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CARACTÉRISATION DES GLYCOPROTÉINES D'ENVELOPPE DES VARIANTS VIRAUX IMPLIQUÉS DANS LA TRANSMISSION DU VIRUS DE L'HÉPATITE C

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To my brother, Nico.

Theory is when you know everything but nothing works. Practice is when everything works but no one knows why. In our lab, theory and practice are combined: nothing works and no one knows why!
(Albert Einstein)

The greatness of a nation and its moral progress can be judged by the way its animals are treated.
(Mahatma Gandhi)

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The PhD period is probably the moment for a researcher to test herself and the passion she has for this job. There are many moments of happiness for successes but there may be numerous moments of dejection for failures too. But, if even Albert Einstein did not understand some of his experiments' results, then you can say yourself that there may be hope for you too! Then, the desire to understand and to move forward remains. That is why I would like to thank those people without whom this thesis work should not have been possible.

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Abstract

The hepatitis C virus (HCV) infection is a world health burden, with three to four million new infections per year and no vaccine currently available. The identification of viral variants capable of establishing new infections and definition of the phenotypic requirements for transmission would facilitate the design of preventive strategies.

To address this issue, we compared HCV variants found in three health care workers, who developed acute hepatitis C after a needlestick accident, to HCV variants found in the corresponding chronically infected donor patients. We mapped the genetic bottleneck event leading to productive clinical infections by single-genome amplification (SGA) of viral envelope glycoprotein E1E2 sequences, direct amplicon sequencing and phylogenetic analysis. We found that infections were successfully established by a single variant in two out of three cases, or five variants in the third case. We could show that E1E2 amino acid sequences of the transmitted variants were identical or closely related to those found in the donor quasispecies. The transmitted variants harbored a common key substitution at position 394 within the hypervariable region 1 of E2. Surprisingly, these E1E2 variants conferred no greater capacity for entry than the E1E2 derived from non-transmitted variants, in lentiviral pseudoparticle assays. Moreover, the selection of the transmitted variants was not influenced by the presence of neutralizing antibodies in donor serum. Fitness parameters affecting the selective outgrowth of post-transmission HCV variants in an immunocompetent host may thus be more complex than previously described using chimeric mice models. Two human monoclonal antibodies directed against HCV envelope glycoproteins effectively cross-neutralized the lentiviral particles bearing E1E2 derived from transmitted variants. Combination of monoclonal antibodies may thus further increase the genetic barrier for resistance and offers a promising prevention strategy.

Altogether, our data shed light on the selective transmission of HCV quasispecies during the early stage of infection, a period where the virus may be most vulnerable to elimination by vaccines or immunotherapies.

Key words: *HCV transmission, needlestick accidents, viral quasispecies, SGA, neutralizing antibodies, envelope glycoproteins*

Résumé

Le virus de l'hépatite C (VHC) est un problème de santé publique à l'échelle mondiale, avec trois à quatre millions de nouvelles infections par an et aucun vaccin actuellement disponible. L'identification des variants viraux capables d'établir de nouvelles infections et la définition des propriétés phénotypiques à la base de la transmission virale faciliteraient la conception de stratégies de prévention.

Pour explorer cette problématique, nous avons étudié les variants du VHC circulant chez trois représentants du personnel soignant ayant développé une hépatite C aiguë après s'être piqués avec une aiguille contaminée. Ces variants viraux ont été comparés avec ceux présents chez les patients chroniquement infectés à l'origine des contaminations. Nous avons analysé le goulot d'étranglement génétique subit par le virus lors de la transmission en réalisant une amplification de génomes uniques («Single Genome Amplification» ou SGA) sur les séquences des glycoprotéines d'enveloppe E1E2, suivie d'un séquençage direct des amplicons et d'une analyse phylogénétique. Dans deux cas sur trois, nous avons constaté qu'un seul variant était à l'origine de l'infection. Dans le troisième cas, nous avons identifié jusqu'à cinq variants transmis. Nous avons pu montrer que les séquences protéiques E1E2 des variants transmis étaient identiques ou étroitement liées à celles de certains variants retrouvés dans les quasi-espèces des donneurs. Ces variants transmis avaient en commun une substitution clé en position 394 dans la région hypervariable 1 de la glycoprotéine d'enveloppe E2. De manière inattendue, les séquences E1E2 portant cette substitution ne conféraient pas une capacité accrue d'entrée virale par rapport à celles dérivées des variants non transmis. L'entrée virale a été testée avec le système des pseudo-particules VHC (VHCpp) basé sur l'utilisation de lentivirus. Par ailleurs, nous avons également pu montrer que la sélection des variants transmis n'était pas influencée par les anticorps neutralisants circulant dans le sérum des donneurs. Les mécanismes affectant l'amplification sélective de certains variants viraux, après transmission chez un hôte immunocompétent, pourraient donc être plus complexes que ceux décrits précédemment dans les modèles de souris chimériques. Deux anticorps humains monoclonaux dirigés contre les glycoprotéines d'enveloppe du VHC se sont montrés très efficaces dans la neutralisation des VHCpp portant les séquences E1E2 dérivés des variants transmis. La combinaison d'anticorps monoclonaux pourrait donc augmenter la barrière génétique pour la résistance virale et offrir une stratégie de prévention prometteuse.

En conclusion, nos données apportent des éléments de compréhension sur la transmission sélective des quasi-espèces du VHC et sur la phase précoce de l'infection, une période pendant laquelle le virus pourrait être plus vulnérable à l'élimination par les vaccins ou les immunothérapies.

Mots clés : *transmission du VHC, accidents d'exposition au sang, quasi-espèces virales, SGA, anticorps neutralisants, glycoprotéines d'enveloppe.*

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I. INTRODUCTION

A. HEPATITIS C: OVERVIEW

In the late 1960's, viral hepatitis was originally believed to consist of two types only, infectious or type A hepatitis (HAV) and serum or type B hepatitis (HBV). From the beginning of the 1970's, however, cases of transfusion-acquired hepatitis, associated neither to HAV nor to HBV, put the emphasis on the possibility that a not yet known pathogen could be the origin of these infections. Then, the term of "non-A non-B hepatitis" (NANBH) was coined for it (Feinstone, Kapikian, Purcell, Alter, & Holland, 2001). Proof that the hepatitis C virus (HCV) was responsible for transfusion-related hepatitis followed a decade and a half later, when a cDNA library was constructed using RNA of the putative etiological agent of NANB hepatitis (Choo et al., 1989). Analysis of these sequences allowed the classification of HCV within the *Flaviviridae* family (Miller & Purcell, 1990), including spherical, enveloped viruses of about 50 nm of diameter, and harboring a single-stranded, positive-sense RNA genome. Furthermore, because of its singularity, especially its transmission pathway that does not require arthropod vectors, the HCV was classified as the unique type member of the genus *Hepacivirus*.

According to the WHO, HCV is to date estimated to infect 170 million people worldwide (3% of the world's population). The infection, presenting itself as an acute hepatitis 4 to 12 weeks after contamination, is often asymptomatic, although it can be accompanied by classic signs of hepatic illness (fatigue, flu-like symptoms, jaundice). While part of the patients can clear the virus, up to 80% of cases may evolve into chronic hepatitis (persistence of the viral genome in the serum 6 months after the acute phase of infection), accompanied by several serious complications, such as cirrhosis, fatty liver

disease and hepatocellular carcinoma (Pawlotsky, 2004) (Fig. 1). Unfortunately, there is no effective vaccine to fight this virus which has become the leading cause of liver transplantation in Europe and in the United States, and which is characterized by one of the highest mortality rates in the world (Center for Disease Control, CDC).

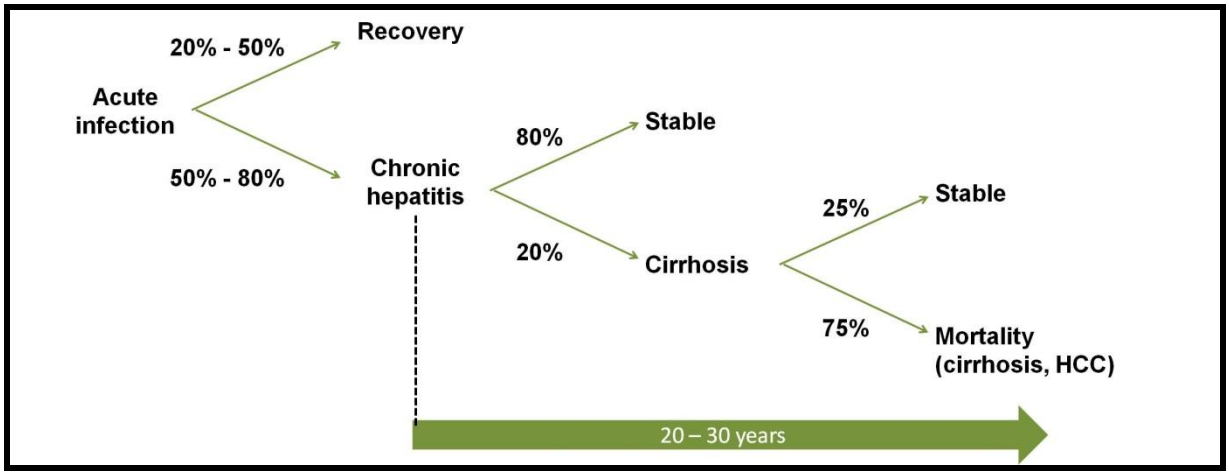


Fig. 1. Schematic representation of the natural history of hepatitis C virus infection.

Until May 2011, the standard of care (SOC) for chronic HCV infection of all genotypes was the combination of pegylated-inerferon-α (PEG-INF-α) and ribavirin (RBV). Unfortunately, this treatment is effective in only approximately 45% of cases, with a strict dependence on the race (more effective in Caucasians than in African patients) (Melia *et al*., 2011), and on the virus genotype (not very effective against HCV genotype 1, the most highly represented in Western countries) (Schoggins & Rice, 2013). Recently, new molecules targeting specific viral proteins have been approved whilst others are in clinical trials. Among them, the two NS3/4 protease inhibitors, Boceprevir and Telaprevir are currently approved by the Food and Drug Administration (FDA) and by the European Medicine Agency (EMA), for the treatment of patients with chronic genotype 1 HCV infection (Kwo *et al*., 2010; McHutchison *et al*., 2010). Although these drugs are

effective against HCV infection, leading to a significant improvement in cure rates in relapsers, in partial, and in null responders to the SOC, they are associated with serious side effects, in addition to those caused by PEG-INF-α and RBV (Schlutter, 2011). More than 50 other drugs and different candidate vaccines are, however, in the development, but as long as neither the former nor the latter will be available to all people living with HCV, understanding of the molecular mechanisms underlying the biology of the virus remains essential.

B. THE HEPATITIS C VIRUS (HCV)

1. Structure of the HCV Particle

The actual morphological structure of an HCV particle still remains an intriguing mystery because of the difficulty to purify infectious virions to a grade appropriate for such a characterization. As a *Flaviviridae* member, HCV is described as a small particle (~50 nm), composed by an electron-dense nucleocapsid of ~30 nm, holding the viral genome, and surrounded by a lipid bilayer in which the two envelope glycoproteins (E1 and E2) are anchored (Lindenbach & Rice, 2007) (Fig. 2). It has been demonstrated that in sera of HCV infected patients, RNA-containing particles are associated with low and very low density lipoproteins (LDLs, VLDLs), forming the so called lipo-viro-particles (LVPs) (André et al., 2002a) (Fig. 2). One of main features of these circulating LVPs is their great density heterogeneity, which ranges from 1.25 g/ml to below 1.06 g/ml, depending also on the serum sample analyzed (André et al., 2002a; Bartenschlager, Penin, Lohmann, & André, 2011; Thomssen et al., 1992). Interestingly, lower density particles (1.03 – 1.08 g/ml) are associated with a greater infectivity, whilst higher density particles (1.17 – 1.25 g/ml) are less infectious (André et al., 2002a). However, while only very little information on structural and biochemical characterization of lipoprotein-associated virus is available, the hybrid nature of the HCV particle has proved to have a profound impact on the viral infectious cycle, suggesting to play a role, both in increasing the infectious properties of the virion, and in protecting it from immune responses (Lindenbach *et al.*, 2006; Maillard *et al.*, 2006) (see paragraphs B-4, and C-4.b).

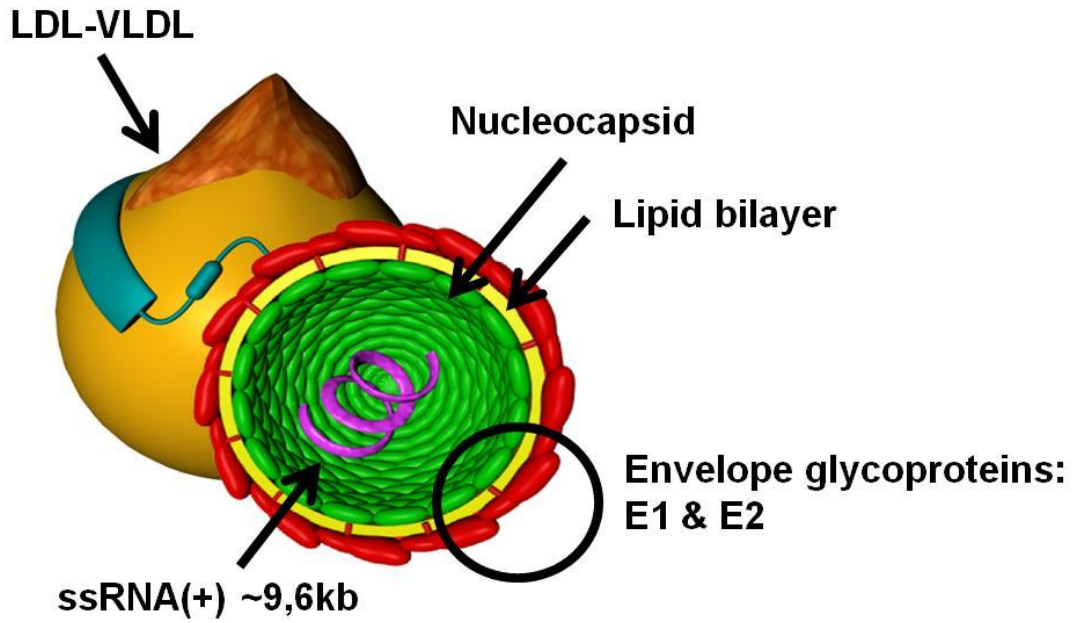


Fig. 2. Lipo-Viro-Particle (LVP) hypothetical structure.

Most circulating and highly infectious HCV particles are associated with low- and very-low-density lipoproteins (apolipoproteins (apo)B, apoE, and apoCs) (Thomssen et al., 1992; Agnello et al., 1999). LVPs are composed by an electron-dense nucleocapsid, holding the viral genome, and surrounded by a lipid bilayer in which the two envelope glycoproteins, E1 and E2 are anchored. (Adapted from Popescu & Dubuisson, 2010).

Regarding their composition, the lower density LVPs are rich in triacylglycerol, and immunocapture studies suggest that they contain the non-exchangeable apolipoprotein (apo)B, the exchangeable apoE, apoC1, apoC2 and apoC3, the viral nucleocapsid, formed by the Core viral protein, and the RNA genome, as well as the viral glycoproteins, E1 and E2 (André *et al.*, 2002; Andréo *et al.*, 2007; Chang *et al.*, 2007; Meunier *et al.*, 2008; Nielsen *et al.*, 2006). On the other hand, the high-density RNA-containing LVPs are non-enveloped nucleocapsids, complexed with patient-derived immunoglobulins, which possibly neutralize HCV entry, thus explaining decreased infectivity of the high-density pool of particles (Bartenschlager *et al.*, 2011; Choo, So, Cho, & Ryu, 1995; Hijikata *et al.*, 1993; Thomssen *et al.*, 1992).

2. HCV Genome Organization and Viral Proteins

The HCV genome is composed of a single positive-strand RNA molecule of about 9.6 kb, characterized by a unique open reading frame (ORF) encoding a polyprotein of ~3,000 amino acids (Bartenschlager et al., 2011; Moradpour, Penin, & Rice, 2007). The ORF is flanked at its 5' and 3'ends by untranslated regions (UTRs), which are essential for polyprotein translation and RNA replication (Fig. 3). The 5'-UTR is highly conserved and folds into a complex secondary structure (domains numbered I to IV) forming, together with a portion of the Core-coding domain, an internal ribosome entry site (IRES). The latter is crucial for cap-independent translation of the viral RNA (Friebe, Lohmann, Krieger, & Bartenschlager, 2001). The HCV 3'UTR is composed of an about 40-nucleotide variable region, a poly(U/UC) tract, which has a heterogeneous length, and a highly conserved 98-nucleotide element, termed X tail or 3'X (Friebe, & Bartenschlager, 2002). It has been shown that these structures are essential for both replication in cell culture (Friebe, & Bartenschlager, 2002), and *in vivo* (Yanagi *et al*., 1998). The nascent polyprotein is co- and post-translationally processed into the mature structural (Core protein, C; envelope glycoproteins 1 and 2, E1, and E2), and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Fig. 3 and Table 1). The structural proteins are processed by the endoplasmic reticulum (ER) signal cellular peptidase whereas the non-structural proteins are processed by two viral proteases, the NS2-3 protease and the NS3-4A serine protease (Moradpour, Penin, & Rice, 2007).

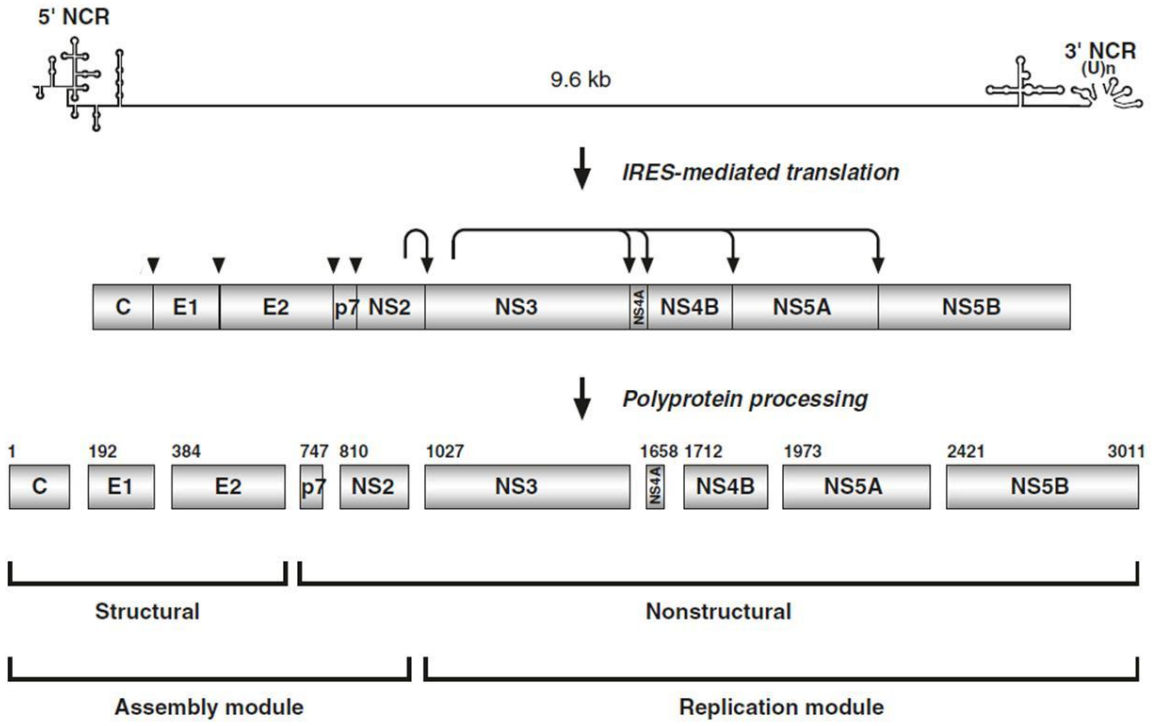


Fig. 3. Genetic organization and polyprotein processing of HCV.

The 9.6 kb positive-strand RNA genome, composed of a unique open reading frame (ORF), flanked by 5' and 3' untranslated regions (UTRs), is schematically depicted at the top. Internal ribosome entry site (IRES)-mediated translation yields a polyprotein of about 3,000 amino acids, that is processed into the ten structural and non-structural proteins. Amino acid numbers are shown above each protein (HCV H strain; GenBank accession number AF009606). Solid arrowheads indicate cleavage by the endoplasmic reticulum signal peptidase. Arrows denote cleavage by the viral NS2 and NS3-4A proteases. (From Moradpour & Penin, 2013).

3. Genetic Variability of HCV

HCV infection is a highly dynamic process with a viral half-life of only a few hours (2.7h) and a production and clearance of an estimated 10^{12} virions per day in a given individual (Guedj, Rong, Dahari, & Perelson, 2010; Neumann *et al*., 1998). This high replicative activity, together with the lack of a proof-reading function of the viral RNA-dependent RNA polymerase (RdRp), for which an *in vivo* mutation rate from about 10^{-4} to up 10^{-5} mutations per nucleotide per replication cycle has been estimated (Ribeiro *et al*., 2012), is the basis of the high genetic variability of HCV. This genetic diversity is shaped by both "Darwinian" and "neutral" evolution processes. The expression "Darwinian evolution", describes the process of adaptive changes of organisms, in response to external, sometimes changing, selection pressures. This phenomenon occurs

HCV protein	Function	Apparent molecular weight (kDa)
Core	Nucleocapsid	23 (precursor) 21 (mature)
ARFP	?	16-17
E1	Envelope Fusion domain ?	33-35
E2	Envelope Receptor binding Fusion domain ?	70-72
p7	Calcium ion channel (viroporin)	7
NS2	NS2-3 autoprotease	21-23
NS3	Component of NS2-3 and NS3-4A proteinases NTPase/helicase	69
NS4A	NS3-4A proteinase co-factor	6
NS4B	Membranous web induction	27
NS5A	RNA replication by formation of replication complexes	56 (basal form) 58 (hyperphosphorylated form)
NS5B	RNA-dependent RNA-polymerase (RdRp)	68

Table 1. HCV Viral proteins and their functions.

A complete list of all the viral proteins is shown here (For a detailed review about functions of these proteins, see Moradpour, Penin & Rice, 2007).

when random mutations introduced during copying of the genetic material in the replication process might confer, entirely by chance, an improvement in organism fitness, allowing the mutated gene to spread and, eventually, to predominate in the population (Simmonds, 2004). One possible example of adaptive changes in HCV is the rapid evolution of the hypervariable region 1 of the E2 envelope glycoprotein to prevent recognition by antibodies produced by infected hosts (see paragraph C-4.b). Beside the Darwinian evolution, nucleotide changes might also be fixed, always by chance, in coding and non-coding sequences with no or little effects on organism fitness. This is referred to as "neutral evolution", first proposed by Kimura in 1983. It is now widely accepted that this type of evolution accounts for most of the genetic variability found in organisms (Simmonds, 2004). Under the influence of these two evolutionary pathways, we can distinguish different levels of genetic variability for HCV. First, there is the genetic divergence of HCV clades (or "genotypes"), with HCV variants showing specific geographical distributions in the human population, and associations with particular risk groups of infection (Simmonds *et al*., 1993; 2005). Below this level, we can observe variability between strains within each genotype ("subtypes"), and finally, HCV diversifies enormously within an infected individual over time, forming what has been termed a "quasispecies".

a) HCV Variability around the World: Genotypes and Subtypes

Following the VIIIth report of the International Committee for the Taxonomy of Viruses (ICTV), six major phylogenetic groups ("genotypes"), numbered from 1 to 6, are recognized around the world (Simmonds *et al*., 2005; Trinks, Gadano, & Argibay, 2012), although a seventh genotype was recently proposed (Murphy *et al*., 2007). Consistent with the phylogenetic methods stipulated by the report, nucleotide sequences differing from each other

by 31 to 33% over the complete genome are classified as different genotypes. More variability is concentrated in regions encoding the E1 and E2 glycoproteins, whereas sequences of the Core and the NS3 genes are more conserved. Conversely, the lowest sequence variability is found in the 5'UTR (Simmonds, 2004). Genotypes contain a variable number of more closely related strains, or "subtypes", labeled with lower case letters (a, b, c, etc.), and differing from each other by 20-25% in their nucleotide sequences (Simmonds, 2004).

With the onset of epidemiology studies conducted after the discovery of the transfusion-acquired NANBH, two distinct patterns of sequence differences were found around the world. According to these patterns, genotypes 1a, 1b, 2a, and 3a, have become very widely distributed over the past 50 to 70 years in the USA, Europe, Australia, and East Asia (Japan, Taiwan, Thailand, and China), as a result of transmissions through blood transfusion and other invasive medical procedures (Trinks *et al*., 2012). Needle sharing between injecting drug users (IDUs) also has contributed to the spread of HCV in Western countries, where genotypes 1a, 1b, and 3a are now the most highly represented (Fig. 4) (Simmonds, 2004). It was postulated that most of the variability observed between such strains since the introduction of HCV into these new risk groups in the 20th century might reflect a neutral sequence drift. Later, other variants showing a much greater diversity were discovered in specific geographical regions. For example, in the Middle East, almost all anti-HCV-positive individuals were found to be infected primarily by genotype 4. HCV types 5 and 6 show even highly restricted distribution, being apparently confined to South Africa and Southeast Asia, respectively (Trinks *et al*., 2012) (Fig. 4). The three HCV isolates included in genotype 7 were amplified from one Canadian and two Belgian patients which, however originated from the Democratic Republic of Congo, where they are supposed to have been infected (Abstracts, 2007). These findings have led to the hypothesis that HCV was likely

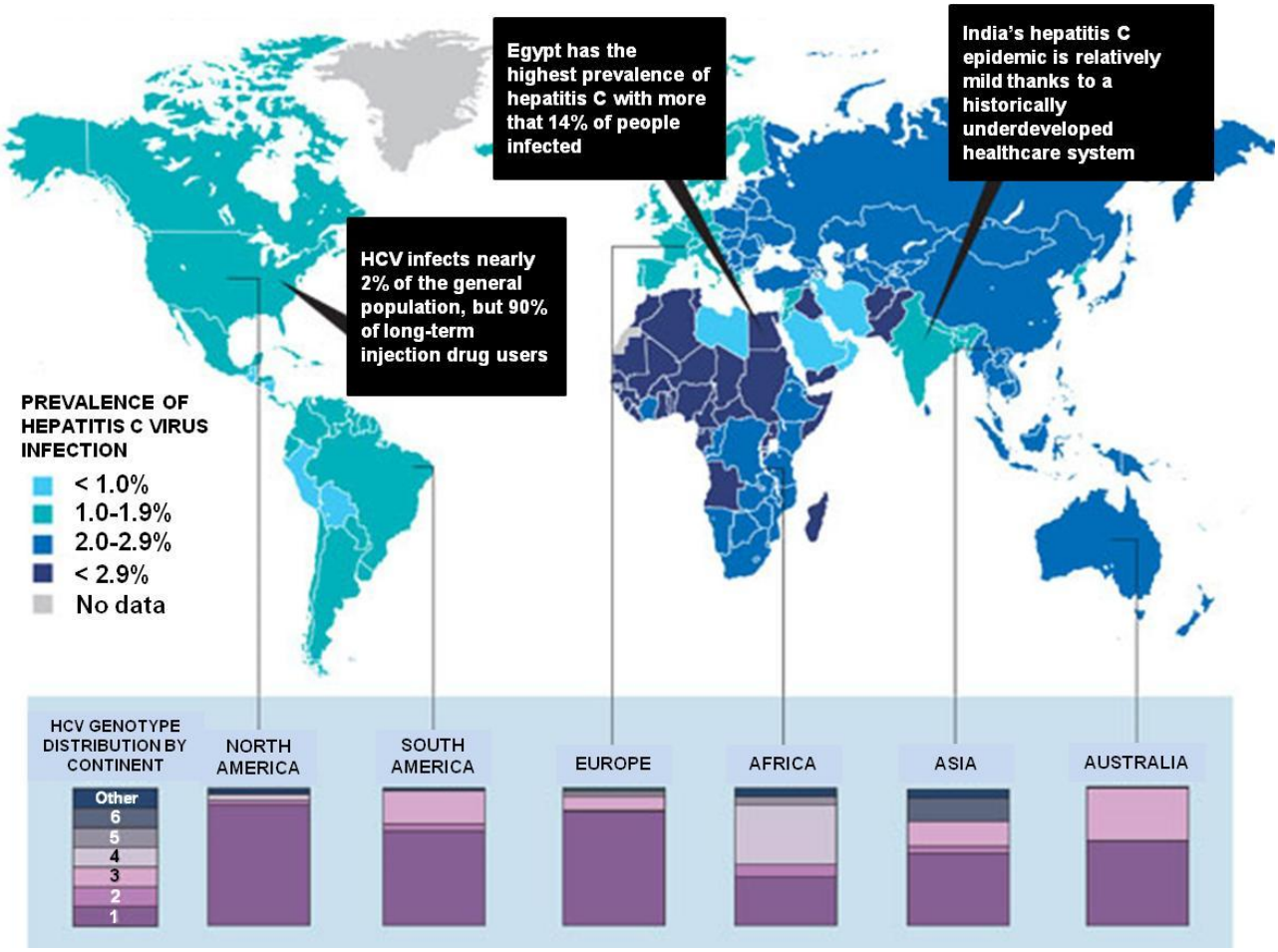


Fig. 4. Prevalence of hepatitis C virus around the world.

(From http://www.natap.org/2011/HCV/080211_01.htm).

present in those populations for a very long time, whilst spread to Western countries should be a relatively recent event.

Despite the fact that all HCV genotypes share main features, such as particle structure, life cycle, transmission and ability to establish persistent infection, there is growing evidence for genotype-specific differences in other properties, such as viral persistence and interactions with the immune system of infected hosts. For example, genotype 1 HCV seems to be more inclined to establish persistent infections and cause more severe liver disease, compared with genotypes 2 and 3 (Franchini *et al*., 2001; Mazzeo *et al*., 2003; Resti *et al*., 2003;

Yee, Griffioen, Sabin, Dusheiko, & Lee, 2000). Moreover, only about 50% of individuals chronically infected with genotype 1 HCV can clear the virus when treated with the combination therapy of INF-α/RBV, in contrast to the about 80% of individuals infected with HCV of genotypes 2 or 3 (Pawlotsky, 2003; Zeuzem, 2004). On the other hand, infections with genotype 3 are associated with higher incidence of steatosis (Adinolfi *et al*., 2001; Quadri *et al*., 2000; Roingeard, 2013).

b) HCV Diversity Within an Individual: the 'Quasi-species' Theory

Neutral and adaptive evolution of HCV operate also during the course of infection within a single individual, leading to continuous sequence diversity within the replicating viral population. Since the pioneering studies on the RNA phage Qβ, conducted by Domingo and coworkers (1978), more and more studies have highlighted the extensive sequence heterogeneity of HCV and other RNA viruses, even at the level of one infected individual (Holland *et al*., 1982; Lauring & Andino, 2010; Lauring, Frydman, & Andino, 2013; Fishman & Branch, 2010). As mentioned above, this sequence heterogeneity is the result of three main features of RNA viruses, such as the large population size (due to their short replication times), the high mutation rate (due to the error-prone nature of RNA-dependent RNA polymerases during the replication process), and the small genome size (Holland *et al*., 1982; Lauring, Frydman, & Andino, 2013; Fishman & Branch, 2010). As a consequence, RNA viruses, and thus also the hepatitis C virus, exist in a host as a dynamic population of genetically related viruses, closely distributed around a consensus sequence (or 'master sequence'), which are collectively referred to as "quasispecies" as defined by Eigen in 1971 (Martell *et al*., 1992; Lauring, Frydman, & Andino, 2013; Fishman & Branch, 2010). Viral populations with a quasispecies structure have specific

features with important implications on both the outcome of pathology they cause, and on treatments and prevention strategies. Firstly, the target of evolutionary selection is the quasispecies as a whole rather than the individual viral variant. In this way, and under appropriate conditions, lower fitness variants (where viral fitness could be defined as the capacity of a virus to generate infectious progeny), can survive thanks to the presence of higher fitness variants in the population. This phenomenon is known as the "law of the survival of the flattest vs. the survival of the fittest", where the 'flattest' is the more evolutionarily neutral and highly connected viral variant in the population (or fitness landscape), whereas the 'fittest' is the viral variant located at a higher but narrower fitness peak (Nimwegen *et al*., 1999; Wilke, Wang, Ofria, Lenski, & Adami, 2001). Secondly, as a result of their tiny genomes, large population sizes, and high mutation rates, quasispecies populations can 'experience' all possible neutral mutations diverging from the master sequence (Comas, Moya, & González-Candelas, 2005) (Fig. 5).

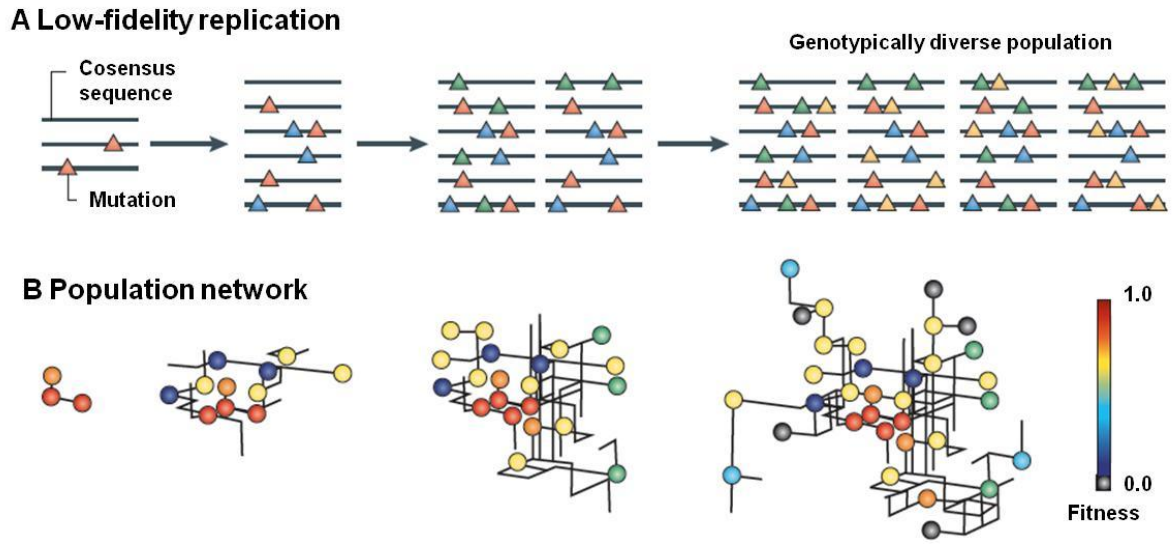


Fig. 5. Representation of a quasispecies viral population.

(A) *In a quasispecies the consensus sequence (or "master sequence") is represented as the average sequence of a highly heterogeneous genetic population. Genetic diversity, which is a characteristic feature of RNA viruses, results from low-fidelity replication ensured by RNA polymerases, which lack proof-reading function. Mutations acquired in each replication cycle are represented by differently colored triangles. In* ***(B)****, RNA virus populations can be depicted as networks in which genetic variants (circles) of varying fitness are connected to each other (From Lauring et al., 2013).*

All these features imply a significant adaptation advantage for a virus, because the simultaneous presence of multiple variant genomes allows for the rapid selection of the mutant(s) with better fitness for any new environment condition (Hayden, Ferrada, & Wagner, 2011; Lauring & Andino, 2010; Lauring *et al*., 2013; Martell *et al*., 1992; Simmonds, 2004). In this way, although continuously balanced by fitness constraints (no all mutants are viable), pre-adapted, or 'exapted' variants in a quasispecies would be able, for example, to escape from neutralizing antibodies, or from cytotoxic T lymphocytes the host produces at a given time point (see also paragraphs C-4.b; D-2). Furthermore, other variants would be able to resist antiviral agents (Wohnsland, Hofmann, & Sarrazin, 2007), or even be able to replicate better in a different tissue or host (Domingo *et al*., 1998; Hayden, Ferrada, & Wagner, 2011;Lauring *et al*., 2013; Schneider & Roossinck, 2001; Vignuzzi, Stone,

Arnold, Cameron, & Andino, 2006). In summary, HCV's extensive genetic heterogeneity plays a crucial role in the rapid and effective adaptation to dynamic environments, and so, it has profound implications in its biological features, such as persistence in infected individuals, resistance to treatment, different tissue tropism, and failure of experimental vaccines.

4. HCV Life Cycle

a) Cell Culture Systems to Study HCV Biology

The cell culture systems allowing an efficient viral propagation *in vitro* have been a major focus of research to understand the complex interplay between HCV and its host cells. The establishment of subgenomic replicons in cultured human hepatoma cells was a first major breakthrough in this field (Lohmann *et al*., 1999). In its first application, it consisted of a bicistronic RNA of genotype 1b (Con1 isolate), lacking the structural genes (Core, E1 and E2), p7 and NS2, and encoding a neomycin resistance gene under the control of the HCV internal ribosomal entry site, followed by a second IRES from encephalomyocarditis virus (EMCV) which controlled the expression of the NS3-NS5B genes (Fig. 6). With this system, the study of the replication process of the HCV life cycle became entirely accessible, and separable from all the other steps of the viral life cycle. Subsequently, the structure of the replicon, as described above, has undergone several changes over time to accommodate the special needs of different research groups (Steinmann & Pietschmann, 2013).

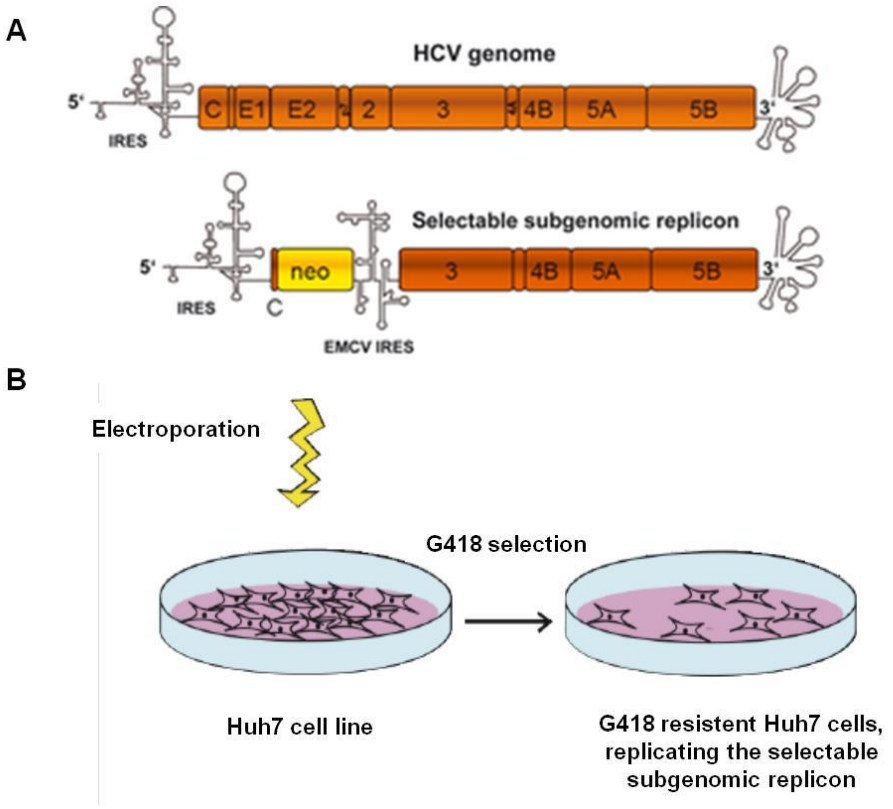


Fig. 6. Subgenomic replicon system.

(A) *HCV replicon systems allow for productive viral RNA replication in cell culture. Bicistronic replicon RNA, encoding a selectable marker (Neo^r) under control of the HCV IRES in the first cistron and the HCV replicase proteins (NS3-NS5B) under control of heterologous IRES from encephalomyocarditis virus (EMCV) in the second cistron, is delivered to Huh7 cell line by electroporation* ***(B)****. Replication of this replicon leads to the production of the selectable marker protein, thus allowing for selection of colonies containing active RNA replication (Lohmann et al., 1999).*

Before the advent of an efficient cell culture system encompassing the entire life cycle of the virus, HCV retroviral pseudoparticles (HCVpps) were the first robust system used to investigate early steps of HCV infection, and to study the role of HCV glycoproteins in virus entry into host cells (Bartosch, Dubuisson, & Cosset, 2003a; Drummer, Maerz, & Poumbourios, 2003; Hsu *et al*., 2003). This system is based on the co-transfection of human embryonic kidney (HEK)-293T cells with expression vectors encoding HCV E1 and E2, the gag-pol proteins of either murine leukemia virus (MLV) or human immunodeficiency

virus (HIV), and a retroviral genome encoding a reporter gene (Fig. 7). When a HCVpp enters a cell, it delivers the retroviral nucleocapsid into the cytosol, which is followed by reverse transcription and integration of the viral genome into the host cell genome. The reporter gene is then expressed from the integrated provirus making detection of a successful entry step into the target cell simple and reproducible. Almost all of the viral entry attachment factors and receptors known today have been identified or confirmed using this system. Indeed, the main application of the HCVpp system remains the study of interactions between a functional HCV E1-E2 complex, incorporated into the envelope of these particles, and cellular receptors. Therefore, this system is also a valid instrument to test neutralization effects of antibodies targeting the viral glycoproteins, as well as of sera sampled from infected patients (Cai *et al*., 2005; Hsu *et al*., 2003). Several studies aimed at the characterization of patient-derived glycoproteins used and still use this simple and robust system (Bartosch *et al*., 2003b; Owsianka, Tarr, Juttla, Lavillette, & Bartosch, 2005; Tarr, Owsianka, Szwejk, Ball, & Patel, 2007; Vieyres & Pietschmann, 2013). Although useful for studying early steps of HCV life cycle, this system has some limitations. The most important is that HCVpps are not associated with any host lipoproteins, because they are produced in a non-liver cell line (HEK-293T) and because they assemble in post-Golgi compartments and/or plasma membrane as retroviruses do. Therefore, these particles cannot overall mimic serum-derived particles (Burlone & Budkowska, 2009).

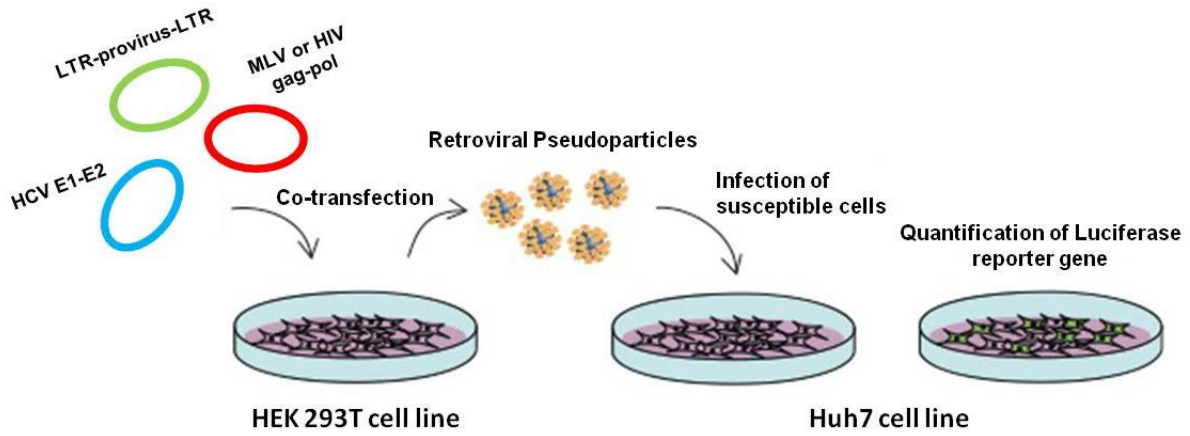


Fig. 7. The HCVpp model system.

The HCV pseudoparticle system provides a method to investigate glycoprotein-mediated early steps of viral life cycle. The principle of this system is based on co-transfection of vectors allowing the assembly of a retroviral particle and a vector encoding the HCV envelope glycoproteins. Entry of those particles into target cells, strictly dependent on the HCV functional glycoproteins on their surface, is followed by the detection of a reporter gene carried by the retroviral vector (Bartosch et al., 2003; Drummer et al., 2003; Hsu et al., 2003).

A few years after the construction by Kato and colleagues in 2001, of a very highly efficient subgenomic replicon derived from a *J*apanese patient suffering from a *f*ulminant *h*epatitis, and thus termed "JFH1" (Kato *et al*., 2001), three groups reported that the complete wild type JFH1 genome (genotype 2a) replicated efficiently in Huh7.5 cells, producing infectious viral progeny both in tissue culture and in animal models, and without the requirement of adaptive mutations (Lindenbach *et al*., 2005; Wakita *et al*., 2005; Zhong *et al*., 2005). These particles were designated cell culture-derived HCV (HCVcc), and since then, they are routinely used in many laboratories. With this system, all steps of the viral life cycle become accessible, including viral entry, replication, genome packaging, virion assembly, maturation, and release. The HCVcc system is based on the transfection of permissive human hepatoma cell lines with the full-length viral JFH1 genome. This leads to translation and replication of the viral RNA, giving rise to the production of viral particles

that are able to infect new target cells, thereby completing the whole viral life cycle of HCV (Fig. 8). This system has two major disadvantages. First, it has been shown that hepatoma cell lines routinely used to produce HCVcc, lack very-low-density lipoprotein secretion (Burlone & Budkowska, 2009; Jammart *et al*., 2013; Meex, Andreo, Sparks, & Fisher, 2011). Therefore, virions produced in cell culture cannot completely mimic actual physicochemical properties of patient-derived LVPs. Indeed, it has been demonstrated that cell culture-derived HCV particles have a lower specific infectivity than viruses produced in infected animals (Lindenbach *et al*., 2006). Actually, Lindenbach and colleagues found that a majority of particles produced *in vitro* had a buoyant density near 1.14g/ml, whereas animal-derived HCV particles had a peak buoyant density ≤1.10g/ml, more close to the density of most infectious LVPs circulating in patient's sera. A second limitation of the HCVcc system is that it is essentially restricted to the JFH1 strain, which belongs to genotype 2a. To overcome this restraint, a comprehensive panel of chimeric genomes was constructed by combining the JFH1 clone with heterologous strains of all the main HCV genotypes (Bungyoku *et al*., 2009; Gottwein *et al*., 2007, 2009; Jensen *et al*., 2008; Kaul, Woerz, Meuleman, Leroux-Roels, & Bartenschlager, 2007; Pietschmann *et al*., 2006; Scheel *et al*., 2008). The best strategy to engineer such chimeras was proven to be the exchange of the region encoding the Core up to NS2 proteins in the JFH1 isolate, which are required for viral morphogenensis, with the corresponding regions derived from other HCV genotypes. With this approach, the proteins necessary for generating the replicase complex and the non-translated regions remain those of the highly efficient JFH1 strain. Nevertheless, the construction of viable JFH1-based chimeras in which sequences encoding NS3/4A or NS5A were replaced with homologous sequences of other genotypes has recently been reported (Scheel, Gottwein, Mikkelsen, Jensen, & Bukh, 2011; Scheel, Gottwein, Carlsen, et al., 2011), proving this to be a valuable tool for the study of responses to the recently developed antiviral molecules.

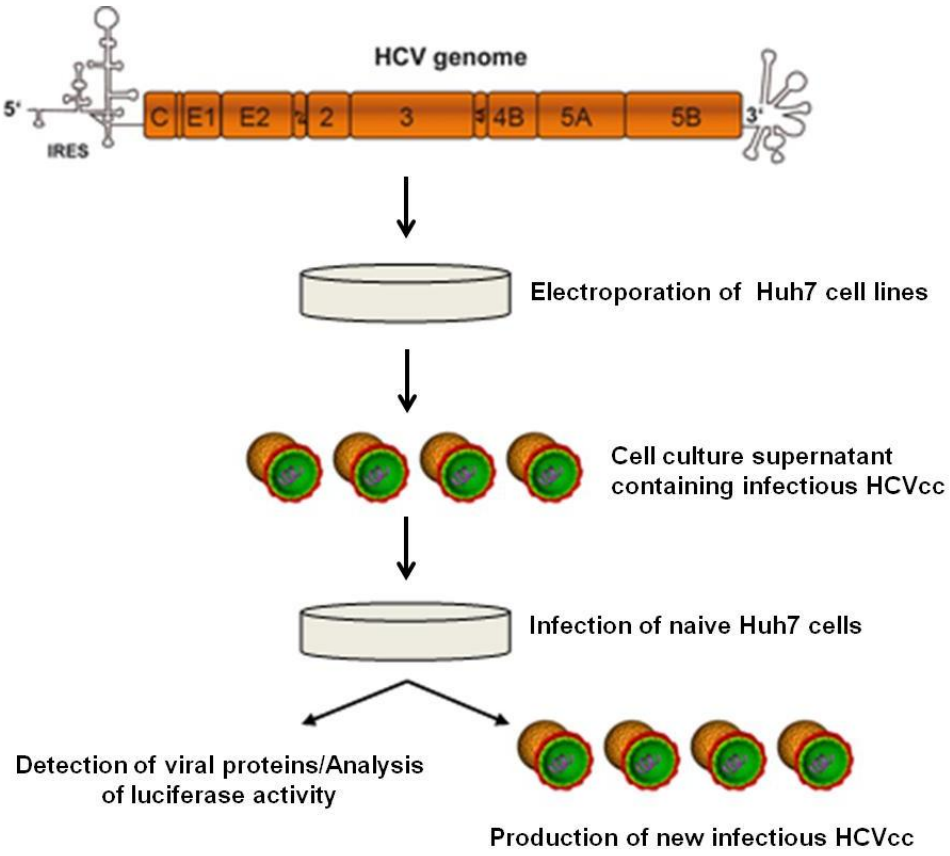


Fig. 8. The HCVcc model system.

The HCVcc system uses either JFH1 HCV genomic RNA or chimeras of this genome harboring heterologous sequences. These genomic RNAs are electroporated into permissive cell lines, leading to production of HCV virions, which can infect naïve cells or animal models. Productive infection can be monitored by detection of the expression of several viral proteins, by a number of reporter genes, or by direct measurement of viral RNA. (Lindenbach et al. 2005; Wakita et al. 2005; Zhong et al. 2005).

b) Early Steps in HCV Life Cycle: from the Bloodstream to the Hepatocyte

In order to establish productive infection within its primary site of replication, the hepatocytes, HCV must be able to transfer from the bloodstream through the sinusoidal liver endothelium and then into the liver. How this process occurs still remains elusive, but increasing evidence is available for what looks like a complex and multistep mechanism. The liver is a big organ composed of many different cell types which may be divided into

parenchymal cells (hepatocytes) and non-parenchymal cells (sinusoidal endothelial cells, Kupffer cells, hepatic stellate and dendritic cells, etc.) (Ishibashi, Nakamura, Komori, Migita, & Shimoda, 2009). The portal vein has the task to bring enriched blood from intestine, splanchnic circulation and other organs to the liver, and it can also be the front gateway for pathogens like HCV (Perrault & Pécheur, 2009). Forming the so called 'sinusoid capillaries', the portal vein surrounds the hepatocytes, which are organized as hexagonal bricks, each composed of approximately 15-25 cells, in the hepatic lobule (Ishibashi et al., 2009). Endothelial cells compose the walls of the hepatic sinusoid capillaries, forming a fenestrated endothelium, from which solutes and pathogens can reach the 'Space of Disse', lined with hepatic stellate cells which play a major role in the production of the hepatic extracellular matrix (ECM) (Perrault & Pécheur, 2009) (Fig. 9A). The liver endothelium plays a central and active role in regulating the exchange of macromolecules, solutes and fluids between blood and liver tissue, according to their size, charge and chemistry (Bartosch & Dubuisson, 2010). A set of capture receptors called C-type lectins, expressed on liver endothelium and/or dendritic cells, have been demonstrated to mediate uptake of several viruses, resulting in their transcytosis across the liver endothelial barrier, and transfer of the pathogen to the target host cells (Bobardt et al., 2007; Breiner, Schaller, & Knolle, 2001; Z. B. Zhu et al., 2004).

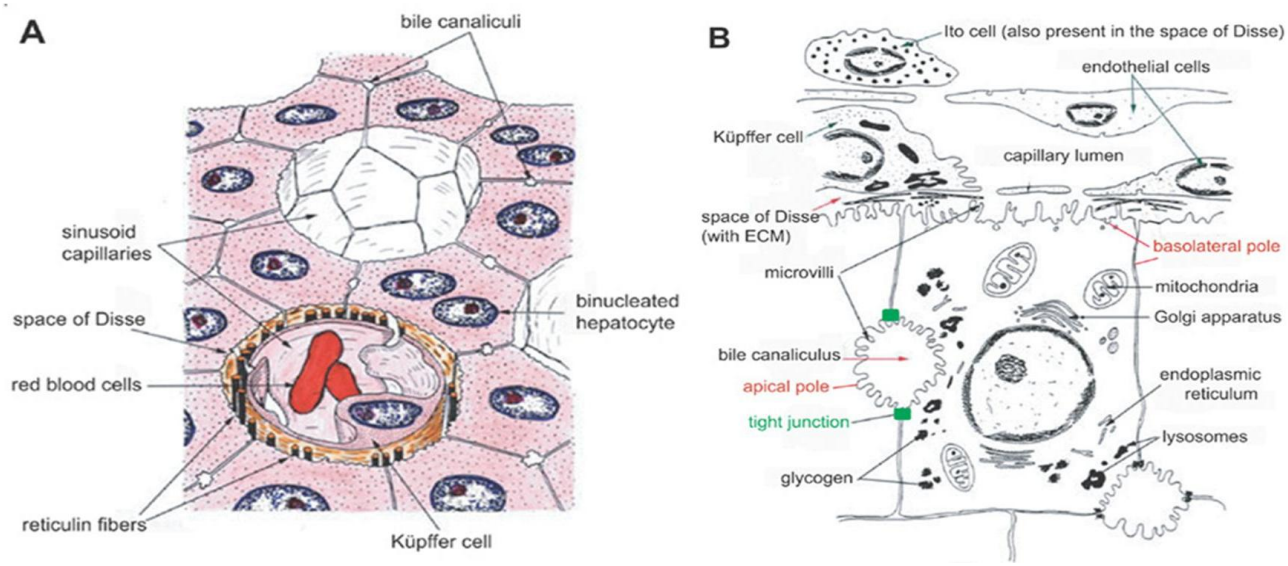


Fig. 9. Hepatic lobule (A) and hepatocytes (B) architecture.

(From Perrault & Pécheur, 2009).

It has been shown that two of these receptors, the dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), and the liver/lymph node-specific intercellular adhesion molecule 3-grabbing integrin (L-SIGN), which recognize high-mannose carbohydrates, can interact with the HCV glycoproteins, suggesting a role in capture of HCV from the blood and in transcytosing it to the underlying hepatocytes. In this way, HCV should be able to come close to its target cells (Cormier *et al*., 2004; Falkowska *et al*., 2006; Gardner *et al*., 2003; Lozach *et al*., 2004) (Fig. 10). Hepatocytes represent a unique kind of epithelial cell because of their polygonal architecture (Perrault & Pécheur, 2009). Each cell has two highly structured and specialized sides, one in contact with sinusoidal endothelium (sinusoidal face or basolateral pole), characterized by abundant microvilli, and the other in contact with neighboring hepatocytes (lateral face), of whom a portion is modified to form bile canaliculi, thus constituting the apical membrane delimited by tight junctions (TJs) (Perrault & Pécheur, 2009) (Fig. 9B). Therefore, once passed through the liver sinusoidal endothelium, HCV must cross the 'space of Disse', which contain the ECM (Bedossa & Paradis, 2003), and which is a

zone of active exchange between blood and hepatocytes. Hepatic ECM is mainly composed of collagens, glycoproteins, glycosaminoglycans (GAGs) and proteoglycans such as heparin-sulfates (HSPG) (Perrault & Pécheur, 2009). Interestingly, several viruses were found to interact with elements of the ECM (Spillmann, 2001; Vivès, Lortat-Jacob, & Fender, 2006), and among *Flaviviridae*, the *Dengue virus* and the *Classical swine fever virus* were reported to bind to HSPG (Germi *et al*., 2002; Hilgard & Stockert, 2000). Highly sulfated HSPG were also reported to play a role in HCV entry, maybe through direct interaction with the envelope glycoproteins and the apoE (Barth *et al*., 2006; Haberstroh *et al*., 2008; Jiang *et al*., 2012) (Fig. 10). Then, the hybrid nature of the HCV particle closely associated to serum β-lipoproteins drove Agnello and colleagues to evaluate the role of low-density lipoprotein receptor (LDL-R) as an HCV co-factor (Agnello, Abel, Elfahal, Knight, & Zhang, 1999). Indeed, physiologically, LDL-R transports cholesterol-rich LDL particles from extracellular medium into cells by clathrin-dependent endocytosis. More recently, the role of LDL-R in early steps of HCV infection was also demonstrated using plasma HCV particles and primary human hepatocytes (PHH) (Molina *et al*., 2007). However, LDL-R expression is not restricted to hepatocytes and its ectopical expression does not restore cell susceptibility to HCV infection, thus indicating that other 'players' are necessary to mediate HCV entry (Bartosch *et al*., 2003b; 2003c; Hsu *et al*., 2003). The scavenge receptor class B type 1 (SR-BI), also called CLA-1, functions as a lipoprotein receptor at both basolateral and apical membranes of hepatocytes. It mediates selective uptake of cholesteryl ester from apoA containing lipoproteins (in particular high density lipoprotein, HDL), by a process of cellular internalization, leading to removal of cholesterol and recycling of the protein part of HDL (Connelly, 1999; Silver, Wang, Xiao, & Tall, 2001). Cross-linking studies using a soluble form of E2 (sE2), have lead to the identification of SR-BI as a direct binding partner of E2, an interaction requiring the hypervariable region 1 (HVR1) of the protein (Scarselli *et al*., 2002).

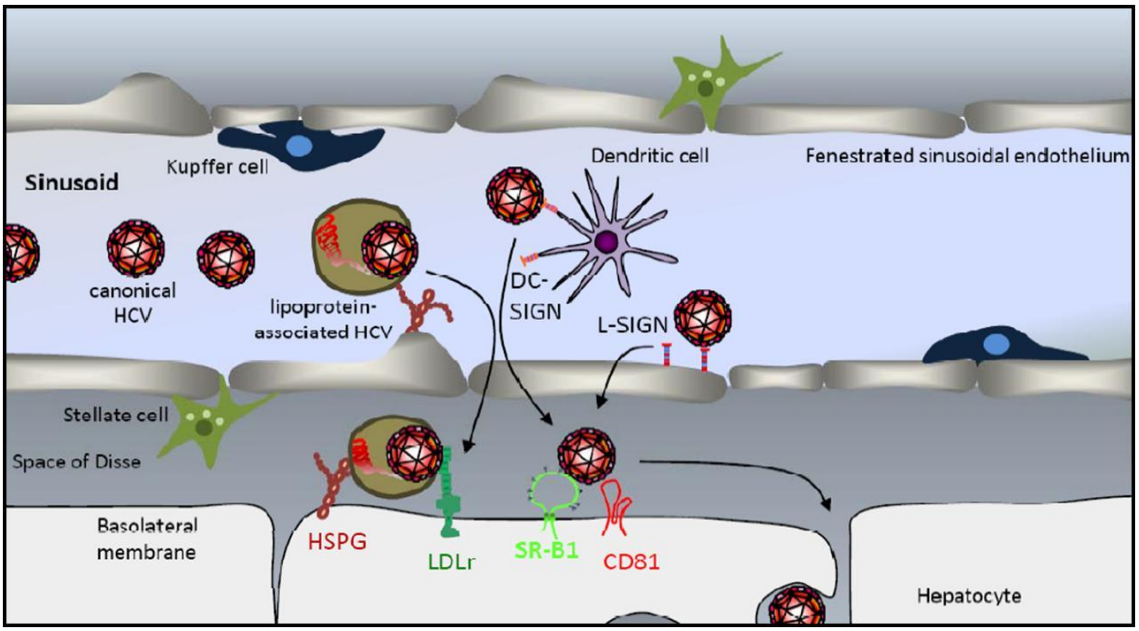


Fig. 10. HCV liver uptake.

C-type lectins (DC-SIGN and L-SIGN), expressed on liver endothelium and/or dendritic cells mediate uptake of HCV, resulting in its transfer to the target host cells via transcytosis. Within the 'space of Disse', at the basolateral membrane of hepatocytes, HCV interacts with glycosaminoglycans (GAGs), heparin-sulfate proteoglycans (HSPG), low-density loporotein receptor (LDL-R) and more specifically with the scavenger receptor calls B type 1 (SR-B1) and the CD81 tetraspanin. (From Bartosch & Dubuisson, 2011).

Two recent studies showed that SR-BI acts at different steps during the HCV entry process. Indeed, SR-BI might first interact with the lipoprotein component of the viral particle, and in an E2 independent manner, while later during the entry process, the interaction between SR-BI and HCV would be directly dependent on the glycoprotein (Dao Thi *et al*., 2012; Zahid *et al*., 2013). In summary, several binding studies using infectious viral particles suggested that HSPG, LDL-R, and SR-BI contribute to non-specific viral attachment (Albecka *et al*., 2012; Dao Thi *et al*., 2012; Koutsoudakis *et al*., 2006), thus forming a docking area at the hepatocytes membrane, from which the virus could then specifically interact with other cellular receptors, leading to a series of membranous molecular rearrangements resulting in the virus internalization (see below) (Fig. 10).

c) HCV Entry into Hepatocytes

The first HCV factor shown to be required for HCV cell entry was the CD81 (Pileri, 1998). CD81 is a member of the tetraspanin superfamily and is ubiquitously expressed. Key feature of tetraspanins is the formation of an extended network at the cell surface, called "tetraspanin web", which is thought to structure the membrane by coordinating interactions between the tetraspanins themselves and several other classes of proteins. In the liver, CD81 is expressed both on sinusoidal endothelium and on hepatocytes, where it is mainly localized in the basolateral membrane (Reynolds *et al*., 2008). CD81 ectodomain is formed by two extracellular loops, a smaller (SEL) and a larger (LEL) one, with the latter involved in the interaction with specific regions of E2 (see paragraph C-1) (Pileri, 1998). Several studies reported that infection of PHH or hepatoma cell lines was inhibited by anti-CD81 antibodies (Bartosch *et al*., 2003b; Hsu *et al*., 2003; Lindenbach *et al*., 2005; Molina *et al*., 2007; Wakita *et al*., 2005) and by downregulation of the tetraspanin using a siRNA approach (Zhang *et al*., 2004a). Others showed that ectopic expression of CD81 in CD81-negative hepatoma cell lines rendered them permissive to HCVpp bearing envelope glycoproteins representative for all genotypes (Bartosch *et al*., 2003b; Lavillette *et al*., 2005; Zhang *et al*., 2004a), and moreover, it increased HCV infectivity in already permissive cell lines (Koutsoudakis *et al*., 2006). Altogether, these data confirmed that CD81 is an essential factor for HCV entry. Yet, at which step of the HCV entry process the tetraspanin CD81 is involved remains unclear. Recently, the idea is emerging that HCV internalization could involve a complex interplay between CD81 and SR-BI. Indeed, both molecules were found to act in a cooperative manner to govern internalization of HCVcc in Huh7 cells (Kapadia, Barth, Baumert, McKeating, & Chisari, 2007). Further investigations showed that HCVcc binds to cells non-permissive for HCV infection only when SR-BI was expressed, but not CD81 (Evans *et al*., 2007). Moreover, using antibodies against CD81 and SR-BI, Zeisel and

colleagues showed that the kinetics of the inhibition of HCV infection were comparable for both antibodies, suggesting that activity of both CD81 and SR-BI are closely linked to mediate the HCV entry step (Zeisel *et al*., 2007). Taken together, these data suggest that a first contact with SR-BI might be necessary before the particle interacts with CD81. Interestingly, a protein expressed ubiquitously but not in hepatocytes, able to interact with CD81, has been found to inhibit HCV-CD81 interactions, thus blocking HCV cell entry (Rocha-Perugini *et al*., 2008). This is so far the only cellular co-factor that could explain the hepatotropism of HCV.

Besides SR-BI and CD81, two additional factors have been recently identified as essential for HCV entry, claudin-1 (CLDN1) and occluding (OCLN) (Evans *et al*., 2007; Ploss *et al*., 2009). Both, CLDN1 and OCLN are components of the cellular TJs, which act as seals between neighboring cells, separating apical from basolateral membranes of hepatocytes (Fig. 9B). The precise role of CLDN1 and OCLN in HCV cell entry is not yet well understood; indeed, no direct interaction between HCV virions and the two proteins has been demonstrated. Nevertheless, blocking experiments with antibodies directed against CLDN1 indicated that this protein is used late in the entry process, at the time of virion internalization, probably soon after or concomitantly to virus interaction with CD81 (Evans *et al*., 2007; Fofana *et al*., 2012; Krieger *et al*., 2010). On the other hand, expression of human OCLN in mouse cells removed the species-specific restriction of HCV entry and allowed development of a mouse model susceptible to HCV infection (Dorner *et al*., 2011). The involvement of OCLN in the HCV entry process was further demonstrated by two other studies using HCVcc and a knock down approach, which showed that the expression of OCLN at TJ region is necessary for the HCV membrane fusion process (Benedicto *et al*., 2009; Liu *et al*., 2009). Since, in polarized hepatocytes, both CLDN1 and OCLN are mainly sequestered at TJ complexes between adjacent hepatocytes, which are not accessible to HCV circulating in the

bloodstream, it is not clear how such proteins become accessible to incoming virions. Recently, the mechanism by which group B coxsackieviruses (CVB) initiate infection in the highly polarized epithelial cells has been described (Coyne & Bergelson, 2006). In particular, the virus attachment to DAF (decay-accelerating factor), located at the accessible apical membrane of epithelium, activates Abl kinase, triggering Rac-dependent actin rearrangements that permit virus movement to the TJ. Within the junction, interaction with CAR (coxsackievirus and adenovirus receptor), promotes conformational changes in the virus capsid, essential for virus entry and release of viral RNA. Similarities with the polarized structure of hepatocytes have lead to the hypothesis that a similar mechanism might underlie the entry process of HCV. Interestingly, CD81 engagement, either by CD81 antibodies or soluble E2 protein, has been shown to activate Rho GTPase, which mediates actin-dependent relocalization of such CD81 complexes to TJ regions (Brazzoli *et al*., 2008). Furthermore, Lupberger and colleagues recently reported that two receptor tyrosine kinases, the epidermal growth factor (EGFR) and the ephrine receptor A2 (EphA2), regulate the formation of CD81-CLDN1 complexes essential for HCV entry and membrane fusion (Lupberger *et al*., 2011), proving that HCV might exploit CD81-CLDN1-mediated signaling pathways to surmount the TJ barrier. Finally, since the HCV virion is rich in cholesterol, the role of cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) was recently investigated and it was identified as an additional entry factor, although its exact role remains to be determined (Sainz *et al*., 2012). Actually, it has been shown that NPC1L1 silencing or its antibody-mediated blocking can impair HCVcc infection (Sainz *et al*., 2012). To summarize, altogether, these data suggest the following hypothetical HCV cell entry model (Fig. 11): early and non-specific interactions with molecules such as DC-SIGN, L-SIGN and then GAG, HSPG and LDL-R, allow the passage of HCV particles, associated with lipoproteins, from the bloodstream to hepatocytes. These early interactions likely have the task of concentrating

virions at the basolateral membrane of the hepatocytes. As a result, more specific interactions between HCV and the co-receptors SR-BI and CD81 lead to the activation of intracellular signaling pathways, with the consequence of an actin-dependent transport of the virion into the TJ region, where endocytosis might be mediated by interactions with CLDN1 and OCLN (Ploss & Evans, 2012; Zeisel, Felmlee, & Baumert, 2013).

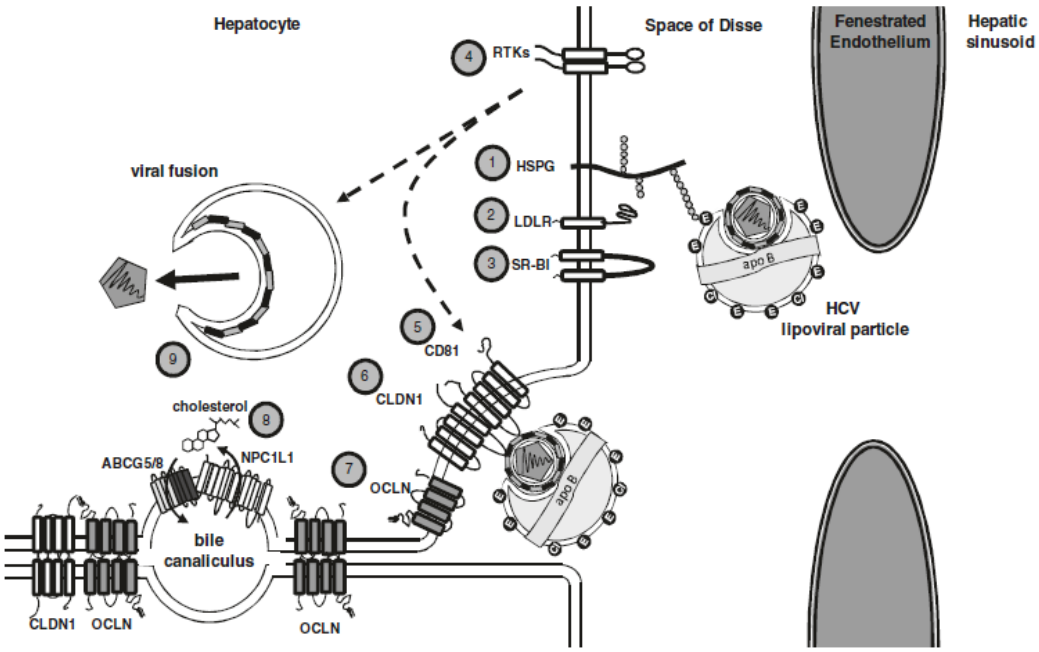


Fig. 11. Hypothetical HCV entry mechanism into hepatocytes.

Early, non-specific interactions first with DC-SIGN, LC-SIGN and GAGs in the liver endothelium, and then with HSPG and LDL-R in the "Space of Disse" facilitate the recruitment of HCV particles to the basolateral membrane of hepatocytes. Here, virions can specifically bind to several receptors such as SR-BI and CD81. Such interactions should activate intracellular signaling pathways via receptor tyrosine kinases as EGFR and EphA2, which allow the accessibility to TJ proteins, CLDN1 and OCLD, resulting in the internalization of virions. (From Zeisel et al. 2013)

d) HCV Internalization and Membrane Fusion

Similar to other flaviviruses, HCV entry is thought to be mediated by clathrin-dependent endocytosis, with subsequent delivery of the nucleocapsid via early endosomes (Fig. 12). Indeed, this mechanism has been demonstrated by using siRNAs targeting clathrin (Blanchard *et al*., 2006; Meertens, Bertaux, & Dragic, 2006). Furthermore, use of endosome acidification inhibitors showed that HCV entry is pH dependent (Blanchard *et al*., 2006; Hsu *et al*., 2003; Koutsoudakis *et al*., 2006; Meertens *et al*., 2006; Tscherne *et al*., 2006). Interestingly, exposure of virions bound to a cell surface to acid pH, followed by a return to neutral pH, does not affect HCV infectivity (Meertens *et al*., 2006; Tscherne *et al*., 2006), suggesting that, besides low pH exposure, additional triggers or modifications are required to activate the fusion machinery. Although controversial, it is currently thought that HCV envelope glycoproteins have some features reminiscent of those of class II fusion proteins (Penin, Dubuisson, Rey, Moradpour, & Pawlotsky, 2004) (see paragraph C-1). Therefore, the mechanism proposed for the HCV fusion process, relies on a rearrangement of the E1E2 heterodimer into its fusogenic form, probably triggered by the acidification of early endosomes, leading to the formation of the fusion pore and release of nucleocapsid, and thus of viral RNA into the cytoplasm (Fig. 12) (Lavillette *et al*., 2006). None of the HCV receptors has been shown to mediate this viral fusion mechanism, but recent data suggested that HCV cell entry is also dependent on an intact microtubule network (Roohvand *et al*., 2009). Translation of the positive-stranded RNA begins immediately afterwards, with the subnsequent formation of the immature polyprotein at the rough endoplasmic reticulum (rER) (see below).

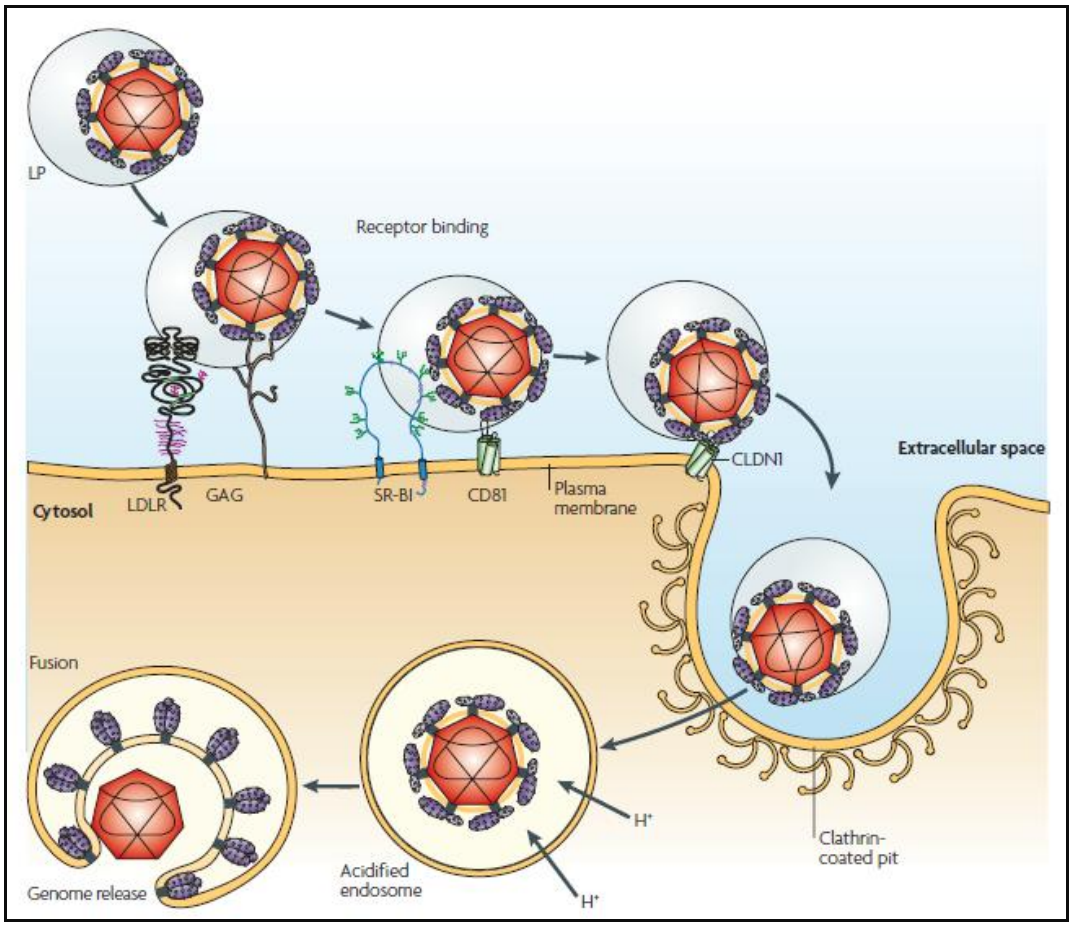


Fig. 12. HCV fusion mechanism.

HCV enters the cell by clathrin-dependent endocytosis and, upon acidification, fusion of the viral envelope, presumably with the membrane of an early endosome, leads to the release of the viral nucleocapsid into the cytoplasm. (From Moradpour, Penin & Rice, 2007).

e) Viral Dissemination via Cell-to-Cell Contacts

It has recently been proposed that upon initiation of hepatocyte infection by 'cell-free' HCV virions entering the liver through the bloodstream, virus dissemination within the liver and establishment of chronic HCV infection could also be achieved by direct viral 'cell-to-cell' transmission between adjacent hepatocytes (Timpe *et al*., 2008). Indeed, it has been reported that HCV cell-to-cell transmission *in vitro* should be more efficient than cell-free particle entry (Brimacombe *et al*., 2011; Timpe *et al*., 2008). Moreover, in contrast to

cell-free HCV particle transmission, this process seems to be more resistant to the majority of neutralizing antibodies, probably contributing to evasion from the host humoral immune responses (Brimacombe *et al*., 2011; Timpe *et al*., 2008) (see also paragraph C-4.b). Regarding the molecular mechanism, HCV cell-to-cell transmission appears to require almost all the host factors so far described for the cell-free entry process, namely CD81, SR-BI, CLDN1, OCLN, EGFR, EphA2, and possibly NPC1L1 (Brimacombe *et al*., 2011; Lupberger *et al*., 2011; Sainz *et al*., 2012; Timpe *et al*., 2008). However, it is worth noting that, in contrast to cell-free virus entry, cell-to-cell transmission pathways can occur in a CD81-independent manner (Jones *et al*., 2010; Witteveldt *et al*., 2009). Conversely, targeting SR-BI allows to inhibit HCV spread (Brimacombe *et al*., 2011; Meuleman *et al*., 2012; Zahid *et al*., 2013), whereas its overexpression in HCV permissive cells increases virus dissemination (Brimacombe *et al*., 2011), thus suggesting a relevant role of this latter co-receptor in HCV cell-to-cell transmission.

f) Replication, Assembly and Particle Release

After infection of a cell, the positive-strand RNA genome of HCV directly serves as the template for translation in the cytosol. Due to the presence of an IRES in the 5'UTR of the viral RNA, the HCV genome can bypass the need for nuclear processing events like capping, thus directly recruiting the translation apparatus of the infected cell to start the translation of the viral polyproteins (Tsukiyama-Kohara, Iizuka, Kohara, & Nomoto, 1992; Wang, Sarnow, & Siddiqui, 1993). Translation takes place at the rER where host and viral proteases catalyze cleavage of the viral polyproteins (Gosert *et al*., 2003). Although the precise mechanism of HCV replication is still poorly understood, the most accredited model proposes the formation of a replication complex (RC) at the ER (Fig. 13A), consisting of the

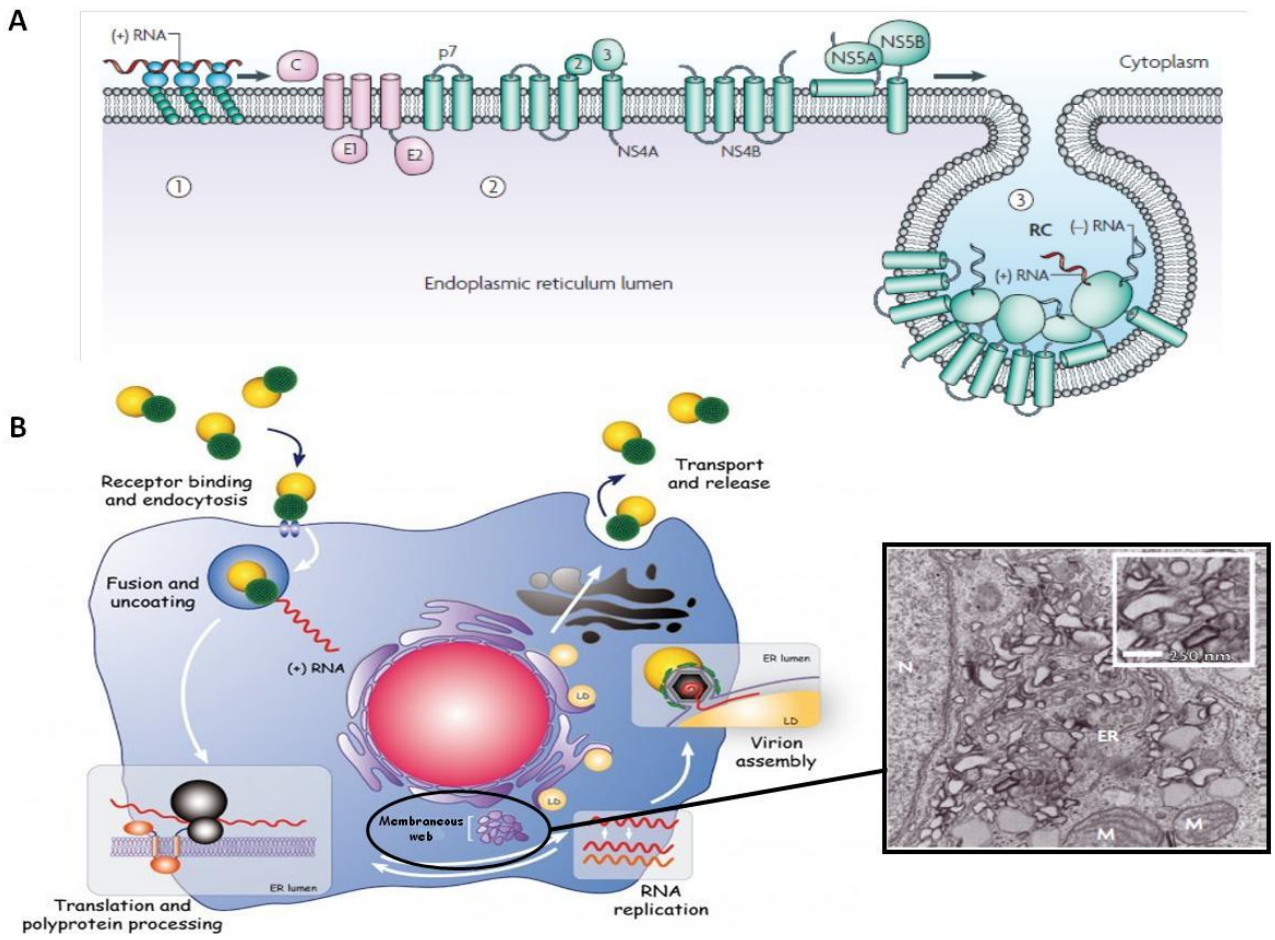


Fig. 13. (A) HCV replication complex and (B) the hypothetical virus life cycle.

(A) *Upon release of viral genomic RNA into the cytosol of an infected cell (1), the viral genome is translated into a polyprotein which is processed by viral and host proteases, thus forming the structural (pink) and non-structural (green) proteins (2). The viral NS4B induces membrane alterations, called "membraneous web", which probably serve as a scaffold for the viral replication complex (RC) (3).* ***(B)*** *HCV LVPs enter the hepatocyte following a tightly controlled multistep process, leading to their endocytosis into early endosomes, and fusion. The released positive-strand viral RNA can be immediately translated into a polyprotein at the ER membrane, where mainly the non-structural proteins NS3 to NS5B form the replication complex, thus altering the ER membrane structure (Gossert et al., 2003). An electron microscopy image of the "membranous web" is presented in the picture. After accumulation of newly synthesized genomic RNA and viral proteins, the HCV particle is assembled in an ER-related compartement, in close connection with the VLDL biogenesis process (see also Fig. 14). Then, HCV particles, associated with lipoproteins are exported through the Golgi and released. (Adapted from (A) Miller & Krijnse-Locker, 2008; (B)Popescu, 2010).*

non-structural proteins NS3-NS5B, both the positive- and negative-strand RNA, and cellular factors (Miller & Krijnse-Locker, 2008). This step of the viral life cycle is associated with a profound alteration of ER membranes, named the 'membranous web' (Appel, Schaller, Penin, & Bartenschlager, 2006; Egger *et al*., 2002; Gosert *et al*., 2003; Moradpour *et al*., 2003), in which the NS4B protein would be particularly involved (Egger et al., 2002; Gao, Aizaki, He, & Lai, 2004) (Fig. 13B). The first step of RNA synthesis generates a negative-strand RNA genome, which serves as template for progeny positive-strand RNA, which will be produced in large excess. The NS5B polymerase, probably in cooperation with additional viral and host factors, assembles at the 3'UTR of the template strand, thus initiating *de novo* synthesis of RNA (Appel *et al*., 2006; Friebe & Bartenschlager, 2002; Harrus *et al*., 2010). Newly synthesized positive-strand RNA is either used for a new round of RNA translation/replication, or is packaged into virus particles (Gu & Rice, 2010).

Little is known about late steps of the viral life cycle, but it likely involves E1E2 glycoproteins resident in the ER (Boson, Granio, Bartenschlager, & Cosset, 2011; Jirasko *et al*., 2010; Ma *et al*., 2011; Phan, Beran, Peters, Lorenz, & Lindenbach, 2009; Popescu *et al*., 2011; Stapleford & Lindenbach, 2011), the recruitment of lipid droplets (LD)-associated Core protein (Appel *et al*., 2008; Depla *et al*., 2010; Masaki *et al*., 2008; Miyanari *et al*., 2007), and several viral and host factors. Therefore, virus particle assembly may be intimately coordinated with the RNA replication process, as it is the case for other members of the *Flaviviridae* family (Khromykh, Varnavski, Petra, Westaway, & Sedlak, 2001; Welsch et al., 2009). Moreover, given the lipid nature of HCV particles, a possible link between VLDL metabolism and viral assembly has been proposed (Miyanari *et al*., 2007; Roingeard, Hourioux, Blanchard, & Prensier, 2008). To support the latter hypothesis, it has been shown that intracellular membranes containing the HCV RC are enriched in microsomal triacylglycerol transfer protein (MTP), apoB100 and apoE, which are

all essential for VLDL maturation and release from hepatocytes (Huang *et al*., 2007). Additionally, Gastaminza and colleagues demonstrated that the inhibition of MTP activity or the apoB down-regulation prevented the assembly of HCV infectious particles at an early stage (Gastaminza *et al*., 2008), thus suggesting an intimate connection between the two processes. For the latest steps of maturation and release of HCV particles, virions bud from the ER membranes, pass through the Golgi apparatus, where envelope glycoproteins undergo a series of biochemical modifications (Vieyres *et al*., 2010) (see also paragraph C-2), and the HCV particles acquire their low buoyant density, undergoing post-synthetic lipidation (Gastaminza *et al*., 2008; Gastaminza, Kapadia, & Chisari, 2006), to be finally released through the plasma membrane into the bloodstream (Fig. 14; 13B).

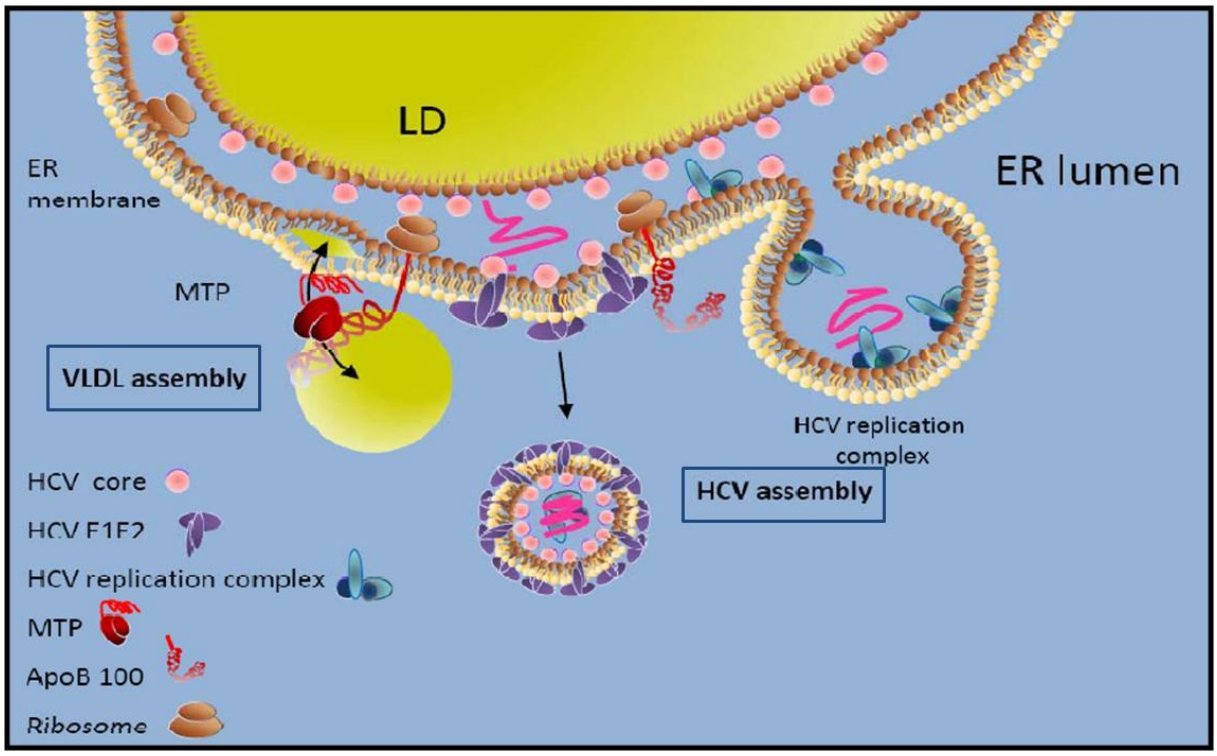


Fig. 14. HCV assembly is related to VLDL metabolism.

HCV assembly and VLDL biogenesis seem to be intimately connected processes. Both need the presence of MTP, apoB100 and apoE. HCV assembly requires at least the Core protein, the envelope glycoproteins and genome RNA. (Adapted from Bartosch & Dubuisson, 2010).

C. HCV ENVELOPE GLYCOPROTEINS E1 & E2

1. E1 and E2 Proteins: from Structure to Function

Anchored in the lipid bilayer, HCV harbors on its surface two glycoproteins, namely E1 (~31kDa) and E2 (~70kDa), respectively composed of about 191 and 361 amino acids. HCV glycoproteins are type I transmembrane proteins with highly glycosylated N-terminal ectodomains (aa 192-351 and 384-661, respectively), and hydrophobic C-terminal transmembrane (TM) domains, each composed of about 30 amino acids (aa 352-382 and 717-745 respectively) (Lindenbach & Rice, 2007; Op De Beeck & Dubuisson, 2003). HCV envelope glycoproteins play pivotal roles at different steps of the viral life cycle, including assembly of the infectious particle, virus entry into target cells, and fusion with the endosomal membrane (Penin *et al*., 2004). Furthermore, HCV envelope glycoproteins represent the main antigenic determinant of the infectious particle recognized by the humoral immune system, thus constituting a good candidate for a vaccine against the virus (Choo *et al*., 1994; Rosa *et al*., 1996). The genes encoding HCV envelope glycoproteins also harbor the most variable nucleotide sequences across the viral genome, varying extensively also within the same individual (Simmonds, 2004), thus rendering determining the rationale for an efficient vaccine a difficult task. For all these reasons, a detailed understanding of the structural and functional features of E1 and E2 proteins seems to be essential to fight HCV infection.

The E2 ectodomain includes the most variable sequences of the entire genome, namely the hypervariable regions HVR1, HVR2 and HVR3, and the intergenotypic variable region (IgVR) (Helle & Dubuisson, 2008; Law *et* al., 2008; McCaffrey, Gouklani, Boo, Poumbourios, & Drummer, 2011; Troesch *et al*., 2006). HVR1 (aa 384-410) is located at the

N-terminus of E2. It shows a very high degree of variation under pressure of the humoral responses of infected hosts (Edwards *et al*., 2012; Prentoe *et al*., 2011; Vieyres, Dubuisson, & Patel, 2011) (see also paragraphs C-4.b; D-2), although, in spite of its apparent hypervariability, HVR1 seems to preserve some positively charged residues at specific positions (Callens *et al*., 2005; Penin *et al*., 2000). Regarding the viral entry process, HVR1 directly interacts with the SR-BI cellular receptor, likely involving serum HDL (Bartosch *et al*., 2005; Dao Thi *et al*., 2012; Dreux *et al*., 2006; Voisset *et al*., 2005; Zeisel *et al*., 2007). A recent study reported that five residues at positions 14, 15, and 25-27 mediate binding of the E2 protein to the SR-BI (Guan *et al*., 2012). Actually, it has been proposed that HVR1 might modulate accessibility of at least two HCV receptors, CD81 and SR-BI, since its deletion increases E2 binding to CD81 (Roccasecca *et al*., 2003) whilst it abrogates the binding to SR-BI (Scarselli *et al*., 2002). In contrast to HVR1, HVR2 (aa 460-485) and the IgVR (aa 570-580) are not known to be a target of the humoral immune response (McCaffrey *et al*., 2011). However, a comprehensive study which investigated the involvement of these three variable regions in the expression and function of the E1E2 heterodimers showed that only deletion of HVR2 and/or IgVR affected E1E2 heterodimerization, and reduced the ability of HCVpp to bind the CD81 cellular receptor. Furthermore, when deleted in a JFH1 context, these two regions were able to abolish virion infectivity whilst they are dispensable for genome replication and translation (McCaffrey *et al*., 2011).Therefore, HVR2 and IgVR likely have principal roles in E1E2 assembly (Albecka *et al*., 2011). An additional highly variable region in HCV E2 glycoprotein is the HVR3 (aa 431-466), although it includes lower mean genetic distance among the three hypervariable regions (Troesch *et al*., 2006). This region has been empirically subdivided into two regions of roughly equal size, respectively termed HVR3a (aa 431-449) and HVR3b (aa 450-466). Regarding physicochemical properties and predicted

antigenicity of HVR3, it has been predicted by several *in silico* prediction algorithms, that the HVR3a might represents an antigenic region, exposed at the surface of E2, thus being accessible and recognizable by antibodies. This region should display a rather hydrophilic central portion flanked by generally hydrophobic sequences (Troesch *et al*., 2006). The ectodomain of E2 includes also the CD81-binding site, consisting of three non-contiguous regions at amino acidic positions 474-494, 522-551, and 612-620 (Drummer *et al*., 2006; Owsianka *et al*., 2006; Roccasecca *et al*., 2003; Rothwangl, Manicassamy, Uprichard, & Rong, 2008; Rychlowska *et al*., 2011). According to a recent reconstruction of the tertiary structure of the E2 glycoprotein, these residues should be closely assembled into the folded protein (Fig. 15) (Krey *et al*., 2010). Furthermore, sequence analyses of the two envelope glycoproteins suggested that E2 sequence might include the fusion peptide (Yagnik *et al*., 2000). Indeed, due to the similarities of HCV genome organization with that of other members of the *Flaviviridae* family, E2 has been proposed to be a class II fusion protein. Thus, as for other class II fusion proteins, the predicted secondary and tertiary structure of E2 revealed a three-domain architecture, with the core domain of the protein, the domain 1 (DI), organized as a 'β-sheet barrel'. This domain should be composed of 8 β-sheets, with an N-terminal extension represented by the HVR1 region, and it should contain most of the CD81-interacting residues. Moreover, DI would be highly glycosylated, including 5 of the 11 putative N-linked glycosilation sites of E2 (see below). Between the third and fourth β-sheets of DI, there should be a less structured domain, called domain 2 (DII), which includes two N-glycosylation sites, three disulfide bonds, the HVR2, and the second CD81-binding region. Furthermore this domain should also include the putative fusion loop (aa 502-520) (Krey *et al*., 2010). This disordered segment is mainly composed of non-charged residues, and is rich in glycine (Krey *et al*., 2010). Linked to the C-terminal side of DI by the IgVR segment, the third domain of the glycoprotein, DIII, should be organized

including three disulfide bonds and the last two glycosylation sites. It is thought that the IgVR region works as a flexible linker indispensable for the fusion process (Albecka *et al*., 2011; Krey *et al*., 2010). DIII also contains the third CD81-binding region, and it is followed by a relatively flexible but conserved region, called the "stem" (aa 652-715), which connects the ectodomain of E2 to its TM domain, and which contains also the last disulfide bond of the envelope protein. Interestingly, this region includes a heptad repeat region likely involved in the fusion process (Rychlowska *et al*., 2011). The architecture of the gene encoding E1 envelope glycoprotein is much less known. Some controversy on the identity of the HCV fusion peptide exists, and it has been postulated that the segment spanning residues 264-290 of the E1 ectodomain should contain a fusion peptide-like motif (Flint *et al*., 1999; Lavillette *et al*., 2007; Li, Huang, Ai, Chuang, & Chen, 2009). However, several discrepancies between HCV envelope proteins and class II fusion proteins remain. Indeed, contrary to what is observed for other class II fusion proteins, there is no evidence that HCV envelope glycoproteins are maturated by a cellular endoprotease during their transport through the secretory pathway (Op De Beeck *et al*., 2004). Moreover, HCV envelope proteins are highly glycosylated, whereas other class II fusion proteins contain a very low number of glycans (Lavie, Goffard, & Dubuisson, 2007).

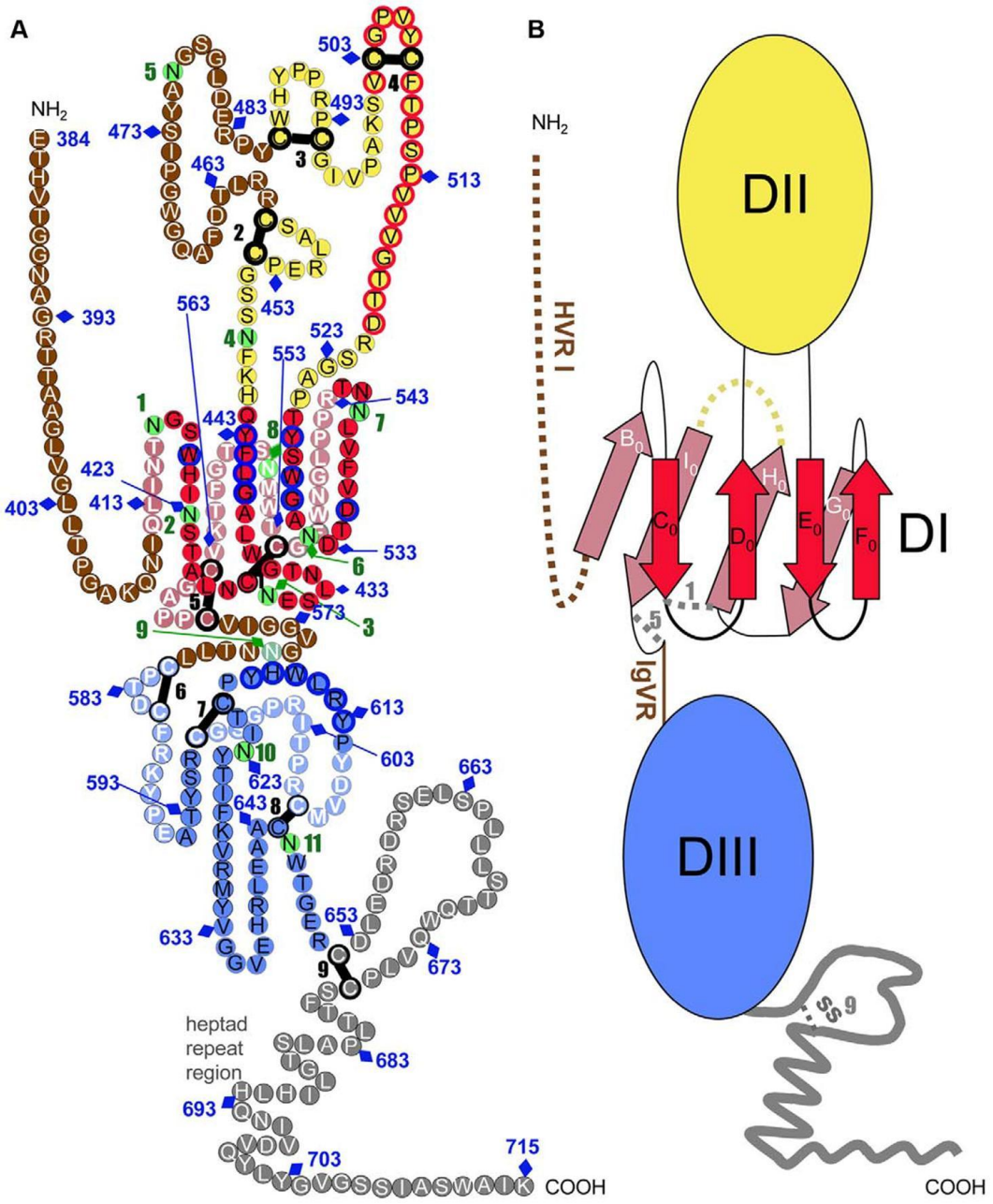


Fig. 15. Hypothetical secondary and tertiary structures of E2 ectodomain.

Sequence similarities between members of Flaviviridae family, and identification of disulfide bonds led to a model of the E2 ectodomain, consisting of three domains ***(B)****. DI consists of 8 β strands and is extended at the N-terminus by HVR1. DII includes HVR2 and the fusion peptide-like motif (aa 502-520). DIII, connected to DI by the IgVR, is in turn connected to the TM domain by the flexible 'stem region'. In* ***(A)*** *the linear sequence of the truncated form of H77 E2, numbered every 10 residues according to the polyprotein depicted. According to the model proposed by Krey and colleagues, disulfide bonds and glycosylation sites are indicated by thick black bars and green circles, respectively, and numbered sequentially. The unstructured segments (HVR1, HVR2 and IgVR) are in white font on a brown background. Residues participating in CD81 binding are contoured in blue, whereas those involved in the putative fusion peptide are contoured in red. (From Krey et al., 2010).*

2. Synthesis and Glycosylation of Envelope Proteins

During polyprotein translation, the two envelope glycoproteins are targeted to the ER by internal signal peptides (Cocquerel *et al*., 2002). Translocation involves an aqueous pore in the ER membrane, called translocon, through which secretory proteins and luminal domains of membrane proteins pass from the cytosol to the ER lumen (Martoglio, Hofmann, Brunner, & Dobberstein, 1995). The signal sequences which direct E1 and E2 proteins to the ER membrane have been identified and are located at N-terminal regions in the precursor polypeptides (Fig. 16A) (Cocquerel *et al*., 2002; Op De Beeck & Dubuisson, 2003). The TM domains of the HCV envelope glycoproteins have proved to be multifunctional, and essential for the E1E2 correct maturation. Indeed, they bring the signal sequence function, and the ER retention signals, but also, they act as membrane anchors, and are involved in E1E2 heterodimerization (Op De Beeck & Dubuisson, 2003). By analyzing E1E2 sequences from a large number of HCV isolates, it has been shown that the TM domains of both envelope glycoproteins are composed of two stretches of hydrophobic residues, separated by a short segment containing at least one fully conserved positively charged amino acid (Fig. 16A) (Cocquerel *et al*., 2000). Before signal sequence cleavage, the charged residue in the TM domain of E1 interacts with the translocon to allow the translocation of E2 across the ER (Fig. 16B) (Op De Beeck & Dubuisson, 2003). The C-terminus of the TM domains of both E1E2 proteins form hairpin structures with a double membrane-spanning topology (Fig. 16C). After cleavage, these regions change their orientation, reorganizing as single-spanning TM domains, with the charged residues being exposed towards the cytosol (Fig. 16B & C) (Cocquerel *et al*., 2000; Cocquerel *et al*., 2002; Op De Beeck & Dubuisson, 2003). As a consequence, signal peptides at the C-termini of E1 and E2 become part of their TM domains, now contributing to new functions, such as membrane anchoring, glycoprotein non-covalent

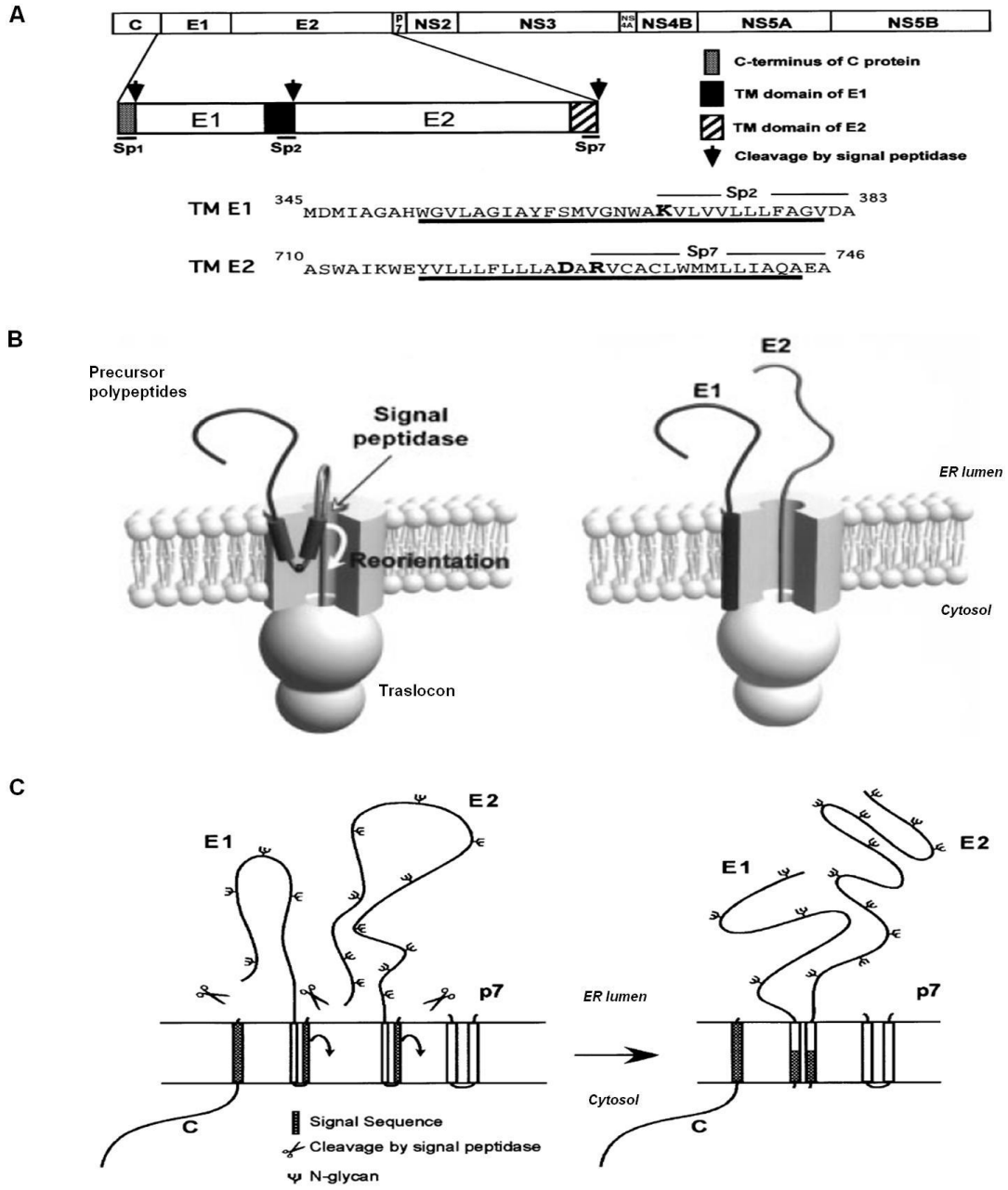


Fig. 16. Biogenesis of HCV envelope glycoproteins.

(A) *E1 and E2 glycoproteins are generated by co-translationally processing of the viral polyprotein. Sp1, Sp2 and Sp7 represent the signal sequences of E1, E2 and p7, respectively, recognized by ER proteases to generate the mature forms of the envelope glycoproteins. The E1 and E2 TM domains are underlined and charged residues are indicated in bold.* ***(B)*** *Before signal sequence cleavage, the charged residue in the TM domain of E1 interacts with the translocon to allow the translocation of E2 across the ER.* ***(C)*** *Topology of the TM domains of HCV envelope glycoproteins before and after signal sequence cleavage. (Adapted from (A) Cocquerel et al., 2002; (B)* Op De Beeck & Dubuisson, 2003; (C) *Op De Beeck et al., 2001).*

heterodimerization, and ER retention (Cocquerel *et al*., 2000; Cocquerel *et al*., 2002; Michalak *et al*., 1997; Op De Beeck *et al*., 2000).

As mentioned, ectodomains of E1 and E2 proteins are highly glycosylated. N-linked glycosylation is one of the most common types of protein modification, providing proteins with important structural and functional features. Glycosylation of E1 and E2 ectodomains starts soon during their synthesis, when they are targeted to the ER lumen, where the translocon-associated oligosaccharyltransferase enzyme transfers a $Glc_3Man_9GlcNAc_2$ oligosaccaride from a lipid intermediate to an Asn residue in the consensus 'sequon' Asn-X- Ser/Thr of the nascent polypeptides, where X is any aminoacid except Pro (Goffard & Dubuisson, 2003). Although it is believed that this enzyme has access only to nascent peptides, other data have revealed that the glycosylation of E1 is a very slow process (Goffard & Dubuisson, 2003), which may occur post-translationally (Duvet *et al*., 2002), and whose efficiency depends greatly on the co-expression of E2 (Goffard & Dubuisson, 2003). Probably due to differences in accessibility of potential glycosylation sites, it has been reported that mature forms of HCVcc-associated E1E2 proteins can be linked by both high-mannose-type and complex-type glycans (Vieyres *et al*., 2010). The former should be transferred to the glycoproteins in ER compartments, whereas the latter should result in a modification process, which may occur during the exocytosis pathway, through the Golgi. E1 and E2 proteins possess up to 6 and 11 potentially glycosylated sites, respectively (Fig. 17). Sequence analyses of E1 indicated that five potential N-glycosylation sites (196, 209, 234, 305 and 325) are strongly conserved among HCV genotypes (Goffard & Dubuisson, 2003; Zhang *et al*., 2004), but only four have high chances to be modified. Indeed, site 325 has a highly conserved proline residue at the Y position of the sequon Asn-X-Ser-Y, which is unfavorable for glycosylation (Goffard & Dubuisson, 2003). Interestingly, an additional potential glycosylation site at position 250 has been found to be extremely conserved in E1 sequences

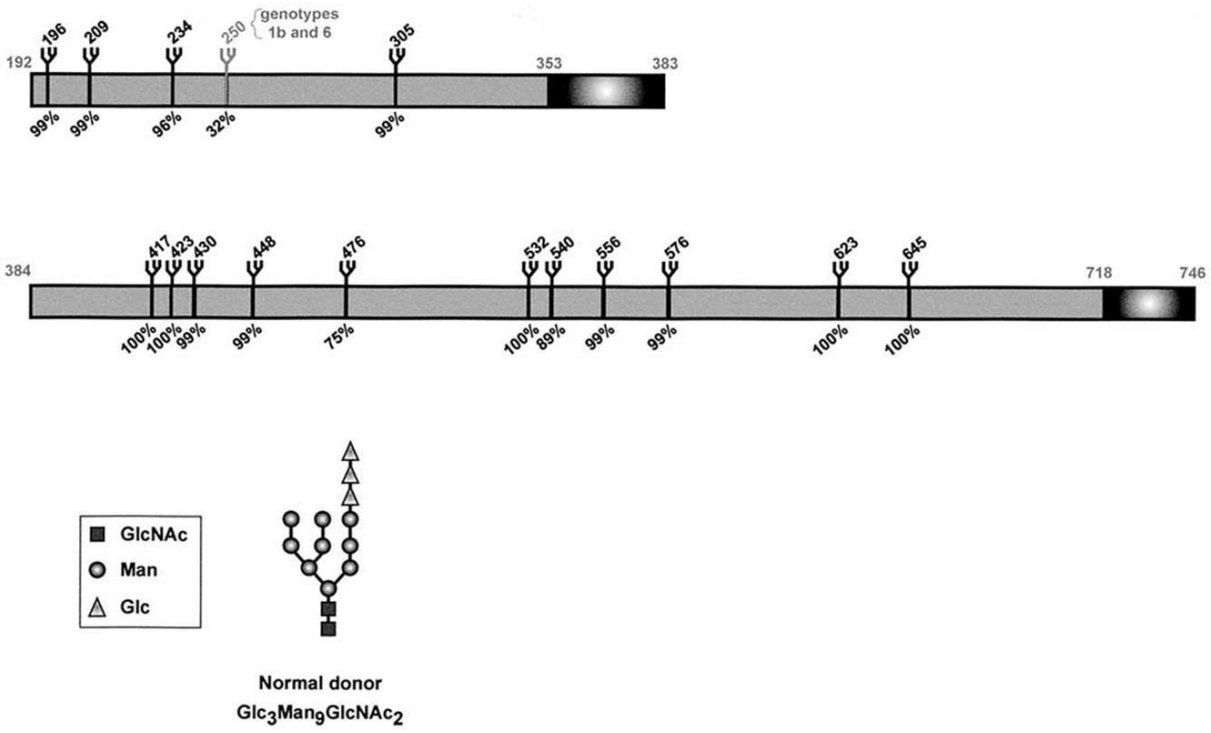


Fig. 17. HCV envelope proteins are highly N-glycosylated.

E1 and E2 possess up to 6 and 11 potentially glycosylated sites, respectively. Particularly, four and eleven potentially glycosylated sites are extremely conserved in all HCV genomes, respectively in E1 and in E2 sequences. The percentages of conservation of the glycosylation sites are indicated below each site. The position 250, particularly conserved just in 1b and 6 HCV genotypes is also shown. The numbers reported in the figure correspond to the positions in the polyprotein of reference strain H (EMBL access number, AF009606). At the bottom of the figure, the N-linked core $Glc_3Man_9GlcNAc_2$ oligosaccaride is indicated. (Adapted from Goffard & Dubuisson, 2003).

of genotypes 1b and 6 (97 and 100% respectively), whilst it is rarely recovered in all other HCV genotypes (Dubuisson *et al*., 1994; Goffard & Dubuisson, 2003). On the other hand, sequence analysis of the potential glycosylation sites in E2 indicated that nine of the 11 sites are strongly conserved (Fig. 17). The two remaining sites (476 and 540), showed a level of conservation of 75 and 89%, respectively (Goffard & Dubuisson, 2003; Zhang *et al*., 2004). It has been demonstrated that different N-linked glycans may have specific implications in envelope protein functions. Indeed, during the early secretory pathway, they might play several roles in protein folding, in quality control and in certain

sorting events, whilst it has also been demonstrated that N-glycans linked to viral E1E2 envelope proteins are extremely important for the HCV entry step, as well as for modulating the immune response of the host. By mutating one by one all the 4 and 11 putative N-glycosylation sites, respectively in E1 (Meunier *et al*., 1999) and E2 (Goffard *et al*., 2005) sequences, it has been shown that in the HCVpp system some glycans seem to be essential for the proper folding of the envelope proteins (E1N1, E1N4, E2N8 and E2N10), whereas others (E2N1, E2N2, E2N4, E2N5, E2N6, E2N11) are directly involved in HCVpp entry, without affecting E2 folding or its incorporation in the pseudoparticles. Particularly, E2N4, E2N6 and E2N11 can reduce the access of E2 to a soluble form of CD81, thus also reducing the sensitivity of HCVpp to antibody neutralization (François Helle et al., 2007). Glycans associated with HCV envelope proteins might also be involved in specific interactions between virions and the lectins DC-SIGN and L-SIGN, which recognize mannose residues, thus contributing to the establishment or persistence of infection either by delivering virions to the hepatocytes, or by modulating dendritic cell functions (Cormier *et al*., 2004; Lozach *et al*., 2004). Finally, as described for HIV envelope glycoproteins, N-linked glycans can also modulate the humoral immune response (Wei *et al*., 2003). However, conversely to HIV, which can reorganize envelope glycans to limit its recognition by neutralizing antibodies while still preserving the ability to interact with CD4 and other co-receptors, HCV envelope proteins show highly conserved glycosylation sites (Wei *et al*., 2003; Zhang *et al*., 2004).

3. E1E2 Folding and Heterodimerization

Despite extensive research, many details about the structure and functions of HCV glycoproteins are not fully understood (Rychlowska *et al*., 2011). Actually, it is possible to identify two types of E1E2 complexes (Vieyres *et al*., 2010): intracellular forms assembled as

non-covalent heterodimers, which should correspond to the functional and prebudding complexes; and large covalent aggregates stabilized by intermolecular disulfide bonds, which show a lower infectivity (Deleersnyder *et al*., 1997; Dubuisson *et al*., 1994; Flint *et al*., 2004; Op De Beeck *et al*., 2004). Interestingly, when studied using the HCVcc system, the latter is found to be preferentially associated to the purified particles (Vieyres *et al*., 2010). Thus, it has been speculated that, as was observed for alphaviruses (Forsell *et al*., 2000) and flaviviruses (Gastaminza *et al*., 2006), these more stable covalent complexes might play a key role in the budding process of the viral particle (Vieyres *et al*., 2010). Studying the early steps of oligomerization of the HCV envelope glycoproteins is essential to understand several steps of the viral infectious cycle, such as the fusion mechanism with the endosomal membrane (Op De Beeck *et al*., 2000). Although precise interactions between envelope glycoproteins and the involvement of covalent bonds in the formation of heterodimer complexes still remain unclear, results obtained with alanine scanning insertion mutagenesis, led to the indentification, in the TM domain of E1, of two consecutive GXXXG motifs (Gly^{350}-Gly^{354} and Gly^{354}-Gly^{358}), known to be involved in helix-helix interaction in membrane proteins (Ciczora *et al*., 2007; Op De Beeck *et al*., 2000). Therefore, it has been postulated that these two glycine motifs in E1, might play an essential role in early contacts between the envelope TM domains, thus allowing the two glycoproteins to get closer, so as to enable further links between their N-terminal domains (Op De Beeck, Cocquerel, & Dubuisson, 2001). It has been found that the ectodomains of E1 and E2 respectively contain 8 and 18 cysteine residues, known to be involved in disulfide bonds (Krey *et al*., 2010). Folding of the HCV envelope glycoproteins is believed to be initially due to the formation of intra-chain disulfide bonds, catalyzed by the ER protein disulfide-isomerase (PDI), and promoted by the oxidizing environment of the ER lumen (Dubuisson *et al*., 1994). Assisted by three chaperones, calnexin, calreticulin and BiP, which show an affinity for monoglycosylated

N-linked oligosaccharides (Lavie *et al*., 2007), most of the envelope glycoproteins, whose coexpression seems to be mutually necessary to reach their proper folding, slowly form nonionic detergent-stable heterodimer complexes (Brazzoli *et al*., 2005; Cocquerel *et al*., 2003; Michalak *et al*., 1997).

4. Neutralizing Antibodies against the Envelope Glycoproteins

a) Adaptive Immune Responses in HCV Infection

Adaptive immunity refers to antigen-specific defense mechanisms including humoral and cell-mediated immunity, designed to remove a specific antigen (Fig. 18). Humoral immunity involves the production of specific antibodies molecules, and is mediated by B-lymphocytes, whereas the cell-mediated immunity principally involves CD4+ and CD8+ T-lymphocytes (Quaranta, Mattioli, & Vella, 2012). One of the key characteristics of the HCV infection is the delayed adaptive immune responses despite the early increase in HCV RNA titer occurring within few days from infection, as well as the induction of the innate immune system, which constitute the first line of defense against pathogens (Bowen & Walker, 2005; Rehermann, 2009). Virus-specific T cells and antibodies are usually detected respectively around 5-9 weeks and 8-20 weeks from the start of HCV infection (Rehermann, 2009). Antibodies may be targeted against epitopes within both structural and non-structural viral proteins, although they do not exert any antiviral activity in most cases (Neumann-Haefelin & Thimme, 2013). Indeed, only the neutralizing antibodies (nAbs), which have been mapped to the E1E2 envelope glycoproteins (Johansson *et al*., 2007; Keck *et al*., 2004a; 2008; Meunier *et al*., 2008; Owsianka *et al*., 2005; Perotti *et al*., 2008) are able to prevent viral infection and spread (Neumann-Haefelin & Thimme, 2013). First evidence for a protective role of nAbs came from the chimpanzee animal model of HCV

infection, for which vaccination with envelope glycoproteins or passive immunoprophylaxis resulted in a partial protection of the animal against a homologous viral challenge (Farci *et al*., 1994). In humans, the role played by nAbs in HCV natural infection is debated. Indeed, it has been reported that viral clearance can occur in the absence of neutralizing antibodies, in agammaglobulinaemic patients (Neumann-Haefelin & Thimme, 2013). In immunocompetent patients, neutralizing antibodies are initially transient and isolate specific. Subsequently, they increase in titer and breadth, and typically exhibit crossreactivity against multiple HCV genotypes once chronic HCV infection is established (Bartosch, *et al*., 2003b; Logvinoff *et al*., 2004; Meunier *et al*., 2005; Rehermann, 2009). Evidence that anti-HCV antibodies may passively protect humans from the HCV infection arose from a retrospective study by Feray *et al*. (1998) on patients who became protected against the virus after receiving non-screened HBV polyclonal immunoglobulin (Edwards *et al*., 2012). Moreover, a recent study in which patients were infected with known viral inoculum in a single-source outbreak of hepatitis C, revealed that whilst acute-resolving infection was associated with an early development of nAbs, persistent infection was instead associated with a delayed induction of nAbs (Pestka *et al*., 2007). In contrast to antibodies, HCV-specific T cells responses are critical for HCV clearance (Bowen & Walker, 2005; Neumann-Haefelin *et al*., 2005; Rehermann, 2009). Particularly, virus-specific CD8+ T cells are key players in the antiviral immune responses, showing to be the main adaptive effector cells involved in HCV clearance (Neumann-Haefelin & Thimme, 2013). HCV-specific CD8+ T cells mediate their antiviral effects through two different mechanisms. They can kill infected target cells (mainly hepatocytes) presenting viral antigens on their surface by HLA (human leukocyte antigen) class I molecules by cytolysis, which is mediated either by cell-bound receptors such as FAS and its ligand FAS-L, or by paracrine secretory factors such as perforine (Fig. 18). HCV-specific CD8+ T cells can also secrete antiviral cytokines such as Interferone-gamma

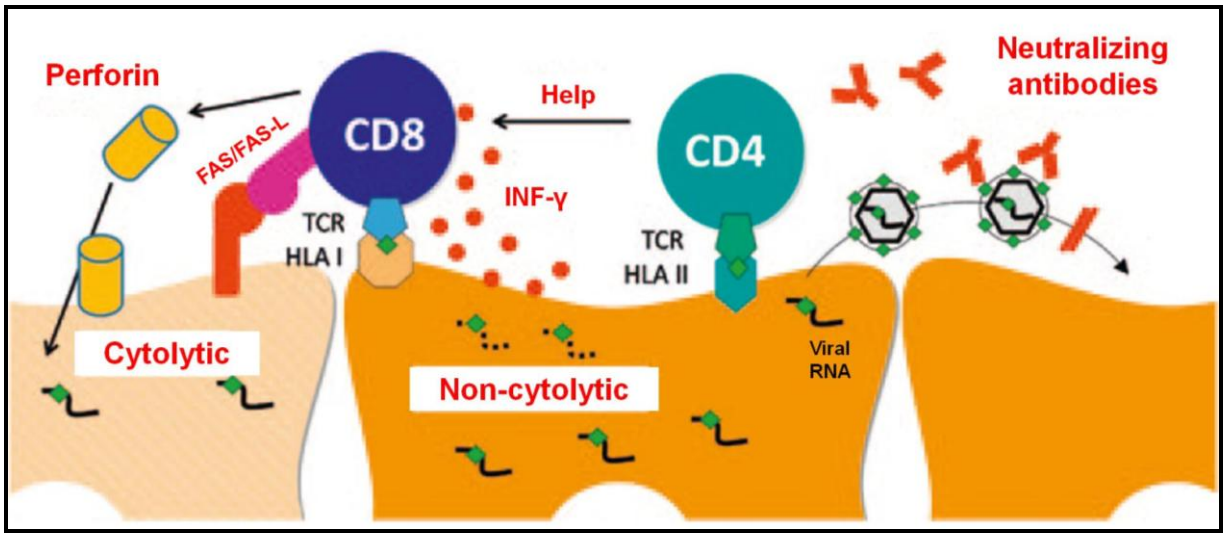


Fig. 18. Adaptive immune responses against HCV.

Adaptive immunity refers to antigen-specific defense mechanisms including humoral and cell-mediated immunity, designed to remove a specific antigen. Humoral immunity can produce neutralizing Abs which have important functions in virus binding and entry; the cell-mediated immunity principally involves CD4+ and CD8+ T-lymphocytes. CD8+ T cells mediate their antiviral effects through cytolytic and non-cytolytic mechanisms. They can kill infected target cells presenting viral antigens on their surface by HLA class I molecules by cytolysis, mediated either by cell-bound receptors such as FAS and its ligand FAS-L, or by paracrine secretory factors such as perforine. HCV-specific CD8+ T cells can also secrete antiviral cytokines such as Interferon-gamma (INF-γ). HCV-specific CD4+ T cells have important helper functions orchestrating efficient adaptive immune responses. (From Neumann-Haefelin & Thimme, 2013).

(INF-γ) and Tumor Necrosis Factor-alpha (TNF-α) (Neumann-Haefelin & Thimme, 2013). The latter non-cytolytic effector mechanism, principally mediated by the IFN-γ production, has been reported to be the dominant function exerted by the HCV-specific CD8+ T-lymphocytes to control viral replication (Jo *et al*., 2009). However, CD8+ T cells responses are initially delayed, becoming detectable only when HCV-specific CD4+ T cells responses develop, with a consequent viral titer decrease (Rehermann, 2009). A vigorous proliferation of HCV-specific CD4+ T cells early in infection has been directly correlated with viral clearance in humans, whereas virus-specific CD4+ responses are absent or weak in patients who subsequently develop chronic infection (Diepolder *et al*., 1995; Missale *et al*., 1996; Rehermann, 2009; Schulze Zur Wiesch *et al*., 2012). Furthermore, CD4+ depletion studies in

the chimpanzee model have revealed the important role of HCV-specific CD4+ T-lymphocytes in viral control (Neumann-Haefelin & Thimme, 2013). Indeed, it has been reported that two immune chimpanzees reinfected after antibody-mediated depletion of CD4+ T cells, failed to control viremia despite functional CD8+ T cells responses (Grakoui *et al*., 2003), thus supporting the concept that HCV-specific CD8+ T-lymphocytes are the main antiviral effector cells, while HCV-specific CD4+ T cells have important helper functions, preventing viral escape from the CD8+ T cells response (Neumann-Haefelin & Thimme, 2013). Detailed understanding of the HCV-specific immune responses and of its failure in persistent infection have important implication in vaccine design. For example, current knowledge about HCV-specific adaptive immunity highlighted how vaccine-induced CD8+ T cell responses should fail if sufficient CD4+ T cell help is missing (Neumann-Haefelin & Thimme, 2013). A promising approach, based on recombinant adenoviral vectors expressing the non-structural HCV proteins, has been reported in healthy volunteers (Barnes *et al*., 2012). In this assay, adenoviral vectors were able to prime sustained HCV-specific CD4+ and CD8+ T cell responses, which targeted multiple viral proteins derived from heterologous strains (Barnes *et al*., 2012). Finally, antibodies could be used as post-exposure prophylaxis as reported for experimentally challenged animals (Bartosch *et al*., 2003b; Dorner *et al*., 2011; Farci *et al*., 1996; Forns *et al*., 2000; Meuleman *et al*., 2008; 2011; Vanwolleghem *et al*., 2008).

b) Immunogenic Domains of E1 and E2 Proteins

Since evidences for a protective role of neutralizing antibodies against HCV infection are available, characterization of the best immunogenic domains of the two envelope glycoproteins proteins is essential in the design of an effective B cell-based HCV vaccine

(Keck *et al*., 2012). The main difficulty encountered in this field of research covers the identification of conserved epitopes in this highly variable virus, able to elicit protective antibodies. nAbs targeting E1 and E2 glycoproteins have been mapped in both linear and conformational discontinuous epitopes. These Abs have important functions in virus binding and entry, as well as in postattachment steps (Sabo *et al*., 2011). HVR1 represents the main immunogenic region in the E2 sequence. Linear antibodies targeting HVR1 are present in nearly all HCV infected patients (Ray *et al*., 2010; Wang, Keck, & Foung, 2011), and because the wide sequence variability of this region, they tend to be highly strain-specific, thus not constituting a valid immunogenic epitope for a vaccine design (Bartosch, *et al*., 2003b; Edwards *et al*., 2012; Vieyres, Dubuisson, & Patel, 2011). However, broadly neutralizing HVR1 antibodies, reactive against several HCV genotypes, have also been reported (Wang *et al*., 2011). Indeed, a neutralizing monoclonal antibody (nMAb), the rat 9/27 MAb, specifically targeting the more conserved C-terminal region of HVR1 (aa 396-407), inhibiting pseudotype viruses infection, has been characterized (Hsu *et al*., 2003). This MAb is also able to neutralize HCV cell-to-cell transmission, suppressing the interaction between E2 and the cellular receptor SR-BI (Brimacombe *et al*., 2011). Interestingly, it has been shown that viral mutants lacking HVR1 region are much more susceptible towards neutralization by both patient sera and MAbs targeting CD81-binding sites (Bankwitz *et al*., 2010; Prentoe *et al*., 2011). These data lead to postulate that HVR1 may operate by stimulating a strong antibody response which does not result in viral clearance, but in driving the virus towards selection of antibody-escape mutants (see below) (Edwards *et al*., 2012; Ray *et al*., 1999). Simultaneously, early during infection, it constitutes a kind of shield which protects viral entry determinants within E2 from neutralization (Bankwitz *et al*., 2010). CD81-binding epitopes show a weak immunogenicity compared to HVR1, but they are more conserved, thus constituting better candidates in the development of a B-cell based vaccine,

being able to neutralize a broad range of HCV genotypes (Broering *et al*., 2009; Johansson *et al*., 2007; Law *et al*., 2008; Owsianka *et al*., 2005; Perotti *et al*., 2008). The region immediately downstream of HVR1 includes a linear epitope, spanning aa 412-425, known to be targeted by two MAbs, the mouse AP33 (Owsianka *et al*., 2005) and the rat 3/11 (Tarr *et al*., 2006). These antibodies display a broad neutralization towards HCVpp bearing envelope glycoproteins from genotype 1 to 6 (Broering *et al*., 2009; Tarr *et al*., 2006). However, less that 5% of patients who cleared HCV infection showed AP33- or 3/11-like antibodies, thus suggesting that this epitope should be poorly immunogenic in humans (Tarr *et al*., 2007). Conversely, the majority of human MAbs (HMAbs) targeting the CD81-binding site are directed against conformational epitopes. In particular, it has been shown that the E2 envelope glycoprotein contains three immunogenic domains, namely A, B, and C, of which the latter two include neutralizing HMAbs, whereas the domain A contains non-neutralizing HMAbs (Keck *et al*., 2004a). These findings suggested that residues in the domains B and C are directly involved in interactions between E2 and CD81 (Keck *et al*., 2004a). Abs targeting the antigenic domain B showed broadly neutralizing activities against different genotypes and subtypes E2 proteins expressed with the HCVpp system (Keck *et al*., 2008a; 2008b; Owsianka *et al*., 2008). Interestingly, the majority nAbs were directed against a region between residues 529 and 535, including the absolutely conserved G530 and D535 residues (Keck *et al*., 2012), which fold into the DI of E2, according to the 3D model proposed by Krey *et al*. A further difficulty encountered in the rational design of an effective vaccine concerns the emergence of viral escape mutants under neutralizing antibodies selection (von Hahn et al., 2007). Two domain B Abs, HC-11 and HC-1, demonstrated to target very essential residues into CD81 binding domain of the E2 protein. Indeed, selective pressure of the HC-11 led to progressive single or double substitutions in the E2 sequence associated with a loss of viral fitness, whereas the HC-1 Ab

Envelope Glycoprotein	EPITOPE		Ex. of nAb
	Linear	Conformational	
E2	HVR1 (aa 396-407) SR-BI binding site		9/27 Mab (Hsu *et al*., 2003; Brimacombe *et al*., 2011)
	Downstream HVR1 (aa 412-425)		AP33 (Owsianka *et al*., 2005) 3/11 (Flint *et al*., 1999b)
		Domain B, C and D CD81-binding site	Domain B MHAb: CBH-5 (Keck *et al*., 2004a) HC-11 (Keck *et al*., 2008) Domain C HMAb: CBH-7 (Keck *et al*., 2004a) Domain D HMAb: HC-84 Abs (Keck *et al*., 2012)
		Antigenic Region 3 (AR3) CD81-binding site (aa 394-424; 437-447; 523-540)	AR3A (Law *et al*., 2008)
E1	^{192}YEVRNVSGVYH^{211}		H-111 (Keck *et al*., 2004b)
E1E2 Heterodimer		AR4 and AR5 CD81-binding site (aa D698 and R639)	AR4A and AR5A (Giang *et al*., 2012)

Table 2. Summary of the envelope glycoproteins antigenic domains targeted by nAbs.

completely suppressed viral replication generating no escape mutants at a critical concentration of 10 μg/ml (Keck *et al*., 2011). Recently, it has been reported a new cluster of conformational epitopes, overlapping CD81 binding residues, which differ from the antigenic domain B residue patterns, and named domain D (Keck *et al*., 2012). All the nine HMAbs characterized for this immunogenic domain, and referred to as HC-84 Abs, were able to neutralize interaction between the CD81 receptor and the E2 glycoprotein expressed by HCVcc of different genotypes. Interestingly, no escaping virus emerged during long-term HCVcc *in vitro* propagation (Keck *et al*., 2012). By alanine scanning analysis of the E2 regions known to be involved in CD81 binding (aa 410-446, aa 526-540, aa 611-617), the authors showed that the HC-84 epitopes centered at aa 441-443, with the majority including a contact residue at aa 616, all being 100% conserved in 1, 2, 3 and 4 HCV genotypes, thus rendering these epitopes very attractive for a vaccine design (Keck *et al*., 2012). In another study, three antigenic regions (AR1, AR2 and AR3) were identified by competition analysis

with known MAbs (Law *et al*., 2008). Particularly, AR3-specific antibodies were able to broadly neutralize HCVpp bearing envelope glycoproteins from genotype 1 to 6. Epitope mapping identified AR3 as formed by three discontinuous E2 segments, aa 394-424, aa 437-447, and aa 523-540, involved in the interaction with the CD81 receptor (Law *et al*., 2008). AR1 is not conserved and probably not exposed on the viral surface because AR1 Abs bind only genotype 1a HCV and do not have significant neutralizing activity (Law *et al*., 2008). AR2 is distal from CD81 binding domain and is exposed on E2 because MAb AR2A can neutralize several HCV isolates (Giang *et al*., 2012). Using a strategy termed "exhaustive panning of a phage-display antibody repertoire", particularly suitable for discovery of rare MAbs (Tsui *et al*., 2002), two new distinct antigenic regions (AR4 and AR5) have been identified on the HCV E1E2 heterodimers (Giang *et al*., 2012). All but one AR4 and AR5 Abs epitopes recognized in this study is distinct from AR1, AR2 and AR3 regions. Analyzing the properties of such Abs in binding to E1E2 heterodimers, the authors found that whilst the AR1 to AR3 Abs specifically targeted epitopes on E2 glycoprotein alone, mainly affecting the interaction between the CD81 tetraspanin and the E2 envelope, the newly identified AR4 and AR5 MAbs did not significantly interfere with the CD81 interaction (Giang *et al*., 2012). Furthermore, the latter Abs bound only correctly folded E1E2 heterodimers (Giang *et al*., 2012). By using single and double alanine-scanning mutants of both E1 and E2 proteins, two extremely conserved E2 envelope glycoprotein residues (D698 and R639) have been identified, respectively targeted by the MAbs AR4A and AR5A. The E2 D698 residue is located within the membrane proximal external region, about 20 residues upstream of the E2 TM domain. Interestingly, the AR4A MAb was able to cross-neutralize HCVpp and HCVcc bearing envelope glycoproteins from 1 to 6 HCV genotypes (Giang *et al*., 2012). The authors speculated that this MAb likely interfere with the rearrangement of the E2 TM domain probably required for membrane fusion and virus cell

entry, as reported for HIV-1 (Song *et al*., 2009). Moreover, it has been shown that this MAb could also provide protection against HCV infection in a mouse model challanghed with 1b or 2a HCVcc isolates (Giang *et al*., 2012).

Neutralizing Abs outside the E2 region have been also reported. Actually, antibodies targeting epitopes within the envelope glycoprotein E1 have been identified in some patients, although they are rare (Pestka *et al*., 2007). Identifying immunogenic epitopes in E1 protein is not technically easy. Indeed, the E1 protein misfolds if expressed without its partner E2 (Dubuisson *et al*., 1994). Furthermore, the presence of E2 glycoprotein could either mask E1 epitopes or be immunologically dominant (Garrone *et al*., 2011). Despite these difficulties, the MAb H-111 has been reported to be able to bind the N-terminus epitope ^{192}YEVRNVSGVYH^{211} of E1 genotypes 1a, 1b, 2b, and 3a (Keck *et al*., 2004b). Interestingly, a recent study showed that anti-E1 antibodies elicited in a chimpanzee immunized with a recombinant form of E1 protected the animal from a heterologous HCV challenge, and the protective effect of this E1-based vaccine would be more effective than that of an E2-based vaccine (Verstrepen *et al*., 2011).

c) Strategies for HCV Persistence in the Presence of nAbs

One of the most intriguing features of the HCV biology is its ability to persist in a host even in the presence of broadly neutralizing responses. The most widely reported evasion mechanism in this sense is the mutational escape of HCV variants (Edwards *et al*., 2012). Indeed, as we have already seen, HCV is present in an infected host as a swarm of viral variants forming a quasispecies population, in which new variants are constantly generated and able to circumvent both B- and T-cell responses (Dowd *et al*., 2009a; Farci *et al*., 2000; von Hahn *et al*., 2007). Although evidence for viral evolution driven by nAbs still remains

controversial, two recent works showed that antibodies from sequentially collected sera neutralized more efficiently earlier viral variants than those circulating contemporarily to the serum samples (Dowd *et al*., 2009a; von Hahn *et al*., 2007). In favor of a positive selection by the neutralizing response of the host, there is evidence of reduced evolution of the quasispecies in immunocompromised humans, thus suggesting that the progressive change of viral variants in a quasispecies should need selective pressures (Herring *et al*., 2005). Actually, it is thought that evolution of the HVR1 genetic sequence during acute infection, more than any other genomic region, might be used as an immunological marker to predict whether the hepatitis will be resolved or whether the infection will evolve into chronic hepatitis (Edwards *et al*., 2012; Farci *et al*., 2000; Ray *et al*., 1999). Indeed, it has been found that acute resolving hepatitis was associated with relative evolutionary stasis of the HVR1 sequences represented in viral quasispecies, whereas progressing hepatitis was correlated with a more marked genetic variability (Farci *et al*., 2000). It has been shown that this genetic variability was the result of positive selection of variants presenting a high Ka/Ks ratios (= nonsynonymous nucleotide substitutions per nonsynonymous site (Ka)/synonymous nucleotide substitutions per synonymous site (Ks)), thus corroborating the idea for viral evolution driven by nAbs (Farci *et al*., 2000).

Beside mutation of viral variants as strategy to escape from neutralizing antibodies, glycosylation of viral envelope (Falkowska *et al*., 2007; Helle *et al*., 2007), non-neutralizing antibodies (Zhang *et al*., 2007, 2009), cell-to-cell transmission (Baldick *et al*., 2010; Brimacombe *et al*., 2011; Timpe *et al*., 2008) and virion-associated lipoproteins (Thomssen *et al*., 1992) might interfere with antibody-mediated neutralization by masking or reducing the access of neutralizing epitopes. Suggestions that E2 N-linked glycans might contribute to HCV evasion from the humoral response come from studies which, using both the HCVpp and the HCVcc systems, showed how particular glycosylation sites on the E2 protein were

able to modulate sensitivity towards nAbs, and binding to CD81 receptor (Falkowska *et al*., 2007; Helle *et al*., 2007; 2010). Particularly, slightly different data were obtained by the two model systems, probably due to dissimilarities in envelope protein glycosylation between HCVpp and HCVcc (Vieyres *et al*., 2010). Indeed, whilst E2N1, E2N6, and E2N11 glycans were found to be able to reduce the sensitivity of HCVpp towards antibody neutralization, hindering the access to CD81-binding sites (Falkowska et al., 2007; François Helle et al., 2007), the HCVcc system revealed that E2N1, E2N2, E2N4, E2N6, and E2N11 glycans might instead reduce neutralizing sensitivity towards both antibodies purified from sera of patients infected with different HCV genotypes, and towards MAbs (François Helle et al., 2010). Interestingly, among these glycosylation sites, only E2N1, E2N2, E2N4, and E2N6 were more sensitive to inhibition with a soluble form of CD81 (CD81-LEL), whereas a mutation at the E2N11 site did not affect the CD81 binding to E2 at all, in contrast to what was observed using the HCVpp system (François Helle et al., 2010). Looking at the 3D model of the E2 ectodomain (Krey *et al*., 2010), we can easily realize that the four glycosylation sites (E2N1, E2N2, E2N4, E2N6) are all included in, or close to the DI domain.

A third likely mechanism by which HCV might persist in infected hosts despite activation of neutralizing responses relies on the production of non-nAbs together with broadly nAbs in the same host (Zhang *et al*., 2007; 2009). Indeed, Zhang and colleagues identified two epitopes at amino acid positions 412-419 (epitope I), and 434-446 (epitope II), located downstream of the HVR1 of the E2 protein sequence (Zhang *et al*., 2007). They demonstrated that binding of non-neutralizing antibodies at the epitope II site completely hindered the association of nAbs to the epitope I site, thereby allowing the virus to persist even in the presence of an abundance of nAbs (Zhang *et al*., 2007; 2009).

A number of viruses have evolved direct cell-to-cell modes of transmission to maximize particle delivery, protecting it from nAbs (Mothes, Sherer, Jin, & Zhong, 2010). Recently, also HCV has proven to be capable of spreading within its host by a cell-to-cell transmission strategy (Timpe *et al*., 2008; Witteveldt *et al*., 2009). Interestingly, it has been shown that cell-to-cell transmitted HCV were more protected from neutralization by a panel of diverse anti-glycoprotein antibodies than cell-free particles (Brimacombe *et al*., 2011). However, an anti-HVR1 MAb was demonstrated to be highly efficient in reducing cell-to-cell transmission, suggesting that SR-BI, more that other identified HCV co-receptors, plays an essential role in this kind of viral dissemination. Finally, lipid shielding may represent an additional strategy used by HCV to evade the humoral response of the host (Edwards *et al*., 2012). Indeed, beside the evidence that HDL components of human serum are able to enhance HCVpp and HCVcc infectivity *in vitro* (Bartosch *et al*., 2005; Dreux & Cosset, 2007; Meunier *et al*., 2005), Grove and colleagues demonstrated that low-density HCV particles are less sensitive to antibody neutralization due to the possibility that lipoproteins might mask critical epitopes on E2 envelope glycoprotein from nAbs (Grove *et al*., 2008).

D. HCV TRANSMISSION

1. Transmission Routes and Risk Factors

Because the blood-borne nature of HCV, parenteral routes are the most efficient ways of transmitting the hepatitis C infection, though viral RNA has been detected in several body fluids other than the blood, including saliva, menstrual fluid, semen, urine, spinal fluid, and ascites (Henderson, 2003). Until the 80's, intravenous (i.v.) drug abuse and unsafe blood transfusion or blood products treatments accounted for the higher risk factors in developed countries of the world (Lavanchy, 2009). However, since 1992, when blood screening became a routine practice in the Western countries, the risk for hepatitis C infection associated with transfusion was suddenly lowered. Therefore, today the most important behavioral risk factor for the transmission of HCV in developed countries remains the needle sharing among injecting drug users (IDUs), accounting for up to 60% of HCV infections (Henderson, 2003; Lavanchy, 2009). Other risk factors include patients in chronic haemodialysis, (prevalence of 10-33%), sexual transmission (less than 5% of cases), perinatal transmission (in 3% to 5% of infants born from HCV or HCV/HIV co-infected mothers), and occupational transmission (Villena, 2006). For the latter, contaminations of health care workers (HCWs) following blood-exposure accidents (BEAs) have been well documented (Hosoglu *et al*., 2003; Liu *et al*., 2006; Morand *et al*., 2001; Noguchi *et al*., 1997; Poignet, Degos, Bouchardeau, Chauveau, & Courouce, 1995; Saito *et al*., 2004; Toda *et al*., 2009), although this mode of transmission is quite rare (Morin *et al*., 2011). Actually, the prevalence of HCV-positive individuals among HCWs is similar to that observed among blood donors (Morin *et al*., 2011). Since neither a preventive vaccine, nor a post-exposure treatment are

currently available, all the people who are daily exposed to potential carriers of HCV (mainly anesthesiologists, nurses and surgeons), are at risk of contracting the infection. Assessing risk factors for HCV occupational transmission to HCWs, it has been found that this kind of transmission occurs prevalently after percutaneous exposure to viremic blood or blood fluids, although transmission cases of HCV as a result of splashes of blood from infected patients onto mucous membranes of HCWs have also been documented (Hosoglu *et al*., 2003). Deep injuries and BEAs involving hollow-bore needle placement in the source patient's vain or artery, where a larger volume of blood is transferred and where the needle contains undiluted blood, seems to increase the risk for occupational HCV transmission (Yazdanpanah *et al*., 2006), as previously reported also for HCWs occupationally exposed to HIV-infected blood (Cardo *et al*., 1997). Moreover, as also found in case of vertical transmission (Okamoto *et al*., 2000), the risk of occupational HCV transmission further increases as a consequence of high HCV RNA titers in the source patient (Yazdanpanah *et al*., 2006).

2. Early Events Surrounding Viral Transmission

As already explained previously, principally due to the error-prone viral replicase of HCV, in an infected host at least 10^9 variants with single and double nucleotide changes are likely to arise in each individual multiple times per day (Guedj *et al*., 2010). Both, a continuous genesis of new variants in a completely stochastic manner (genetic drift), and a purifying selection exerted by host immune pressure and viral fitness (natural selection) result either in extinction or in preferential selection of certain variants (Bull *et al*., 2011). When selection overbalances drift, a genetic bottleneck occurs, defined as an evolutionary event resulting in a reduction of the population's size and of its genetic variation, due to

extinction of a significant proportion of the viral variants. Genetic bottlenecks potentially limit replicative fitness of resultant viruses (Bull *et al*., 2011). Such within-host genetic bottlenecks have been observed upon virus transmission into a new host for a number of RNA viruses, including HIV and HCV (Bar *et al*., 2010; Boutwell *et al*., 2010; Bull *et al*., 2011; Fafi-Kremer *et al*., 2010; Keele *et al*., 2008; Laskus *et al*., 2004; Li *et al*., 2012; Liu *et al*., 2006; Saito *et al*., 2004). After a bottleneck event, RNA viruses undergo rapid evolution, leading to accumulation of deleterious mutations and the occurrence of few fit variants. Then, within-host evolution of these rapidly mutating viruses may be characterized by selective sweeps, resulting in reduction or elimination of certain variants in the viral population due to a strong selection pressures. In this way, only few variants in a quasispecies, derived from the "transmitted/founder" (T/F) viruse(s), emerge to dominate the future population. This phenomenon has been widely documented for HIV (Keele & Derdeyn, 2009; Keele *et al*., 2008; Li *et al*., 2010), and it has been also reported in a few studies for HCV (Bull *et al*., 2011; Li *et al*., 2012; Wang *et al*., 2010). However, for both viruses, it remains unclear whether the transmission bottleneck is attributable to a low number of variants being transferred between hosts, or whether it is the result of early evolutionary events where a larger number of strains are rapidly eliminated as a result of varying fitness constraints (Bull *et al*., 2011). After transmission, the viral population evolves within the new host driven by both viral and host factors, and the rate and the way in which this occurs seems to determine the progression of the infection and the hepatitis outcome (Bowen & Walker, 2005; Bull *et al*., 2011; Farci *et al*., 2000; Farci *et al*., 2006; Kuntzen *et al*., 2007; Merani *et al*., 2011; Post, Ratnarajah, & Lloyd, 2009; Ray *et al*., 2005; Smith *et al*., 2010). Interestingly, it has been reported that early variability of the HVR1 segment alone, known to be involved both in HCV entry and in escape from nAbs, might determine the fate of the new quasispecies (Edwards *et al*., 2012; Farci *et al*., 2000; Ray *et al*., 1999). Differential abilities

of transmitted viral variants to get access to hepatocytes and to escape from the host neutralizing response are thought to be among the main features discriminating between variants which will be selected in the new host, and variants which will not be selected (Fafi-Kremer *et al*., 2010).

a) Studies in Humans

Deciphering the virus-host interactions responsible for HCV transmission, including molecular signatures, as well as phenotypic properties shared by the T/F viruses able of establishing *de novo* infections, is very important for HCV vaccine development. For this reason, it follows an overview of the majority of studies on the HCV transmission in humans and in animal models. Since parenteral routes are the most efficient ways of transmitting the hepatitis C infection, cases of HCV transmission including infection through blood transfusion or parenteral therapies with blood products (Cantaloube, 2003; Herring *et al*., 2005; Laskus *et al*., 2004; Nainan *et al*., 2006; Prati, 2006; Ray *et al*., 2005), and occupational transmission to HCWs following needlestick accidents (Liu *et al*., 2006; Noguchi *et al*., 1997; Saito *et al*., 2004; Yazdanpanah *et al*., 2006) have been reported. Laskus and co-workers had the opportunity to study fifteen patients becoming HCV positive following blood transfusion from 1974 to 1980 (Laskus *et al*., 2004). By analyzing the HVR1 segment of E2 envelope glycoprotein as a more genetically variable region, and the 5'UTR as a more conserved genomic region, they reported that a genetic bottleneck at transmission was far from universal. Indeed, up to 10 of 15 recipient patients showed a post-transfusion E2/HVR1 quasispecies composition which closely matched that found in the donor, and up to 14 of 15 recipient patients acquired all the 5'UTR viral variants present in donors (Laskus *et al*., 2004). Furthermore, the authors emphasized that it was highly unlikely that this could simply

correspond to passive transmission of donor virus because this quasispecies composition persisted for at least several weeks (Laskus *et al*., 2004). The horizontal transmission of HCV through needlestick accidents is a serious issue among HCWs. By using PCR amplification, cloning and sequencing of HVR1, two studies reported the analysis of HCV quasispecies transmission between HCV chronic donor patients and HCWs recipients through needlestick accidents (Liu *et al*., 2006; Saito *et al*., 2004). Both the authors showed a genetic bottleneck with selective transmission of few variants, although none of the recipient's variants was identical to the donor's (Liu *et al*., 2006; Saito *et al*., 2004). Moreover, in the two cases the minor variant of HCV quasispecies in the donor was transmitted to the recipient, becoming the predominant one in the latter after transmission. Also, though in one case the recipient cleared the virus within five months after the contamination (Liu *et al*., 2006), in the other one, the recipient developed chronic hepatitis C (Saito *et al*., 2004). Trying to explain the phenomenon of the HCV selective transmission, Liu and colleagues proposed three likely mechanisms. Firstly, selection of the viral variants might occur at the time of entry. Indeed, as reported for HIV-1 infection for which the different macrophage-tropic abilities of the virus may determine the efficiency of replication in the submucosal space (O'Brien *et al*., 1990), it is possible for HCV that one variant has a selective advantage over the others during viral entry. Secondly, there may be a selective amplification of the variants in the new host. For example, it should be possible that multiple variants enter the new host but that only one or a few of these are selectively amplified to become the dominant strain, because of a biological advantage (Zhu *et al*., 1993). This hypothesis is supported by an HIV report in which patients with hemophilia presumably inoculated with multiple HIV-1 variants, showed homogeneous sequences (Zhang *et al*., 1993). Thirdly, the donor's major variant might be eliminated by the recipient's immune system (Liu *et al*., 2006).

Recently, the enumeration of T/F viruses using more suitable experimental approaches has been reported (Bull *et al*., 2011; Li *et al*., 2012; Wang *et al*., 2010). For instance, using a "Single Genome Amplification" (SGA) approach (for more details see the "MATHERIALS & METHODS" section) and a mathematical model of HCV diversification, Li and colleagues identified T/F viral sequences, including the Core to the NS3 genes, in 17 subjects with acute community-acquired HCV infection (Li *et al*., 2012). Analyzing sera sampled within the initial weeks of infection, before an evident immune selection, they found a greater homogeneity in these sequences compared to those amplified in 14 chronically infected subjects. In about 60 % of cases, 1 to 4 different viruses were transmitted (Li *et al*., 2012). In these subjects, viral lineages showed a star-like phylogeny of the mutated sequences with random nucleotide substitutions which coalesced to the T/F viral genomes. In the remaining part of the studied subjects, six to more than 30 discrete viral lineages were transmitted with a mean of 12 T/F viruses (Li *et al*., 2012). However, the subjects enrolled in this study represented a broad spectrum of HCV infection in the United States, such as IDUs, men who have sex with men, heterosexuals, household acquired infection, which could explain why the authors highlighted a range of T/F viruses from 1 to 37. These results are quite different from those obtained by 454 pyrosequencing reported by two different groups (Bull *et al*., 2011; Wang *et al*., 2010). The pyrosequencing method allows to determine a DNA sequence following a chemiluminescent reaction which detects the pyrophosphate release upon incorporation of each complementary nucleotide. This technology may generate more than 10^8 bases of DNA sequence, while in short fragments of about 300-500 nt (Margulies *et al*., 2005; Ronaghi, Karamohamed, Pettersson, Uhlén, & Nyrén, 1996). A limitation of this method relies on the assembly process of these short reads into longer genome sequences, because this approach has the disadvantage of losing linkage information between amplicons. Moreover, pyrosequencing does not allow easy distinction of sequence

variations authentically present in the viral population from artifactual mutations introduced as a result of the experimental procedure (Wang *et al*., 2010). Wang *et al*. analyzed HCV populations of three subjects following longitudinally during acute HCV infection. Focusing the analysis on four different regions in the HCV genome, from the 5'UTR to the E2 gene, they found that only 1 to 4 viral variants initiated productive infections (Wang *et al*., 2010). Subsequently, they described almost no diversification in the 5'UTR and the greatest sequence divergence in the HVR-1 of the E2 envelope, showing until more than 100 different variants (Wang *et al*., 2010). Bull and colleagues analyzed HCV populations in four subjects followed longitudinally from asymptomatic acute infection until clearance or chronicity. They amplified almost the entire viral genome (Bull *et al*., 2011). Phylogenetic analysis of viral variants obtained at different time points revealed two sequential bottleneck events. The first was associated with the viral transmission, upon which a single HCV variant was sufficient to establish three out of the four infections. Afterwards, a new decline in viral diversity was observed at about 100 days post-infection, likely driven by the emergence of the adaptive immune responses (Bull *et al*., 2011). Although in none of these latter studies the exact date of the beginning of the infections was known, nor the subjects at the origin of the contaminations were available, these experimental approaches could allow to unambiguously identify actual T/F viral genomes and to track single nucleotide diversification from them. In this way a unique opportunity to the analysis of HCV evolution *in vivo* is provided.

Mother-to-child transmission (MTCT) is the leading cause of childhood HCV infection in developed countries, with an estimated transmission rate of about 5% (Farci *et al*., 2006). Why some mothers transmit the virus to their child, while others do not, remains obscure (Dowd *et al*., 2009b; Kudo *et al*., 1997). However, several maternal factors have been shown to play a significant role in HCV vertical transmission. These include high HCV viral load, HIV/HCV co-infection, i.v. drug use, certain HLA types, and the presence of

HCV RNA in maternal peripheral blood mononuclear cells (PBMC) (Dowd *et al*., 2009b; Kudo *et al*., 1997; Pawlowska *et al*., 2012). Nevertheless, the exact mechanism of MTCT has not yet been clarified. As for HCV transmission after LT, studies of MTCT in mother-infant pairs have highlighted bottleneck events in infected babies, with HVR1 sequences in the newborns' quasispecies less heterogeneous than those amplified in the corresponding mothers' quasispecies (Weiner *et al*., 1993; Kudo *et al*., 1997; Manzin *et al*., 2000; Mazza *et al*., 1998; Pollack, Hou, Hughes, & Borkowsky, 2004). However, a greater divergence has been reported between mothers' and infants' envelope sequences in cases of HCV MTCT compared to HIV MTCT events (Mazza *et al*., 1998). Similarly to what has been shown for HCV transmission after LT, transmitted variants in children did not always represent the predominant variant(s) in the mothers' quasispecies (Weiner *et al*., 1993; Kudo *et al*., 1997; Pollack *et al*., 2004). Moreover, as for HIV transmission, for which protection from mother-to-infant virus transmission by the presence of HIV nAbs in seropositive mothers is still controversial (Barin *et al*., 2006; Lynch *et al*., 2011; Russell *et al*., 2011), investigations on the role of maternal nAbs in the prevention of HCV MTCT have produced contradictory results. Indeed, some studies reported that the initial presence of maternal nAbs, and then of infant antibodies, did not prevent the appearance and persistence of high HCV titers in children (Dowd *et al*., 2009b; Farci *et al*., 2006), whereas other authors found a link between maternal humoral response and prevention of HCV transmission in their children (Kudo *et al*., 1997). Interestingly, in this report, compared to the mothers with infected infants, those with uninfected children more frequently had high amount of HCV RNA in the high-density fraction of their sera, which it is known to include antibody-bound HCV particles.

Discourse apart, since the particular circumstances of patients suffering of chronic hepatitis C undergoing liver transplantation, deserve studies of liver grafts reinfection, in

which HCV genetic bottleneck events have also been characterized (Fafi-Kremer *et al*., 2010; Feliu *et al*., 2004; Hughes *et al*., 2004; Schvoerer *et al*., 2007). Liver failure induced by HCV is the leading cause for liver transplantation (LT) in Western countries (CDC), but after transplantation, allografts are universally reinfected by the virus still circulating in the transplanted host, which constitutes an important issue in the management of those patients (Brown, 2005). In all studied cases of LT consequent to HCV infection, a profound homogenization of the viral quasispecies population have been reported, with only a small fraction of viral variants present before transplantation which was selected after LT (Fafi-Kremer *et al*., 2010; Feliu *et al*., 2004; Hughes *et al*., 2004; Schvoerer *et al*., 2007). Whereas Feliu *et al*. and Fafi-Kremer *et al*. found that the predominant variant before transplantation propagated and remained predominant after LT (Fafi-Kremer *et al*., 2010; Feliu *et al*., 2004), this was not the same for Hughes and colleagues, which, conversely, found that the predominant variants in serum of pre-transplanted patients and in postperfusion liver differed greatly (Hughes *et al*., 2004). Interestingly, all reported genetic bottlenecks were associated with decreased genetic complexity and diversity of HVR1 (Fafi-Kremer *et al*., 2010; Feliu *et al*., 2004; Hughes *et al*., 2004; Schvoerer *et al*., 2007). It is likely that the implantation of the new liver (changing environment), as well as the immunosuppressive therapy (lack of selective pressure) lie at the root of the genetic bottleneck event, by selecting those variants in the quasispecies which are able to infect the liver graft more efficiently (Fafi-Kremer *et al*., 2010; Schvoerer *et al*., 2007). Functional investigation of E1E2 envelope sequences harbored by viral variants before and after LT, demonstrated that viral entry into hepatocytes and escape from nAbs likely drove the selection of HCV variants during reinfection of the liver graft (Fafi-Kremer *et al*., 2010). Therefore, the hypothesis that viral variants with more efficient entry capabilities and no sensitivity to nAbs might be preselected in the pre-transplanted patient, has been proposed by Fafi-Kremer and

colleagues. Indeed, in all patients undergoing LT, they found that the strains present after transplantation, and which escaped host neutralizing response, were also the most prevalent strains before LT (Fafi-Kremer *et al*., 2010). Furthermore, analyzing envelope sequences of non-selected and selected variants, Fafi-Kremer and co-workers found that selected E1E2 sequences included some mutations within HVR1 and within a region known to be involved in E2 and CD81 interaction, which could explain the enhanced viral entry (Fafi-Kremer *et al*., 2010). However, it is worth to note that the presence of naturally occurring neutralizing antibodies in transplant patients and post-transplant therapy undoubtedly influence reinfection (Brown *et al*., 2012).

b) Studies in Animal Model Systems

Beside humans, chimpanzees are the only species naturally susceptible to HCV infection (Sandmann & Ploss, 2013). Therefore, before the advent of chimeric mouse models, chimpanzees have been widely used as an HCV model system, although several differences in the natural history of HCV infection between chimpanzees and humans have been reported. Indeed, HCV infection in chimpanzees is milder than in humans; they can clear the virus more often than humans; chronic carriers do not develop cirrhosis or fibrosis; they produce a lower amount of anti-envelope Abs, and in experimentally infected animals, envelope amino acid sequences, even the HVR1, can remain relatively homogeneous during infection (Fernandez *et al*., 2004; MacArthur, Wu, & Wu, 2012; Post *et al*., 2009; Ray *et al*., 2000). However, ethical concerns, high cost, and limited availability have led to a ban on the use of these animals for biomedical research in most countries (Sandmann & Ploss, 2013). Nevertheless, several studies on HCV transmission using chimpanzees have been reported (Farci *et al*., 1996; Nainan *et al*., 2006; Ray *et al*., 2000; Hijikata *et al*., 1995; Sugitani &

Shikata, 1998). I would like to mention two studies in particular, about chimpanzees experimentally infected by inoculation with the same human plasma sample containing HCV quasispecies (Hijikata *et al*., 1995; Sugitani & Shikata, 1998). In both of these studies, the authors found the same HVR1 sequences as predominantly represented within the donor inoculum quasispecies. However, whereas in one study comparison of HVR1 amino acid sequences in the inoculum and in infected chimpanzees revealed that the major quasispecies of the donor inoculum was transmitted to the animals (Sugitani & Shikata, 1998), in the other study a minor variant of the donor inoculum was recovered from the chimpanzees (Hijikata *et al*., 1995). Furthermore, in the latter study, the viral variant transmitted to the chimpanzee was the same amplified in a human lymphocytic cell line infected with the same serum inoculum, suggesting that the same viral strain was selected *in vivo* and *in vitro* (Hijikata *et al*., 1995). According to these data, Sugitani *et al*. hypothesized that although all the viral variants in the inoculum might be infectious, selection in the infected chimpanzees would have allowed the propagation of one HVR1 variant rather than another (Sugitani & Shikata, 1998). Interestingly, another study about five chimpanzees experimentally infected using a commercially prepared HCV-contaminated factor VIII concentrate, and one human patient accidently contaminated through the exposure to the same preparation, reported that among the HCV multi-genotypes included in the factor VIII preparation, only HCV of genotype 1b was able to initiate infection in the patient, whereas of the five experimentally infected chimpanzees, three had a mixed infection with HCV subgenotypes 1a and 1b (Nainan *et al*., 2006).

Recently, a 'SCID/Alb-uPA' chimeric mouse model, generated through the transplantation of normal human hepatocytes into SCID (Severe Combined Immunodeficiency) mice carrying an Urokinase Plasminogen Activator transgene under the albumin promoter (Alb-uPA), has been reported to be susceptible to HCV infection

(Mercer *et al*., 2001). This small animal model has been used to study HCV transmission. Indeed, with this system, HCV infection could be established in human liver cells, and the absence of a functional adaptive immune response provides a good surrogate for the acute phase of HCV infection in humans, when infected patient are still asymptomatic and there is no evidence for host immune responses (Brown *et al*., 2012). By using an SGA-direct sequencing approach, Brown and co-workers were able to dissect HCV transmission events by inoculating a human HCV-positive serum into four recipient SCID/Alb-uPA mice (Brown *et al*., 2012). By analyzing the E1E2 amino acidic sequences derived from the donor inoculum and from recipient mice, these authors found that the majority HVR1 variant in the donor plasma was also the majority HVR1 variant in all recipient mice, but none of the recipient full-length E1E2 envelope sequences was identical to the donor E1E2 variants (Brown *et al*., 2012). By applying a Bayesian coalescent analysis, they hypothesized that a restricted number of donor viral variants established initial infection in the mice, undergoing nonsynonymous substitutions upon the transmission event. Subsequently, using the HCVpp system, the authors performed a functional characterization of the E1E2 sequences harbored by the donor and the recipient viruses. It is interesting to note that, when tested on the Huh7.5 cell line, the HCVpp entry level conferred by both the mouse- and inoculum-derived E1E2 glycoproteins was regularly less than 5% of the entry level conferred by the H77 E1E2 control. The infection values increased by 40% when primary hepatocytes were used as target cells (Brown *et al*., 2012). These results contrast with a previously cited study of HCVpp entry using E1E2 envelope proteins derived from a liver transplant setting, where primary hepatocytes were less efficient at supporting entry than Huh7 cells (Fafi-Kremer *et al*., 2010). Several reasons might account for these discrepancies, for instance hepatocyte donor-specific genetic differences, virus-specific factors allowing different use of entry factors, or also technical factors. However, using both the Huh7.5 or primary hepatocytes, they found that the

transmitted envelope sequences were statistically more infectious than the non-transmitted ones, and that one of the sequence variations which could explain such a different phenotype could involve the loss of a putative N-glycosylation site, outside the HVR1 region (Brown *et al*., 2012). Thus, with these results, they highlighted the fact that focusing functional analysis of E1E2 HCV envelope glycoproteins exclusively on the variability of the HVR1 region might be misleading (Brown *et al*., 2012). Nevertheless, the chimeric mice were experimentally inflected by inoculating 100 µl of HCV-infected serum (2.3×10^6 IU/ml) directly via the intrajugular route. This approach may not mimic the overall process of natural exposure to the virus, in particular the *a priori* small inoculum resulting from needlestick accidents. Therefore, further studies are needed to understand molecular mechanisms underlying HCV transmission in humans.

II. THESIS PROJECT

Authentic transmission events of HCV in humans, before seroconversion, have rarely been investigated to date (Liu *et al*., 2006; Saito *et al*., 2004). This is mostly due to difficulties in identifying primo-infections (70% of acute hepatitis C cases are asymptomatic) (Pawlotsky, 2004), and difficulties to identify source subjects at the origin of transmission events. Therefore, how HCV with multiple variants in an infected individual is transmitted to subsequently cause acute hepatitis in a new recipient is not well understood yet. To address this issue, we had the opportunity to work on three cases of BEAs through contaminated needlesticks between two chronically HCV-infected patients (DA and DB), and three HCWs (RA1, RA2, and RB). Having access to plasma samples collected from the source subjects at the time of the accidents, and exactly knowing the date of contaminations, we proposed to track the virus transmission event in these three cases. The first objective of this thesis project sits on the identification of HCV viral variants in the donors' quasispecies which were able to start the new infection in the naïve hosts. Particularly, we focused our analysis on the E1 and E2 envelope glycoproteins, since they should be a good candidate to understand whether the transmitted viruses bring phenotypic features that could explain their selective transmission. Hence, a major challenge in our study was to detect low frequency viral variants circulating in the donors' and recipients' quasispecies, and to be able to unambiguously identify T/F HCV genomes. The experimental approach we chose for this purpose, which has been previously used to gain insight into HIV-1 transmission (Keele *et al*., 2008; Salazar-Gonzalez *et al*., 2009), was the SGA of end-point diluted plasma viral vRNA/cDNA, followed by direct sequencing of uncloned DNA amplicons. This strategy differs from previous methods applied to HCV genome analysis by several key points. First, it provides proportionally represented gene-wide viral sequences which circulate in human plasma at different frequencies. Second, SGA-direct sequencing eliminates *Taq* polymerase errors since such nucleotide misincorporation is identified as double peaks in sequence chromatograms.

Third, SGA eliminates both template resampling and template recombination events because amplification is initiated from single genomes (Keele *et al*., 2008; Meyerhans, Vartanian, & Wain-Hobson, 1990; Palmer *et al*., 2005; Salazar-Gonzalez *et al*., 2009; Shaw & Hunter, 2012; Simmonds, Balfe, Ludlam, & Brown, 1990).

Very little is known about the phenotypic characteristics of founder viruses in HCV bottleneck transmission. A recent functional characterization of HCV variants emerging after LT in HCV-infected recipients indicated that variants with increased viral entry efficiency and lower neutralizing sensibility were preferentially selected in the post-transplant phase (Fafi-Kremer *et al*., 2010). Moreover, in another study conducted on a murine model of HCV infection, it has been shown that the major post-transmission E1E2 variants conferred an increased capacity for cell entry *in vitro* compared to the major variant present in the human donor inoculum (Brown *et al*., 2012). However, it is worth to note that these envelope sequences generally conferred very low-level infectivity in the HCVpp system used to infect the hepatoma cell line Huh7.5. One can speculate that transmitted viruses might interact with greater avidity with HCV-specific cellular receptors. In a parallel with HIV transmission in humans, is now globally accepted that HIV-1 founder viruses consistently predict at least a CCR5 (C-C chemokines receptor type 5) tropism (Keele *et al*., 2008; Shaw & Hunter, 2012). To assess whether our identified T/F viruses possess critical functional properties that distinguish them from the viral variants circulating in the donor patients, we proposed as the second objective of this thesis project, their functional characterization using the *in vitro* model system of the HCVpp. Data obtained by this genetic and phenotypic analysis shed light on the selective transmission of HCV quasispecies and on the early stage of infection, which is a critical period from a vaccine perspective, since the lowest viral diversity might render HCV most vulnerable to elimination by vaccine-elicited immune responses (Houghton & Abrignani, 2005; Maheshwari, Ray, & Thuluvath, 2008).

III. INTRODUCTION & OBJECTIFS DU TRAVAIL

Selon l'Organisation Mondiale de la Santé, l'infection par le virus de l'hépatite C (VHC) touche près de 170 millions de personnes dans le monde. Elle se manifeste par une atteinte hépatique aiguë 4 à 12 semaines après contamination, le plus souvent asymptomatique. L'hépatite aiguë est spontanément résolutive dans environ 30% des cas. Dans les autres cas, l'hépatite devient chronique (Fig. 1) provoquant une cirrhose chez 10 à 20% des personnes infectées dans les 20 ans suivant la contamination. La maladie peut ensuite progresser vers un carcinome hépatocellulaire avec un taux de transition annuel de 1 à 4% chez les patients atteints d'une cirrhose liée au VHC (Pawlotsky, 2004). Les traitements actuels, reposant sur une bithérapie interféron (IFN) α-pegylé associé à la ribavirine, ont une efficacité variable selon les génotypes : 50% d'échec en cas d'infection par une souche de génotype 1. Cependant, l'évolution des connaissances sur la biologie du virus a récemment permis le développement de nouvelles molécules thérapeutiques ciblant directement les protéines virales. Des inhibiteurs des protéases NS3/NS4A, tels que le Bocéprévir ou Télaprévir, ont ainsi été développés et mis sur le marché d'abord aux Etats Unis, et puis en Europe, à partir de Mai 2011 (Kwo *et al*., 2010; McHutchison *et al*., 2010). Même si ces nouvelles molécules permettent d'améliorer le pronostic de patients en échec thérapeutique, notamment ceux infectés par le VHC de génotype 1b (Bacon *et al*., 2011; Liang & Ghany, 2013), le développement d'un vaccin contre l'hépatite C reste primordial (Barnes *et al*., 2012; Beaumont *et al*., 2013; Law *et al*., 2013).

Le VHC appartient à la famille *Flaviviridae*, et au genre *Hepacivirus*. Sept génotypes majeurs de VHC ont été identifiés dans le monde, numérotés de 1 à 7, avec au sein de certains de ces génotypes une division en sous-types. Les génotypes 1a, 1b, 2a et 3a sont prédominants aux Etats-Unis, en Europe, en Australie, et en Asie de l'Est (Fig. 4) (Trinks *et al*., 2012). Dans les pays en développement, la transmission du virus résulte notamment de l'usage de matériel d'injection non stérile et de transfusions de sang non testé.

En Europe de l'Ouest et en Amérique du Nord, elle a été liée à des transfusions de sang contaminé jusqu'au début des années 1990 et découle actuellement de l'usage de drogues dans la plus part des cas. Il existe également des cas exceptionnels de contamination accidentelle de personnel médical lors d'accidents d'exposition au sang (Liu *et al*., 2006; Noguchi *et al*., 1997; Saito *et al*., 2004; Simmonds, 2004; Yazdanpanah *et al*., 2006).

La véritable structure de la particule virale reste encore mal définie. En tant que membre de la famille des *Flaviviridae*, le VHC est décrit comme une petite particule (~ 50 nm de diamètre) composée d'une nucléocapside dense aux électrons d'environ 30 nm de diamètre, entouré d'une bicouche lipidique dans laquelle sont ancrées les deux glycoprotéines d'enveloppe E1 et E2 (Lindenbach & Rice, 2007). Il a été démontré que les particules virales contenant de l'ARN étaient associées à des lipoprotéines de faible et très faible densité (LDLs et VLDLs) dans le sérum des patients infectés. Cette association aboutit à la formation des 'lipo-viro-particules' (LVPs) (Fig. 2) (André et al., 2002; Bartenschlager et al., 2011; Icard et al., 2009). Le génome du VHC est composé d'une seule molécule d'ARN simple brin, de polarité positive, d'environ 9,6 kb. Il se caractérise par un unique cadre de lecture ouvert, ou ORF, codant une polyprotéine d'environ 3000 acides aminés (Bartenschlager *et al*., 2011; Moradpour *et al*., 2007). Cette ORF est flanquée à ses extrémités 5' et 3' par des régions non codantes, ou UTRs, essentielles pour la traduction de la polyprotéine et pour la réplication de l'ARN (Fig. 3). Comme de nombreux autres virus à ARN, le VHC se caractérise par un haut degré d'hétérogénéité génétique résultant d'un taux élevé de mutations induites par l'ARN polymérase virale, celle-ci étant dépourvue d'activité correctrice. Ce taux de mutation est estimé à 10^{-4} – 10^{-5} mutations par nucléotide et par cycle de réplication (Ribeiro *et al*., 2012). Par ailleurs, la production virale quotidienne peut atteindre 10^{12} virions chez un individu donné (Guedj *et al*., 2010; Neumann, 1998). Par conséquent, le VHC circule chez un individu infecté sous forme d'une population dynamique

de virus génétiquement liés, collectivement appelés « quasi-espèces » (1971) (Fig. 5) (Lauring *et al*., 2013; Fishman & Brunch, 2010; Martell *et al*., 1992). Cependant, l'hétérogénéité génétique n'est pas répartie de façon uniforme tout au long du génome. Les régions les plus conservées sont la région 5'-UTR et la partie terminale de la région 3'-UTR, leur variabilité étant limitée par l'existence de structures secondaires spécifiques. A l'inverse, les gènes codant les deux glycoprotéines d'enveloppe représentent la partie plus hétérogène du génome viral (Lindenbach & Rice, 2007; Simmonds, 2004).

Les glycoprotéines d'enveloppe du VHC (E1 et E2) sont des protéines de membrane de type I avec des domaines extracellulaires N-terminaux fortement glycosylés et des domaines transmembranaires C-terminaux hydrophobes (Lindenbach & Rice, 2007). E1 et E2 jouent un rôle essentiel dans différentes étapes du cycle infectieux du VHC et plus particulièrement, dans l'assemblage de la particule virale, dans l'entrée du virus dans les cellules cibles et dans la fusion avec la membrane endosomale. Par ailleurs, ces glycoprotéines constituent le principal déterminant antigénique de la particule infectieuse reconnu par les anticorps neutralisants. Ainsi, elles sont considérées comme un élément central pour l'élaboration d'un vaccin contre le VHC (Edwards *et al*., 2012). La région N-terminale de E2 contient la séquence la plus variable de l'ensemble du génome viral, à savoir la région hypervariable 1, ou HVR1 (McCaffrey *et al*., 2011). HVR1 est composée d'un segment de 27 acides aminés, essentiellement basiques (aa 384-410 selon la polyprotéine de référence H-77 ; numéro d'accès EMBL: AF009606), qui est supposé être le site d'interaction avec le récepteur cellulaire SR-BI (Scarselli *et al*., 2002). HVR1 contient également un épitope B immunodominant (Ray *et al*., 2010; Wang *et al*., 2011) et son grand taux de mutations semble contribuer à l'évasion du virus de la réponse immunitaire de l'hôte (Edwards *et al*., 2012). Trois autres régions très variables de E2 sont représentées par les régions hypervariables 2 (HVR2) et 3 (HVR3) et la région variable intergénotypique (IgVR)

(McCaffrey *et al*., 2011; Troesch *et al*., 2006). HVR2 (aa 460-485) et IgVR (aa 570-580) ne sont pas connues pour être des cibles de la réponse humorale (McCaffrey *et al*., 2011). Cependant, il a été montré que seule la suppression de HVR2 et/ou de IgVR, et non pas celle de HVR1, affectait l'hétérodimérisation des complexes E1E2, en réduisant la capacité des VHCpp de se lier au récepteur cellulaire CD81 (Albecka *et al*., 2011; McCaffrey *et al*., 2011). HVR3 (aa 431-466), la région la moins variable parmi les quatre citées précédemment, a été empiriquement divisée en deux portions de taille à peu près égale, appelées respectivement HVR3a (aa 431-449) et HVR3b (450-466). À l'aide de plusieurs algorithmes de prédiction *in silico*, il a été supposé que la portion HVR3a correspond à une région antigénique, exposée à la surface de la glycoprotéine E2, étant ainsi accessible et reconnaissable par les anticorps. Cette région devrait comprendre une partie centrale plutôt hydrophile flanquée de séquences hydrophobes (Troesch *et al*., 2006). Par ailleurs, le domaine extracellulaire de E2 contient aussi trois segments discontinus plus conservées (aa 394-424, aa 437-447, aa 523-540), qui forment collectivement un épitope conformationnel constituant le site putatif d'interaction avec un autre récepteur cellulaire, le CD81 (Krey *et al*., 2010; Pileri *et al*., 1998).

Lors de l'infection d'un nouvel hôte par le VHC, il a été démontré qu'il y avait un goulot d'étranglement génétique s'exerçant sur la quasi-espèce présente chez l'individu à la source de la contamination. Ce goulot d'étranglement génétique est défini comme un événement évolutif entrainant une réduction à la fois de la taille da la population virale et de sa variabilité génétique, en raison de la disparition d'une proportion importante de variants viraux (Bull et al., 2011). Ainsi, un seul ou un nombre limité de «virus transmis/ fondateurs» (T/F) émergent pour dominer la population future et établir une infection chez ce nouvel hôte (Bull *et al*., 2011; Li *et al*., 2012; Wang *et al*., 2010). Cependant, il reste difficile de savoir si ce phénomène de goulot d'étranglement génétique est dû à un faible nombre de variants transférés lors de la transmission, ou s'il est le résultat d'événements évolutifs précoces avec

élimination d'un grand nombre de variants liée à l'environnement rencontré chez l'hôte naïf (Bull *et al*., 2011). Après transmission, la population virale se diversifie sous l'influence de facteurs viraux et de facteurs liés à l'hôte, notamment la réponse immunitaire. Ce processus semble déterminer la progression de l'infection (Farci *et al*., 2000; 2006; Kuntzen *et al*., 2007; Merani *et al*., 2011; Post *et al*., 2009; Ray *et al*., 2005; Smith *et al*., 2010). Le mécanisme de transmission du VHC chez l'homme, en particulier la phase précédant la séroconversion, a rarement été étudié à ce jour (Liu *et al.*, 2006; Saito *et al.*, 2004) en raison des difficultés rencontrées pour identifier les individus durant la primo-infection (Pawlotsky, 2004). L'accès aux 'sujets sources' à l'origine des infections est également compliqué en dehors du contexte particulier des accidents d'exposition au sang. Par conséquent, des investigations doivent encore être menées pour tenter d'apporter des éléments de compréhension sur les contraintes subies par le VHC lors de sa transmission à un nouvel hôte. Dans ce travail de thèse, nous avons eu accès à trois cas d'accidents d'exposition au sang (AES), suite à des piqûres avec des aiguilles contaminées, impliquant deux patients chroniquement infectés par le VHC (DA et DB) et trois représentants du personnel soignant (RA1, RA2 et RB). Les sujets RA1 et RA2 ont été contaminés par le même patient DA. Ainsi, nous avons pu suivre dans son intégralité le processus de transmission du VHC dans ces trois cas d'AES en identifiant précisément les variants viraux transmis chez les sujets RA1, RA2 et RB. Nous avons centré notre analyse sur les deux glycoprotéines d'enveloppe E1 et E2, ces protéines étant susceptibles de porter les caractéristiques phénotypiques associées à la transmission sélective de certains variants viraux. L'approche expérimentale que nous avons choisie, l'amplification de génomes uniques (SGA), est un point essentiel de ce projet. Initialement développée pour l'étude da la transmission du VIH-1 (Keele *et al*., 2008; Salazar-Gonzalez *et al*., 2009), cette stratégie diffère des précédentes méthodes appliquées à l'étude de la variabilité du VHC en fournissant des séquences génomiques virales de manière proportionnelle à leur

représentation dans le plasma de l'individu infecté, et cela sans erreur d'échantillonnage ou d'incorporation nucléotidique liée à la *Taq* polymérase (Keele *et al*., 2008; Palmer *et al*., 2005; Salazar-Gonzalez *et al*., 2009; Simmonds *et al*., 1990; Simmonds *et al*., 1990).

Les caractéristiques phénotypiques des virus transmis lors de l'infection par le VHC restent à déterminer. Une étude récente, dans le contexte très particulier de la greffe hépatique, a montré que les variants émergeant après greffe avaient une capacité accrue à entrer dans les cellules cibles. Par ailleurs, ces variants sont moins sensibles aux anticorps neutralisants du patient greffé que les variants éliminés après transplantation (Fafi-Kremer *et al*., 2010). Dans une autre étude menée sur un modèle murin d'infection par le VHC, il a été démontré que les variants majoritaires transmis aux souris conféraient une capacité accrue dans l'entrée cellulaire par rapport au variant viral dominant la quasi-espèce du donneur (Brown *et al*., 2012). Si on fait un parallèle avec la transmission du VIH chez l'homme, il a été largement montré que les glycoprotéines d'enveloppe des virus transmis leur conféraient un tropisme préférentiel pour le corécepteur viral CCR5 (C-C chimokines receptor type 5) (Keele et al., 2008; Shaw & Hunter, 2012). Afin d'évaluer si les virus T/F identifiés dans notre étude possédaient des propriétés spécifiques les distinguant des variants viraux circulant chez les patients donneurs, nous avons analysé leurs propriétés fonctionnelles *in vitro* en utilisant le système des virus pseudo-typés (VHCpp) (Bartosch *et al*., 2003a; Drummer *et al*., 2003; Hsu *et al*., 2003a). Lors de mon travail de thèse, les données obtenues par l'analyse génétique et phénotypique des glycoprotéines d'enveloppe des virus T/F nous ont permis d'apporter des éléments nouveaux sur le processus de la transmission sélective des quasi-espèces du VHC, et sur le stade précoce de l'infection par ce virus, une période critique dans une perspective vaccinale. En effet, la faible diversité virale observée à cette période

pourrait rendre le virus plus vulnérable à l'élimination par la réponse immunitaire induite par la vaccination (Houghton & Abrignani, 2005; Maheshwari *et al*., 2008).

IV. MATERIALS & METHODS

1. Study Participants

The study was conducted on three health care workers (recipients RA1, RA2 and RB), infected by HCV genotype 1b through a documented needlestick exposure to blood from patients with chronic hepatitis (donors DA and DB). All subjects were females, with the exception of donor DB. Recipients RA1 and RA2 shared the same donor DA (Fig. 19) with an interval of 10 months. They became chronic carriers of hepatitis C, but after receiving a similar bitherapy with pegylated interferon and ribavirin they cleared the virus.

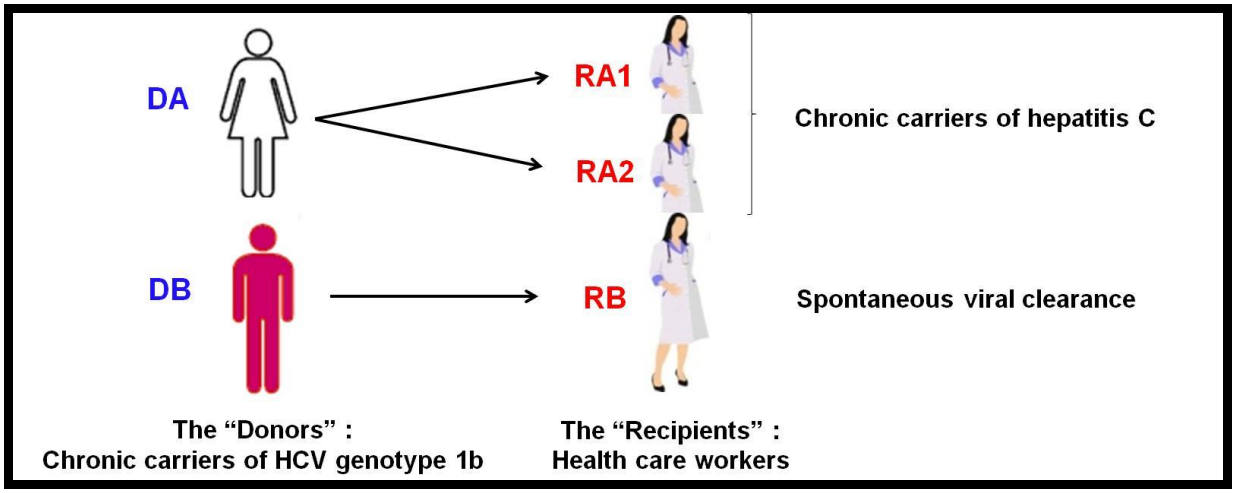


Fig. 19. Three blood-exposure accidents (BEAs) through HCV contaminated needlesticks.

Recipient RB, which was contaminated by donor DB, underwent spontaneous viral clearance without the need of drug therapy. For the transmission pairs DA/RA1 and DB/RB, we obtained plasma samples from donors at the time of the needlestick accident. Unfortunately, no sample was available from donor DA at the time of RA2 contamination, which occurred 10 months after the RA1contamination. All recipients were monitored with sequential sampling of plasma until they became viremia negative after 6, 8, and 12

months, respectively for RA1, RA2 and RB (Fig. 21). All seroconverted after about the first month of infection. HCV antibody testing was performed using the qualitative Abbott Architect anti-HCV chemiluminescent microparticle immunoassay (Abbott). Specific anti-HCV antibodies were confirmed using the RIBA (Recombinant Immunoblot Assay) test (Chiron corporation). Quantitative HCV RNA detection was performed using an Abbott HCV RealTime assay (Abbott). The lower limit of detection for this assay was: 12 IU/ml for the 0.5 ml sample volume. This study was approved by the Institutional Review Board of Tours University Hospital (Comité de Protection des Personnes - CPP), on the basis of written informed consents in accordance with French regulations.

2. Single Genome Amplification of Full-length E1E2 Glycoproteins and Sequencing

Full-length E1E2 sequences (encoding a region including the last 22 amino acids of the Core through the C-terminal end of E2, corresponding to the polyprotein sequence of the reference strain H77 [accession no. NC_004102]) were amplified by an SGA approach. For the donors, plasma sampled obtained at the time of BEAs were used. The earliest time point plasma samples, giving a positive result of amplification, were used for the recipients (Fig. 21). For each sample, viral RNA was extracted using the Qiagen Viral RNA Mini Kit (Qiagen), and then reverse-transcribed into complementary DNA (cDNA) with the antisense primer HCV1b ExtAS 5'-GAGCAGGAGCAGCGGCCAT-3' (nt 2720-2738 H77) using the SuperScript III (Invitrogen, Life Technologies) according to the manufacturer's protocol. cDNA was serially diluted and amplified in 96-multiwell plates by nested-PCR of full-length E1E2 sequence, so as to identify a dilution giving a

maximum frequency of 3/10 PCR-positive reactions. According to the 'Poisson distribution law', at this dilution, most wells contain amplicons derived from a single cDNA molecule (Keele *et al*., 2008; Li *et al*., 2012; Salazar-Gonzalez *et al*., 2009). Nested-PCR was done using the Platinum *Taq* High Fidelity polymerase (Invitrogen, Life Technologies) with the following primers: 1st round sense primer HCV1b ExtS 5'-CGGCGTGAACTATGCAACAGG-3' (nt 821-841 H77) and 1st round antisense primer HCV1b ExtAS (see above), 2nd round sense primer HCV1b IntS 5'-TCTGATGGGTTGCTCTTTCTCTATCTTCC-3' (nt 845-873 H77) and 2nd round antisense primer HCV1b IntAS 5'-AATCAGGCCTCAGCCTGGGCTATCAG-3' (nt 2559-2584 H77). All PCR yields were separated by electrophoresis on 1% sucrose gels, so as only the amplification products which complied with the 'Poisson distribution' were purified and directly sequenced via BigDye terminator chemistry, using an ABI 3130 capillary sequencer (Applied Biosystems, Life Science Technologies). Electropherograms were manually inspected and amplicons exhibiting mixed bases (double peaks), suggesting amplification from multiple templates or a *Taq* polymerase error, were excluded from further analysis (Fig. 20).

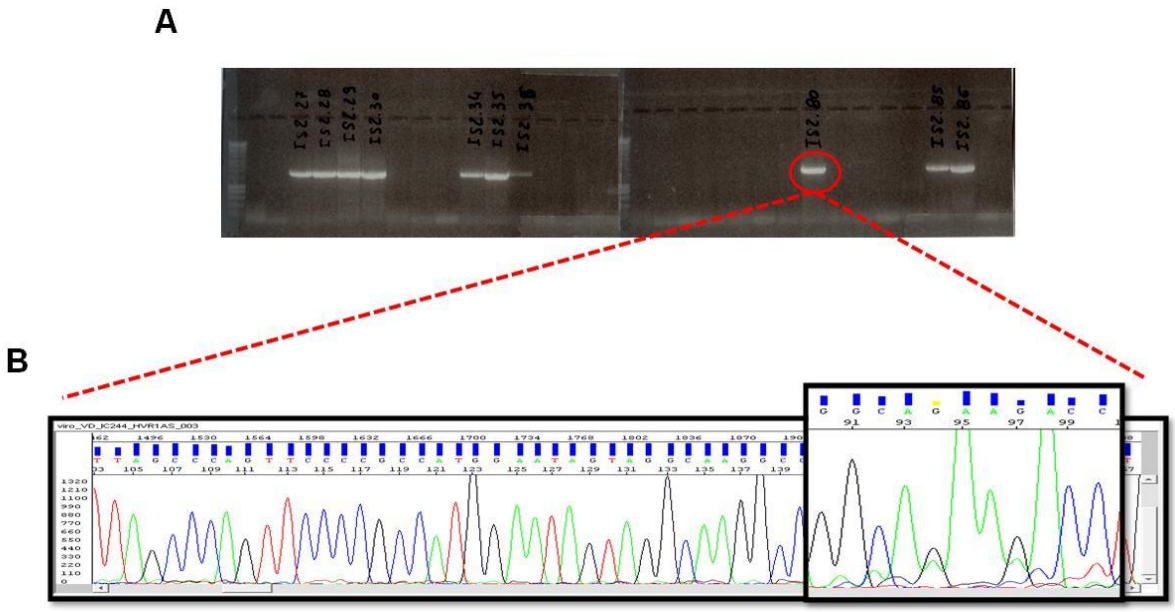


Fig. 20. The SGA principle.

(A) *Amplicons derived from end-point dilutions are separated by electrophoresis to identify the dilution giving a maximum frequency of 3/10 PCR-positive reactions.* ***(B)*** *Electropherograms obtained by direct sequencing of SGA-yields are manually inspected. All PCR amplicons producing double peaks are excluded from further analysis.*

3. Quasispecies Analysis

The obtained nucleotide sequences were aligned using 'ClustalW' in MEGA5 (Tamura *et al*., 2011), and were translated in the corresponding deduced amino acid sequences. Overall phylogenetic relationships between sequences were analyzed using a neighbor-joining tree constructed utilizing the Tamura 3-parameter method, the best suited distance matrix for our sequences following the MEGA5 models test. The rate of variation among sites was modeled with a gamma distribution (shape parameter = 5). Genetic complexity (entropy), defined as the number of viral variants within HCV quasispecies, was evaluated at both the nucleotide and amino acid level, for the recipients and the donors quasispecies. Genetic diversity, defined as the frequency of mutations within the different

isolates of HCV quasispecies, was expressed by the Hamming score, at the nucleotide and amino acid level (Fishman & Branch, 2010; Schvoerer *et al*., 2013). To track amino acid substitutions in the recipient quasispecies that occurred upon transmission, we used the Highlighter tool (http://hcv.lanl.gov/content/sequence/HIGHLIGHT/highlighter.html) which compares transmitted and non-transmitted envelope sequences to a 'master sequence'. In our case, the 'master sequence' corresponds to the sequence of a non-transmitted dominant variant amplified in the corresponding donors' quasispecies (Fig. 24). To check the most significant differences between transmitted and non-transmitted envelope sequences, we used the 'Viral Epidemiology Signature Pattern Analysis' (VESPA) program developed by B. Korber and G. Myers in 1992 (http://hcv.lanl.gov/content/sequence/VESPA/vespa.html), with default settings (Elhefnawi *et al*., 2010). To generate graphical representations of the sequence patterns within the donors and recipients quasispecies, we computed sequence logos using 'WebLogo' (http://weblogo.berkeley.edu/logo.cgi) (Crooks *et al*., 2004).

4. Cloning of the Full-length Envelope Sequences

Following the phylogenetic and bioinformatic analysis of the quasispecies of the three pairs of patients, we chose to characterize the functional properties of 19 transmitted and non-transmitted E1E2 sequences obtained by SGA, representative of the main genetic lineages previously identified. A few additional E1E2 sequences derived from minor variants were also studied. (Fig. 24). To do this, we cloned the corresponding SGA products into the pcDNA3.1/V5-His-TOPO-TA mammalian expression vector (Invitrogen, Life Technologies), according to the manufacturer's protocol. The resulting plasmids were

subsequently used to transform TOP10 chemically competent *E. coli* bacteria. We analyzed positive transformants by PCR using the primers 'HCV1bIntAS' and 'HCV1bIntS' (see above), to further amplify only those plasmids in which E1E2 sequence genes were cloned in the correct orientation. All the amplified clones were then sequenced to confirm that the cloned full-length envelope sequences were identical to the nested-PCR yields used for the genetic analysis, and to check for the presence of the START and STOP codons.

5. Cell Lines

Human hepatoma Huh7.5 cells (Nakabayashi *et al*., 1982), and HEK-293T cells (Graham, Smiley, Russell, & Nairn, 1977) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% antibiotics (100 IU/ml of penicillin and 100 μg/ml streptomycin; Invitrogen).

6. Lentiviral HCV Pseudoparticles Production

To functionally characterize transmitted envelope variants, we used the lentiviral HCVpp system. The principle of this system is to produce a chimeric virus by replacing the parental virus envelope proteins with glycoproteins of heterologous virus. For this thesis project, HCVpps were generated by contransfecting $5.5x10^6$ HEK-293T cells with a 1:1 (6 μg/6 μg) mixture of a reporter virus, the pNL4.3*Luc*R$^-$E$^-$ HIV proviral clone, lacking

HIV glycoproteins and hosting the firefly luciferase reporter gene, and an expression vector bearing donor or recipient-derived E1E2 glycoproteins (Connor *et al.*, 1995). Control HCVpp bearing the vesicular stomatitis virus glycoprotein (VSV-G), the well characterized UKN1B5.23 HCV envelope, or no-envelope (ΔE1E2) were produced similarly (Brown *et al.*, 2012; Hsu *et al.*, 2003). Transfections were performed using the Fugene6 reagent (Promega) according to the manufacturer's instructions (Brown *et al.*, 2007; 2012; Hsu *et al.*, 2003). Cells were then maintained in DMEM supplemented with 10% FCS, 1% antibiotics, and 2% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). HEK-293T cells supernatants were collected 48h post-transfection, and purified by filtration through a 0.45µm-filter. The determination of the HIV p24 Core antigen concentration, performed by a commercially available ELISA kit assay (Innotest HIV Antigen MAb kit, Innogenetics), was used to quantify the pseudo-viral particles production to be able to normalize the input of each HCVpp tested in entry and neutralization assays (see below).

7. HCVpp Entry and Neutralization Assays

For HCVpp entry assays, Huh7.5 cells were seeded the day before assays in 96-well plates at a density of $5x10^3$ cells per well. Viral entry was determined by transducing the Huh7.5 cells with a small volume of serial five-fold dilutions of p24-normalized HCVpp-containing supernatants. HCVpps were added to target cells, tested in triplicate, and incubated for 4 hours at 37 °C and 5 % CO_2, in order to facilitate the pseudoparticle entry. Four hours post-transduction, DMEM supplemented with FCS and antibiotics was added to transduced cells so as to reach 250 µl final volume.

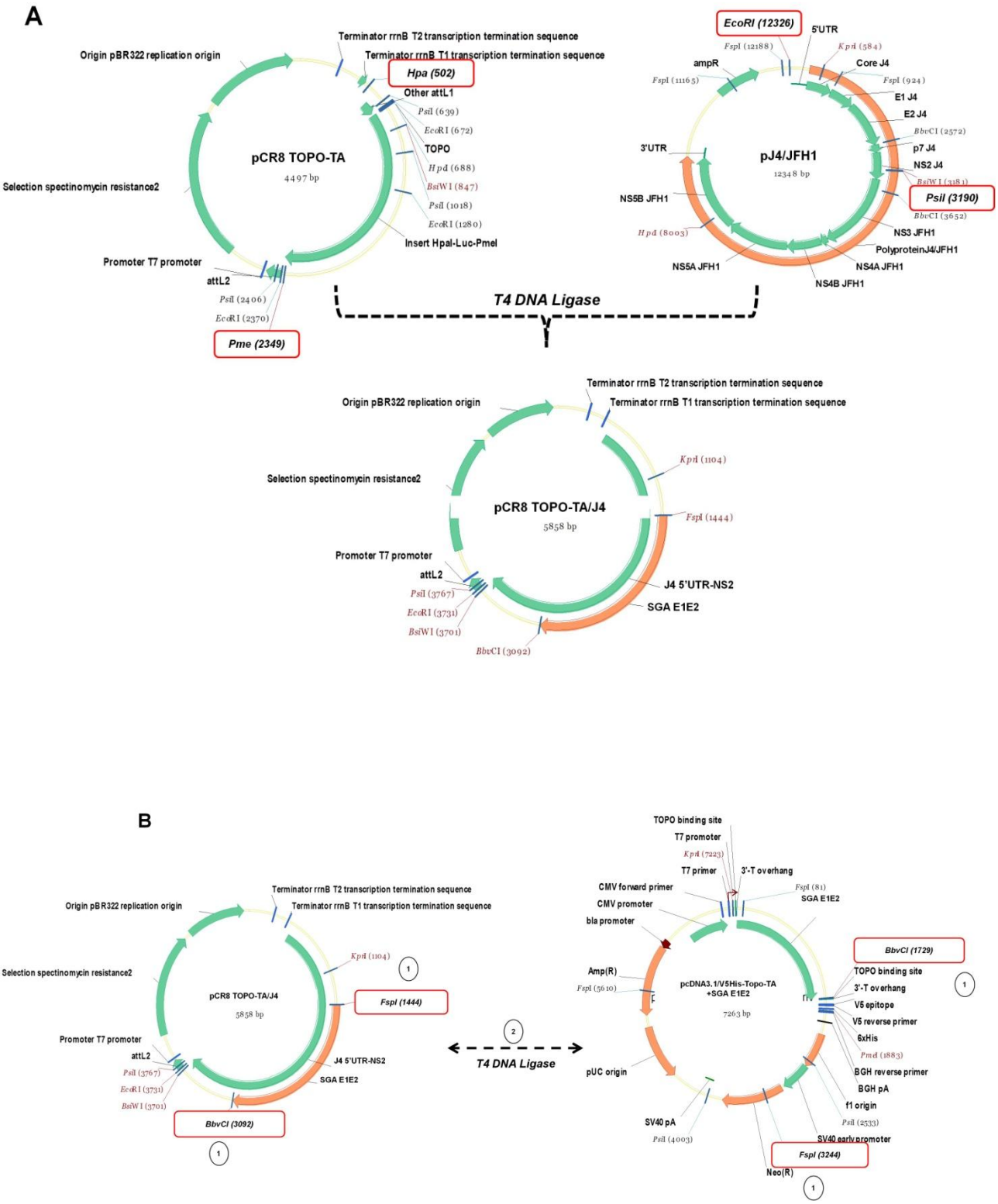
Seventy-two hours post-transduction, the Huh7.5 cells were rinsed once in Phosphate Buffered Saline (PBS), and lysed for 30 minutes at 4 °C using the luciferase cell culture lysis buffer (Promega). Relative Light Units (RLUs) were then measured. The RLUs were quantified using the Centro LB 960 luminometer (Berthold Technologies). The detection limit for positive luciferase reporter protein expression was 10 x 10^3 RLUs/assay, corresponding to the mean ± 3SD of background levels obtained using cells infected with ΔE1E2 pseudoparticles. Antibody-mediated neutralization of HCVpp was assessed by the reduction of luciferase activity in the transduced Huh7.5 cells in the presence of human sera or neutralizing MAbs. HCVpps were mixed with dilutions of the same donor sera used for the SGA amplification, dilutions of four pools of heterologous HCV-positive sera (each composed of three sera collected from patients chronically infected with HCV genotype 1a, 1b, 2a, and 3a), dilutions of a negative control serum (consisting of a pool of three anti-HCV-negative sera), as well as dilutions of two anti-E2 MAbs AR3A (M. Law et al., 2008), HC-11 (Keck *et al.*, 2008a), or the irrelevant isotype control IgG R04. The HCVpp-antibodies mixtures were preincubated for 1h at 37 °C and then added to Huh7.5 cells seeded the day before in 96-well plates at a density of 5x10^3 cells per well. Each experiment was performed in triplicate. The cells were harvested at 72h post-transduction, and the RLUs were measured within the cell lysates as described above. For each antibody dilution, the percentage of neutralization was calculated as 100- [100 x (infectivity of HCVpp in the presence of serum or MAb/infectivity of HCVpp in the presence of anti-HCV-negative control sera or irrelevant isotype control IgG)] (Fafi-Kremer *et al.*, 2010). The neutralization titer of each serum was defined as the last dilution of the sample that conveyed a ≥ 50 % reduction of HCVpp entry. The antibody concentrations that reduced ≥ 50% (IC_{50}) of HCVpp infectivity were assessed by testing MAbs at 20, 2, 0.2 or 0.02 μg/ml.

8. Envelope Glycoproteins' Incorporation into the HCVpps

E1E2 envelope glycoproteins incorporation into the pseudoparticles was assessed by western blotting of sucrose cushion-purified HCVpps. For this, 600 µl of HCVpp-containing supernatant, normalized to a same concentration of HIV p24 Core antigen, was pelleted through a 30 % (wt/v) sucrose cushion in TBS (Tris Buffered Saline) at 124,740 x g for 2 hours. After centrifugation, supernatants were removed leaving about 100µl of pellets, in which 5X Laemmli/β-mercaptoethanol sample buffer (60 mM Tris-Cl pH 6.8; 2 % SDS; 10 % glycerol; 5 % β-mercaptoethanol; 0.01 % bromophenol blue) was five-fold diluted. Virus pellets were heated for 5 minutes at 96 °C and denatured proteins were separated on a 10 % SDS-PAGE (Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis) gel, and then transferred to nitrocellulose membranes (Amersham) at 4 °C. Blocking of non-specific binding sites was achieved by placing the membranes in 5 % non-fat dry milk, diluted in TBS-0.1 % Tween 20, for 1 hour at room temperature. Proteins were detected by the 3/11 anti-E2 rat MAb (Wang *et al*., 2011) and an anti-HIV p24 rabbit polyclonal Ab (NIBSC AIDS reagents) as primary antibodies, respectively diluted at 1/200 and 1/2000. The incubation was performed over night, at 4 °C. After rinsing the membranes to remove unbound primary antibodies, the membranes were incubated 1 hour at room temperature with either horseradish peroxidase-linked anti-rat or anti-rabbit secondary antibodies. Protein detection was achieved by production of insoluble, brown-black precipitate obtained through the reaction between 3,3'-Diaminobenzidine (DAB) and hydrogen peroxide catalyzed by the peroxidase enzyme (SIGMAFAST DAB tablets, Sigma).

9. Engineering of J4/JFH1-Based Chimeric Viruses

To be able to express our transmitted and non-transmitted E1E2 sequences in a HCVcc context, we engineered a J4/JFH1-based chimeric genome for which we replaced the E1E2 genes in the pJ4/JFH1 plasmid (Gottwein *et al*., 2009) with the corresponding sequences we obtained by the SGA approach. The experimental strategy we used included an intermediary step in which a greater J4/JFH1 fragment was first cloned into a pCR8 TOPO-TA vector. First, the pJ4/JFH1 and pCR8 TOPO-TA plasmids were digested by EcoRI-pSiI and HpaI-PmeI respectively (Fig. 21A). The pJ4/JFH1 digested product was treated with the 'Klenow enzyme' (New England BioLabs) to produce blunted ends, whilst the pCR8 TOPO-TA-Hpa digested product was treated with Calf Intestinal Phosphatase (CIP) to prevent self-ligation (New England BioLabs). After agarose gel electrophoresis separation, the J4 5'UTR-NS2 insert fragment (~3,210bp) and the pCR8 TOPO-TA cloning vector were purified and fused by the T4 DNA ligase enzyme (New England BioLabs) (Fig. 21A). In a second step, the pcDNA3.1/V5His-TOPO-TA expression vector hosting our full-length envelope sequences, and the resulting pCR8 TOPO-TA/J4 plasmid were digested with BbvcI and FspI restriction enzymes (New England BioLabs) in order to replace the J4 full-length envelope genes by our corresponding E1E2 sequences (Fig. 21B). Finally, digestion with BsiWI and KpnI restriction enzymes (New England BioLabs) allowed us to insert our E1E2 sequences into the pJ4/JFH1 original plasmid (Fig. 21C).


A
pCR8 TOPO-TA
4497 bp
Origin pBR322 replication origin
Terminator rrnB T2 transcription termination sequence
Terminator rrnB T1 transcription termination sequence
Hpa (502)
Other attL1
PsiI (639)
EcoRI (672)
TOPO
HpaI (688)
BsiWI (847)
PsiI (1018)
EcoRI (1280)
Insert HpaI-Luc-PmeI
Selection spectinomycin resistance2
Promoter T7 promoter
attL2
PsiI (2406)
EcoRI (2370)
Pme (2349)
pJ4/JFH1
12348 bp
EcoRI (12326)
FspI (12188)
5'UTR
KpnI (584)
Core J4
FspI (924)
E1 J4
E2 J4
BbvCI (2572)
p7 J4
NS2 J4
BsiWI (3181)
PsiI (3190)
BbvCI (3652)
NS3 JFH1
PolyproteinJ4/JFH1
NS4A JFH1
NS4B JFH1
NS5A JFH1
HpaI (8003)
NS5B JFH1
3'UTR
ampR
FspI (11165)
T4 DNA Ligase
pCR8 TOPO-TA/J4
5858 bp
Terminator rrnB T2 transcription termination sequence
Terminator rrnB T1 transcription termination sequence
Origin pBR322 replication origin
Selection spectinomycin resistance2
KpnI (1104)
FspI (1444)
Promoter T7 promoter
attL2
PsiI (3767)
EcoRI (3731)
BsiWI (3701)
BbvCI (3092)
J4 5'UTR-NS2
SGA E1E2
B
pCR8 TOPO-TA/J4
5858 bp
Terminator rrnB T2 transcription termination sequence
Terminator rrnB T1 transcription termination sequence
Origin pBR322 replication origin
Selection spectinomycin resistance2
KpnI (1104)
FspI (1444)
Promoter T7 promoter
attL2
PsiI (3767)
EcoRI (3731)
BsiWI (3701)
BbvCI (3092)
J4 5'UTR-NS2
SGA E1E2
1
2
T4 DNA Ligase
pcDNA3.1/V5His-Topo-TA
+SGA E1E2
7263 bp
TOPO binding site
T7 promoter
KpnI (723)
T7 primer
3'-T overhang
CMV forward primer
CMV promoter
bla promoter
FspI (81)
SGA E1E2
Amp(R)
FspI (5610)
pUC origin
SV40 pA
PsiI (4003)
Neo(R)
SV40 early promoter
FspI (3244)
f1 origin
PsiI (2533)
BGH reverse primer
BGH pA
PmeI (1883)
V5 reverse primer
6xHis
V5 epitope
3'-T overhang
TOPO binding site
BbvCI (1729)

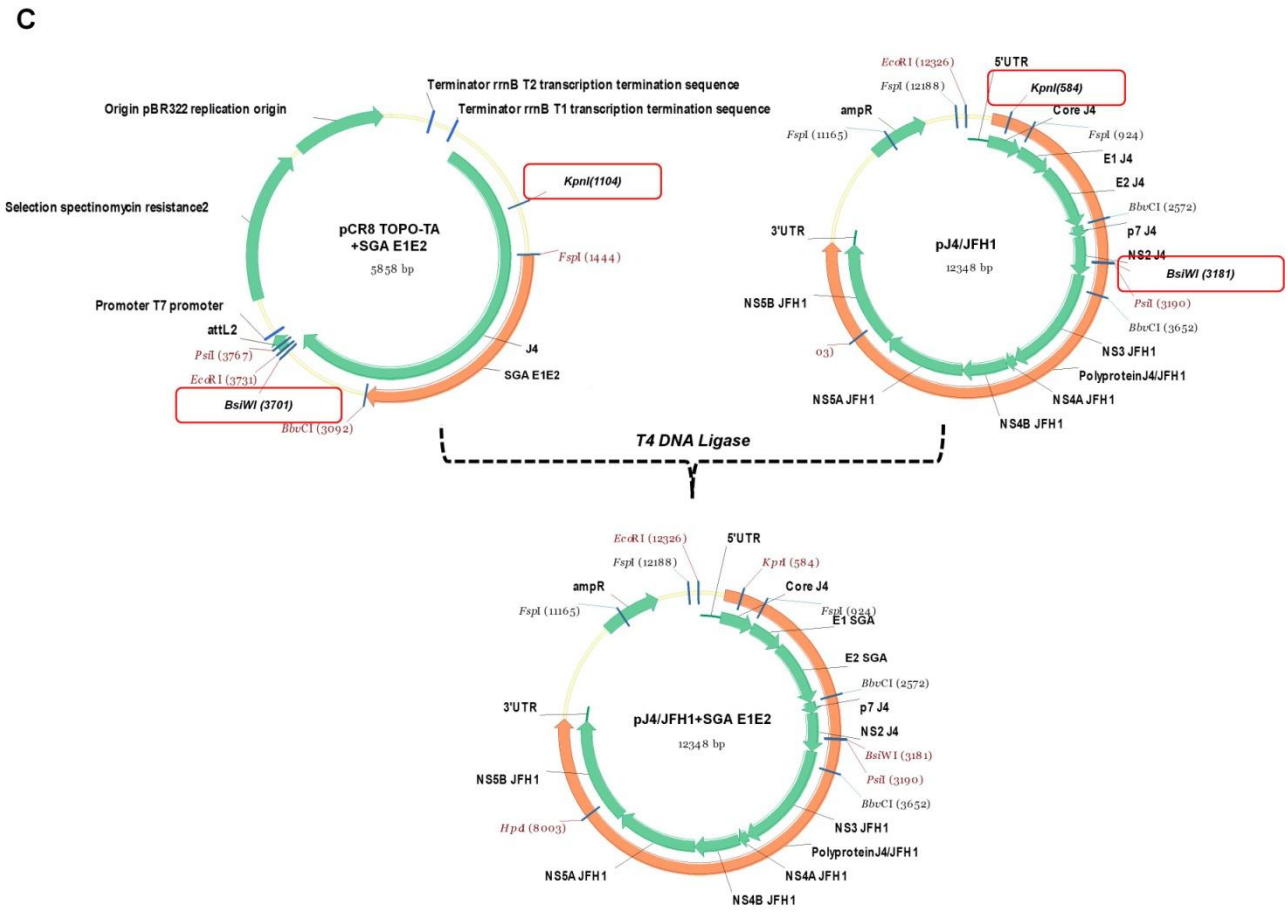


Fig. 21. Cloning strategy to engineer J4/JFH1-based chimeric genomes expressing our full-length E1E2 SGA sequences.

The cloning strategy consists of three steps. Firstly, a region of the pJ4/JFH1 plasmid, including sequences from the 5'UTR up to the NS2, is cloned into the shuttle vector pCR8-TOPO-TA ***(A)****. Hereinafter, genes coding the J4 envelope glycoproteins are replaced by the E1E2 sequences amplified with the SGA protocol* ***(B)****. Finally, the SGA E1E2 sequences are fused into the plasmid pJ4/JFH1, downstream the JFH1 5'UTR* ***(C)****.*

V. RESULTS & DISCUSSION

1. Results

a) Study Subjects and Single-Genome Sequencing

To study the genotypic and phenotypic E1E2 envelope glycoprotein determinants underlying HCV transmission, we investigated viral quasispecies evolution between three HCWs (recipients RA1, RA2 and RB) and the corresponding chronically infected patients at the origin of the contaminations (donors DA and DB) (Fig. 19). RA1 and RA2 shared the same donor, DA, although infected 10 months apart. All recipients were monitored following the needlestick injury with sequential sampling of plasma until they became viremia negative (Fig. 22). An SGA approach was employed, followed by direct sequencing, to provide

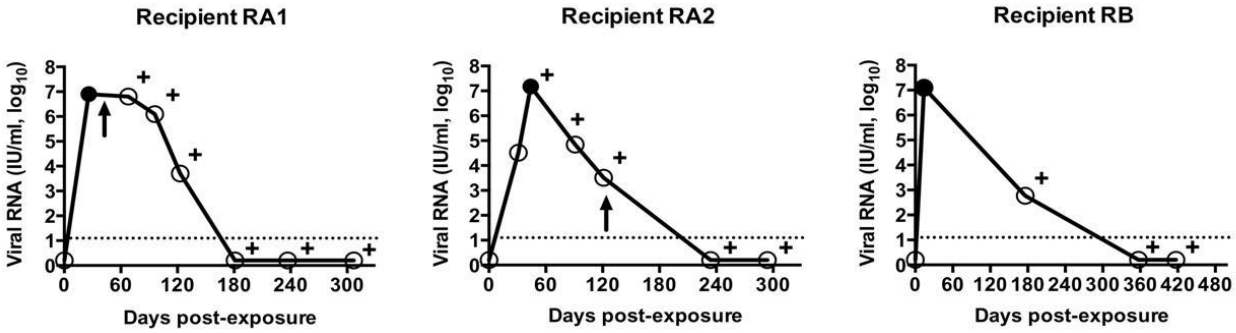


Fig. 22. Follow-up of recipients RA1, RA2 and RB after needlestick exposure to infected blood.

Viremia was monitored after the needlestick injury, by sequential plasma samples, repeated until the virus could no longer be detected. The solid line with circled values corresponds to plasma viral RNA quantified with the Abbott HCV RealTime assay. The dotted line indicates the lower limit detection. The black dots correspond to the recipient viremic time point analyzed by SGA and '+' signs denote positivity for anti-HCV antibody. An arrow indicates the start of treatment for subjects RA1 and RA2.

proportional representation of viral variant frequency in the patients' quasispecies, and E1E2 sequences that are not confounded by template resampling or *Taq* polymerase errors (Keele *et al*., 2008; Li *et al*., 2012). SGA was performed on donor plasma obtained at the time of needlestick accident for the transmission pairs DA/RA1 and DB/RB. For RA1 and RB, SGA was conducted before antibody seroconversion, on the first viremic plasma sample collected 25 and 14 days post infection, respectively. In case of RA2, only the second sequential sample collected 44 days post infection, at the time of viremia peak and antibody seroconversion, allowed a successful amplification of E1E2 sequences (Fig. 22). A total of 155 full-length E1E2 nucleotide sequences were derived from the two donors and three recipients (range, 24 to 37 per subject) (Table 3).

	Donor DA		Recipient RA1		Recipient RA2		Donor DB		Recipient RB	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Total number of « SGA » E1E2 sequences analyzed *	33	31	37	36	28	27	33	30	24	23
Number of viral variants/quasispecies (Entropy)	33	20	16	8	14	11	33	19	7	3
Mean within-subject diversity (%) (Hamming score)	1.57	0.819	0.049	0.081	1.487	1.012	1.944	1.859	0.047	0.032
Number of T/F viruses			1		5				1	

Table 3. Composition and diversity of donors' and recipients' quasispecies.

**The differences between the number of nucleotide (nt) and amino acid (aa) sequences resulted from the occurrence of stop codons or deletions altering the open reading frame.*

b) Evolution of HCV Quasispecies between Donors and Recipients

The E1E2 nucleotide sequences derived from donor and recipient subjects were first submitted to a neighbor-joining phylogenetic analysis including reference sequences of various genotypes (Fig. 23). Infection by HCV genotype 1b was confirmed for all the subjects enrolled in the study. Nucleotide sequences corresponding to each transmission pair clustered together with high bootstrap support, confirming the epidemiological linkage. Noticeably, the E1E2 nucleotide sequences of RA2 were interspersed with those of DA and RA1, whereas the RA1 and RB sequences formed a lineage characterized by an extremely low diversity (Fig. 23). These phylogenetic data were consistent with the mean within-subject diversities, expressed by the 'Hamming score' of 1.49% (range 0-2.6%) for RA2 sequences and 0.05% for RA1 or RB sequences (range 0-0.18%) (Table 3). As expected, sequences from the chronically infected subjects DA and DB showed broader genotypic heterogeneities with mean within-subject sequence diversities of 1.57 % (range 0.18-2.9 %) and 1.94 % (range 0.18-3.44 %), respectively (Table 3). Together, these results showed a decrease of diversity (a bottleneck effect) in the recipients' quasispecies after transmission, which was strongly marked in the cases of RA1 and RB.

To further characterize the transmission process, the deduced E1E2 amino acid sequences derived from donors and recipients were analyzed within each transmission pair using a combination of neighbor-joining phylogenetic tree reconstructions, and Highlighter plots showing the nonsynonymous substitutions which had occurred upon transmission (amino acid changes) (http://hcv.lanl.gov/content/sequence/ HIGHLIGHT/highlighter.html). Genetic relationships within the E1E2 sequences derived from DA and RA1 are represented in Fig. 24A. The sequence used as reference, at the top of the tree and Highlighter plot, corresponds to the dominant variant of donor DA which, in this case was not transmitted to

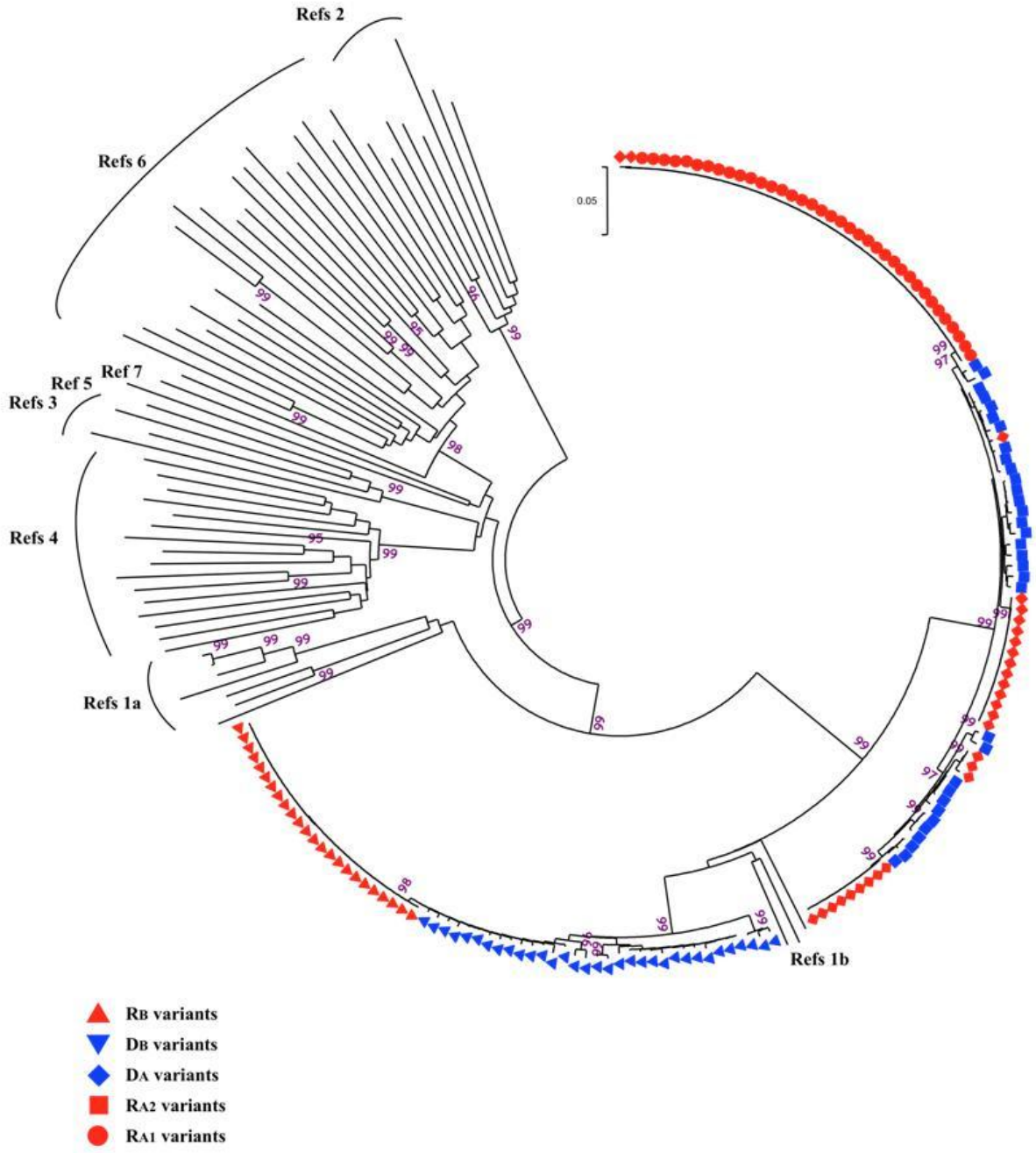
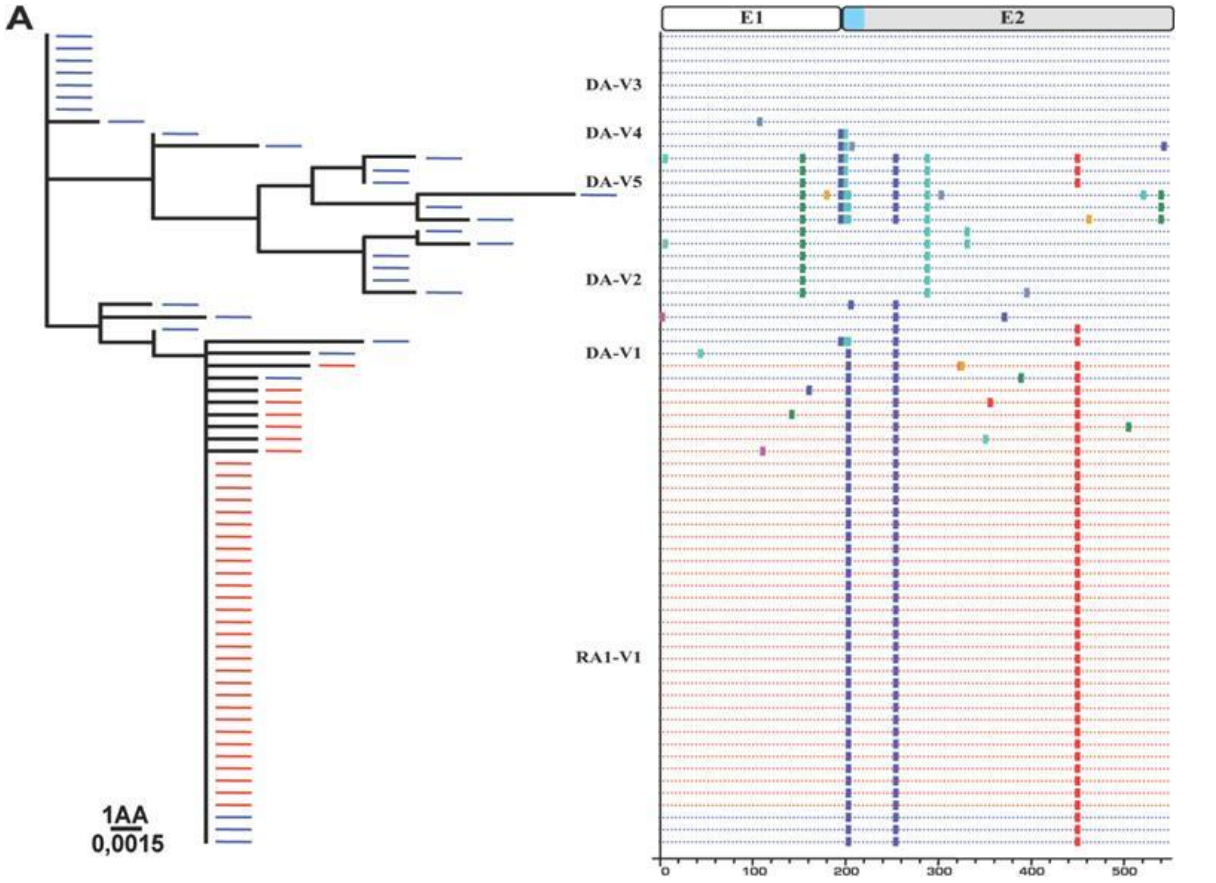
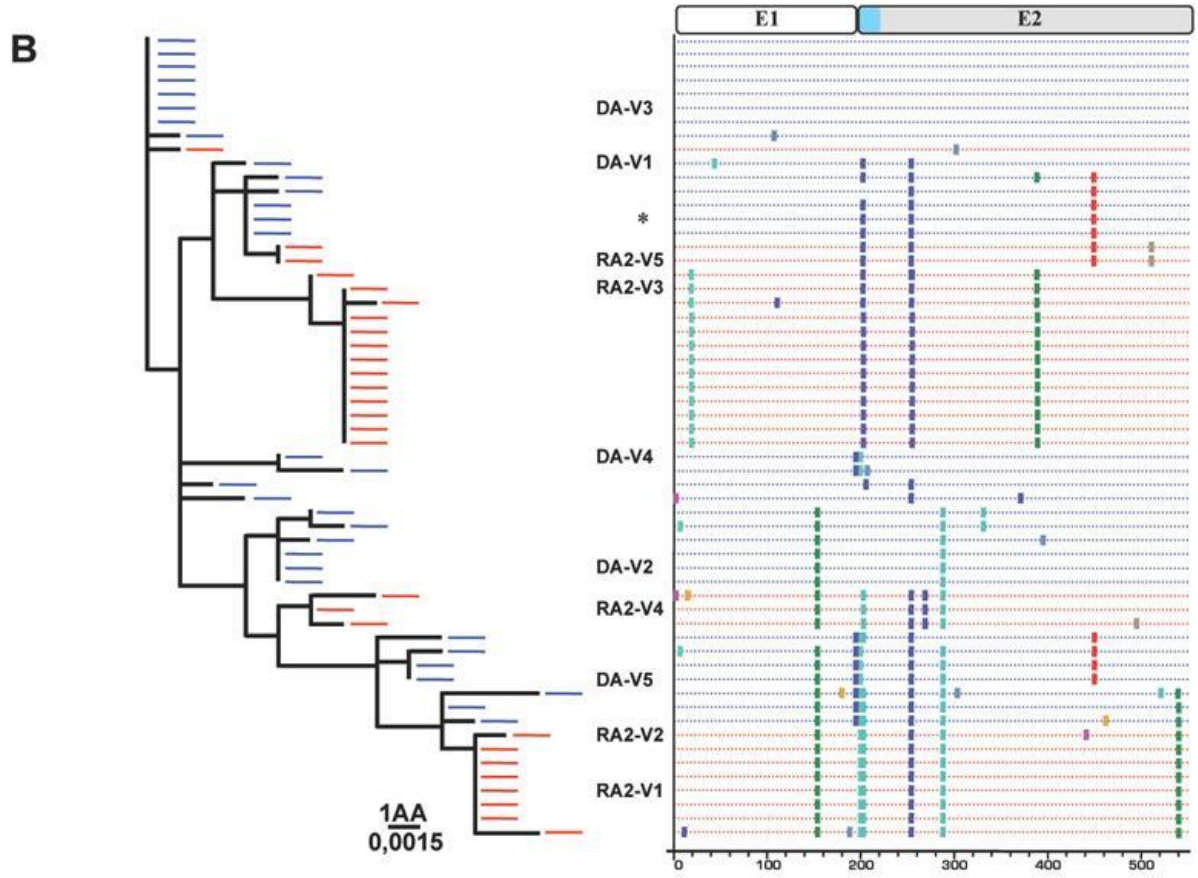


Fig. 23. Combined phylogenetic analysis of donors' and recipients' quasispecies.

A neighbor-joining tree constructed with all the E1E2 nucleotide sequences available from DA (blue squares), DB (blue triangles), RA1 (red circles), RA2 (red diamonds), and RB (red triangles) along with HCV genotype 1 to 7 reference sequences (Los Alamos National Laboratories HCV database) is shown. The arrow indicates a RA2 E1E2 sequence closely related to that harbored by the RA1 T/F virus. Only bootstrap values over 95 are presented. The scale bar corresponds to 0.05 nucleotide substitutions per site.


A
E1
E2
DA-V3
DA-V4
DA-V5
DA-V2
DA-V1
RA1-V1
1AA
0,0015
0
100
200
300
400
500


B
E1
E2
DA-V3
DA-V1
*
RA2-V5
RA2-V3
DA-V4
DA-V2
RA2-V4
DA-V5
RA2-V2
RA2-V1
1AA
0,0015
0
100
200
300
400
500

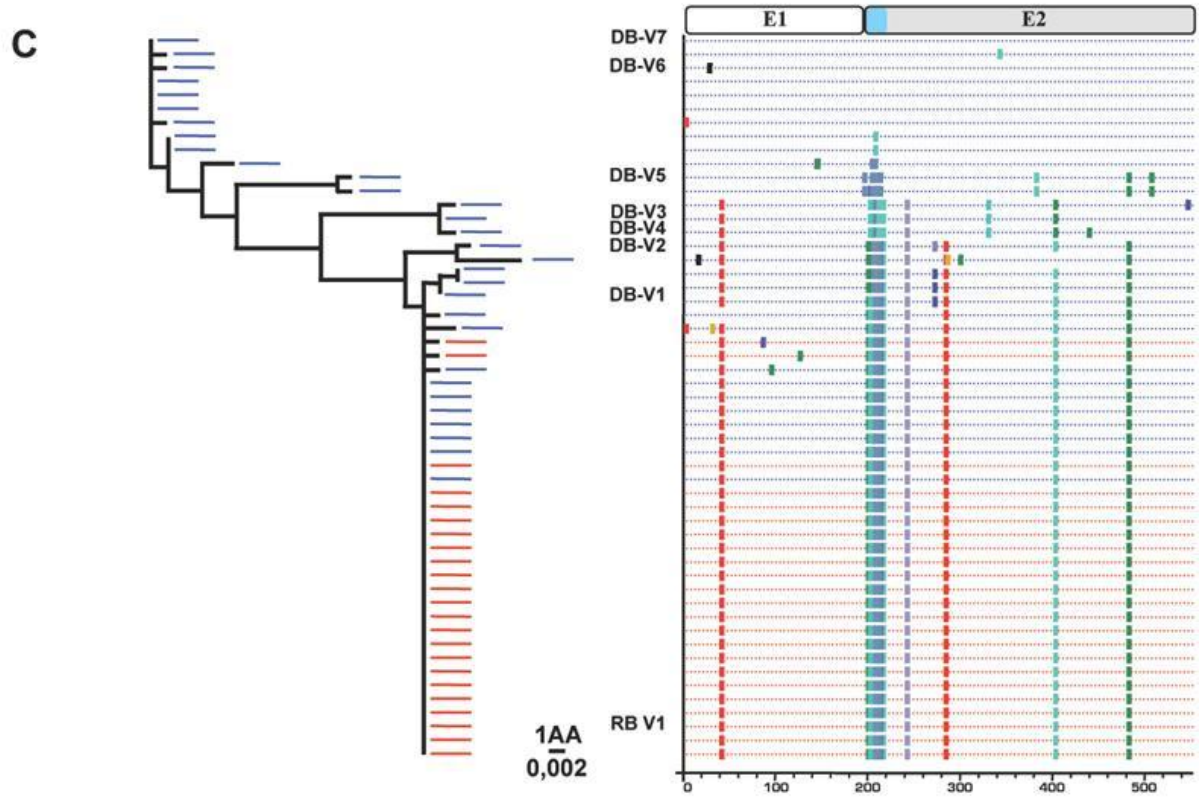


Fig. 24. Phylogenetic relationships and amino acid substitutions in HCV E1E2 quasispecies sequences between the transmission pairs.

E1E2 amino acid sequences derived from the recipients RA1 ***(A)****, RA2* ***(B)****, and RB* ***(C)*** *(in red) were analyzed with the pre-transmission donor sequences (in blue) by neighbor-joining trees (left panels) and Highlighter plots (right panels). Polymorphisms are indicated by a colored tick mark specific for each amino acid, according to the color scheme of BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A schematic diagram of the E1E2 proteins, showing the location of the HVR1 segment of E2 in light blue, is provided above the Highlighter plots. The scale bars are proportional to the genetic distance and represent 0.0015 (in A and B) or 0.002 (in C) amino acid substitutions per site. On the right side of the Highlighter plots, the E1E2 sequences chosen for further phenotypic analyses are identified. The E1E2 sequence indicated by an asterisk in the Highlighter plot B corresponds to the RA1 transmitted variant, 'RA1-V1'.*

the recipient. Twenty-nine out of 36 sequences derived from RA1 were identical, though the remaining sequences differed only by 1 or 2 randomly distributed substitutions. This strong genetic homogeneity indicates that RA1 was productively infected by a single T/F virus (Table 3). Furthermore, the E1E2 amino acid sequence of this T/F virus was found to be identical to that of a minor variant present in the DA quasispecies. Fig. 24B extends the latter

analysis to the pair DA/RA2, for which the same DA dominant variant sequence was used as reference to track nonsynonymous substitutions which had occurred upon the transmission event. The sequences derived from RA2 were clearly separated into 5 lineages whose consensus sequences differed from each other by 3 or more nonsynonymous substitutions (Fig. 24B). This observation suggests that 5 genetically distinct T/F viruses initiated the productive infection in this case (Table 3). We do not believe that the sampling of RA2 at the time of seroconversion may have confounded this identification of T/F variants, since at that time the recipient was positive for anti-Core antibodies but still negative for anti-E2 antibodies (the RIBA assay was negative), thus rendering unlikely a positive selection on the envelope gene sequences. No common E1E2 sequence was identified between the DA and RA2 quasispecies, but the consensus sequences representative of the five transmitted lineages differed only by one to four amino acid substitutions from that of the closest variants present in the donor quasispecies. Genetic relationships among the E1E2 sequences present in donor DB and recipient RB are depicted in Fig. 24C. The sequence used as reference, at the top of the tree and Highlighter plot, corresponds to one of the two major variants of the donor, which was not transmitted to RB. Twenty-one out of 23 sequences derived from the RB quasispecies were identical, with the remaining sequences differing only by one substitution. As previously observed, this strong genetic homogeneity shows that a single T/F virus productively infected RB (Table 3). However, in this case, the E1E2 amino acid sequence of the T/F virus was found to be identical to that of a major variant from the donor. In summary, these data demonstrated that the donor quasispecies underwent a strong genetic bottleneck with only a single variant from the inoculum yielding a productive infection in two out of three recipients studied. In these two cases, the E1E2 amino acid sequences of the transmitted variant were fully conserved upon the transmission process whereas synonymous substitutions occurred in the corresponding nucleotide sequences (Fig. 25). Interestingly, five T/F viruses were

detected in the second recipient infected by the donor DA, which demonstrate the occurrence of a less stringent selective process in this case.

c) Molecular Determinants Underlying HCV Transmission

The neighbor-joining phylogenetic tree and Highlighter plot analyses showed the preferential transmission of specific variants from the donor quasispecies to the recipients (Fig. 24). To further identify specific molecular determinants related to the genetic bottlenecks observed, amino acid differences between sequences derived from donors and the corresponding recipients were examined using VESPA (Elhefnawi *et al*., 2010). VESPA calculates the frequency of each amino acid at each position in an alignment for the query (recipient) and reference sets (donor), and selects the positions for which the most common character in the query set differs from that in the background set. Six signature amino acids (corresponding to positions 394, 399, 401, 445, 476 and 641 in the polyprotein sequence of the reference strain H77) were found by VESPA to be significantly different between donors and recipients E2 glycoprotein sequences, three of which being located within the HVR1. Interestingly, the amino acid substitution at position 394 (H394R or H394Y) is a shared feature of the transmitted variants in the 3 transmission pairs (with the exception of a single E1E2 sequence derived from RA2) whereas the other signature amino acids selected are pair-specific (Fig. 26A). None of the amino acid substitutions located outside HVR1 affects directly a residue known to participates in CD81 binding or a potential N-glycosylation site (Boo *et al*., 2012; Goffard *et al*., 2005; Owsianka *et al*., 2006). With the aim to further visualize results obtained by the VESPA analysis, the frequencies of each combination of signature amino acids in the E1E2 sequences derived from the 3 transmission pairs are

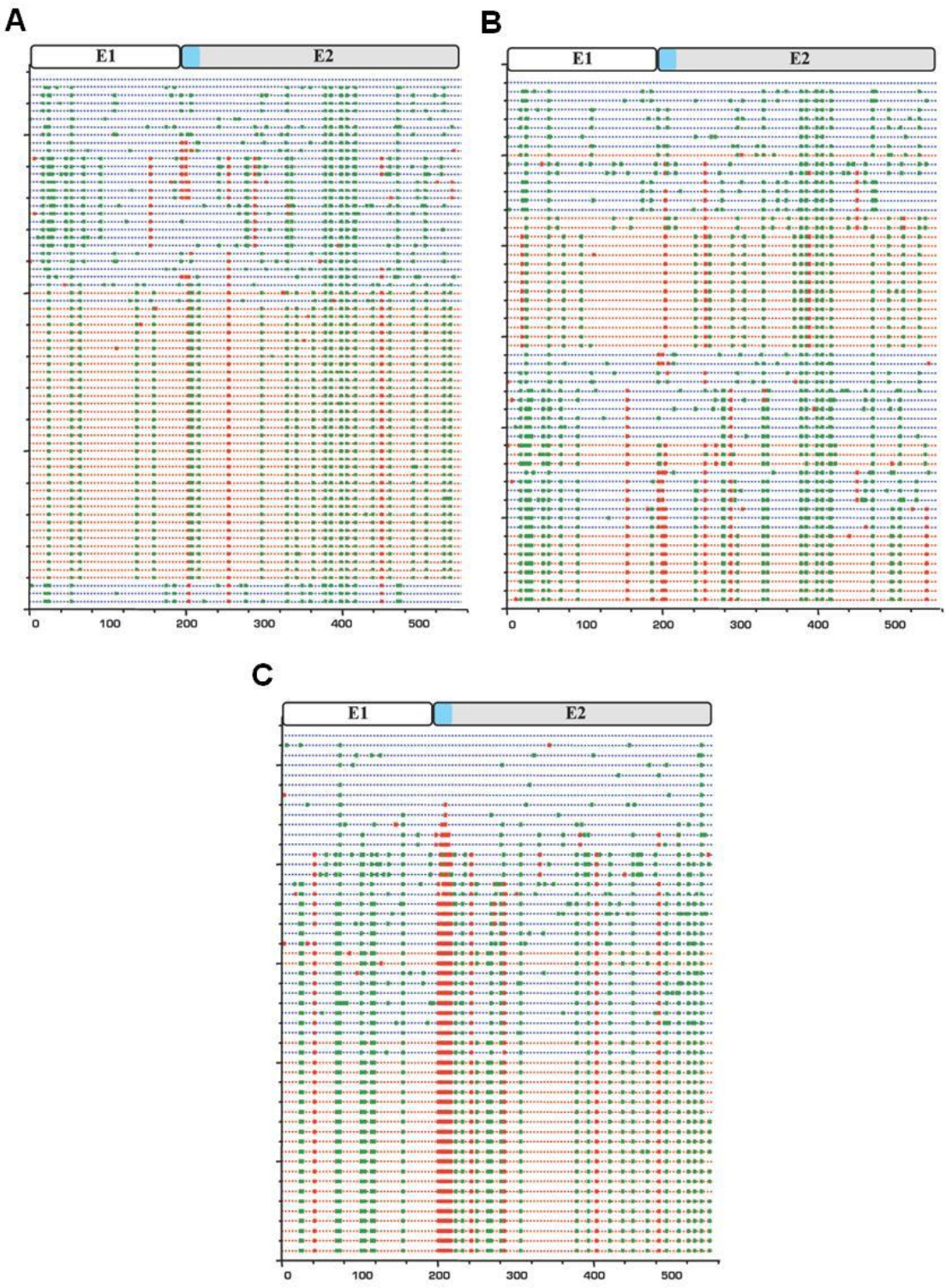


Fig. 25. Highlighter plots of E1E2 nucleotide sequences from recipients RA1 (A), RA2 (B) and RB (C) (red) with the pre-transmission donor sequences (blue).

The sequences are presented in the same order as in Fig. 24. Nucleotide polymorphisms are indicated by a colored tick mark (green bars: synonymous substitutions; red bars: non-synonymous substitutions). A schematic diagram of the E1E2 sequence, showing the location of the HVR1 segment of E2 in light blue, is provided above the Highlighter plots.

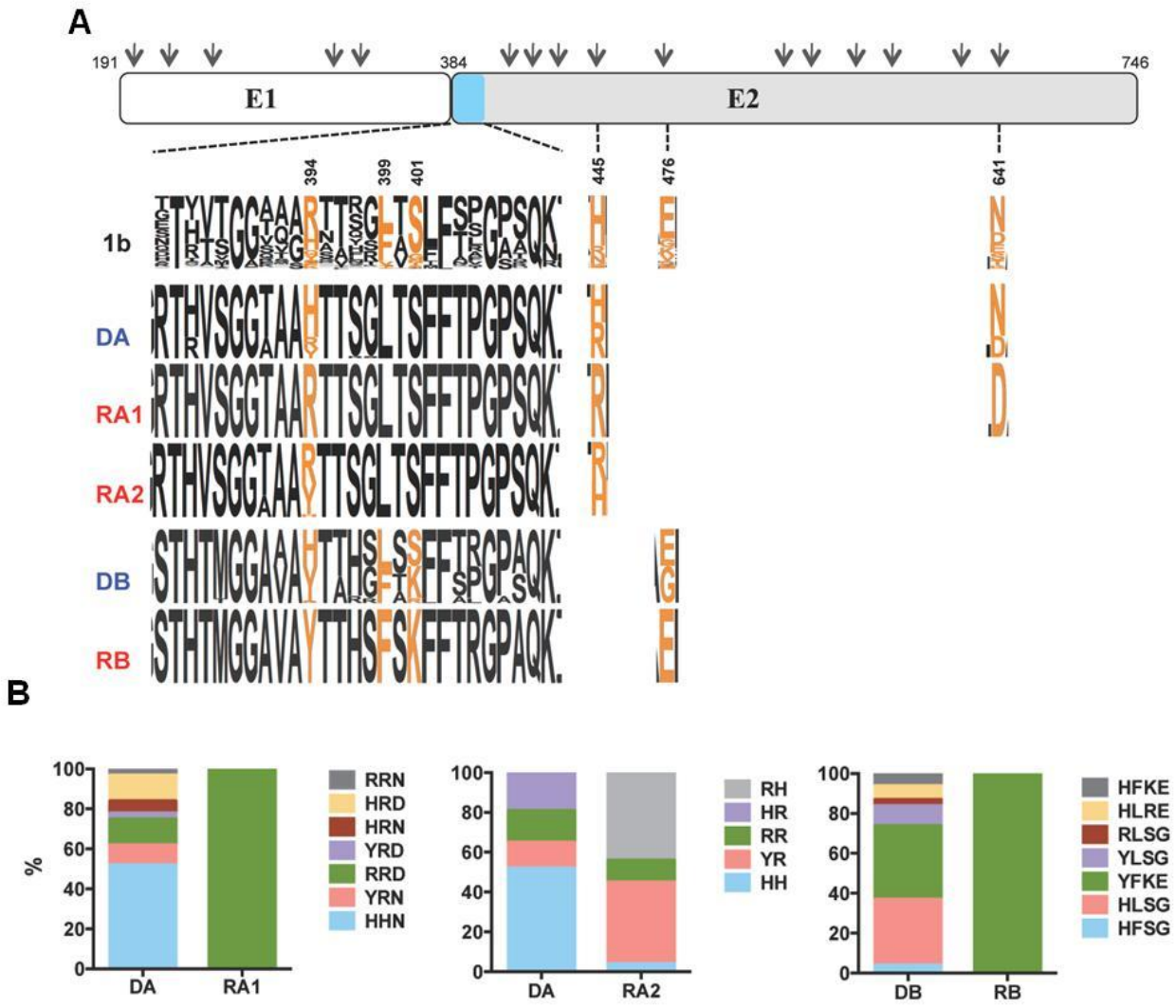


Fig. 26. Identification of the key residues of the E1E2 envelope glycoproteins characterizing the recipients' quasispecies.

(A) *Sequence logo depiction of signature amino acids specific to the transmitted E1E2 variants. A schematic representation of the E1E2 proteins, showing the location of HVR1 (in light blue), and the putative glycosilation sites (arrows) is provided at the top. The positions of the signature amino acids, identified with VESPA in the three transmission pairs, are indicated below the schematic representation of E1E2 proteins. The numbers indicate positions relative to the H77 polyprotein (accession number NC_004102). The 1b reference logo (top row) was obtained with an alignment of 340 full-length HCV 1b genotype sequences from the Los Alamos National Laboratories HCV database. Sequence logo indicating the variability of each amino acid in sequences derived from donors and recipients are shown below. The height of each single-letter amino acid code is proportional to its frequency in the given position.* ***(B)*** *Frequencies of key residues combinations identified within donors' and recipients' quasispecies.*

summarized in Fig. 26B. Thus, a specific combination of signature amino acids characterizes the E1E2 sequence of the RA1 and RB T/F virus (i.e. R394-R445-D641 for RA1 and Y394-F399-K401-E476 for RB), whilst, for RA2, the combination of signature amino acids allowed to distinguish 4 out of the 5 genetic lineages previously identified with the Highlighter tool, with two of these lineages sharing the same combination of signature amino acids (i.e. Y394-R445). Together, these data suggest that, during transmission or the initial phase of infection, a selective advantage might have been provided to donor variants depending on a common key amino acid located at position 394 in HVR1, together with additional signature amino acids specific to each transmission pair.

d) Influence of Viral Entry and Donor nAbs on Variant Selection

To investigate whether T/F viruses possess functional properties which might explain their selective transmission in our three BEAs cases, we determined the relative entry efficiency conferred by E1E2 amino acid sequences representative of the main genetic lineages previously identified within donors' and recipients' quasispecies (Fig. 24). A few additional E1E2 sequences derived from minor variants were also included in the study. Thus, lentiviral HCVpps bearing E1E2 glycoproteins were generated and tested for their ability to transduce Huh7.5 cells as described in the "Material and Methods" section. The expression of 15 out of 19 selected E1E2 sequences resulted in the production of infectious HCVpps (Fig. 27A & B). As shown by the western blot in the Fig. 27C, the E1E2 glycoprotein levels of incorporation into HCVpps were similar, thus suggesting that differences in HCVpp entry efficiency resulted from intrinsic E1E2 features. E1E2 sequences derived from the recipient quasispecies always produced functional HCVpps with the exception of the RA2-V2

sequence (Fig. 27A). However, this particular sequence diverged by a single amino acid change from that of a dominant RA2 variant, which conversely resulted in infectious HCVpps (Fig. 24B). The E1E2 sequences corresponding either to the RA1 T/F virus (RA1-V1) or the three main genetic lineages identified in the RA2 quasispecies (i.e. RA2-V1, RA2-V3 and RA2-V4) conferred high levels of infectivity but still within the same order of magnitude than those observed for the majority of the non-transmitted variants (including the DA dominant sequence, V3) (Fig. 27A). For the transmission pair DB/RB, the E1E2 sequence harbored by the RB T/F virus, which includes a Y at position 394, conferred a level of infectivity one order of magnitude lower than that harbored by the infectious DB variants, presenting a H394 residue (Fig. 27B). These data suggest that the key residue H394, which is found only in the non-transmitted variants, does not correlate with the infectivity levels obtained using the HCVpp model system. Since the anti-HCV antibodies present in the donors inocula might also influence the transmission process, we carried out additional neutralization experiments using the same DA and DB sera exploited for the SGA analysis. These experiments were restricted to those E1E2 sequences, which conferred a good HCVpps transduction efficiency (with a signal-to-background ratio >10) in order to minimize variability between assays and errors in the calculation of antibody titers attributable to background signal noise. HCVpps were preincubated with serial dilutions of the donors' sera, their subsequent entry levels in Huh7.5 target cells were quantified, and the neutralization titers were calculated following the formula described in the "Material and Methods" section. As shown in Fig. 28A, HCVpp bearing E1E2 sequences derived from transmitted variants were neutralized in a similar range than those of non-transmitted variants by the antibodies present in the corresponding donor serum. Thus, these findings argue against the involvement of the donor neutralizing antibodies in the selection of T/F viruses.

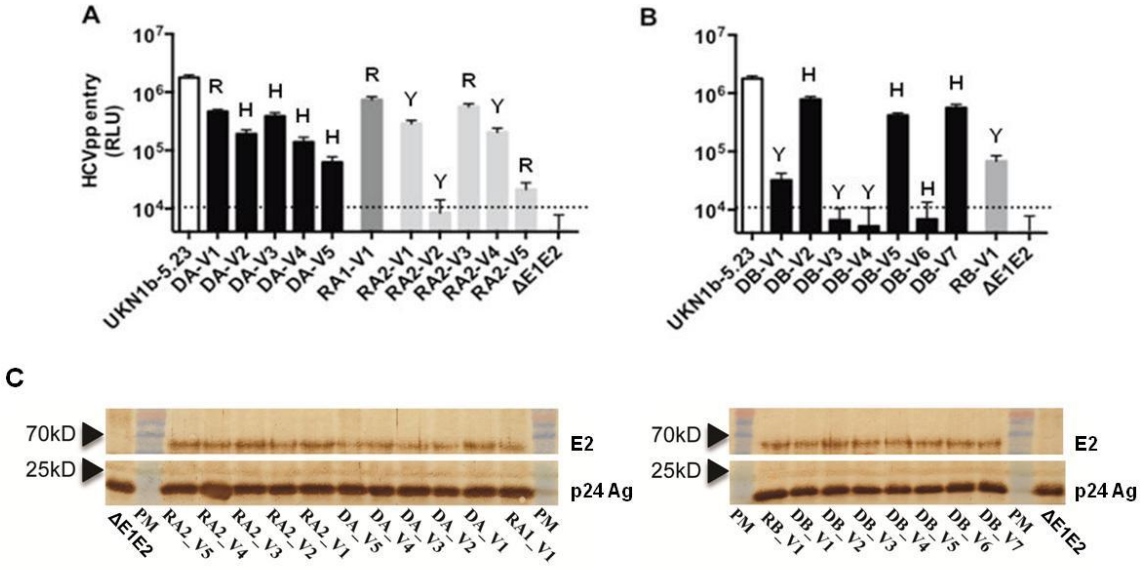


Fig. 27. Comparative analysis of HCVpps entry bearing donors' and recipients' E1E2 envelope glycoproteins.

HCVpp entry efficiency conferred by non-transmitted E1E2 variants (black bars) was compared with that conferred by the transmitted variants (gray bars) in the transmission pairs DA/RA1 or DA/RA2 ***(A)*** *and DB/RB* ***(B)****. Successful HCVpp entry in the Huh7.5 target cells was evaluated through the activity of the luciferase reporter gene. Results are expressed in relative light units (RLUs) plotted on a logarithmic scale. The UKN1B5.23 envelope was used as an external reference. The dotted line indicates the threshold for detectable HCVpp entry, corresponding to* $10x10^3$ *RLUs/assay, i.e. the mean ± SD of the background levels obtained with cells transduced with ΔE1E2 pseudoparticles. Means ± SD from four independent experiments (performed in triplicate) are shown. The letter above each bar indicates the amino acid at position 394 for each E1E2 variant tested.* ***(C)*** *Western blot analysis of envelope glycoprotein incorporation into HCVpps. Particles were pelleted through a 30% sucrose cushion and analyzed by western blotting. HCV E2 glycoprotein and the capsid protein of HIV were respectively detected with the 3/11 rat MAb, and an anti- HIV p24 rabbit polyclonal antibody. Bound antibodies were revealed using the DAB peroxidase substrate. PM, protein marker.*

e) Entry Inhibition of Transmitted Variants by Cross-neutralizing Polyclonal or Monoclonal Antibodies

In a final set of experiments, we sought to assess whether targeting entry of transmitted variants by cross-neutralizing antibodies is a suitable approach for the prevention of HCV infection. To this scope, as described in the "Materials and Methods" section, we tested pools of HCV-positive sera derived from patients chronically infected by different HCV genotypes, i.e. 1a, 1b, 2a, and 3a HCV genotypes. Similar experiments were conducted with two well known anti-E2 broadly nMAbs, AR3A (M. Law et al., 2008) and HC-11 (Keck *et al*., 2008a). As shown in Fig. 28B, the serum pools inhibited to a varying degree the entry of HCVpps bearing E1E2 sequences derived from both transmitted and non-transmitted viral variants. The genotype 1b serum pool displayed the highest neutralizing activities against all the variants, which is in accordance with the fact that donors and recipients were infected by HCV genotype 1b. The anti-E2 MAbs also efficiently inhibited entry into target cells of HCVpps bearing E1E2 sequences derived from both transmitted and non-transmitted viral variants, showing IC_{50} values from 0.1 to 1.04 μg/ml for AR3A, and from 0.19 to 1.67 μg/ml for HC-11 (Fig. 29). This efficient neutralization of transmitted HCV variants by cross-neutralizing antibodies suggests that targeting viral entry by MAbs should be a promising strategy for preventing HCV transmission.

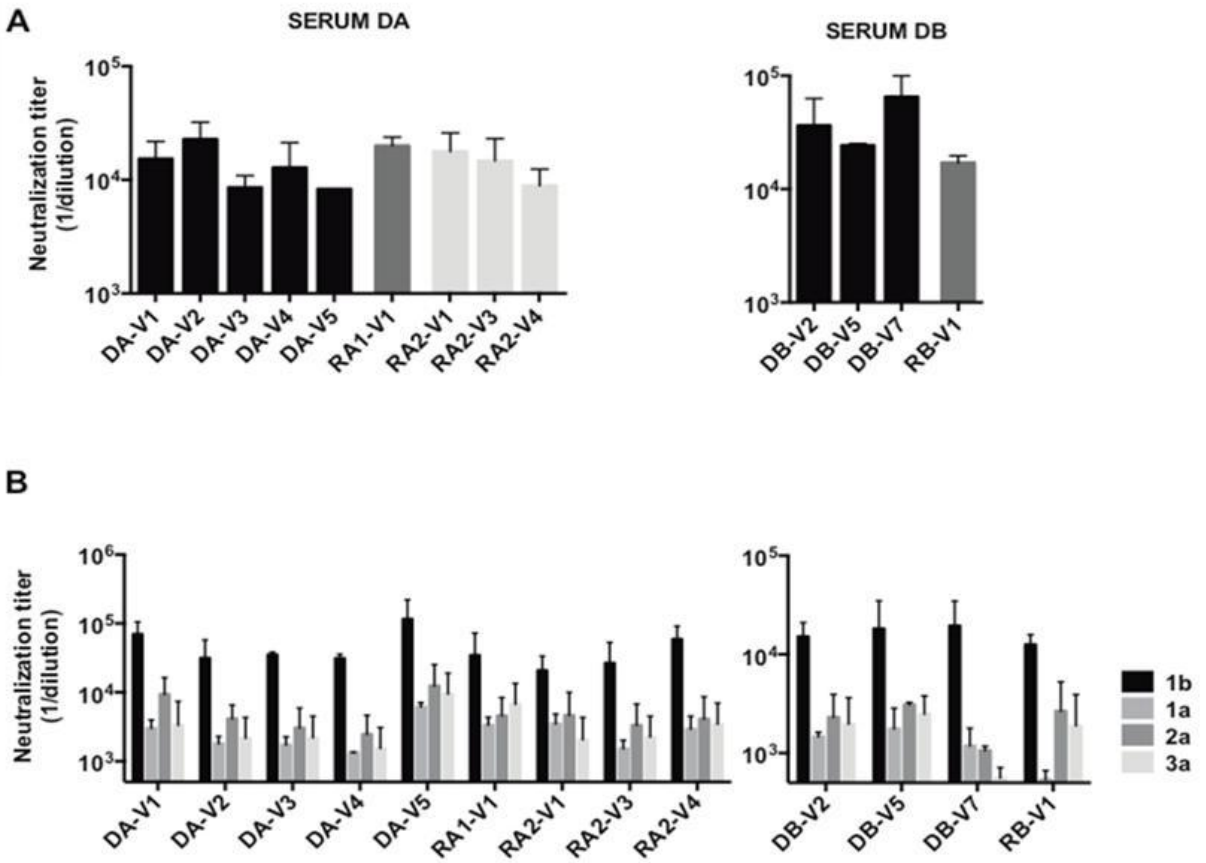


Fig. 28. Sensitivity of HCVpp bearing donors and recipients E1E2 envelope glycoproteins to neutralization by donors' sera (matched by transmission pair) or heterologous pools of sera.

HCVpps were incubated with serial dilutions of donors' sera ***(A)*** *or genotype-specific serum pools* ***(B)****. HCVpp-antibody complexes were then added to Huh7.5 cells, and entry assays were performed as previously. Neutralization titers were calculated as described in the 'Materials and Methods' section. End-point dilution titers reducing HCVpp entry of ≥50% are reported for donors- (black bars) and recipients-derived (gray bars) E1E2 variants. Data are mean values from two independent experiments performed in triplicate.*

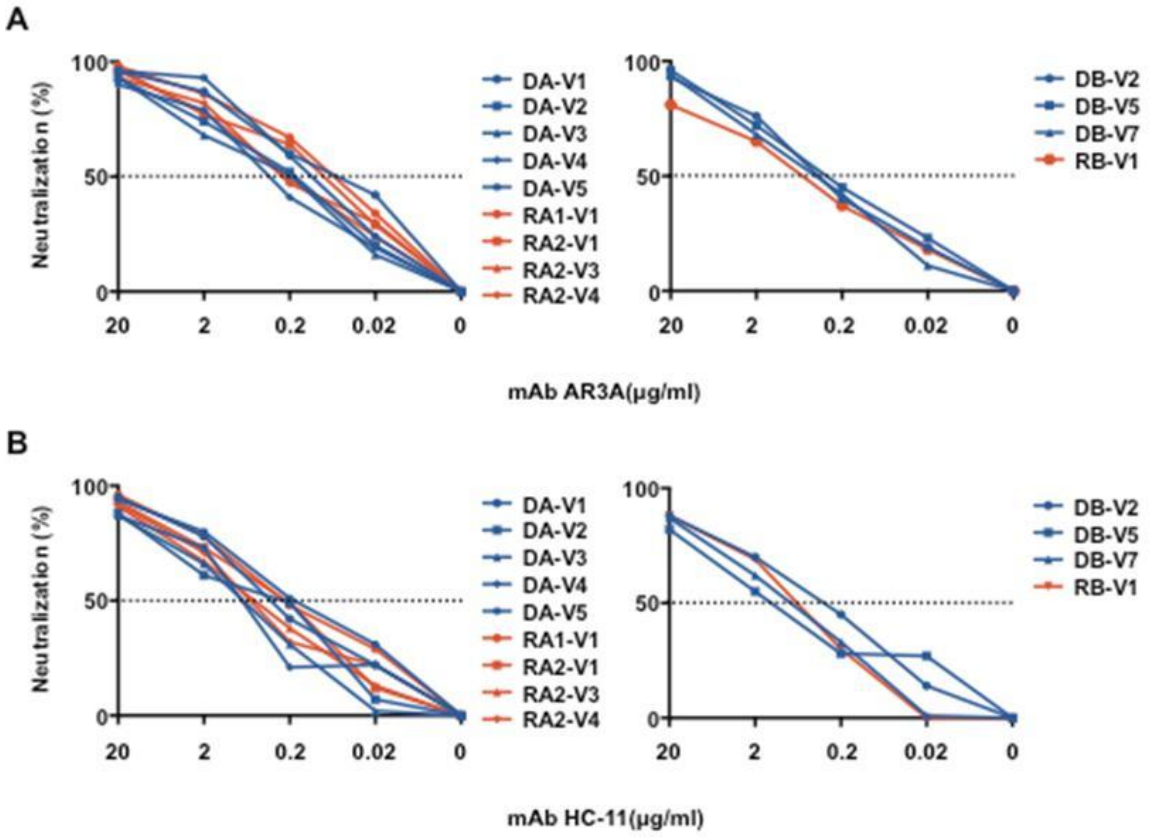


Fig. 29. Sensitivity of HCVpps bearing donors' and recipients' E1E2 envelope glycoproteins to neutralization with MAbs.

HCVpp neutralization sensitivities were assessed with the human anti-E2 nMAbs AR3A ***(A)*** *and HC-11* ***(B)****. Percentage neutralization was calculated as described in the 'Materials and Methods' section. Neutralization curves are shown for each donor- (blue) and recipient-derived (red) E1E2 variant. The data are mean values from 1 representative experiment performed in triplicate. The dashed line indicates 50 % neutralization of HCVpp entry.*

2. Discussion

The present study provides insights into the genotypic and phenotypic properties of HCV variants selectively transmitted to a new host from swarms of viruses constituting the quasispecies of source subjects. The HCV transmission process from donor to recipient has been rarely investigated due to the difficulty of recruiting patients at early stages of acute infection and to identify the source of contamination. In addition, the few reports describing experimental inoculation in the chimpanzee model or accidental contamination in humans with an available donor sample were mostly based on the genetic analysis focused on the HVR1 of E2 glycoprotein, without phenotypic characterization (Hijikata *et al*., 1993; Liu *et al*., 2006; Saito *et al*., 2004; Sugitani & Shikata, 1998). The methodology used to accurately identify T/F viruses and assess the full spectrum of genetic diversity at various stages of infection has also evolved with the development of the SGA, which has become a gold standard in the HIV field but has only recently been applied to HCV studies (Brown *et al*., 2012; Keele *et al*., 2008; Li *et al*., 2012; Salazar-Gonzalez *et al*., 2009). Therefore, for this study, we chose the SGA approach to provide a detailed investigation of HCV transmission between three HCWs, who developed acute hepatitis C after a needlestick accident, and the corresponding chronically infected donor patients. We focused our attention on HCV envelope glycoproteins, which are the most likely candidates for harboring phenotypic determinants that confer fitness for transmission. Full-length E1E2 sequences were amplified from donor plasma obtained at the time of needlestick accident for the transmission pairs DA/RA1 and DB/RB. For the recipients, SGA were conducted on the first sequential sample allowing a successful amplification of E1E2 sequences after contamination, which corresponded to the viremia peak (Fig. 21). Phylogenetic analyses demonstrated that the change of host environment resulted in a strong genetic bottleneck with a single T/F virus

yielding a productive infection in RA1 and RB (Fig. 24A & C). The genetic bottleneck observed was less stringent in the second recipient (RA2) infected by the donor DA with an estimate of five T/F viruses (Fig. 24B). These findings are quite consistent with the recent study based on SGA in which 10 of the 17 acutely infected subjects examined were found to have a range of one to four T/F viruses (Li *et al*., 2012). A similar range of T/F viruses was also determined by the 454 pyrosequencing approach in seven acutely infected subjects reported by two different groups (Bull *et al*., 2011; Wang *et al*., 2010). These recent studies on the T/F variant enumeration and their early diversification reported the occurrence of a genetic bottleneck upon HCV transmission without addressing the question of the molecular phenomenon underling this reduced genetic diversity of the donor quasispecies. By analyzing donor plasma sampled at the time of transmission, we could demonstrate that the T/F E1E2 amino acid sequences derived from the recipients RA1 and RB were respectively identical to that of a minor or a major variant in the corresponding donors' quasispecies. No identical E1E2 amino acid sequence was shared between the DA quasispecies and the 5 T/F virus enumerated in recipient RA2, which could be due to the ten-months interval between the sampling of DA and the needlestick accident of RA2. However, the E1E2 sequences obtained from both subjects remain closely related to each other with sometimes just a single amino acid difference between DA- and RA2-derived variants. It remains yet unclear whether the selective transmission of T/F virus results from a founder effect with a single or low number of variants being transferred between hosts, or whether it is attributable to early evolutionary events where a larger number of variants are selectively swept due to varying fitness constraints (Bull et al., 2011a). No specific genetic signature characterizes each of these scenarios. In a recent report based on the experimental transmission of HCV to a chimeric SCID/Alb-uPA mouse model with transplanted human hepatocytes, the occurrence of a selective sweep was put forward to explain that undetectable inoculum variants bearing an

advantageous E1E2 motif became the major variant circulating in the four infected mice (Brown *et al*., 2012). However, the *a priori* small inoculum resulting from needlestick accidents as well as the varying proportions of T/F E1E2 sequences present in the donor quasispecies complicate the transposition of the previously described scenarios to each of our transmission pairs. For instance, the T/F virus identified in recipient RB may be resulting either from a selective sweep favoring the inoculum variant harboring the E1E2 sequence with the 'YFKE' genetic signature, or it could also be arisen through a founder effect of a single major variant in the donor inoculum transferred to RB. Moreover, the recipients RA1 and RA2 provided us with the rare opportunity to comparatively investigate HCV transmission in two subjects sharing the same source subject. The difference of T/F virus enumerated for each recipient in our cohort is coherent with the source/recipient-specific clades observed in a previous study of a common-source outbreak, where HCV was found to evolve differently in 22 women accidently contaminated with the same inoculum (Ray *et al*., 2005).

Genetic and phenotypic features owned by T/F E1E2 sequences identified by SGA were investigated, to the best of our knowledge, only in the previously cited study on experimental transmission of HCV in a chimeric mouse model (Brown *et al*., 2012). This study showed that the transmitted variants harbored key substitutions in E1E2 sequences outside HVR1, including the lost of a potential N-linked glycosylation site in E2. By contrast, we found that three out of the six signature amino acids revealed by the VESPA analysis (positions 394, 399 and 401) characterizing the T/F viruses were located in HVR1 (Fig. 26). Importantly, the amino acid substitution at position 394 (H394R or H394Y) is a shared feature of the T/F variants in the 3 transmission pairs studied. HVR1 plays a major role in both HCV cell entry and immune evasion (Bartosch *et al*., 2003b; 2005; Dowd *et al*., 2009a; Guan *et al*., 2012; Scarselli *et al*., 2002). Despite strong amino acid variability, the

chemicophysical properties and conformation of HVR1 are well conserved and HVR1 is globally a basic stretch with most basic residues observed at positions 386, 394, 397, and 410 (corresponding to positions 3, 11, 14 and 27 in HVR1) in HCV genotype 1b (Callens *et al*., 2005; Penin *et al*., 2000). The basic residues H and R are the most frequently observed at position 394, which does not preclude the presence of other amino acids including the non-basic Y. The presence of basic residues in HVR1 was reported to facilitate virus entry. This effect can be modulated depending on the basic residue present at each position (Callens *et al*., 2005). Thus, the substitution affecting the residue 394 is potentially involved in phenotypic changes impacting the virus replicative fitness during the transmission process or the early acute infection. None of the signature amino acids identified outside HVR1 (positions 445, 476 and 641) affects directly a residue that participate in CD81 binding or a potential N-glycosylation site (Boo *et al*., 2012; Goffard *et al*., 2005; Owsianka *et al*., 2006). Of note, the residue 476 was previously described as a potential glycosylation site in the genotype 1a reference strain H77. However, this site has a percentage of conservation below twenty percent in the genotype 1b and it was absent from the E1E2 sequences studied here (François Helle et al., 2007).

In the chimeric mouse model, the major post-transmission E1E2 variant with the key substitutions outside HVR1 was found to confer an increased capacity for HCVpp entry compared to the major variant present in the inoculum (Brown *et al*., 2012). A similar conclusion was raised in a previous report on HCV evolution in a liver transplant setting, in which the uPA-SCID mouse model was used to support the hypothesis that the viral entry becomes an important determinant of relative fitness in the host, when the cell-mediated immune system is taken out of the equation (Fafi-Kremer *et al*., 2010). However, we found here that the E1E2 sequences corresponding to the RA1 T/F virus or to the three main genetic lineages identified in the RA2 quasispecies (i.e. RA2-V1, RA2-V3 and RA2-V4), which

included the signature residue R394, conferred levels of infectivity within the same order of magnitude as those observed for the majority of the non-transmitted variants (H394) (Fig. 27A). Moreover, the E1E2 sequence derived from the RB T/F virus (Y394) displayed a level of infectivity one order of magnitude lower than that of the infectious DB variants bearing a H394 residue (Fig. 27B), likely due to the loss of a basic residue (Callens *et al*., 2005). Thus, our results are not in agreement with the increased capacity of entry characterizing the post transmission variants raised in the chimeric mouse model, although this discrepancy is probably not surprising considering the different settings encountered by HCV between the chimeric mouse model and an immunocompetent patient. Also, these data suggested that the signature residue at position 394 is not correlated with the infectivity levels of transmitted and non-transmitted E1E2 variants, at least in the HCVpp model system. Indeed, while the HCVpp system usually employed to assess differential entry abilities of E1E2 sequences isolated from a variety of sources is sensitive and well validated, it may not capture the whole impact of the envelope glycoprotein properties in the natural HCV infection. The selection of T/F virus was not influenced by the neutralizing antibodies present in the donor sera, either. Drawing a parallel with the HIV, it is well established that sexual transmission across intact mucosal barriers is invariably associated with a viral population bottleneck, with transmission of a single T/F virus from the swarm of viral variants present in the donor (Shaw & Hunter, 2012). Studies designed to compare HIV envelope glycoproteins obtained from acutely infected individuals and the corresponding chronically infected donors generally found that transmitted envelopes include fewer putative N-linked glycosilation sites, and shorter sequences (Alexander *et al*., 2010; Derdeyn *et al*., 2004; Isaacman-Beck *et al*., 2009; Sagar *et al*., 2009). A study comparing envelope function in cell entry, co-receptor tropism, CCR5 interaction efficiencies, primary CD4+ T cell tropism, fusion kinetics, and neutralization sensitivities in the context of

pseudotype viruses, was designed to assess the possibility that T/F envelopes might differ in some phenotypic properties from chronic envelopes (Wilen *et al*., 2011). However, the results reported failed to identify a major transmission phenotype, with HIV T/F and chronic derived envelopes which have proved to be phenotypically equivalent (Wilen *et al*., 2011). More recently, it has been developed a method to clone full-length T/F genomes, obtained with an SGA approach, which produce replication-competent viruses (Li *et al*., 2010; Parrish *et al*., 2013; Salazar-Gonzalez *et al*., 2009). Using this method, the authors compared biological properties of T/F and chronically derived viruses that would be expected to influence viral fitness during the transmission process. They revealed that T/F viruses shared common traits likely enhancing their fitness in crossing mucosal barriers and promoting the establishment of a productive initial infection (Parrish *et al*., 2013). Indeed, their data indicated that T/F viruses were, on average, nearly two times more infectious and contained two times more envelope proteins than viruses cloned from chronically infected patients. A further interesting finding was that the HIV T/F viruses replicated to higher titers than the chronic controls when propagated in the presence of IFN-α, thus suggesting a relative INF-α resistance, although this property was not shared by all the HIV clades studied (Parrish *et al*., 2013). Considering these data on HIV together with previous findings on HCV, it remains conceivable that some HCV E1E2 variants contribute to the selective process for instance by differently attenuating the innate immune responses observed during the early phase of infection (Rehermann, 2009). Indeed, the E2 glycoprotein has already been shown to interfere with IFN-stimulated genes such as protein kinase R or to induce functional changes in natural killer cells (Crotta, Brazzoli, Piccioli, Valiante, & Wack, 2010; Taylor, 1999). Another important parameter that could contribute to the selective transmission of HCV variants in a viral quasispecies is the genetic interaction between virus and host. Indeed, several studies reported HCV compartmentalization in more than one tissue in the same

patient, for instance serum and brain (Radkowski et al., 2002), serum and saliva (Roy *et al*., 1998), serum and PBMCs (Ducoulombier *et al*., 2005), suggesting that HCV may be sensitive to the genetic, biochemical or metabolic background of the host cells (Nainan *et al*., 2006; Penin *et al*., 2000). If we compare again to HIV mucosal transmission, Parrish and colleagues speculated that the genetic bottleneck observed could result from several factors including inherent physical barriers and virus-host cell interactions necessary for early virus infection and replication. Thus, even slightly advantages of T/F viruses might increase their transmission fitness (Parrish *et al*., 2013).

Finally, our findings have important implication for the development of strategies preventing HCV transmission. Various cross-neutralizing anti-E1 or E2 MAbs are capable of neutralizing genetically diverse HCV isolates, and some of them were shown to protect against heterologous HCV quasispecies challenge in a HCV animal model (Edwards *et al*., 2012; Giang *et al*., 2012; Keck *et al*., 2012; Keck *et al*., 2008a; Law *et al*., 2008; Meunier *et al*., 2008). Polyclonal anti-HCV antibodies isolated from chronically HCV-infected patients can also protect against an *in vivo* challenge with different HCV genotypes (Meuleman *et al*., 2012). We confirmed here the sensitivity of our T/F HCV isolates to cross-reactive monoclonal or polyclonal Abs *in vitro*. In addition, our data show that the anti-E2 MAbs tested here have sufficient cross-reactivity to neutralize T/F variants derived from the three recipients. Combination of antibodies may thus further increase the genetic barrier for resistance and offer a viable and promising strategy to prevent HCV transmission.

VI. CONCLUSIONS ET PERSPECTIVES

To summarize, for the first time to the best of our knowledge, we presented here a project on HCV transmission where the genetic analysis of full-length E1E2 sequences amplified in immunocompetent humans was coupled to the study of their functional properties. We studied three cases of BEAs in a cohort of five patients, i.e. three HCWs and the corresponding chronically HCV infected source patients. We analyzed the donors and recipients' quasispecies evolution upon transmission by amplifying 155 SGA E1E2 sequences, 147 of which presenting preserved ORFs. The originality of our work lies in several points. Firstly, the rare possibility to compare E1E2 sequences amplified in recently infected patients with those amplified in the subjects at the origin of contamination. We exactly knew the date of each contamination event, and therefore we were able to analyze envelope glycoproteins sequences before the appearance of anti-E2 protein antibodies. Secondly, the SGA approach enabled us to identify proportionally represented viral variants in the patients' quasispecies (Bull et al., 2011). Compared with the classical bulk PCR, cloning and sequencing, the SGA methodology avoids the introduction of *Taq* polymerase errors into cloned products, and non-proportional representation of target sequences resulting from template resampling or unequal template amplification and cloning (Keele *et al*., 2008; Palmer *et al*., 2005; Salazar-Gonzalez *et al*., 2008; 2009). In this way, we were able to unambiguously identify and determine the exact E1E2 nucleotide sequences of T/F viruses in the three recipients of our study cohort. Thus, we reported that a single T/F virus was sufficient to initiate a productive infection in two out of three recipients studied. In these two cases, the E1E2 amino acid sequences of the T/F virus were fully conserved upon the transmission process, and they respectively corresponded to a minor and a major variant in the donors' quasispecies. Conversely, in the third contamination case, we observed a less stringent selective transmission, with five T/F viruses detected in the recipient RA2. With the aim to find molecular determinants

underlying the selective transmission process of the T/F viruses, we showed that the histidine in position 394 (corresponding to a position in the polyprotein sequence of the reference strain H77), which was highly represented in the donors' quasispecies, was never present in the recipients' quasispecies, except for one E1E2 variant in the RA2 quasispecies. For this thesis project, we also sought to investigate functional properties owned by the T/F viruses with the aim of highlighting the phenotypic features which could affected the selective transmission of E1E2 sequence variants in the three recipients. To this end, we used the HCVpp model system. Despite the limited number of cases we studied, our findings argue against a greater entry efficiency into target cells of HCVpp harboring E1E2 sequences from transmitted viruses compared to the non-transmitted one. Furthermore, using the same model system, the hypothesis of an involvement of the donor neutralizing antibodies also failed to explain the phenomena of selective transmission, which we nevertheless revealed by the genetic analysis. Beside this, we showed efficient neutralization of transmitted E1E2 variants by both heterologous anti-HCV-positive sera and broadly neutralizing MAbs, thus suggesting their suitability for preventing HCV infection.

Since the HEK-293T cell line used for the production of HCVpp does not synthesize lipoproteins, envelope glycoproteins expressed on the HCVpps are not associated with such lipid components (Burlone & Budkowska, 2009). However, a lot of evidences have highlighted the importance of lipoprotein association as a strategy for HCV virions to escape the host adaptive immunity, as well as their involvement in the early steps of virus entry. Therefore, although the HCVpp system constitutes a valid and simple system for the study of viral entry and fusion with cell membranes, it cannot mimic serum-derived particles. For this reason, it would be interesting to further investigate functional properties of transmitted and non-transmitted viruses using the *in vitro* model system of

cell-culture derived HCV (HCVcc) (Lindenbach *et al*., 2005; Wakita *et al*., 2005; Zhong *et al*., 2005). To do this, during the last months of this thesis project, we engineered J4/JFH1-based chimeric genomes (Gottwein *et al*., 2009) (Fig. 21), in which we replaced the J4 E1E2 region by the E1E2 sequences we sought to phenotypically characterize. In this way envelope sequences derived from our study participants (all infected by 1b HCV genotype) should be expressed in a 1b background. In the near future, this tool will be used to investigate functional properties of T/F E1E2 sequences both in cell entry and in neutralization assays, as a normal continuation of this thesis project. It would be very interesting to be able to confirm or refute results obtained with the HCVpp system or more, to highlight new biological and chemicophysical properties of E1E2 sequences brought by the identified T/F viruses. However, since the experimental difficulties encountered in working with HCV complete-genome clones, likely it will not be simple to identify a major transmission phenotype shared by T/F viruses.

Studies in chimeric mouse models (Brown *et al*., 2012; Fafi-Kremer *et al*., 2010) reported that transmitted HCV variants conferred an increased capacity for HCVpp entry compared to the non-transmitted sequences. Conversely, we found that the E1E2 sequences corresponding to the recipients T/F viruses conferred a level of infectivity within the same order of magnitude or less that those observed for the majority of the non-transmitted variants. The selection of T/F viruses was not influenced by the neutralizing antibodies present in the donor sera, either, thus leaving unanswered the question posed here about the molecular mechanism underlying the selective transmission of only a few E1E2 sequences included in a heterogeneous quasispecies. Intracellular antiviral mechanisms and the innate immune response can affect HCV transmission, selecting viral variants able to overcome them. Indeed, the E2 glycoprotein has been shown to interfere with INF-stimulated genes thus counteracting its suppressive activity on

the HCV infectious cycle (Crotta *et al*., 2010; Rehermann, 2009), as well as to inhibit Natural Killer cells *in vitro*. Therefore, it should be interesting to test T/F envelope glycoprotein sequences for their interplay with immunocompetent hepatocytes. Actually, the high permissivity of Huh7.5 to HCV replication *in vitro* has been partially attributed to impaired innate immune responses in these cells, because RIG-I (retinoic acid inducible gene I) mutation and inactivation (Jammart *et al*., 2013; Sumpter *et al*., 2005). RIG-I is a pattern recognition receptor that senses HCV double-stranded RNA and triggers type I INF pathways resulting in the expression of interferon stimulated genes (ISGs) (Feigelstock *et al*., 2010). In contrast to Huh7.5, HepG2 human liver carcinoma cell line and the recently characterized Huh7D clone are able to mount an antiviral innate immune response against HCV infection via the production of type I INF, thus constituting more suitable model systems to study T/F envelope glycoproteins properties as regards the innate immune responses (Feigelstock *et al*., 2010; Jammart *et al*., 2013). Finally, also a particular genetic, biochemical or metabolic background of HCV-susceptible host cells may determine the selective transmission of viral variants, for example favoring the emergence of variants with a greater capacity to interact with them (Nainan *et al*., 2006; Penin *et al*., 2000).

Therefore, a thorough characterization of the HCV T/F viruses able to initiate productive infection in new hosts could provide valuable clues for the design of prophylactic or/and therapeutic vaccines, that might be more effective in a period during which the virus may be more susceptible to successful elimination, given the strong genetic homogenization which follows transmission events.

V. CONCLUSIONS & PERSPECTIVES

Pour la première fois à notre connaissance, nous présentons ici les résultats d'une étude sur la transmission du VHC où l'analyse génétique de séquences complètes de glycoprotéines d'enveloppe E1E2 amplifiées chez des patients immunocompétents a été couplée à l'étude de leurs propriétés phénotypiques. Notre étude a porté sur trois cas d'accidents d'exposition au sang (AES) regroupant au total cinq patients : trois patients contaminés, à savoir des représentants du personnel soignant (receveurs RA1, RA2 et RB), et deux patients chroniquement infectés à l'origine de leur contamination (donneurs DA et DB). Nous avons analysé les quasi-espèces virales circulant chez les donneurs et les receveurs par amplification de génomes uniques (SGA ou « Single Genome Amplification »). Cent cinquante cinq séquences codant des glycoprotéines d'enveloppe virale E1E2 ont été obtenues au total, 147 de ces séquences ayant un cadre ouvert de lecture complet. L'originalité de ce travail de thèse repose sur différents points. Tout d'abord, nous avons eu la possibilité exceptionnelle de comparer les séquences E1E2 amplifiées chez des patients très récemment infectés avec celles amplifiées chez les sujets à l'origine de leur contamination. Nous connaissions exactement la date de chaque événement de contamination, et, par conséquent, nous avons pu analyser les séquences des glycoprotéines d'enveloppe avant l'apparition d'anticorps anti-VHC. Ensuite, l'approche expérimentale utilisée, l'amplification de génomes uniques, nous a permis d'identifier les différents variants viraux présents au sein des quasi-espèces (Bull et al., 2011a). Par rapport à la méthode classique d'amplification par PCR, de clonage et de séquençage des clones à étudier, le protocole d'amplification des génomes uniques nous a permis d'éviter l'introduction d'erreurs d'incorporation dans les séquences amplifiées par la *Taq* polymérase et d'obtenir une représentation proportionnelle des séquences cibles dans les quasi-espèces (Keele *et al*., 2008; Palmer *et al*., 2005; Salazar-Gonzalez *et al*., 2008; 2009). De cette façon, nous avons pu identifier sans ambiguïté les séquences

nucléotidiques E1E2 des virus T/F chez les trois receveurs étudiés. Nous avons ainsi pu constater qu'un seul virus T/F était suffisant pour initier une infection productive chez deux receveurs (RA1 et RB) sur trois. Dans ces deux cas, les séquences protéiques des glycoprotéines d'enveloppe portées par les virus T/F ont été conservées lors de la transmission, ces séquences étant issues respectivement d'un variant minoritaire et majoritaire dans les quasi-espèces des donneurs correspondants DA et DB. En revanche, dans le troisième cas de contamination, nous avons mis en évidence une transmission moins sélective, avec cinq virus T/F détectés chez le receveur RA2. Dans le but d'identifier des déterminants moléculaires pouvant expliquer le processus de transmission sélective des virus T/F, nous avons mise en évidence une position d'intérêt dans HVR-1. En effet, la présence d'une histidine en position 394 (selon la numérotation de la séquence de la polyprotéine de la souche de référence H77) était fortement conservée dans les quasi-espèces des donneurs alors qu'elle était remplacée par une arginine ou une tyrosine dans les quasi-espèces des receveurs (à l'exception d'une séquence amplifiée chez RA2). Cette observation suggère le caractère défavorable de l'histidine en position 394 au moment du passage aux nouveaux hôtes. Pour ce projet de thèse, nous avons également cherché à étudier les propriétés fonctionnelles spécifiques des virus T/F dans le but de mettre en évidence des caractéristiques phénotypiques susceptibles d'affecter la transmission sélective des variants E1E2 chez les trois receveurs. À cette fin, nous avons utilisé le système des virus pseudo-typés (VHCpp). Les résultats obtenus avec ce système ne nous ont pas montré une plus grande efficacité dans l'entrée des VHCpps portant les glycoprotéines d'enveloppe E1E2 des virus transmis par rapport à celles des virus non transmis. Ces résultats ne corroborent donc pas ceux obtenus dans les études basées sur le modèle souris chimeriques qui ont montré que les variants du VHC transmis conféraient une capacité accrue à l'entré virale par rapport aux séquences non transmises (Brown *et al*.,

2012; Fafi-Kremer *et al*., 2010). En utilisant le même système des VHCpp, nous avons ensuite pu déterminer que les anticorps neutralisants du donneur n'étaient pas impliqués dans la transmission sélective du VHC. Ces donnés laissent donc sans réponse la question posée ici sur le mécanisme moléculaire qui sous-tendrait la transmission sélective de seulement quelques séquences E1E2 au sein d'une quasi-espèce du VHC. Par contre, nous avons démontré que des sera hétérologues VHC-positifs et des anticorps monoclonaux à large spectre neutralisant étaient efficaces dans l'inhibition de l'entrée associée aux glycoprotéines d'enveloppe dérivées des virus T/F.

Compte-tenu de l'implication des lipoprotéines associées au VHC dans l'échappement du virus à l'immunité adaptative de l'hôte et dans les étapes précoces de l'entrée virale, il serait intéressant d'étudier les propriétés fonctionnelles des séquences des glycoprotéines d'enveloppe portées par les virus transmis et non transmis, en utilisant le système *in vitro* du VHC dérivé de culture cellulaire (VHCcc) (Lindenbach *et al*., 2005; Wakita *et al*., 2005; Zhong *et al*., 2005). En effet, la lignée cellulaire utilisée pour la production des VHCpp (HEK-293T) ne permet pas de produire des pseudo-particules auxquelles sont associées des lipoprotéines de manière similaire au contexte viral naturel. Les propriétés spécifiques des virus T/F pourraient cependant être associées à la présence de ces lipoprotéines (Burlone & Budkowska, 2009). Par conséquent, il serait souhaitable de développer une méthodologie expérimentale permettant de reproduire autant que possible l'ensemble des caractéristiques biologiques des particules circulant dans le sérum des patients. La production des VHCcc est essentiellement restreinte à la souche JFH1 (génotype 2a). Cependant, il a été possible d'obtenir des virus JFH1 chimériques portant les protéines structurales de souches hétérologues dérivées des principaux génotypes du VHC. Les patients de notre étude étant tous infectés par du VHC de génotype 1b, nous avons envisagé de développer une approche similaire en utilisant le

clone viral infectieux intergénotypique J4/JFH1 (1b/2a) (Gottwein *et al*., 2009). Ce clone viral optimisé a été conçu initialement en associant 3 fragments génomiques viraux complémentaires : 'JFH1 5'UTR', 'J4 Core-NS2', et 'JFH1 NS3-3'UTR' (Wakita *et al*., 2005). Les travaux basés sur ce clone ont tout juste été initiés pendant les derniers mois de mon projet de thèse. Nous avons pu construire les génomes viraux chimériques dérivés du clone J4/JFH1 dans lequel nous avons substitué les gènes des glycoprotéines d'enveloppe du virus J4 par ceux codant nos protéines E1E2 caractérisées dans le système de VHCpp. Les travaux de caractérisation de ces virus se poursuivront dans la continuité de mon travail de thèse.

La réponse immunitaire innée doit également être prise en considération dans les éléments susceptibles de moduler la transmission des variants du VHC. En effet, comme nous l'avons évoqué précédamment, il a été montré que la glycoprotéine E2 était capable d'interférer avec les gènes stimulés par l'interféron. Les glycoprotéines d'enveloppe du VHC pourraient aussi avoir un effet inhibiteur sur les cellules 'Natural Killers' (Crotta *et al*., 2010; Rehermann, 2009). Par conséquent, il serait intéressant d'étudier les propriétés des glycoprotéines d'enveloppe des virus T/F dans le contexte de lignées hépatocytaires immunocompétentes. En effet, la grande permissivité des cellules Huh7.5 à la réplication du VHC a été partiellement attribué à une réponse immunitaire innée altérée dans cette lignée cellulaire due à une mutation de RIG-I (retinoid-indiced gene I) (Jammart *et al*., 2013; Sumpter *et al*., 2005). RIG-I est un récepteur intracellulaire qui fonctionne comme une sentinelle reconnaissant des agents viraux tels que les molécules d'ARN double brin. L'activation de RIG-I déclenche ensuite une cascade aboutissant à la production des interférons de type I (INF-α et -β) et à l'activation des gènes stimulés par ces puissantes cytokines antivirales (Feigelstock *et al*., 2010). Contrairement aux cellules Huh7.5, la lignée cellulaire humaine HepG2, dérivée d'un carcinome du foie, et le clone

Huh7D, récemment décrit, sont capables de monter une réponse immunitaire innée dirigée contre l'infection par le VHC par l'intermédiaire de la production d'interféron de type I. Ces cellules constituent ainsi des systèmes modèles plus appropriés pour l'étude des propriétés des glycoprotéines d'enveloppe des virus T/F en ce qui concerne cet aspect de la réponse innée (Feigelstock *et al*., 2010; Jammart *et al*., 2013). Des travaux sur les cellules 'Natural Killers' seront également à envisager. Enfin, dernier point, les propriétés génétiques, biochimiques ou métaboliques particulières des cellules hôtes sensibles au VHC, autres que les hépatocytes, pourraient affecter la transmission sélective de variants viraux en favorisant par exemple l'émergence des variants présentant une plus grande capacité d'interagir avec leur molécules de surface (Nainan *et al*., 2006; Penin *et al*., 2000).

En conclusion, une caractérisation approfondie des virus T/F, capables d'initier une nouvelle infection dans un hôte naïf, pourrait fournir des précieux indices pour la conception de vaccins prophylactiques et/ou thérapeutiques. En effet, ces derniers pourraient être plus efficaces dans une période pendant laquelle le virus peut être plus sensible à l'élimination, compte-tenu de la forte homogénéisation génétique qui suit les événements de transmission.

VIII. BIBLIOGRAPHY

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IX. ANNEXES

Annexe 1 : Original Article

Article submitted to ‘PLoS Pathogens’.

Sequence and Functional Analysis of the Envelope Proteins of Hepatitis C Virus Quasispecies Selectively Transmitted in Primary Infection

Short title: primary HCV transmission

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Abstract

Hepatitis C remains a challenging public health problem worldwide, with an estimated three to four million new infections per year. The identification of viral variants capable of establishing *de novo* infections and definition of the phenotypic requirements for transmission would facilitate the design of preventive strategies. We explored the

transmission of HCV quasispecies in three cases of acute hepatitis following needlestick accidents. We used single-genome amplification of glycoprotein E1E2 gene sequences to map the genetic bottleneck upon transmission accurately. We found that infection was established by a single variant in two cases and five variants in the third case. Studies of donor samples showed that the transmitted variant E1E2 amino-acid sequences were identical or closely related to those of variants among the donor quasispecies. The transmitted variants harbored a common key substitution at position 394, within hypervariable region 1 of E2. Surprisingly, these E1E2 variants conferred no greater capacity for entry than the E1E2 derived from non transmitted variants, in lentiviral pseudoparticle assays. The selection of the transmitted variants was not influenced by the presence of neutralizing antibodies in donor serum. The fitness parameters affecting the selective outgrowth of HCV variants after transmission in an immunocompetant host may thus be more complex than suggested by chimeric mouse models. Human monoclonal antibodies directed against HCV envelope glycoproteins effectively cross-neutralized the lentivial particles bearing E1E2 derived from transmitted variants. These findings provide insight into the molecular mechanisms underlying HCV transmission and suggest that viral entry is a viable target for the prevention of HCV infection.

Author summary

Three to four million of new hepatitis C virus (HCV) infections occur every year worldwide. These primary infections are typically asymptomatic and commonly result in chronic hepatitis, with a strong tendency to progress toward life-threatening liver diseases. A better knowledge of the HCV responsible for the spread of the infection could illuminate key aspects of the transmission process and early stages of infection, during which the

virus may be most vulnerable to elimination by preventive strategies. We addressed this issue by using single genome sequencing to identify envelope glycoproteins harbored by HCV selectively transmitted to health care workers contaminated through needlestick accidents. Numbers of transmitted viruses leading to productive clinical infection ranged from 1 to 5. A key amino acid substitution was identified in the envelope glycoproteins upon transmission. Surprisingly, the selection of the transmitted HCV was not associated to a greater capacity for entry or the presence of neutralizing antibodies in the contaminating blood. The parameters affecting transmission in an immunocompetant host may thus be more complex than suggested by animal models. We finally demonstrated that these early-transmitted viruses were efficiently cross-neutralized by monoclonal antibodies, which could thus represent a viable and promising strategy for preventing HCV transmission.

Introduction

The World Health Organization has estimated that 150 million people are chronically infected with hepatitis C virus (HCV) worldwide, with three to four million new infections occurring annually. About 30% of acute HCV infections resolve spontaneously, but most infections become chronic, with a strong tendency to progress toward life-threatening liver diseases, such as cirrhosis and hepatocellular carcinoma [1,2].

HCV is transmitted mostly via direct blood-to-blood contact [3]. Occupational hepatitis C is occasionally reported in healthcare workers [4,5], and cases resulting from well monitored needlestick accidents provide rare opportunities for tracking the entire transmission process from donor to recipient [6,7]. *In vivo*, HCV replicates rapidly, using an error-prone viral RNA

polymerase, resulting in the generation, within each infected individual, of a group of related but genetically different viral variants called quasispecies [8]. The genetic bottleneck generally observed after transmission indicates that productive infections may be initiated by one or a small number of viral variants [9-12]. These variants are then subjected to constant immune pressure, and the role of the immune response in the clearance of HCV infection or the establishment of chronic hepatitis C has been thoroughly investigated. However, little is known about the transmitted variants responsible for the spread of the disease, principally because it is difficult to recruit patients early enough in acute infection for such studies. Moreover, studies of the viruses transmitted in humans have essentially focused on genetic identification of the transmitted/founder (T/F) variants and their early diversification, through phylogenetic and mathematical approaches, without direct comparison with the donor quasispecies [9-11]. A key question in the rational design of strategies for preventing HCV infection concerns the phenotypic determinants conferring fitness for transmission in T/F viruses. The HCV envelope glycoproteins, which are involved principally in virus attachment and entry into target cells, are the most likely candidates for such transmission-related signatures.

We addressed this issue by exploring HCV transmission in healthcare workers (the recipients) who developed acute hepatitis C after needlestick accidents, comparing findings for these individuals with those for the corresponding chronically infected patients from which the virus was transmitted (the donors). We mapped the genetic bottleneck leading to productive clinical infection, by single-genome amplification of viral envelope glycoprotein E1E2 sequences, direct amplicon sequencing and phylogenetic analyses [11,13]. The recovery of full-length E1E2 sequences from donors and recipients made it possible to investigate the phenotypic properties of these proteins potentially relevant to transmission. Our data provide insight into the selective transmission of HCV quasispecies

and early stages of infection, during which the virus may be most vulnerable to elimination by preventive vaccines or immunotherapies.

Results

Study subjects and single-genome sequencing

Three healthcare workers (recipients RA1, RA2 and RB) and the corresponding chronically infected patients (donors DA and DB) were studied (Figure 1). Recipients RA1 and RA2 shared the same donor, DA, but were infected 10 months apart. Single-genome amplification (SGA) was performed on donor plasma samples obtained at the time of the needlestick accident for the transmission pairs DA/RA1 and DB/RB. For recipients RA1 and RB, SGA was conducted before antibody seroconversion, on the first viremic plasma sample, collected 25 and 14 days post infection, respectively. For RA2, E1E2 sequences were successfully amplified only from the second sequential sample collected 44 days post infection, at the time of peak viremia and antibody seroconversion. In total, 155 full-length E1E2 nucleotide sequences were derived by single-genome sequencing (i.e. SGA followed by direct amplicon sequencing) from the two donors and three recipients (range, 24 to 37 per subject) (Table 1).

Change in HCV quasispecies distribution between donors and recipients

The E1E2 nucleotide sequences derived from donor and recipient subjects were first subjected to neighbor-joining phylogenetic analysis (see Figure S1). Infection by a 1b

genotype was confirmed for all the subjects enrolled in the study. Nucleotide sequences corresponding to each transmission pair clustered together with high bootstrap support, confirming the epidemiological link. In particular, the E1E2 nucleotide sequences of recipient RA2 were interspersed with those of donor DA and recipient RA1, whereas the sequences of recipients RA1 and RB formed a lineage characterized by extremely low diversity (bootstraps ≥ 98). These phylogenetic data were consistent with the mean within-subject diversities, which were of 1.49% for RA2 sequences and 0.05% for RA1 or RB sequences (Table 1). As expected, sequences from the chronically infected subjects DA and DB displayed broader genotypic heterogeneities, with mean within-subject sequence diversities of 1.57% and 1.94%, respectively. There was thus a decrease in quasispecies diversity (bottleneck effect) after transmission, which was very pronounced in recipients RA1 and RB.

For further characterization of the transmission process, we analyzed the pattern of nonsynonymous substitutions (amino-acid changes) within each transmission pair, by a combination of neighbor-joining phylogenetic tree reconstruction and Highlighter plots (Highlighter for Amino Acid Sequences, http://hcv.lanl.gov/content/sequence/HIGHLIGHT/highlighter_top.html). Polymorphisms within the E1E2 amino-acid sequences derived from donor DA and recipient RA1 are represented in Figure 2A. The sequence used as the reference, at the top of the tree and the Highlighter plot, corresponds to the dominant variant of donor DA, which was not transmitted to recipient RA1. Twenty-nine of the 36 sequences obtained from RA1 were identical, the remaining sequences differing by only one or two randomly distributed substitutions. This strong genetic homogeneity indicates that a single T/F virus was responsible for productive infection in RA1. The T/F virus E1E2 amino-acid sequence was found to be identical to that of a minor variant present among donor DA quasispecies. The

corresponding nucleotide sequences could be distinguished on the basis of patterns of polymorphisms, with synonymous mutations specific to DA or RA1 (see Figure S2). We then extended this analysis to recipient RA2, using the same dominant variant sequence from the donor DA as a reference (Figure 2B). The 27 sequences derived from recipient RA2 clearly formed five lineages with consensus sequences differing by at least three nonsynonymous substitutions. This suggests that five genetically different T/F viruses were responsible for initiating productive infection in RA2. No E1E2 sequence common to donor DA and recipient RA2 was identified, but the consensus sequences of the transmitted lineages differed by only one to four amino acids from those of the closest variants present in donor quasispecies. The genetic relationships between the E1E2 sequences present in donor DB and recipient RB are depicted in Figure 2C. The sequence used as the reference, at the top of the tree and the Highlighter plot, is that of one of the two major variants from donor DB, which was not transmitted to recipient RB. Twenty-one of the 23 sequences derived from recipient RB were identical, the remaining sequences differing by only one random substitution. This strong genetic homogeneity indicates that a single T/F virus was responsible for productive infection in recipient RB. However, in this case, the T/F virus E1E2 amino-acid sequence was identical to that of a major variant from donor DB. Thus, the donor quasispecies went through a strong genetic bottleneck, with only a single variant from the inoculum generating productive infection in two of the three recipients studied. In these two cases, the E1E2 amino-acid sequences of the transmitted variant were fully conserved during transmission, although synonymous substitutions nevertheless occurred in the corresponding nucleotide sequences. Interestingly, five T/F viruses were detected in the second recipient infected by donor DA, suggesting that a less stringent selective process occurred in this case.

Molecular determinants underlying HCV transmission

For identification of the signature of molecular determinants related to the observed genetic bottleneck, we investigated amino-acid differences between the sequences derived from donors and those obtained from the corresponding recipients, with VESPA [14]. VESPA calculates the frequency of each amino acid at each position in an alignment for the query (recipient) and reference set (donor), and selects the positions for which the most common character in the query set differs from that in the background set. Six signature amino acids were identified in the E2 glycoprotein, three of which were located in hypervariable region 1 (HVR1) (Figure 3). The amino-acid substitution at position 394 in HVR1 (H394R or H394Y) was common to the transmitted variants in all three transmission pairs (with the exception of a single E1E2 sequence derived from recipient RA2) (Figure 3A). The frequencies of each combination of signature amino acids are summarized in Figure 3B. A specific combination of signature amino acids characterized the E1E2 sequences of the transmitted variants identified in recipients RA1 and RB (i.e. R394-R445-D641 for recipient RA1 and Y394-F399-K401-E476 for recipient RB). In recipient RA2, combinations of signature amino acids identified four of the five genetic lineages previously identified with the Highlighter tool; two of these lineages shared the same combination of signature amino acids (i.e. Y394-R445). These data suggest that, during transmission or early in infection, a common key amino acid located at position 394 in HVR1, together with additional signature amino acids specific to each transmission pair, conferred a selective advantage to the transmitted variants.

Influence of viral entry and donor neutralizing antibodies on variant selection

We investigated the mechanism of selection operating during HCV transmission, by determining the relative entry efficiency conferred by E1E2 amino-acid sequences representative of the main genetic lineages previously identified in donor and recipient quasispecies. A few additional E1E2 sequences derived from minor variants were also studied (see Figures 2 and S3). Lentiviral HCV pseudoparticles (HCVpp) bearing E1E2 glycoproteins were generated and their ability to infect Huh7.5 cells was assessed, as described in the Materials and Methods. The expression of 15 of 19 selected E1E2 sequences resulted in the production of infectious HCVpps (Figure 4). Levels of E1E2 glycoprotein incorporation into HCVpp were similar in each case (Figure S4). The E1E2 sequences corresponding to the recipient RA1 T/F virus or the three main genetic lineages identified in recipient RA2 quasispecies (i.e. RA2-V1, RA2-V3 and RA2-V4) conferred high levels of infectivity, but of the same order of magnitude as observed for of the variants not transmitted to recipients (including the dominant sequence from DA, V3) (Figure 4A). These data suggest that the selective effect on key residue H394 is not directly linked to greater infectivity in the HCVpp system. This finding was supported by results for the transmission pair DB/RB, for which the E1E2 sequence derived from the recipient RB T/F virus had a level of infectivity one order of magnitude lower than that of the infectious DB variants bearing the H394 residue (Figure 4B).

The anti-HCV antibodies present in the inoculum might also influence the transmission process. We therefore carried out neutralization experiments with donor serum. HCVpp were incubated with serial dilutions of the donor serum; their subsequent infection levels were quantified and neutralization titers were calculated as described in the Materials and Methods. HCVpp bearing E1E2 sequences derived from transmitted variants were

neutralized to a similar extent to those carrying non transmitted variants, by the antibodies present in the corresponding donor serum (Figure 5A). None of the donor sera neutralized HCVpp bearing the control VSV-G (data not shown). These findings suggest that donor neutralizing antibodies are not involved in the selection of T/F viruses.

Entry inhibition of transmitted variants by polyclonal or monoclonal antibodies

In a final set of experiments, we sought to assess whether the entry of transmitted variants by neutralizing antibodies could be targeted to prevent HCV infection. HCVpp were incubated with genotype-specific (1a, 1b, 2a, 3a) serum pools and their neutralization was studied, as described in the Materials and Methods. Similar experiments were conducted with the anti-E2 broadly neutralizing mAbs AR3A and HC-11 [15,16]. The serum pools inhibited infection with HCVpp bearing E1E2 sequences derived from both transmitted and non transmitted viral variants, to various extents (Figure 5B). The genotype 1b serum pool displayed the strongest neutralizing activities against all the variants, consistent with the known infection of both donors and recipients with HCV genotype 1b. The anti-E2 mAb also efficiently inhibited infection with HCVpp bearing E1E2 sequences derived from both transmitted and non transmitted viral variants (IC_{50} 0.1-1.04 μg/ml for AR3A, and 0.19-1.67 for HC-11; Figure 6). This efficient neutralization of transmitted HCV variants by cross-neutralizing antibodies suggests that targeting viral entry with mAbs is a promising strategy for preventing HCV transmission.

Discussion

This study provides insight into the genotypic and phenotypic properties of the HCV variants selectively transmitted to a new host from the swarm of viruses present in the donor quasispecies. HCV transmission has rarely been investigated, due to the difficulty of

recruiting patients at early stages of acute infection, which is usually clinically silent [1]. Furthermore, the few reports describing experimental inoculation in the chimpanzee model or accidental contamination in humans, for which a donor sample was available, have been based mostly on genetic analysis of HVR1 of the E2 glycoprotein, with no phenotypic characterization [6,7,17]. Methods for accurately identifying T/F viruses and assessing genetic diversity at various stages of infection have also evolved with the development of SGA, which is now the gold standard in the HIV field, but has only recently been applied to HCV studies [11-13,18-20]. We used SGA for the detailed investigation of HCV transmission in three cases of acute hepatitis acquired through needlestick accidents. Phylogenetic analyses demonstrated that a change in host environment resulted in a strong genetic bottleneck with a single T/F virus responsible for productive infection in recipients RA1 and RB (Figures 2 and 3). The genetic bottleneck observed was less stringent in the second recipient (RA2) infected by donor DA, with an estimated five T/F viruses differing by at least three nonsynonymous substitutions. We do not think that sampling from RA2 at the time of seroconversion confounded this identification of T/F variants. Indeed, in a recent report, no potential immune escape were detected in E1E2 among 17 acutely infected subjects followed during the initial 6-8 weeks of infection [11]. Our findings for the number of T/F viruses are consistent with this report, in which 10 of the 17 acutely infected subjects examined had one to four T/F viruses. A similar number of T/F viruses was also found by 454 pyrosequencing approaches in seven acutely infected subjects reported by two different groups [9,10].

These recent studies on T/F variants did not address the question of the selection mechanism reducing the genetic diversity of the donor quasispecies. By studying donor samples, we demonstrated that the T/F E1E2 amino-acid sequences derived from recipients RA1 and RB were identical to those of a minor or a major variant from the DA and DB

quasispecies, respectively. No identical E1E2 amino-acid sequence was common to the donor DA quasispecies and the five T/F viruses from recipient RA2, possibly due to the ten-month interval between sampling for DA and the needlestick accident resulting in the infection of RA2. However, the E1E2 sequences obtained from the two subjects remained very similar. It remains unclear whether the selective transmission of T/F viruses results from a founder effect, with one or a small number of variants being transferred between hosts, or whether it is due to early evolutionary events, with larger numbers of variants undergoing a selective sweep due to differences in fitness constraints [9]. In a recent report of the experimental transmission of HCV to chimeric SCID/Alb-uPA mice with transplanted human hepatocytes, the occurrence of selective sweeps was put forward to explain the finding that undetectable inoculum variants bearing an advantageous E1E2 motif became the major variants circulating in the four infected mice [12]. However, the *a priori* small inoculum resulting from needlestick accidents and the different proportions of T/F E1E2 sequences present in donor quasispecies complicate the transposition of previously described scenarios to each of our transmission pairs. For instance, the T/F virus identified in recipient RB may result from a selective sweep favoring the inoculum variant harboring E1E2 with the genetic signature YFKE, but it may also reflect a founder effect, due to the transfer of a single major variant of the inoculum to recipient RB. RA1 and RA2 provided us with a rare opportunity to compare HCV transmission in two subjects with the same source of infection. The difference in the number of T/F viruses between these recipients is consistent with the source/recipient-specific clades observed in a previous study of a common-source outbreak [21].

Genetic and phenotypic signatures associated with the transmission of true T/F E1E2 obtained by SGA were investigated, to the best of our knowledge, only in the previously cited study on experimental transmission of HCV in a chimeric mouse model [12]. This

study showed that the transmitted variants harbored key substitutions in E1E2 outside HVR1. By contrast, we found that three of the six signature amino acids (positions 394, 399 and 401) characterizing our T/F viruses were located in HVR1 (Figure 3). Importantly, the amino-acid substitution at position 394 (H394R or H394Y) was common to the T/F variants of all three transmission pairs studied. HVR1 plays a major role in both HCV cell entry and immune evasion [22-26]. Despite strong amino acid variability, the chemicophysical properties and conformation of HVR1 are well conserved and HVR1 is globally a basic stretch of amino acids, the most basic of which are found at positions 386, 394, 397 and 410 (corresponding to positions 3, 11, 14 and 27 in HVR1) in HCV genotype 1b [27,28]. The basic residues H and R are the most frequently observed at position 394, but this does not exclude the possibility of other amino acids, including the non basic Y, occupying this position. The presence of basic residues in HVR1 has been reported to facilitate virus entry [27]. We therefore thought that the substitution at residue 394 might lead to phenotypic changes affecting the replicative fitness of the virus during transmission or early in acute infection. None of the signature amino acids identified outside HVR1 (positions 445, 476 and 641) was known to participate in CD81 binding or a potential N-glycosylation site in HCV genotype 1b [29-31]. The residue 476 was previously described as a potential glycosylation sites in the genotype 1a reference strain H77. However, this site has a percentage of conservation below 20% in the genotype 1b and it was absent in the E1E2 sequences studied here [32].

In the chimeric mouse model study, the major posttransmission E1E2 variant with key substitutions outside HVR1 conferred a greater capacity for HCVpp entry [12]. A similar conclusion was drawn in a previous report on HCV evolution in a liver transplant setting, in which the uPA-SCID mouse model was used to support the hypothesis that viral entry is an important determinant of relative fitness in immunodeficient hosts [33]. However, we

found that the E1E2 sequences corresponding to the recipient RA1 T/F virus or the three main genetic lineages identified in the recipient RA2 quasispecies conferred similar levels of infectivity to those of most of the untransmitted variants (Figure 4A). These data suggest that the selective effect of residue H394 is not linked to an increase in entry capacity, at least in the HCVpp system. This observation was confirmed for the transmission pair DB/RB (Figure 4B). This discrepancy is not surprising, given the different settings encountered by HCV in chimeric mice and immunocompetent patients. In addition, the HCVpp entry assay generally used to generate viruses displaying E1E2 isolated from various sources is sensitive and well validated, but may not capture the full impact of the properties of E1E2 on natural HCV infection. The selection of T/F viruses was not influenced by the neutralizing antibodies present in donor serum. However, E1E2 variants may, conceivably, contribute to the selective process, through differential attenuation of the innate immune responses observed during the early phase of infection [34]. The E2 glycoprotein has been shown to interfere with IFN-stimulated genes and to induce functional changes in natural killer cells [35,36]. Furthermore, if assuming some similarity to HIV infection, in which transmission across intact mucosal barriers is invariably associated with a viral population bottleneck, T/F viruses replicate to higher titers than are observed in chronically infected controls, if propagated in the presence of IFN-α [20].

Finally, our findings have important implications for the development of strategies for preventing HCV transmission. Various cross-neutralizing anti-envelope glycoprotein mAbs neutralize genetically diverse HCV isolates, and some have been shown to protect against heterologous HCV quasispecies challenge in an HCV animal model [15,16,37-40]. Polyclonal anti-HCV antibodies isolated from chronically HCV-infected patients can also protect against *in vivo* challenge with different HCV genotypes [41,42]. We confirmed the

sensitivity of HCV primary isolates, represented by our T/F variants, to cross-reactive monoclonal or polyclonal Abs *in vitro*. Furthermore, our data indicate that the anti-E2 mAbs tested here display sufficient cross-reactivity to neutralize T/F variants derived from the three recipients. By combining antibodies, it may be possible to strengthen further the genetic barrier for resistance, in a viable and promising strategy for preventing HCV transmission.

Materials and Methods

Study participants

Three healthcare workers (recipients RA1, RA2 and RB), infected with HCV genotype 1b through documented needlestick exposure to blood from patients with chronic hepatitis (donors DA and DB), were enrolled in the study. All the subjects other than DB were female. Recipients RA1 and RA2 shared the same donor, DA. They received similar bitherapy with pegylated interferon and ribavirin, to achieve viral clearance. Recipient RB was contaminated by donor DB and displayed spontaneous viral clearance. This study was approved by the Institutional Review Board of Tours University Hospital (*Comité de Protection des Personnes* - CPP), and written informed consent was obtained, in accordance with French regulations.

HCV RNA and antibody assays

HCV antibody testing was performed with the qualitative Abbott Architect anti-HCV chemiluminescent microparticle immunoassay. Quantitative HCV RNA detection was performed with an Abbott HCV RealTime assay (lower limit of detection: 12 IU/ml for a sample volume of 0.5 ml).

Single-genome amplification and sequencing of HCV E1E2 envelope glycoprotein genes Full-length E1E2 sequences (encoding a region including the last 22 amino acids of core through the end of E2) were amplified by SGA from the plasma of donors and recipients. For each sample, viral RNA was extracted with the Qiagen Viral RNA Mini Kit (Qiagen). The extracted RNA was reverse-transcribed to generate cDNA, with the antisense primer ExtAS 5'-GAGCAGGAGCAGCGGCCAT-3' (nt 2720-2738, reference strain H77 [accession no. NC_004102]) and SuperScript III (Invitrogen). The cDNA was serially diluted and amplified in 96-multiwell plates, by nested-PCR of the full-length E1E2 sequence, so as to identify the dilution giving a maximum frequency of 3/10 PCR-positive reactions. At this dilution, most of the wells contain amplicons derived from a single cDNA molecule [11,13]. Nested-PCR was carried out with Platinum *Taq* High Fidelity polymerase (Invitrogen), as previously reported [11], with the following primers: 1st round sense primer ExtS 5'-CGGCGTGAACTATGCAACAGG-3' (nt 821-841 H77) and antisense primer ExtAS (see below), 2nd round sense primer IntS 5'-TCTGATGGGTTGCTCTTTCTCTATCTTCC-3' (nt 845-873 H77) and antisense primer IntAS 5'-AATCAGGCCTCAGCCTGGGCTATCAG-3' (nt 2559-2584 H77).

All products were directly sequenced with BigDye Terminator chemistry, using an ABI 3130 capillary sequencer (Applied Biosystems). Electrophoregrams were manually inspected and amplicons displaying mixed bases (double peaks), suggesting amplification from multiple templates or a *Taq* polymerase error, were excluded from further analysis.

Quasispecies analysis

Nucleotide sequences were aligned, using Clustal W in MEGA 5 [43], and the alignments were manually adjusted to ensure open reading frame maintenance. The overall phylogenetic relationships between sequences were analyzed by constructing a neighbor-joining tree by the Tamura three-parameter method. The number of viral variants amplified from each quasispecies and their genetic diversity (Hamming score) were determined at the nucleotide and amino-acid levels. The changes in quasispecies composition following transmission were investigated with the Highlighter tool (http://hcv.lanl.gov/content/sequence/HIGHLIGHT/highlighter_top.html). We checked for specific signature sequence variations with the viral epidemiology signature pattern analysis (VESPA) program (http://hcv.lanl.gov/content/sequence/VESPA/vespa.html) and default settings. [14]. Sequence logos were computed with WebLogo (http://weblogo.berkeley.edu/logo.cgi) [44].

Peudoparticle production, infection and neutralization assays

E1E2 SGA products selected for further phenotypic analyses were inserted into the pcDNA3.1 mammalian expression vector (Invitrogen). Lentiviral pseudoparticules were

generated by the cotransfection of 293-T cells with pNL4-3.Luc.R$^-$E$^-$ and expression vectors encoding either E1E2 glycoproteins derived from donors and recipients, vesicular stomatitis virus glycoprotein (VSV-G), UKN1B5.23 HCV envelope, or no-envelope (ΔE1E2) control, as previously reported [12,45,46]. Viral supernatants were collected 48 h later and purified by passage through a filter with 0.45-μm pores. E1E2 incorporation was assessed on sucrose cushion-purified HCVpp. We centrifuged 600 μl of supernatant through a 30% sucrose cushion (wt/vol) in TBS (Tris-buffered saline) at 124,740 × *g* for 2 h. Virus pellets were analyzed by western blotting with the 3/11 anti-E2 mAb [47] and with a specific rabbit antiserum against HIV-1 p24 (ARP432; Programme EVA Centre for AIDS Reagents).

For HCVpp infectivity assays, Huh7.5 cells were used to seed 96-well plates at a density of 5×10^3 cells per well, the day before assays. Viral infectivities were determined by infecting the Huh7.5 cells with serial five-fold dilutions of p24-normalized viral supernatants (Innotest HIV Antigen mAb kit, Innogenetics) in DMEM supplemented with FCS and antibiotics. Each experiment was performed in triplicate. The cells were harvested at 72 h postinfection, and relative light units (RLU) were measured within the cell lysates, with the luciferase assay system (Promega). RLUs were quantified with a Centro LB 960 luminometer (Berthold Technologies). The detection limit for positive luciferase reporter protein expression was 10 x 10^3 RLU/assay, corresponding to the mean ± 3 SD of background levels obtained with cells infected with ΔE1E2 pseudoparticles.

The antibody-mediated neutralization of HCVpp was assessed by determining the decrease in luciferase activity in Huh7.5 cells infected with HCVpp in the presence of human sera or neutralizing monoclonal antibodies. These experiments were restricted to E1E2 sequences conferring a high level of infectivity on HCVpp (signal-to-background

ratio >10), to minimize variability between assays and errors in the calculation of antibody titers attributable to background infectivity. HCVpp were mixed with dilutions of donor sera, genotype-specific serum pools (consisting of pools of three sera containing antibodies specific for HCV genotype 1a, 1b, 2a, or 3a), a control serum (consisting of a pool of three anti-HCV antibody-negative sera), anti-E2 mAbs AR3A, anti-E2 mAb HC-11, or the irrelevant isotype control, IgG R04 [15,16]. The mixtures were incubated for 1 h at 37°C and added to Huh7.5 cells used to seed 96-well plates at a density of 5×10^3 cells per well the day before the assay. Each experiment was performed in triplicate. The cells were harvested 72 h postinfection, and RLU were quantified for the cell lysates, as described above. For each dilution, the percentage of neutralization was calculated as 100 - [100 × (infectivity of HCVpps in the presence of serum or mAb/infectivity of HCVpps in the presence of anti-HCV–negative control sera or irrelevant isotype control IgG)] [33]. The neutralization titer of the sera was defined as the last dilution resulting in a decrease in HCVpp infectivity of at least 50% compared with an equivalent dilution of control serum. The antibody concentrations decreasing HCVpp infectivity by at least 50% were assessed by testing mAbs at 20, 2, 0.2 or 0.02 µg/ml.

Supporting information

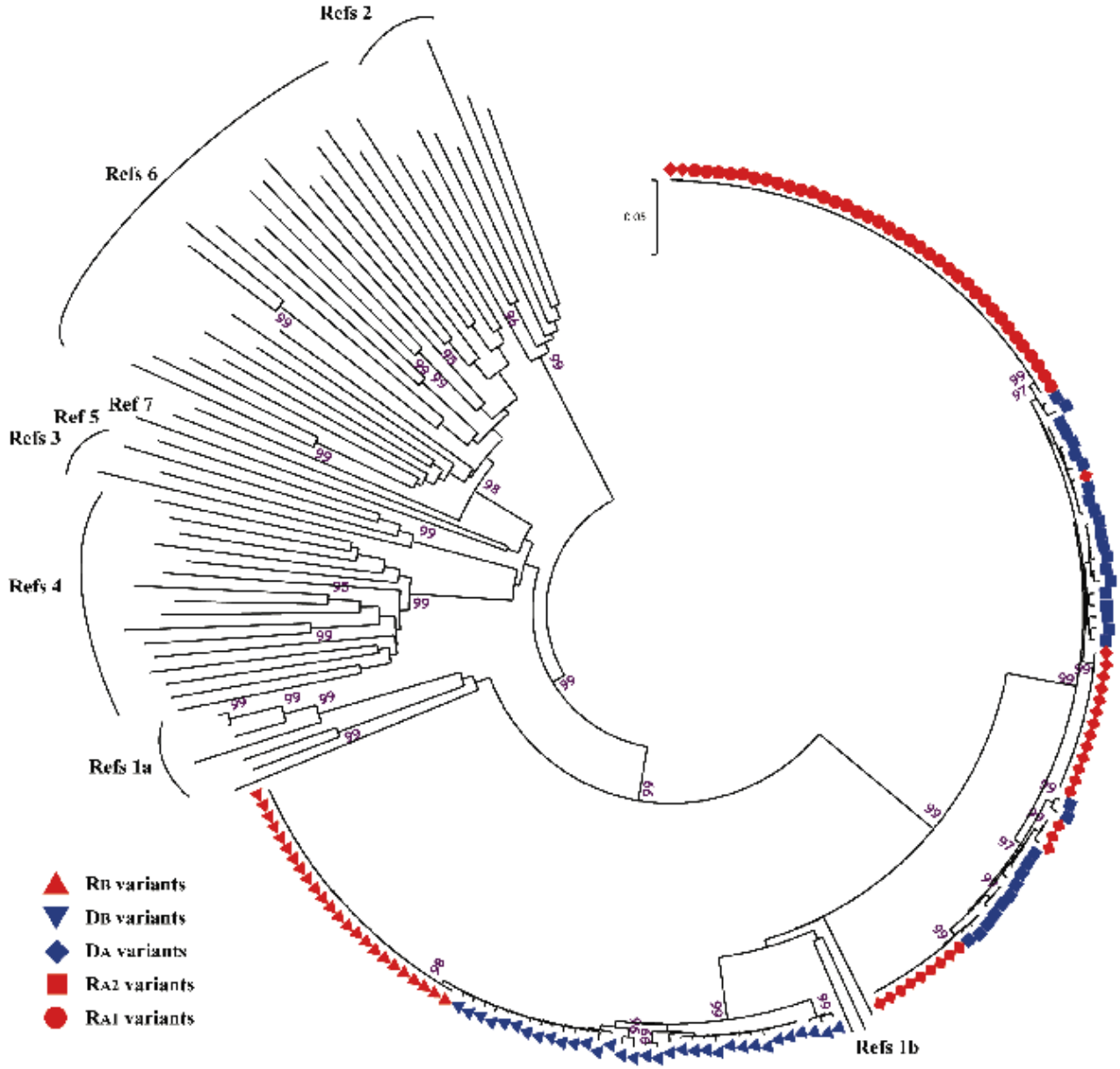

Figure S1. Combined phylogenetic analysis of donor and recipient quasispecies. A neighbor-joining tree constructed with all E1E2 nucleotide sequences available from donor DA (blue square), recipient RA1 (red circle), recipient RA2 (red diamond), donor DB (blue triangle) and recipient RB (red triangle) along with HCV genotype 1 to 7 reference sequences (Los Alamos National Laboratories HCV database) is shown. Only bootstrap values over 95 are presented. The scale bar corresponds to 0.05 nucleotide substitutions per site.

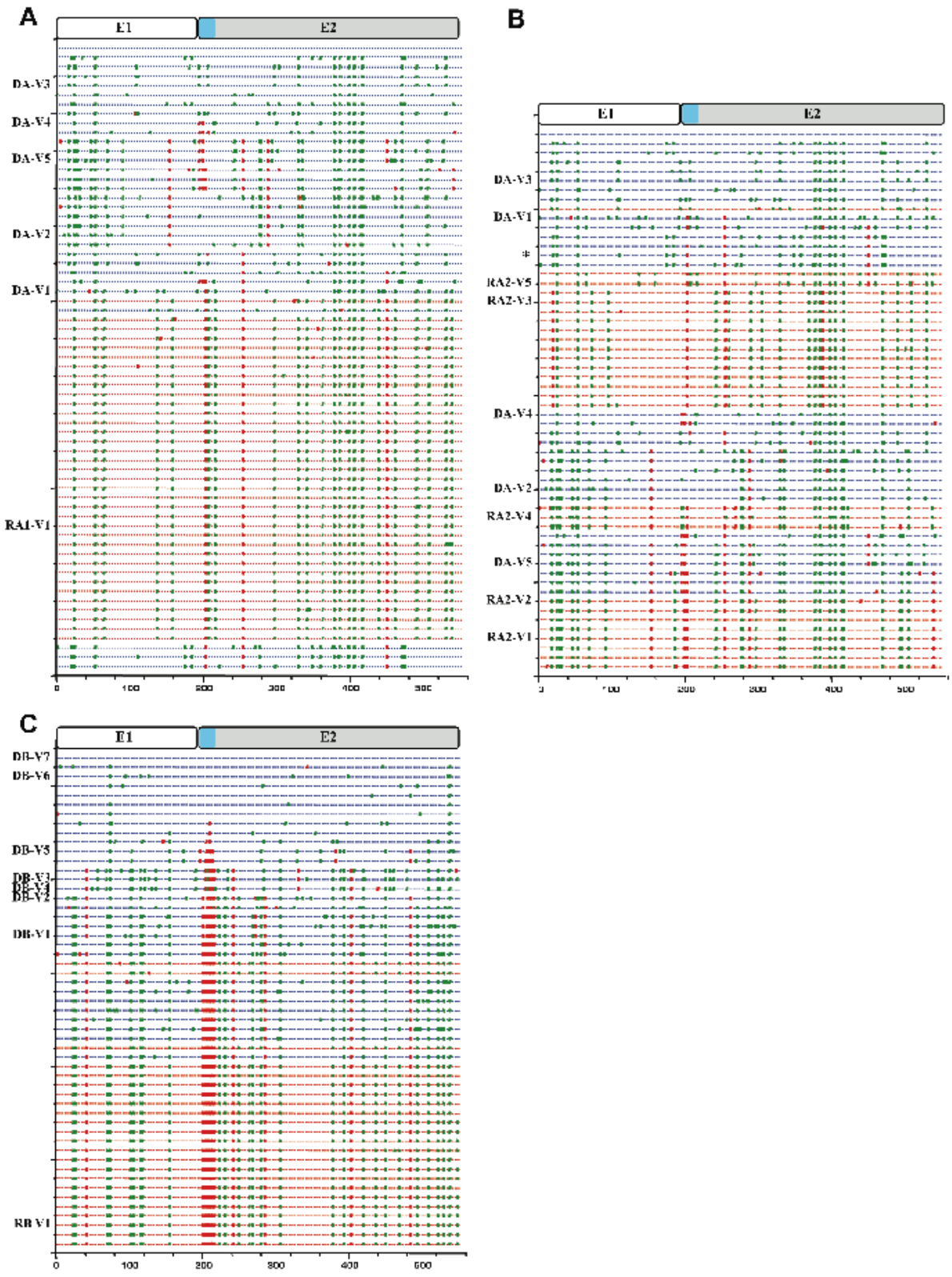

Figure S2. Highlighter plots of E1E2 nucleotide sequences from recipient RA1 (A), RA2 (B) and RB (C) (red) with pretransmission donor sequences included (blue). The sequences are presented in the same order as in Fig. 2. Nucleotide polymorphisms are indicated by a colored tick mark (green bars: synonymous substitutions, red bars: non synonymous substitutions). A schematic diagram of the E1E2 sequence, showing the location of hypervariable region 1 (HVR1) of E2 in light blue, is provided above the Highlighter plots.

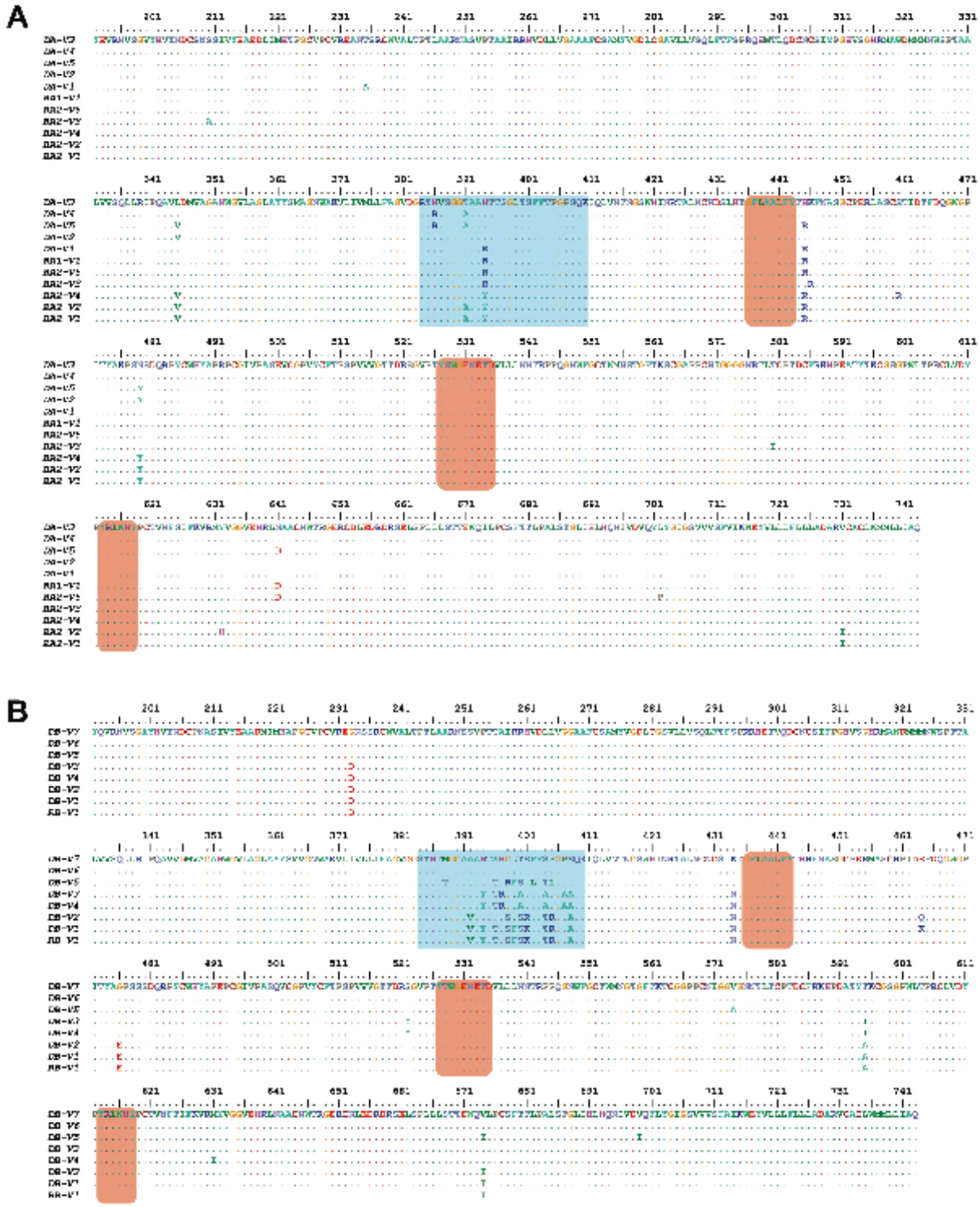

Figure S3. Comparative alignment of amino-acid sequences of E1E2 envelope glycoprotein variants selected for phenotypic analyses. (A) Amino-acid sequences of E1E2 variants derived from donor DA and recipients RA1/RA2. (B) Amino-acid sequences of E1E2 variants derived from donor DB and recipient RB. The sequences are presented in the same order as in Fig. 2. Dots indicate amino-acid identity. Mutations are indicated in the amino acid one-letter code. Amino-acid sequences encompassing HVR1 (highlighted in blue) and CD81 binding domains (highlighted in red) are highlighted [29-31].

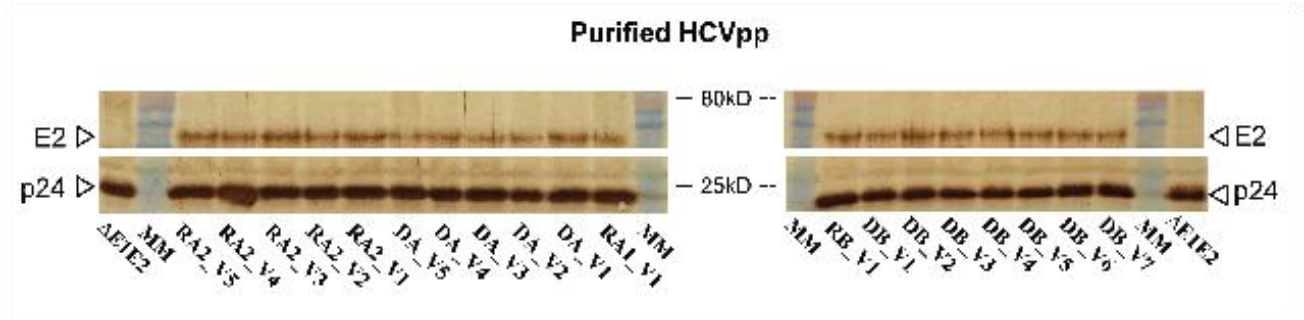

Figure S4. Western blot analysis of envelope glycoprotein incorporation into HCVpp produced by 293T cells. The upper panel shows the blot of E2 glycoproteins derived from the variants selected for phenotypic analyses. These E2 glycoproteins were detected with the mAb 3/11 (see Materials and Methods). The blot was stripped and reprobed with a specific rabbit antiserum against HIV-1 p24 (lower panel). The positions of E2 and p24 are shown on the side of the panels. MM, molecular markers.

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Author Contributions

Conceived and designed the experiments: VDA AM VG EB FD PR DB. Performed the experiments: VDA AM EB ER. Analyzed the data: VDA AM EB LDA VG CGG BG JCP

AG DB. Contributed reagents/materials/analysis tools: VG LDA CGG BG FD JCP AG. Wrote the paper: VDA AM EB PR DB.

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Figure legends

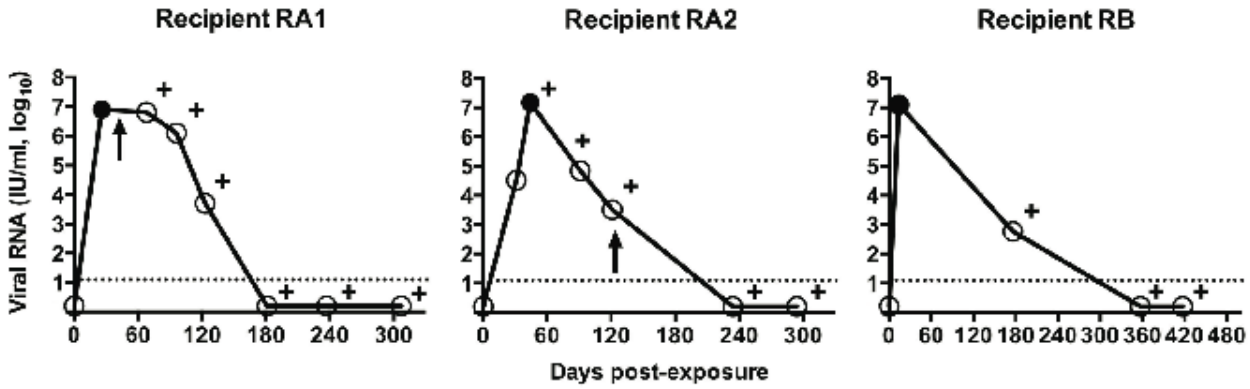

Figure 1. Virological course of recipients RA1, RA2 and RB after needlestick exposure to infected blood. Viremia was monitored after the needlestick injury, by sequential plasma samples, repeated until the virus could no longer be detected. The solid line with circled values corresponds to plasma viral RNA quantified with the Abbott HCV RealTime assay (see Materials and Methods). The dotted line indicates the lower limit of detection. The black dots correspond to the recipient viremic time point analyzed by SGA and plus signs denote positivity for anti-HCV antibody. An arrow indicates the start of treatment for subjects RA1 and RA2.

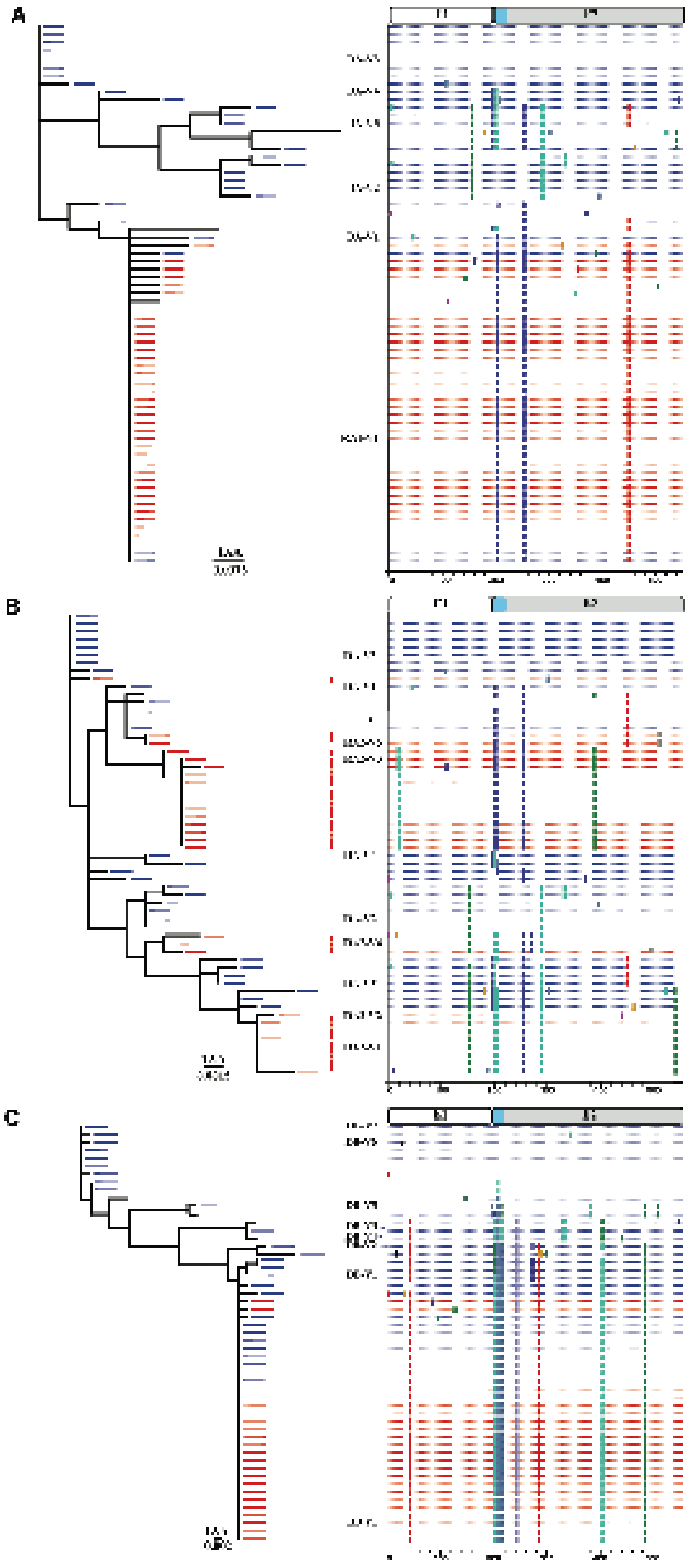

Figure 2. Phylogenetic relationships and patterns of substitution in HCV E1E2 sequences between the transmission pair quasispecies. E1E2 amino-acid sequences derived from recipient RA1 **(A)**, RA2 **(B)** and RB **(C)** (red) were analyzed by neighbor-joining trees (left panels) and Highlighter plots (right panels), with pretransmission donor sequences included (blue). Polymorphisms are indicated by a colored tick mark specific for each amino acid, according to the color scheme of BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A schematic diagram of the E1E2 proteins, showing the location of HVR1 of E2 in light blue, is provided above the Highlighter plots. The scale bar is proportional to the genetic distance and represents 0.0015 (in A and B) or 0.002 (in C) amino-acid substitutions per site. The E1E2 sequences identified on the left side of the Highlighter plots were selected for further phenotypic analyses. The sequence indicated by an asterisk in the Highlighter plots B corresponds to the transmitted variant V1 of recipient RA1.

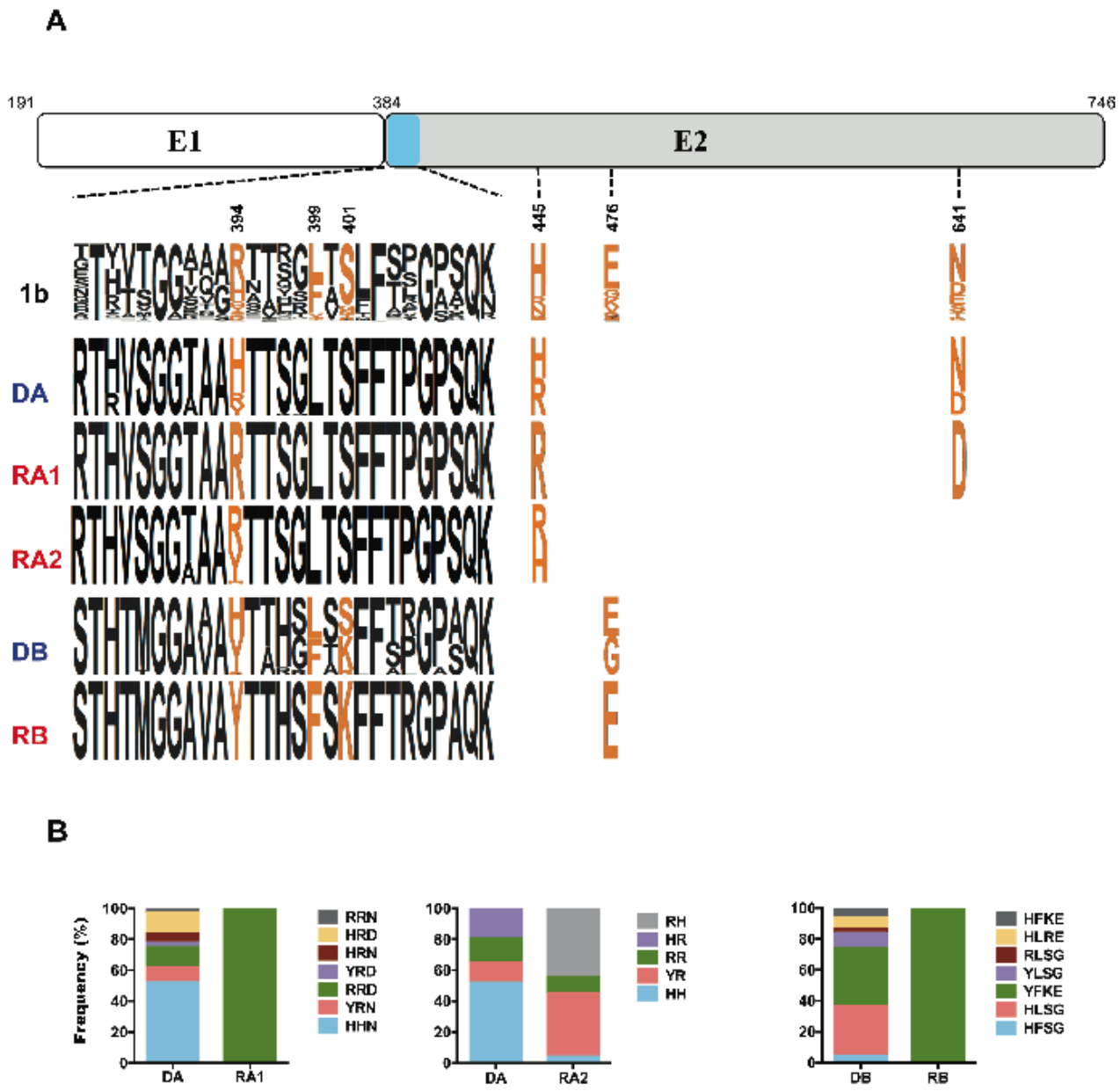

Figure 3. Identification of the key residues of the E1E2 envelope glycoprotein characterizing the recipient quasispecies. (A) Sequence logo depiction of signature amino acids specific to the transmitted E1E2 variants. A schematic representation of the E1E2 proteins, showing the location of the hypervariable region 1 (light blue), is provided at the top. The positions of the signature amino acids, identified with VESPA[14], in the 3 transmission pairs, are indicated below the schematic representation of E1E2 proteins. The numbers indicate positions relative to the H77 polyprotein [accession no. NC_004102]. The 1b reference sequence logo (top row) was obtained with an alignment of 340 full-length HCV subtype 1b sequences from different sources (Los Alamos National Laboratories HCV database). Sequence logos indicating the variability of each amino acid in sequences derived from donors and recipients, are shown below[44]. The height of each single-letter amino acid code is proportional to the representation of that amino acid at the position concerned. **(B)** Frequencies of key residue combinations circulating within donors and recipients in each transmission pair.

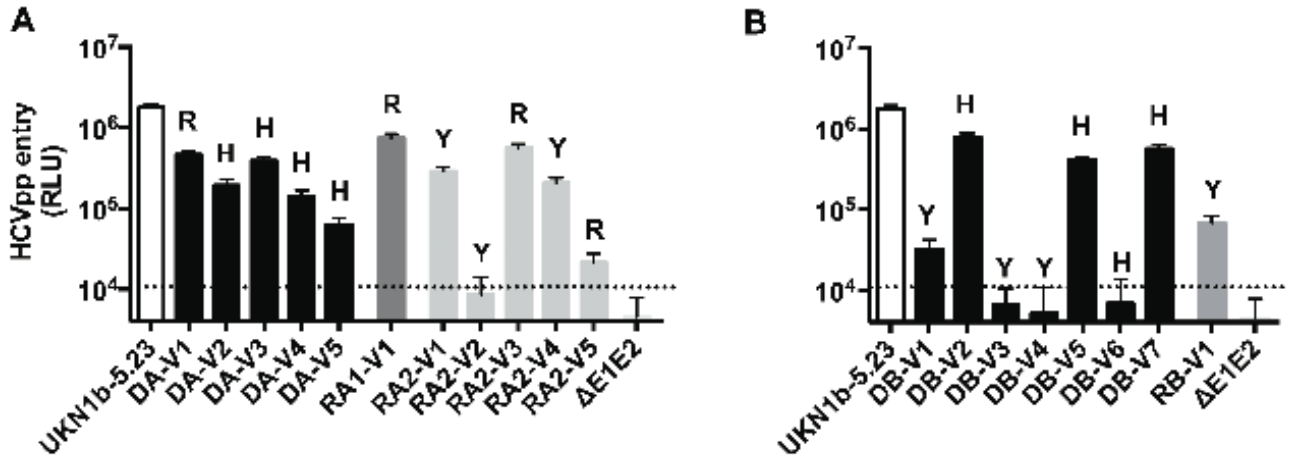

Figure 4. Infectivities of HCVpp bearing donor and recipient E1E2 envelope glycoproteins. The infectivity of HCVpp conferred by major or minor non transmitted E1E2 variants was compared with that conferred by the variants transmitted in the transmission pairs DA/RA1 or DA/RA2 **(A)** and DB/RB **(B)**. Infection assays with the luciferase reporter gene were performed with target Huh7.5 cells. Similar amounts of viral particles were used in each experiment. Results are expressed in relative light units (RLU) plotted on a logarithmic scale. The UKN1B5.23 envelope was used as an external reference. The dotted line indicates the threshold for detectable infection in this system. The detection limit for positive luciferase reporter protein expression was 10 x 10^3 RLU/assay, corresponding to the mean ± 3 SD of the background levels obtained with cells infected with ΔE1E2 pseudoparticles. Means ± SD from four independent experiments (performed in triplicate) are shown. The letter above each bar indicates the amino acid at position 394 of each E1E2 variant.

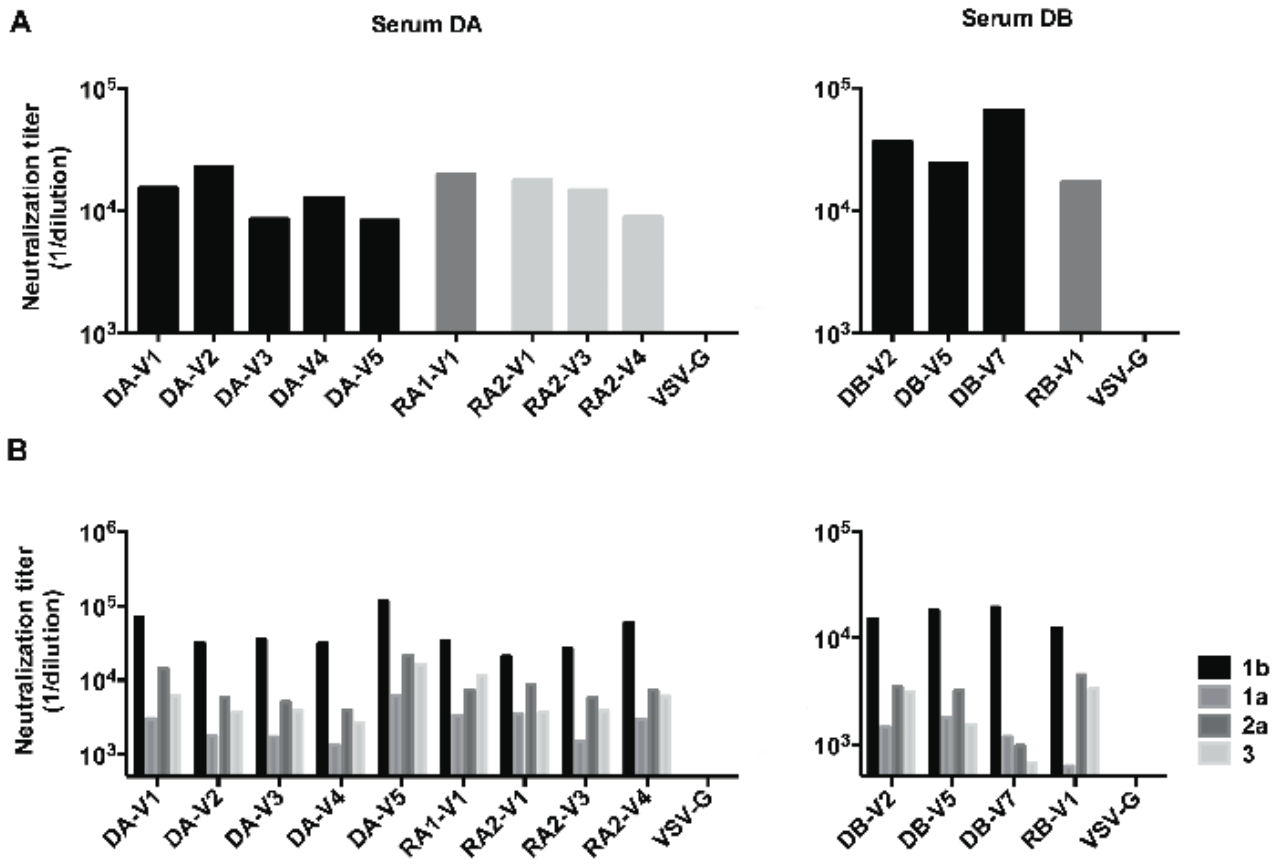

Figure 5. Sensitivity of HCVpp bearing donor and recipient E1E2 envelope glycoproteins to neutralization by donor sera (matched by transmission pair) or heterologous pools of sera. HCVpps were incubated with donor serum (**A**) or genotype-specific (1a, 1b, 2a, 3a) serum pools (**B**), in serial dilutions, for 1 h at 37°C. HCVpp–antibody complexes were then added to Huh7.5 cells, and infection assays were performed with the luciferase reporter gene. Neutralization titers were calculated as described in the Materials and Methods. End-point dilution titers are indicated for each E1E2 variant derived from a donor (black) or recipient (gray). Means ± SD from two independent experiments (performed in triplicate) are shown.

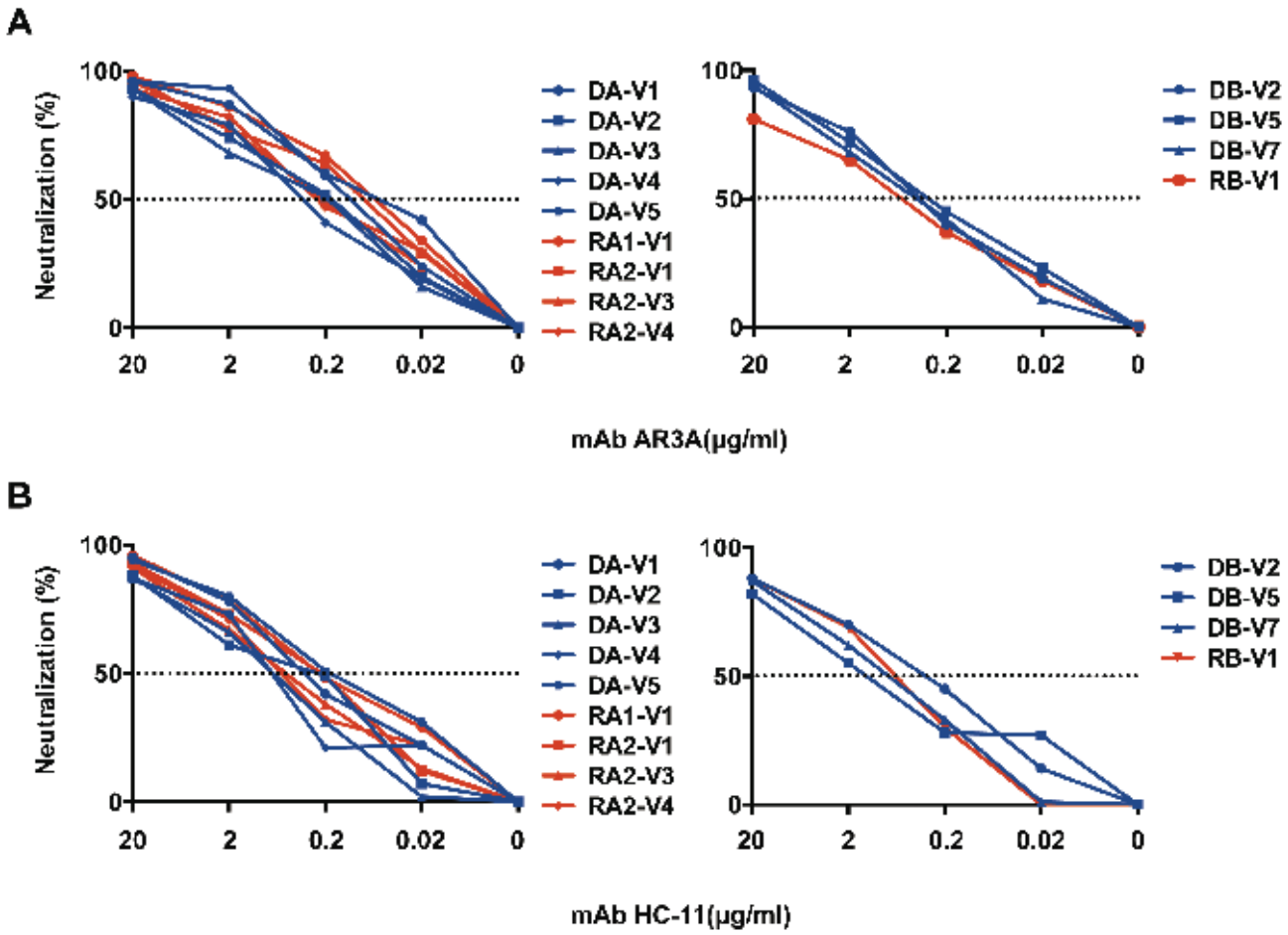

Figure 6. Sensitivity of HCVpp bearing donor and recipient E1E2 envelope glycoproteins to neutralization with neutralizing mAb. HCVpp neutralization sensitivities were assessed with the human anti-E2 neutralizing mAbs AR3A **(A)** and HC-11 **(B)**. The percentage of neutralization was calculated as described in the Materials and Methods. Neutralization curves are shown for each E1E2-derived variant from donors (blue) or recipients (red). The data shown are mean values from 1 representative experiment performed in triplicate. A dashed line indicates 50% neutralization of HCVpp entry.

Table 1: Composition and diversity of the HCV E1E2 sequences derived by SGA from donor and recipient viral quasispecies

	Donor DA		Recipient RA1		Recipient RA2		Donor DB		Recipient RB	
	nt.	aa.	nt.	aa.	nt.	aa.	nt.	aa.	nt.	aa.
Total number of E1E2 SGA sequences analyzed*	33	31	37	36	28	27	33	30	24	23
Number of viral variants	33	20	16	8	14	11	33	19	7	3
Mean genetic diversity (%) (Hamming score)	1.57	0.819	0.05	0.081	1.49	1.012	1.94	1.859	0.05	0.032

Abbreviations: nt, nucleotides; aa, amino acids.

*The differences between the number of nucleotide and amino-acid sequences result from the occurrence of stop codons or deletions altering the open reading frame.

Valentina D'ARIENZO

CARACTÉRISATION DES GLYCOPROTÉINES D'ENVELOPPE DES VARIANTS VIRAUX IMPLIQUÉS DANS LA TRANSMISSION DU VIRUS DE L'HÉPATITE C

Résumé

Les variants viraux impliqués dans la transmission du VHC ont rarement été étudiés en raison des difficultés rencontrées pour recruter des patients au stade de la primo-infection. Pour mener cette étude, nous avons analysé le goulot d'étranglement génétique subit par les quasi-espèces virales au cours de 3 accidents d'exposition au sang impliquant des représentants du personnel soignant contaminés par piqûre d'aiguille. En utilisant la technique d'amplification de génomes uniques nous avons obtenu les gènes codant les glycoprotéines d'enveloppe virales E1E2 des variants viraux présents dans ces quasi-espèces. Ces gènes ont été séquencés et soumis à une analyse phylogénétique. Nous avons ensuite pu étudier les propriétés phénotypiques des glycoprotéines d'enveloppe dérivées de variants qui apparaissent au stade très précoce de l'infection. Pendant cette période, le VHC pourrait être plus vulnérable à l'élimination par des vaccins préventifs ou par des immunothérapies.

Mots clés : *VHC, transmission sélective, quasi-espèces, SGA, piqûre d'aiguille, glycoprotéines d'enveloppe.*

Résumé en anglais

Little is known about the transmitted variants responsible for the spread of HCV infection, principally because of the difficulties to recruit patients early enough in infection. To address this issue, we proposed to track the genetic bottleneck event in HCV quasispecies, leading to productive clinical infection in three health care workers accidentally contaminated through needlestick accidents. By using a single genome amplification (SGA) approach we identified genes coding the viral envelope glycoprotein E1E2 which composed these quasispecies. The E1E2 sequences were then directly sequenced and subjected to a phylogenetic analysis. By cloning these full-length E1E2 sequences, we investigated the phenotypic properties of the envelope glycoproteins potentially involved in selective HCV transmission and early stage of infection, a period during which the virus might be most vulnerable to elimination by preventive vaccines or immunotherapies.

Key words: *selective transmission, HCV quasispecies, SGA, needlestick accident, vaccine.*