Review
Future Virology

Clinical relevance of genetic heterogeneity in HCV

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Infection by HCV affects an estimated 170 million people worldwide and it represents one of the major causes of liver transplantation and a heavy burden to healthcare systems. As with many other RNA viruses, HCV is characterized by very high levels of genetic variation, which have been associated to differences in disease progression and efficiency of antiviral treatment. Studies show many contradictory results and little consensus on such associations. Nevertheless, some general guidelines translating research results to clinical practice have been postulated. Here, we review the main research results obtained on HCV variation so far and explore the reasons for their lack of congruence under a population genetics framework. Understanding the factors responsible for the variable dynamics of HCV diversity in human populations and variation within infected individuals is even more necessary in face of the soon-to-arrive new HCV therapies.

HCV is a positive ssRNA virus representative of the genus *Hepacivirus* in the *Flaviviridae* family. It is estimated that HCV infects over 170 million people worldwide [1,2], many of whom are unaware of anti-HCV positivity. The prevalence of anti-HCV antibodies varies widely among and even within countries, reaching up to 18% of the total population in Egypt, where anti-HCV positivity can rise to 55% in specific populations and age groups [3]. The main replication site of HCV is the liver, and most acute infections evolve to chronicity [2,4]. A significant proportion of chronically infected patients will develop the most severe consequences of the infection, such as cirrhosis, end-stage liver disease, hepatocellular carcinoma and liver failure [4]. Once liver function is compromised, the only reliable therapeutic intervention is liver transplantation. Unfortunately, a recurrent chronic infection is almost invariably early established in the new liver, and the progression of HCV disease post-transplantation is accelerated, as compared with the nontransplanted patient [5].

The HCV genome comprises approximately 9600 nucleotides, coding for a single polyprotein (of ~3000 amino acids) that is cleaved after translation by cellular and viral proteases into ten peptide chains. Three peptide chains are structural proteins, one core and two envelope glycoproteins (E1 and E2), and one protein between the structural and nonstructural parts is an ion channel (p7), while six are nonstructural proteins (NS2–NS5B) [6]. In addition, both flanking portions of the genome (5′ and 3′ untranslated regions [UTRs]) are functionally relevant at the level of viral replication and polypeptide translation (**Figure 1**).

Similar to other RNA viruses, one prominent feature of HCV is its enormous genetic variability. The lack of proofreading activity of the HCV RNA-dependent RNA polymerase (NS5B) is responsible for a viral replication process that leads to an increased mutation rate that is several orders of magnitude higher than that of DNA-based organisms. Short generation times and large population sizes during the infection process contribute to the maintenance of the newly generated genetic diversity, thus increasing the opportunities for the action of natural selection driving the evolution of the viral population in order to adapt to new environmental conditions.

A detailed appreciation of the consequences of genetic variation in HCV is essential for understanding many aspects of the infection, epidemiology and evolution of this virus. This scope is important for designing antiviral drugs, developing vaccines, understanding and, hopefully, anticipating responses to treatment, disease outcome and progression of chronic disease. However, it is also essential to consider that the infection occurs in individuals who are genetically and phenotypically different, with a wide range of variation and an as yet insufficiently explored space of possible interactions between the host and the virus. Hence, it is not surprising to find very few strong generalizations, with universal validity, in the results of processes involving HCV infection. This article is aimed at providing a theoretical and factual overview of how the continuous generation

Keywords
genetic variability ■ HCV genotypes ■ HCV subtypes ■ hypervariable regions ■ immune response ■ interferon ■ nonstructural regions ■ positive selection ■ purifying selection ■ ribavirin ■ STAT-C

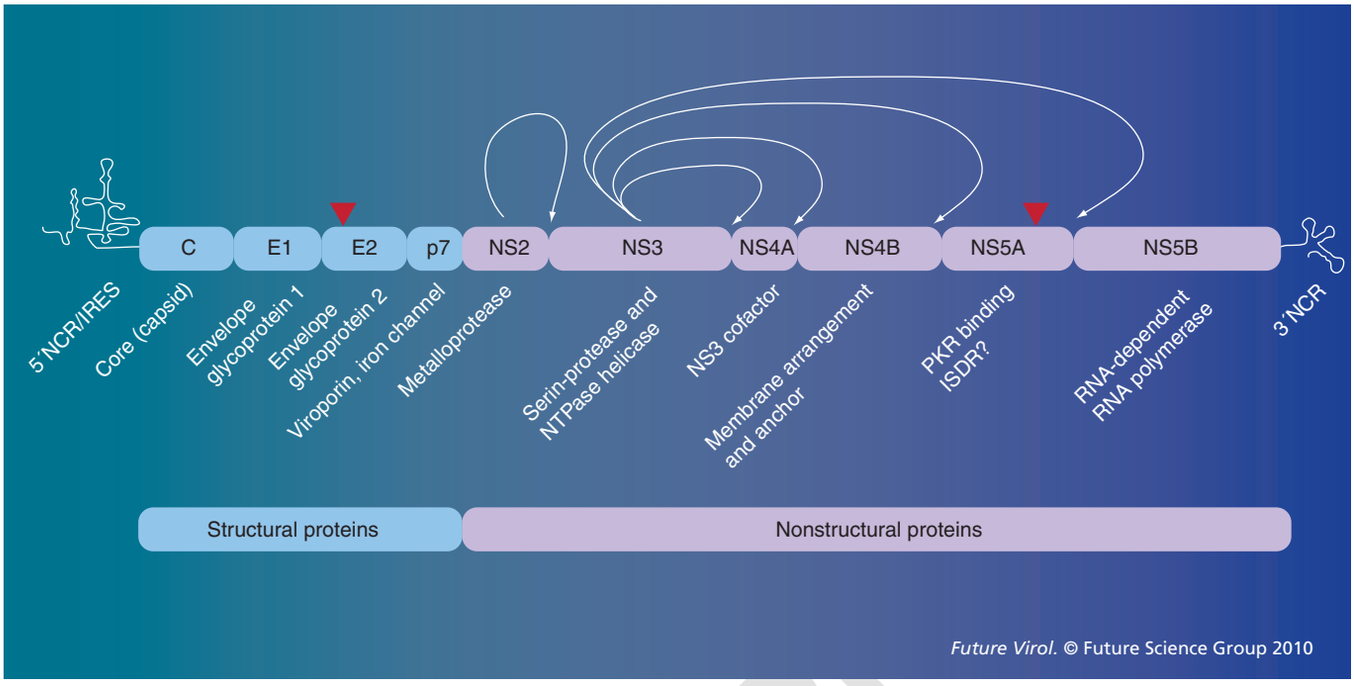

Figure 1. HCV genes and the viral polyprotein, with structural (blue) and nonstructural (purple) proteins. With the exception of the two NCRs in the 5′ and 3′ ends of the viral genome, the viral RNA is transcribed into a polyprotein of approximately 3000 amino acids. Both host and viral peptidases cleave the polyprotein. The NS2 protease cleaves the polyprotein in the NS2–NS3 junction, whereas the NS3-codified serine-protease domain cleaves the rest of the viral polyprotein at the NS3–NS4A, NS4A–NS4B, NS4B–NS5A and NS5A–NS5B junctions. Triangles indicate highly variable regions: the hypervariable regions [1–3] in *E2* envelope gene and the V3 region of NS5A.
IRES: Internal ribosome entry site; ISDR: Interferon sensitivity determining region; NCR: Noncoding region.

of new genetic variation in the HCV genome is fundamental to understand this life-threatening infection and to devise effective prophylactic and therapeutic interventions.

Dynamics of variation

Genetic variability in a population does not inevitably increase with time, since it is the result of factors acting in opposite directions: some processes introduce new variation in the populations while others remove it (Figure 2). Two main processes deplete variation from viral populations: selection and drift. By increasing the proportion of viral particles that carry particular high-fitness variants, selection may transitorily reduce genetic variability in populations, while the effect of drift is continuous and affects all variants equally in the population, regardless of their effect on the viral fitness.

There are three main forms of action of natural selection at the molecular level. First, selection can act to purge new variants arising in the population, diminishing some fitness-related properties, such as replication capacity or encapsidation efficiency. This form is known as purifying or negative selection and it has a moderate effect in reducing genetic variability, because it will mainly eliminate the negative variants, and those associated with them in the same genomic molecule. Second, the opposite form of action is known as positive selection: it favors a new mutant that rises in frequency at the expense of other, less fit variants, leading to a temporary reduction of genetic variation in the population. This reduction is not at random; only those variants that incorporate the selected mutations will increase in frequency until they become fixed, an effect that will extend to the other variants linked to the advantageous mutation (a process usually known as hitchhiking selection). Finally, the third form of action of natural selection at the molecular level actually contributes to and promotes increased levels of genetic variation. This is known as diversifying selection and corresponds to those cases in which the virus directly benefits from generating new variation at particular genome sites, for instance, in the epitopes presented to MHC class I molecules [7].

Genetic drift is the result of the sampling process that occurs in every population in which the total number of individuals is limited. This limit can be very high (billions or trillions, in the case of viral populations) or very low, for example, when a cell is infected by a single viral particle. In the

former case, the reduction in genetic variability is almost imperceptible and it is easily compensated by the continuous generation of new genetic variation. By contrast, extreme reductions in population size, especially during the transmission from one infected host to a new one (or after liver transplantation), result in a drastic elimination of genetic variability after which only a few of the initially present variants are represented in the newly established population (Figure 3). In this case, the variants that originate from the new population are drawn at random from those initially present, and the particular variants transmitted are not necessarily associated with increased fitness; they can be more, equally or less fit than those of the average population they derive from.

Although usually overlooked, if not ignored, in the study of genetic variation in viruses, the neutral theory of molecular evolution sustains that most variation found at the molecular level does not have an impact on viral fitness and, as a consequence, is neutral in terms of natural selection [8,9]. The original proposal was subsequently expanded by incorporating the evolutionary consequences of slightly deleterious mutations whose fate does not depend exclusively on the relative reduction in fitness they produce, but on the size of the population where they arise [10,11]. Stochastic processes, usually associated with genetic drift, will dominate the fate of these mutations if effective population size is lower than the reciprocal of the corresponding selection coefficients. When population sizes or selection coefficients are larger and the above inequality no longer holds, then deterministic processes will dominate and selection will be the main evolutionary force in the population. Given the large population sizes associated with viruses, it is usually considered that genetic drift is not as important as selection in determining evolutionary change in viral populations. However, this is not the case during transmission or during chronic infection. Effective sizes for HIV intrapatient populations have been estimated to be approximately 10^3 [12,13]. These values imply that stochastic factors play an important role in the evolution of viral populations at this level. One additional, often overlooked, aspect of the quasineutral theory is that it also applies to slightly favorable mutations. Whilst some mutations may confer increased fitness, their dynamics (stochastic or deterministic) will be determined by the relationship between the effective population size and the selection coefficient; a slightly advantageous mutation may easily disappear from a small population while it will likely increase in frequency in a large one.

Generation of variation

Mutation is the ultimate source of generic variation in every organism. However, there are other processes that can affect the levels of variation found in natural populations. Genetic drift and natural selection usually act to diminish genetic variability whereas migration and recombination usually increase it, and will substantially affect the rate of substitution – the rate at which a new variant is fixed (or nearly so) – in a viral population. ssRNA viruses, such as HCV, are characterized by very high mutation rates, usually estimated in the range of 10^{-13}–10^{-14} mutations per site and replication [14]. This is the result of the lack of proofreading capacity of RNA-dependent RNA polymerases. Using a new approach, Cuevas *et al.* have recently estimated a spontaneous mutation rate of HCV to be $1.15 \pm 0.29 \times 10^{-14}$ per site, within the usual range for ssRNA viruses [14–17]. Given the rate of generation of new HCV particles during chronic infection, with a turnover rate of approximately 10^{12} virions per day [18], the mutation rate is high enough to potentially generate every single and most double mutants in the viral genome every single day. Nevertheless, functional constrains may limit the amount of variation actually accessible to RNA viruses, such as HCV. RNA genomes form secondary structures with important functional roles in the virus lifecycle, such as the internal ribosomal entry sites involved in protein translation. Furthermore, recent data based on thermodynamic prediction of RNA secondary structure [19,20] and on hybridization accessibility and atomic-force microscopic imaging of RNA transcripts [20] have revealed extensive genome-scaled ordered RNA structures in several RNA viruses, including HCV. This may be an evolutionarily conserved feature

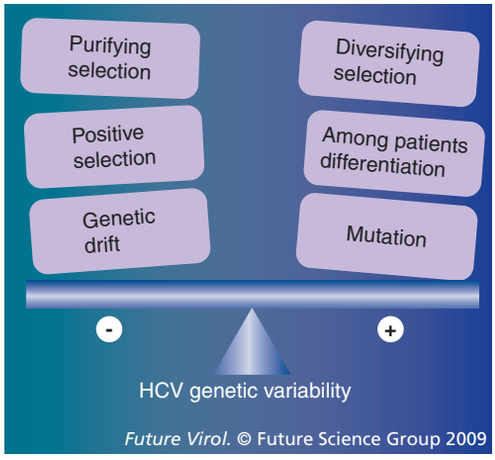

Figure 2. Balance of forces acting on HCV genetic variability.

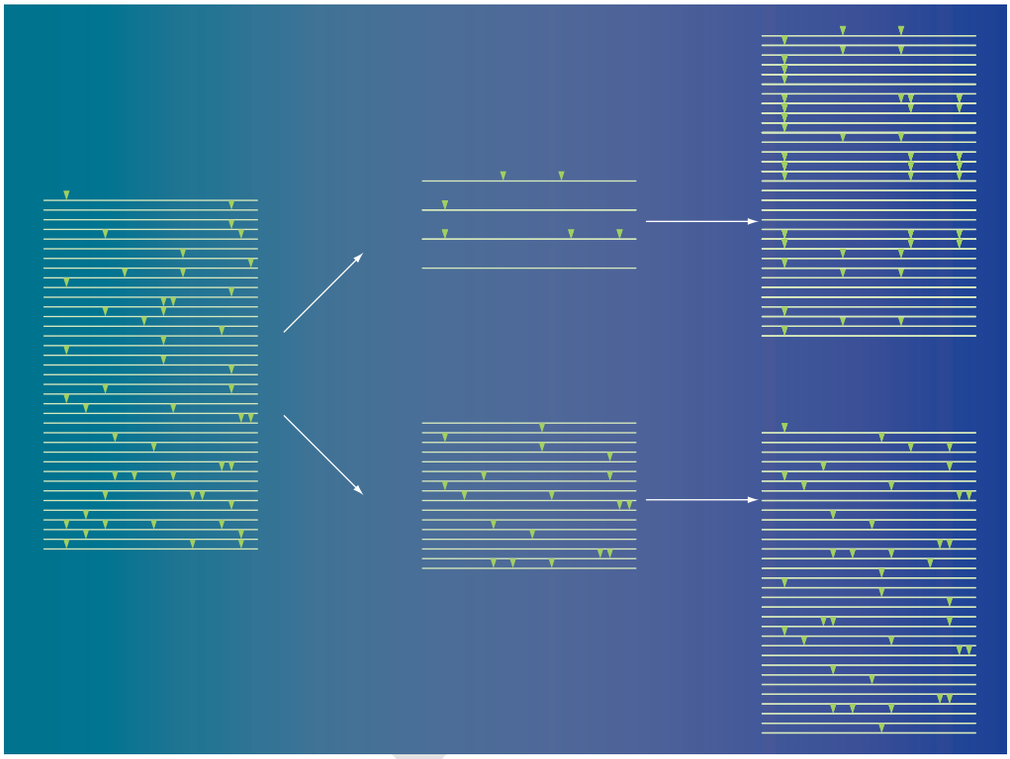

Figure 3. Effects of genetic drift on HCV genetic variability. Genetic drift acts in every population with a finite population size, but its effects are more intense the smaller the number of individuals giving rise to the next generation. Its effects are purely stochastic and it may help or counter those of selective forces acting simultaneously on the same population.

because the presence of genome-scaled ordered RNA structures is invariably associated with the capacity of ssRNA viruses to establish a persisting infection. Therefore, nucleotide positions involved in such secondary structures may be less permissive to variation.

An alternative mechanism generating genetic variation in most RNA viruses is recombination. Until recently, evidence of HCV recombination between different genotypes/subtypes has been scarce [21], suggesting that these events are rare *in vivo* and that the resulting recombinants are usually not viable [22–24]. In the last few years, a few natural intergenotypic recombinants of HCV have been identified (RF1_2k/1b, RF2_1a/1b and RF3_2b/1b) with crossover points mapped to the *NS2*, *NS5B* and *NS3* regions, respectively [25–29]. A recent study involving more than 17,000 viral sequences from 111 patients has detected a relatively low frequency of intra-subtype recombinant sequences in samples from patients with a variety of clinical situations and outcomes, thus reinforcing the idea that HCV recombinants are usually not advantageous [30]. The difficulties for detecting recombinant strains of HCV [30] along with the relative low incidence of dual infections (with different genotypes or subtypes) [24] may explain the little attention that recombination has received, and possibly deserves, as a factor generating genetic variation in HCV, despite the fact that at least one of these recombinant strains has started to spread [31].

Quantification of variation

Genetic variation can be described and quantified at different levels and with different purposes. In general, only appropriate statistical measures of variability are credible for making scientifically valid comparisons and deriving consistent conclusions. In certain contexts, the absolute numbers of variants present in a sample or population may be informative, but this is very unlikely to provide a valid parameter to compare different samples or populations, since these simple variables are highly dependent on sample or population sizes. This caveat can be solved easily by computing relative measures of variability, such as entropy (complexity) or genetic diversity. Both can be applied from the most fundamental unit of comparison, a specific nucleotide position, to the most encompassing one, the complete genome, and they are derived from the relative frequencies of the different alternatives for the corresponding unit. Whilst

the former is derived from information theory and it is very popular in ecology and physical systems, the latter is more closely connected to population genetics. Nevertheless, both measures consider only the frequencies of the different variants and do not incorporate any information on the nature or extent of the differences among them, and these are critical factors for making inferences about evolutionary processes.

Nucleotide diversity (π) is the most commonly used parameter to summarize and compare genetic variability in population genetics. It incorporates information on the frequency of the variants and their relatedness, and can be customized to include only certain types of variants (e.g., synonymous or nonsynonymous). This is one of the most important parameters in molecular population genetics theory because it is closely related to θ, the population mutation rate, which determines the levels of genetic variability or the expected frequencies of neutral variants in a population at mutation–drift equilibrium.

One of the advantages of using well-founded statistical parameters to quantify genetic variability is that they can be used to partition and compare the contribution of different levels of biological organization to the total genetic variation observed in a population. For instance, it is possible to assign the relative contributions of viral variation within and between individuals to the total variation in a population of patients, or within and between cells to the total variation in an individual patient. This is achieved under an analysis of variance framework [32], which was extended by Excoffier *et al.* to account for genetic differences [33]. This method is known as analysis of molecular variance.

Levels of variation in HCV

The different levels of HCV variation, specifically, between genotypes, subtypes or patient isolates (polymorphism and genetic diversity) versus within individuals (genetic complexity), is an important distinction that should be made when designing, performing and interpreting research results. Unfortunately, very often, genetic diversity and complexity variables are considered at the same level while they provide information on quite different properties of viral variation.

Between patients

HCV is currently classified, on the basis of genetic differences and phylogenetic analysis, into six major genotypes and a few dozen subtypes [34]. Differences between genotypes at the nucleotide level are in the 30–35% range whereas those between subtypes within the same genotype are between 20 and 25%. These differences are relatively constant along the complete genome and allowed the classification of HCV variants from the analysis of different subgenomic regions, with core/E1 and NS5B the most commonly used. Nevertheless, some strongly conserved regions, in the *Core* gene for instance, may not harbor enough variation to reliably discriminate between relatively similar subtypes (e.g., 1a and 1b) if only a few prototype reference sequences are used for each subtype [González-Candelas F, Unpublished Data]. Assignment to subtype or genotype on the basis of the basic local alignment search tool (BLAST) searches with partial genome sequences can be misleading and an appropriate phylogenetic analysis is always advisable. There are already a few servers implementing this kind of analyses over the internet [201].

The distribution of HCV genotypes and subtypes is not geographically uniform. Some genotypes are usually restricted to African (genotypes 2 and 4) and southeast Asian countries (genotype 6) while others (e.g., genotypes 1 and 3) have dispersed worldwide from their original areas of distribution [35]. Epidemiological factors (i.e., blood transfusion and intravenous drug use) are more likely to explain this distinctive dispersion than intrinsic differences in transmissibility among genotypes/subtypes [34]. In western countries, the distribution of different HCV genotypes is changing, with an increase in new infections by genotypes 1a and 3 linked to intravenous drug use, which is the main current mode of transmission [1].

When complete genomes are compared, HCV genotypes, subtypes and strains differ on average by 30–35, 20–25 and less than 20% at the nucleotide level, respectively. These genetic differences extend beyond epidemiological and evolutionary features. They are also clinically relevant because the viral genotype is used in therapeutical decision-making and their potential implications in disease outcome and progression.

Within individual patients

The high rate of mutation of HCV and its large population size are responsible for the extremely high genetic variability detected in HCV populations. The term quasispecies has usually been applied to describe highly heterogeneous viral populations, composed of a swarm of closely related variants, many of which may have an ephemeral persistence in the population and whose behavior and dynamics result not from the

sum of those from the individual components, but rather from the population as whole [36]. Although several aspects of quasispecies theory are hotly debated [37], there is ample evidence that viral populations usually present the high genetic variation levels characteristic of viral quasispecies. Nevertheless, the actual dynamics of *in vivo* HCV populations is governed, as detailed later, by processes and mechanisms that cannot be fully accommodated or explained only by quasispecies theory [38].

Genetic variability is not distributed uniformly along the HCV genome. There are regions of low variability, such as the 5′ NCR and 3′ NCR and the *Core* gene, while others are more variable, for example, the *E2* gene. Within any region, there are relatively well-conserved stretches with low variation, and others are highly variable. For instance, three hypervariable regions have been described in the *E2* gene [39–41], which are interspersed among relatively conserved stretches [42]. Even within any of these regions, variation is uneven, with some nucleotides being more variable than others, partially reflecting the degenerate nature of the genetic code. These differences in variability levels are usually associated to differences in function and intensity and mode of action of selection. Very conserved positions or regions are strongly constrained owing to purifying or negative selection, eliminating any variation that might diminish fitness of the corresponding genome. By contrast, positive selection acts to favor variants at specific regions or positions, thus increasing their rate of evolution. This can result either in the fixation of beneficial (from the virus point of view) variants or in a continuous diversification of alternative variants. The former alternative will also result in a temporary reduction of genetic variability whereas the latter will maintain high levels of variation.

One fundamental tenet of evolutionary theory is that mutations occur at random with regard to the immediate or future 'needs' of the organisms. This does not mean that all mutations are equally likely or that they appear with the same frequency in all positions. However, it is assumed that, in a given organism, there is no correlation between the frequency of a mutation and its effects on fitness. However, once the mutation appears, as mentioned earlier, the fate of a mutation will depend on two main factors: the effective size of the population and its associated selection coefficient. The latter will vary depending on different factors such as the specific genome where the mutation emerges, the immune pressure of the host and other environmental pressures such as antiviral treatment. Not all these factors have always the same effect on the fitness of different mutations. For some positions, their effect is almost null (a new mutation can be either lethal or favorable), and for these mutations it seems reasonable to consider that their fitness is (almost) an intrinsic property, independent of other factors.

As mentioned before, in a given individual patient HCV will be under strong evolutionary forces of positive, negative and purifying selection, depending on the genomic region examined. The main evolutionary forces driving HCV genetic variation within individual patients can be classified as intrinsic (viral functional constrains), and extrinsic, both natural (innate immunity, antibody and T-cell responses) and artificially induced (antiviral and immunosuppressive drugs). Different features of HCV infection have been associated to genetic variation and 'selection of the fittest', such as immune escape and response to antiviral treatment. Following HCV infection, a variety of innate and adaptive cellular and humoral responses occur. For instance, acute infection will either resolve or become persistent, mainly depending on the interaction between the virus and the host immune response, and probably on viral immune escape from innate, humoral and cellular responses [43]. The rate of success of interferon (IFN)-based treatment differs among viral genotypes, but there is still controversy on whether specific subtype 1b strains may be resistant to IFN or whether within-patient ongoing viral variation influences response rates [44]. These lines of research have encouraged an intense search for specific mutations and viral genetic patterns responsible for the different clinical phenotypes. Their identification would allow improvements in diagnostic, treatment and clinical management of infected patients.

Between-patient variation

HCV genotypes & infection outcome

After acute infection, clearance or persistence rates are probably not influenced by the HCV genotype. In chronic infection, the clinical presentation or the severity of the disease, defined by the stage of liver fibrosis, is not associated with a particular HCV genotype, although in several studies a correlation was found between genotype 1b and risk of hepatocellular carcinoma [45]. The more severe outcome associated with genotype 1, in particular with subtype 1b, could in fact be due to a cohort effect, with those patients infected with subtype 1b having

acquired the infection in the community, being older and with longer disease duration, as suggested, for instance, in studies from Spain where the percentage of 1b-infected patients correlated with severe liver disease but also increased with age [46]. However, evidence has accumulated for an association between HCV genotype 3 infection and liver steatosis (owing to the blocking of lipoprotein secretion in hepatocytes), a true genotype-related HCV-induced cytotoxic lesion [47].

Another interesting research question is whether differences in pathogenicity between strains of the same viral subtype really exist [48]. In a cohort study of subtype 1b-infected patients, there was a correlation between HCV genetic similarity in *Core* and *NS5B* genes and similarity of fibrosis progression in nontransplanted patients, but not in transplanted patients, presumably indicating a pathogenic role for specific subtype 1b strains, and that immune suppression after orthotopic liver transplantation may hide the effect [49,50].

HLA selection at the population level

One possible explanation for the differences observed between strains of the same HCV subtype is that polymorphism occurs owing to HLA selection and adaptation in each individual host, causing diversifying selection. The cellular immune response performs a critical role in the immune control of viral infections, including those caused by highly variable viruses, such as HIV and HCV. For both HIV and HCV infections, the host–virus interaction drives the virus to escape from the T cells by mutation in target epitopes [51,52], and escape mutations in T-cell epitopes vary between patients depending on the HLA haplotype of the host [53–56]. Thus, the contribution of HLA selection may be important in shaping HCV variation and adaptation between patients, and at least some of the polymorphisms observed between isolates of the same subtype may be driven by T-cell pressure. As an example, in HIV infection, escape mutations that reduce viral fitness revert if the T-cell pressure is removed, for instance, by transmission to another host with a different HLA haplotype [57]. By contrast, other mutations with low or no fitness costs may not revert after transmission, and remain as a polymorphism [56]. As a consequence, HIV variability between hosts may be shaped by HLA selection and adaptation in the host population, and this hypothesis was recently extended to HCV [58].

Envelope variants, tissue tropism & viral entry

The high genetic variation in HCV envelope proteins may facilitate a process of continuous adaptation to target cells. Viral entry is believed to occur through receptor-mediated endocytosis after the interaction of the HCV envelope proteins E1 and E2 with the cellular surface. Several receptors have been implicated in HCV entry: the low-density lipoprotein receptor, the CD81 tetraspanin, the scavenger receptor BI, mannose-binding lectins such as DC-SIGN and L-SIGN, glycosaminoglycans, the asialoglycoprotein receptor and tight-junction proteins claudin-1, -6 or -9 [59]. HCV entry into target cells may be a slow and complex process involving several steps and the presence of different entry factors. Recent data demonstrate that occludin is a new HCV entry factor, involving the tight-junction complex in the viral entry process [60].

The hypervariable region (HVR)-1 in the E2 protein is probably involved in the interaction of HCV with cellular receptors, such as CD81, and the appearance of mutations conferring a better tropism for target cells may facilitate viral entry, replication, persistence and even adaptation to different tissues and cell types. Thus, it is possible that different HCV strains with potentially different tissue tropism and entry affinities may be involved in disease progression. HCV genomes have been detected in several tissues apart from the liver [61], such as lymphocytes, monocytes, dendritic cells, peripheral lymph nodes, bone marrow and the brain [62,63]. Although available data indicate that HCV is a noncytopathic virus, an alteration of the cellular immune response by viral replication in lymphocytes and the infection of immunological privileged sites constitute additional potential escape mechanisms; however, liver damage is thought to be immune mediated [64]. The potential role of envelope variation on extrahepatic viral replication between different HCV strains that may confer enhanced or expanded tissue tropism and entry remains to be elucidated.

Between-patient variation & antiviral therapy

The current standard-of-care treatment for chronic hepatitis C is based on a combined therapy with pegylated IFN (PEGIFN) and ribavirin (RBV), which is effective in approximately 30–50% of patients. The rate of success is different depending on the viral genotype, from approximately 80% in patients infected with

HCV genotypes 2 and 3 to less than 50% for those infected with genotype 1 [65]. Similar differences have been found in patients co-infected with HIV-1 [66]. Moreover, PEGIFN/RBV therapy is costly and has many side effects. Research efforts in this field have focused on the search for predictors of response to antiviral treatment, aiming at optimizing antiviral therapy regimens. Genetic variation in HCV proteins has also been associated with the success of IFN therapy. Since an initial report by Enomoto and coworkers [67], they and others have found a correlation between particular amino acid variations in HCV subtype 1b with sensitivity to IFN-α [68]. These variations occur in a small stretch of the NS5A protein, the putative IFN sensitivity determining region (ISDR) [67,69–71]. These studies compare HCV sequences from clinical isolates to a single reference sequence (that of HCV-J, a Japanese prototype strain for subtype 1b), but the amino acid sequence of NS5A is different between HCV subtypes. Additional studies have found no association with IFN resistance for other HCV subtypes, such as 1a [72,73], 2b [74] or 3a [69].

The NS5A protein has been involved in several interactions with cellular proteins. The most relevant one is the interaction with the IFN signaling pathway, through direct inhibition of the IFN-inducible PKR, which is normally activated by dsRNA to trigger a cellular antiviral state by switching off RNA transcription through elongation factor (eIF)2α [75]. The portion of NS5A interacting with the cellular PKR is known as PKR binding domain, whose first 40 amino acids include the putative ISDR. After several years of research, no clear association has been established between response to therapy and variability of the *NS5A* gene, despite sequencing more than 300 HCV isolates in Japan, Europe and North America [68]. Local differences in viral strains and/or treatment doses or schedules may influence the discrepant results. Three meta-analysis studies have found a correlation between the prototype ISDR sequence and resistance to IFN treatment in subtype 1b viruses [76–78]. Two very recent Japanese studies add more controversy, with data suggesting that amino acid substitutions in the Core (amino acids 70 or 91) and NS5A ISDR are predictive of the virological response to therapy in patients infected with genotype 1b and high viral load [79,80].

Another portion of the HCV genome involved in PKR interaction is located within the E2 protein, denoted as PePHD. This is a small segment of 12 amino acids very similar to the autophosphorylation sites of both PKR and eIF2α and binds to PKR *in vitro* [81]. Recent studies have questioned the predictive value of PePHD genetic diversity before treatment in HCV subtype 1b-infected patients, since a strong conservation of this motif has been observed regardless of the response to therapy [82].

Ribavirin is a base analog with an *in vitro* mutagenic effect, but the *in vivo* mode of action, conferring synergism with PEGIFN, remains poorly understood. Mutations G404S and E442G in NS5A and F415Y in NS5B have been associated with resistance to RBV in the HCV replicon system, but their clinical relevance is controversial. Some studies found no evidence that RBV selects for particular amino acids in NS5A or NS5B [83,84], but other studies have shown higher overall mutational frequencies in NS3 and NS5B [85] and early increased mutation rates in NS5B [86] in patients receiving RBV monotherapy. The latter study explored the hypothesis that RBV causes lethal mutagenesis by enhancement of the extremely error-prone replication of HCV. The authors concluded that RBV therapy was associated with an early but transient increase in the mutation rate of HCV [86]. Using a new method to estimate mutation rates based on the analysis of nonsense mutations, a threefold increase in mutation rate and a significant shift in mutation spectrum were observed in samples from nonresponders after 6 months of IFN plus RBV treatment [15].

The most comprehensive studies on HCV genome variability and response to standard therapy come from recent genome-wide analyses before, during and after therapeutical intervention. It is worth noting that sequences from patients with early response (>3.5 $\log_{10}$ declines in viral RNA levels by day 28) were more variable in NS3 and NS5A for genotype 1a and in Core and NS3 for genotype 1b than those from poor responders (declines of <1.4 $\log_{10}$). These correlations were still significant when T-cell epitopes were excluded from the analyses, suggesting that the effect was independent of the T-cell response [87]. Extensive covariation and network analyses on genome-wide data have shown that coordinated substitutions in the polyprotein can be organized into a network [88], and that covarying positions are common and linked together into networks that differ in their response to PEGIFN plus RBV therapy [89]. These data can potentially provide a framework to predict treatment response (see later).

The lack of a satisfactory therapy and the advent of HCV replicon systems to assess the antiviral efficacy of candidate compounds have boosted the development of HCV-specific antivirals (specifically targeted antiviral therapy for HCV [STAT-C]). The NS3/4A protease, NS5A and the NS5B RNA-dependent RNA polymerase have concentrated most research efforts, and inhibitors of the HCV NS3/4A protease and NS5B polymerase are the most advanced in clinical trials [81,90]. Early reports have demonstrated the selection of HCV variants that are resistant to active-site inhibitors mediated by amino acid substitutions, both *in vitro* [91] and *in vivo* [92].

Variability of the HCV NS3/4A protease was studied early in a limited number viral genotypes, patients and geographical regions [93,94]. The natural polymorphism of the HCV NS3/4A protease was also analyzed in a large number of worldwide HCV isolates deposited in public sequence databases until June 2007 (n = 380, including all six viral genotypes) [95]. A relatively large number of polymorphic sites in HCV NS3/4A proteases in all six viral genotypes were found, with subtype 1a and 1b proteases being the most polymorphic. This probably reflects a biased representation of these two subtypes in the databases and not a truly higher chance of finding polymorphism (275/380 NS3 sequences in the databases). New data, not included in that study, from a large cohort of North American and European patients (n = 570) also demonstrated that most subjects carried wild-type variants in the NS3 protease domain [96]. Another large study investigating 507 North American patients extended the analysis to the NS5B polymerase and found that resistance mutations were also rare in the protease and the polymerase, but present in up to 2.8% of patients globally and in up to 8.6% of patients infected with the HCV subtype 1a [97]. Finally, a more recent study analyzed both NS3 protease and N5B polymerase sequences in patients from Australia, Switzerland and the UK (n = 405), finding that up to 21.5% of subtype 1a viruses (n = 259) shared genetic variants at known drug-resistance sites [98]. The role of naturally occurring variations, which include several compensatory mutations and resistant mutations, on inhibitor binding will be an important issue in the design of broad-spectrum STAT-C inhibitors and deserves further exploration, especially because most compounds, including NS3, NS5A and NS5B inhibitors, are based on antiviral activities determined in genotype 1 HCV-replicon models [95]. However, determination of the relevance of sequence polymorphisms in non-1 genotypes waits for resistance data in nongenotype 1 isolates or cell culture systems.

Within-patient variation

Immune response & infection outcome

The HCV NS3/4A protease may be implicated in the silencing of the innate immune response of the host, ablating *RIG-I* gene signaling to block the downstream IFN antiviral pathway [99–102]. Furthermore, the HCV NS3/4A protease cleaves an adaptor protein for Toll-like receptor (TLR)3 signaling, which recruits cellular activation kinases critical for the induction of IFN regulatory factor 3 and NF-κB and, therefore, for the innate response antiviral state in infected cells [103]. However, this role of the HCV NS3 protease has been questioned by a recent molecular analysis [104]. Another HCV protein, NS5A, has also been implicated in breaking the innate response by inhibiting PKR, which is involved in the blocking of HCV RNA translation [105]. Whereas there are no data available suggesting that HCV variation influences the interaction with RIG-I and TLR3 signaling, several reports have suggested that mutations in the PKR-binding domain of HCV NS5A proteins may be involved in viral resistance to IFN (discussed earlier).

The antibody response to HCV is mainly directed to solvent-exposed residues of the envelope proteins. Evidence indicates that the highest rate of genetic variation of HCV during ongoing infection located in the three HVRs of the envelope proteins is due to antibody pressure [42,44]. For example, the timing between seroconversion and variation in the HVR-1 of E2 after acute infection [106,107], and the accumulation of fewer mutations in the HVR-1 in individuals with defects in the antibody response, such as hypo-γ-globulinemic patients, as compared with immunocompetent controls [108]. These data and seminal works by Farci *et al.* have led to the conclusion that HVR-1 is the main target for neutralizing antibodies aiming to clear the infection [109,110]. However, the role of neutralizing antibodies stills remains elusive. Recent studies in serial longitudinal samples using *in vitro* infection models of retroviruses pseudotyped with HCV envelope proteins have shown that the HCV 1a prototype strain H77 continuously escapes from neutralizing antibody responses by mutation, and that anti-envelope antibodies in a given timepoint fail to

neutralize HCV pseudotypes with the target peptide sequence *in vitro* [111]. Thus, mutation under antibody pressure is probably the main mechanism by which HCV subverts the host antibody response, not only by positive selection of antibody escape variants, but also by the generation of multiple envelope variants that elicit a wide range of non-neutralizing antibodies interfering with an efficient neutralization [112].

Other factors probably involved in HCV persistence may include viral inhibition of antigen processing and presentation, immunological tolerance, infection of immunological privileged sites, and resistance to or downregulation of T-cell-derived cytokines [64]. Both $CD8^+$ and $CD4^+$ HCV-specific T-cell immune responses are barely detectable in most patients with chronic HCV infection [113–115], as compared with specific responses to other viruses, such as Epstein–Barr virus, cytomegalovirus or influenza [116]. The mechanisms by which appropriate HCV-specific $CD8^+$ and $CD4^+$ cellular immune responses are not elicited and/or maintained remains to be determined. It is believed that evolution to chronic infection may be a consequence of mutational escape of the virus from a weak and narrowly focused T-cell immune response. A variety of studies has shown viral mutation and escape in different T-cell epitopes from different HCV immunogenic proteins [43].

In chronic HCV infection, amino acid variation in different HCV $CD8^+$ T-cell epitopes appear to be more frequent in patients with a detectable $CD8^+$ response, as compared with those without [117]. Mutations in target epitopes for T cells may lead to an impaired presentation of antigenic peptides and even to antagonism of T-cell responses, as described in HIV-1 and HBV infections. HCV escape mutations on $CD8^+$ cytotoxic T-lymphocyte (CTL) epitopes have been well documented, both in experimentally infected chimpanzees and in humans [118]. Presumably, the emergence of HCV CTL variants provides a mechanism for the establishment of chronicity early after acute infection [118]. Acute resolving HCV infection is associated with a strong, multispecific CTL response detectable against several viral antigens, and the likelihood of simultaneous viral mutations in different epitopes may be reduced. By contrast, in those individuals evolving to chronicity, weak or inadequate cellular immune responses at early stages of acute infection may allow a gradual and slow selection of viral variants, which ultimately outpace the cellular immune response of the host [119]. Unfortunately, both the nature of a T-cell response, which will eventually select escape variants, and the role of escape mutants in the severity of chronic infection are largely unknown to date. However, the determination of factors governing mutational escape is fundamental for the design of vaccines targeting the T-cell response.

In summary, HCV genetic variation within individual patients probably overrides the host's immune responses when establishing chronic infection. Mutational escape in epitopes targeted by T cells occurs early after infection, whereas escape mutations in epitopes targeted by neutralizing antibodies may accumulate over time. The role of escape mutations in the outcome of HCV infection still requires further investigation and is of paramount interest for the rational design of therapeutic and prophylactic vaccines.

Viral evolution & progression of the liver disease

Contrary to most flaviviruses, HCV is probably noncytopathic for the infected cells, while the immune response of the host is probably the main player in HCV pathogenesis [64]. The role of viral mutational escape from the humoral and cellular immune response in disease outcome has been explored by several studies correlating viral variation within individual patients with the outcome and the severity of liver disease. The early evolution (8–15 weeks) of HVR-1 envelope variants was suggested to predict the outcome of acute infection [109,120], and the long-term evolution to a homogenous and poorly diverse viral repertoire of envelope variants correlated with the severity of liver disease [121]. Another relevant study demonstrated that biochemical evidence of hepatic injury after perinatal infection was associated with a mono- or oligoclonal HVR-1 repertoire whereas mild or no liver damage correlated with the early emergence of HVR-1 variants, indicating that the evolution of HCV HVR-1 variants correlates with hepatic injury [110].

Several cross-sectional studies have reported apparently contradictory results. For instance, no correlation was found between the number of HCV envelope variants and the degree of liver disease in subtype 1b-infected patients [122], but other studies reported a significant correlation for HCV genotype 2-infected cases [123]. It is probably not the number of viral variants at a given timepoint in a cross-sectional study that is directly correlated with the degree of liver

disease, but the nature and long-term evolution of these variants with time. In a 9-year longitudinal study on a hemophiliac cohort, viral complexity of Core and HVR-1 regions narrowed over time in patients with severe outcomes, whereas it increased in nonprogressors [124]. Similarly, subjects with fast fibrosis progression had a higher rate of viral evolution than subjects with slow progression in a small but control-matched cohort [125].

Antiviral resistance & selection of resistance over time

In chronic HCV infection, it seems reasonable to assume that viral persistence is facilitated by alterations in the innate and adaptive immune responses. The simultaneous presence of multiple genome variants, together with a high replication rate, may allow for the rapid selection of the fittest viral genomes to face the immune pressure of the host or new environmental conditions and challenges such as antiviral treatment. Viral genotype and serum concentration of HCV RNA have been identified as factors predicting sustained response to either IFN alone or IFN–RBV combination therapy, and both are important variables in current clinical guidelines for HCV treatment. By contrast, conflicting results have been reported on the influence of genetic variability within single individuals and the response to IFN with or without RBV, either in the HVR-1 [46,73,126] or in other viral regions, such as Core, NS3, NS5A [68,73,127] or NS5B [128]. Cuevas *et al.* recently analyzed the three hypervariable regions in E2, and the PKR-binding and V3 domains in NS5A by sequencing 25–100 clones at baseline and end-of-treatment samples from 22 nonresponder patients to IFN-α-2a plus RBV therapy [129]. They failed to detect a common adaptive mechanism for the lack of response, but a wide range of HCV variant structures were observed within patients, suggesting that several different changes, or their combination along the HCV genome, may confer viruses the ability to overcome selective pressures imposed by nonspecific treatment. These findings are consistent with more comprehensive, genome-wide network interaction studies indicating that the HCV genome evolves as a coordinated unit that produces genome-wide signatures of IFN sensitivity or resistance [88,89]. Furthermore, whilst analyzing the full HCV genome in a set of responders and nonresponder patients, pretherapy interpatient diversity in the viral *NS2* and *NS3* genes was found to be higher among relapsers than in the nonresponders, whereas pretherapy diversity among relapsers is intermediate between those of nonresponders and responders to therapy [130]. These and similar studies analyzing the complete HCV genome may be very helpful to ultimately predict which HCV strains may be intrinsically resistant to IFN-based therapy.

A different problem may arise by the selection of viral variants during treatment of patients with STAT-C inhibitors. Undetectable, preexisting resistant variants at low frequency in the viral population may pose a threat to new STAT-C regimens. Protease inhibitors rapidly select for resistant minor variants, and resistant NS5B variants hidden in the viral population may also cause reduced sensitivity to polymerase inhibitors, especially to non-nucleoside compounds [131]. Once the resistant variants increase their frequency under the pressure of the drug, viral rebound can be unavoidable, although some resistant strains may remain sensitive to PEGIFN/RBV [132]. With regard to analogy to HLA selection and reversion (see earlier), it is tempting to speculate that under STAT-C antiviral drug pressure mutations selected by a particular drug may revert to wild-type after finishing dosing. This hypothesis may provide an incorrect basis for justifying monotherapy treatment regimens. In fact, during monotherapy with the NS3 protease inhibitors telaprevir [132] or boceprevir [133], some drug-resistance variants arising during treatment persisted in the viral population after the end of dosing (no total reversion), and represented a significant amount of viral genomes, sometimes during a quite long follow-up period. Thus, a combination of a protease inhibitor with a polymerase inhibitor (especially nucleoside analogs), as well as other agents and, probably, PEGIFN and/or RBV will minimize the emergence of resistance.

Conclusion

HCV is a highly variable virus with enormous capacity of adaptation, but subjected to different forces of selection in human populations and within individual patients. The dynamics of genetic variation in HCV is the result of several factors, among which purifying, positive and diversifying selection, as well as genetic drift play a prominent role.

Appropriate methods to quantify the variability of HCV include analyzing nucleotide diversity and population mutation rates by using molecular variance approaches. HCV has distinguishable levels of variation: between

genotypes or between infected individuals (polymorphism and genetic diversity) and within individuals (genetic complexity), which are important distinctions essential to interpreting research data.

Available data suggest that ongoing selection (not only the presence of selection in a given timepoint) for a less diverse population of HCV variants is a correlate of more severe and/or accelerated liver disease, presumably because of a less efficient immune response to the virus.

HCV genetic variation is very likely to be involved in several escape mechanisms from the immune response of the host, such as interference with innate immunity, escape from neutralizing antibodies and T-cell escape.

The influence of HCV variation on the success of current standard treatment with PEGIFN/RBV is still controversial, but it appears that not only particular mutations in the HCV genome determine virological response, but a more complex pattern of variation, which may differ between individuals.

Several HCV-specific drugs are in clinical development, and some with encouraging results, but the high capacity for genetic variation of this virus anticipates that viral resistance to STAT-C compounds will represent a clinical problem in the near future.

Future perspective

Genetic heterogeneity of HCV is an essential feature of this virus that plays a major role in different evolutionary, epidemiological and clinical aspects. It is of paramount importance to understand its dynamics and its influence on the different steps and processes of the infection. The increasing availability of data obtained from next-generation sequencers will provide the empirical bases required for new studies characterizing and quantifying genetic variation of infecting viral populations. However, developments in human genetics and molecular biology derived from next-generation sequencers, proteomics and other 'omics' methods will facilitate the integral study of the virus–host system in large enough samples to reveal the contribution of each partner of the system, as well as that of their interaction.

The incorporation of population genetics concepts to such analyses will extend beyond the mere quantification of parameters, such as the number of mutations, genome complexity and nucleotide diversity. Understanding the interplay between deterministic (selection) and stochastic (drift) forces governing viral dynamics and their relationship to intrinsic and circumstantial features of the host will translate into more efficient, individualized therapies. Hence, future research needs to include prospective, longitudinal studies on HCV variation in well-characterized cohorts.

Whether HCV achieves immune escape or not may influence the outcome of acute infection and the degree of escape variants may influence disease outcome in chronic infection. The identification of the determinants of immune escape and immune control is necessary for a rationale design of prophylactic and therapeutic vaccines. It is likely that such determinants result from different combinations of viral and host factors, which might be revealed with the aforementioned systems approach.

HCV resistance to new STAT-C treatments will be less likely to emerge if protease inhibitors are given in combination with drugs targeting other viral enzymes, such as the viral NS5B RNA-dependent RNA polymerase. Owing to the expected emergence of viral resistance, it is accepted that other nonspecific drugs, such as IFN and/or RBV, will remain necessary components of future HCV therapies. Close surveillance of the dynamics and evolution of viral populations in patients undergoing therapy will benefit not only individuals who are not responding to treatment but also the global human population, since it is likely that escape mutations to drugs targeted to more specific viral proteins and infection processes will also be efficient in new hosts. The epidemic spread of resistant viruses will be a new worry for public health officials in the fight against HCV infection.

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Executive summary

Dynamics of variation

- HCV continuously generates new genetic variants that provide ample opportunities for viral adaptation to environmental conditions.
- The dynamics of genetic variation depends on several factors, among which selection and drift are the most important ones.

Generation of variation

- Mutation is the main source of new variation in HCV. Using a new approach, the mutation rate of HCV was estimated to be $1.15 \pm 0.29 \times 10^{-14}$ per site and replication. This rate is very high and could generate all possible single and double mutations in an infected individual in a single day.

Quantification of variation

- There are several parameters describing and quantifying genetic variability in viral populations. Using appropriate measures is essential to analyze and understand the dynamics of variation at different levels.

Levels of variation in HCV

- There are two main levels of genetic variation in HCV, between patients and within patients. Both levels can be further grouped and subdivided to provide a full description of the total variation in this virus.
- Differences between genotypes, subtypes and strains extend beyond evolutionary and epidemiological dynamics and they are also relevant for clinical features, such as treatment response, disease outcome and progression.
- The high levels of genetic variation usually found within patients increase, but not necessarily ensure, the evolution of viral adaptive solutions to the challenges derived from the patient's immune responses and from antiviral therapies.

Between-patient variation: HCV genotypes & subtypes

- There is not a consensus view on the relevance of viral genotype or subtype on disease progression, severity of the disease or viral pathogenicity, with the exception of genotype 3. A few studies suggest some influence of viral genetic composition on these and other traits, but more analyses with a better and more complete genetic characterization of the infecting viral populations are necessary to establish firm conclusions.

Between-patient variation: envelope variants, tissue tropism & viral entry

- HCV entry into target cells probably involves several steps and entry factors. One of the most variable regions in the HCV genome, hypervariable region-1 in the envelope glycoprotein, E2, is probably involved in interactions with cellular receptors. Thus, variation may potentially contribute to better tropism and adaptation to different cells and tissues.

Between-patient variation & antiviral therapy

- Several genomic regions in HCV have been associated with resistance and efficiency of current antiviral treatment, with pegylated-interferon plus ribavirin. Most studies have concentrated on a few viral subtypes and geographic locations, which might partially explain the observed correlations.
- Lessons learned during the introduction of effective multiple antiretroviral therapies against HIV-1 infection should be considered in the use of new HCV-specific targeted antiviral drugs once they reach the clinic.

Within-patient variation, immune response & infection outcome

- There is a significant correlation between generation of genetic variation in several viral genome regions and the effectiveness of the immune responses by the host. Progression to chronic infection may be a consequence of mutational escape from a weakened and narrowed T-cell immune response.
- Spontaneously resolving HCV infection is associated with a strong, multispecific cytotoxic T-lymphocyte response against several viral antigens, thus making the simultaneous appearance of several escape mutations more unlikely.

Within-patient variation: viral evolution & progression of the liver disease

- Genetic variation in hypervariable region-1 has been associated to the outcome of acute infection, but a genetically homogenous viral population has been correlated to severity of liver disease in chronic infection. However, there are several contradictory reports that might be reconciled after better characterization of the genomic variation in the viral population together with the genetic and immunological characteristics of the infected patients.

Within-patient variation, antiviral resistance & selection of resistance over time

- Conflicting results have been reported regarding the influence of genetic variability at several viral genome regions within single individuals and the response to interferon with or without ribavirin. A recent study has revealed the multiplicity of adaptive solutions adopted by HCV against antiviral therapy, with a likely role for the interaction with different hosts factors.
- Pre-existing resistance mutations and strong selective pressures may compromise the efficiency of new HCV-specific therapies, which should be administered in mixed cocktails and not in an individual sequence to prevent and limit the emergence and extension of resistance.

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