this, lipid transfer from the virus particle or lipoproteins *in trans*, may facilitate membrane fusion and/or subsequent entry steps. Which step(s) are influenced by lipid transfer remains to be

fully established. It is possible that transfer of fatty acids and cholesterol changes membrane properties and in turn the capacity of HCV to fuse with the host cellular membrane. There is some evidence that the target cell lipid membrane composition and the density of HCV particles both influence HCV membrane fusion [66]. Alternatively, or in addition, the membrane trafficking of HCV and cellular entry factors may be influenced by changes of the lipid membrane composition. High-resolution proteomic analyses and live-cell imaging studies may shed light on these open questions in the future.

Despite of these uncertainties regarding the mechanisms by which lipoproteins enhance infection, there is firm evidence from multiple laboratories that lipoproteins facilitate HCV escape from neutralizing antibodies. Again, this escape from antibodies can occur *in trans* upon addition of lipoproteins such as HDL [67–69]. Furthermore, HCV antibody escape can be modulated by manipulating the availability of ApoE during intracellular virus production and/or by supplementation of ApoE-containing lipoproteins after virus production, suggesting that the abundance of ApoE within lipo-viroparticles influences the degree of viral escape from antibodies [36,70]. Kinetic analyses of HCV cell entry suggest that lipoproteins accelerate uptake and fusion of HCV particles, thereby reducing antibody neutralization [68]. Conceivably, the mechanisms described above may be mediated by an acceleration of infection. Further to this, it was speculated that lipids and/or apolipoproteins may mask and prevent access to viral neutralizing epitopes and thereby afford antibody escape [36,70]. To address this question, one study incubated HCV particles with excess ApoE-containing culture fluids. While this treatment clearly enhanced particle infectivity and reduced neutralization by monoclonal antibodies against diverse HCV E2 neutralizing epitopes, it did not alter precipitation of HCV particles by these antibodies [36]. While these results do not exclude epitope masking by lipoproteins as one mechanism of antibody evasion, they provide additional evidence for the importance of lipoproteins in immune evasion. Furthermore, ApoE direct interaction with E2 [31], its incorporation in the viral particle during assembly [71] and after egress [36–38], and its facilitation of neutralization escape [36,70] are important considerations for vaccine design. Thus, Gomez-Escobar et al. incorporated ApoE together with the chimeric HCV/HBV glycoproteins in their candidate subviral particle vaccine. This resulted in changes in E2 folding, as probed by antibody binding, possibly correlating with a slightly improved quality of the anti-E2 neutralizing response [72]. On the opposite, Li et al. suggested that preventing interaction with serum ApoE might increase the immunogenicity of HCV vaccines harboring recombinant glycoproteins [38].

Our current understanding of the functional relevance of lipoproteins for HCV cell entry and immune evasion is limited by specific experimental limitations, which should be kept in mind when discussing the relevance of these mechanisms for HCV persistence. First, most studies used cell culture-derived HCV particles or HCV pseudoparticles for assessment of the functioning of lipoproteins. These particles may not faithfully recapitulate the behavior of patient-derived HCV particles. As stated above, HCV particles from patients differ in their density distribution from HCVcc and it seems that they incorporate a greater amount of ApoE [36]. Thus, our cell culture models may underestimate the functions and the impact of lipoproteins during infection. Second, most studies in vitro use poorly differentiated cells grown in two-dimensional cellular monolayers. These limitations may preclude a comprehensive appreciation of the role of lipoproteins during HCV trafficking in exposed cells. Recent advances in imaging technologies and use of complex HCV tissue culture models such as, for instance, *ex vivo* organotypic culture of hepatic slices obtained from human liver explants or liver organoid cultures, could prove very useful in this regard [73–75]. Finally, the lack of a tractable immune-competent animal model of HCV has precluded validation of the importance of lipoproteins for in vivo infection and persistence. Such animal models would be precious to answer yet one additional important and open question: does incorporation of lipoproteins into HCV particles modulate immunogenicity of HCV envelope proteins? The animal infection model based on the rodent hepacivirus (RHV) from *Rattus norvegicus* (RHV-rn) could prove useful to test this, because viruses derived from this isolate (RHV-rn1) share key biophysical properties of HCV particles and they are infectious in vitro and in mice and rats [76–79]. In humans, HCV clearly does induce neutralizing antibodies and early induction of neutralizing antibodies correlates with acute HCV clearance [80]. However, most exposed individuals progress to chronic infection and many patients mount only modest neutralizing antibodies. Therefore, answering this question may be interesting from both the basic science perspective and the view of translational science and vaccine development.

Data Availability

Data will be made available on request.

Declaration of Competing Interest

Both authors declare that they have no conflict of interest. The funders that supported the work in their

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- • of special interest
- •• of outstanding interest

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