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Highlights

- To attain IFN-resistance HCV has to confront a multifactorial selective constraint.
- Multiple host and viral factors intervene in IFN resistance of HCV.
- There is no coherent picture of resistance mutations associated with treatment failure.

Molecular basis of interferon resistance in hepatitis C virus

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Abstract

Resistance to interferon (IFN) in hepatitis C virus (HCV) differs from resistance to standard, directly-acting antiviral (DAA) agents in that the virus confronts a multicomponent antiviral state evoked by IFN. This renders unlikely the selection of the same specific mutations that confer an IFN-resistance phenotype. Comparison of amino acid sequences of viral proteins in HCV that replicates in the presence of IFN *in vivo* or in cell culture (with entire virus or subgenomic replicons) reveals very few common candidate IFN resistance substitutions. Multiple host and viral factors contribute to divergent responses to IFN. The environmental heterogeneity in which exogenous IFN is expected to exert its selective effect may increase as a result of incorporation of new DAAs in therapy.

Highlights

- To attain IFN-resistance HCV has to confront a multifactorial selective constraint.
- Multiple host and viral factors intervene in IFN resistance of HCV.
- There is no coherent picture of resistance mutations associated with treatment failure.

Introduction

The interferons (IFNs) are a family of cytokines that have been divided in three major groups: I (IFN- α , IFN- β , IFN- ϵ , IFN- κ , IFN- ω , IFN- ν); II (IFN- γ or immune IFN), and III (IFN- λ s), which bind to different receptors that trigger the IFN response in cells. They are components of the innate immune response against viruses, in particular hepatitis C virus (HCV) [1•,2•,3]. A combination of pegylated IFN- α and the purine nucleoside analogue ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been the standard of care for treatment of chronic HCV infections for over a decade [4-6]. Only about 60% of treated patients show a sustained viral response (SVR) to standard of care

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4 treatment, and several host and viral factors appear to contribute to treatment efficacy [7,8].
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6 The availability of several directly acting antiviral agents (DAAs) for HCV –mainly
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8 inhibitors of NS3 (protease), NS5A and NS5B (polymerase) [9-12]– has raised the
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10 possibility of IFN-free anti HCV treatments. Despite such prospects, the issue of IFN-
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12 resistance (or decreased IFN sensitivity) of HCV is likely to have continued importance for
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14 two main reasons. One is the introduction of type III interferons (IFN- λ s) in clinical
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16 practice because of their reduced side effects as compared with type I IFNs, by virtue of the
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18 restricted expression of IFN- λ receptors to some epithelial cells. The second reason is that
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20 *in vitro* assays and some clinical trials have documented increased HCV suppression and
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22 SVR rates when IFN is included together with DAAs in therapy [13,14].
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28 Experience gained with other viral diseases, particularly HIV-1 infections,
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30 anticipates that resistance to DAAs may unfold and result in the successive incorporation of
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32 new inhibitors in therapy, with waves of standard and salvage treatments, unless virus
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34 elimination from chronically infected patients is widespread (as a result of new treatments
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36 available worldwide). Particularly difficult cases that may require IFN-based treatments are
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38 HIV-1 coinfecting patients, infections by certain HCV genotypes or subtypes, and patients
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40 with advanced liver fibrosis. If IFNs remain components of some treatment protocols, HCV
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42 IFN resistance will remain a factor of treatment failure. Unfortunately, IFN resistance is
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44 proving a far more complex issue than DAA resistance, due to both physiological and
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46 evolutionary reasons.
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52 **DAA- and IFN-escape mutants**

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55 For antiviral compounds that target a specific viral protein, a number of amino acid
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57 substitutions that mediate different degrees of resistance to the corresponding antiviral
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59 compound are often identified consistently in the target protein. They can be catalogued
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4 and ideally their presence –either as dominant genomes or in minority components of the
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6 mutant spectrum– can be taken into consideration for treatment planning. The restricted
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8 number of mutations (or combination of mutations) found associated with resistance to
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10 inhibitors is a consequence of the limited number of biological solutions available in the
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12 form of viral escape mutants that respond to a focused selective constraint. This is expected
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14 to be the case for the DAAs in use or under development for HCV infections, as it was for
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16 antiretroviral agents in the case of HIV-1, and other antiviral inhibitors.
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21 The picture for IFN-escape mutants is far more complex, primarily due to the
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23 nature of the antiviral response evoked by IFN. IFNs are induced as a consequence of virus
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25 sensing by pattern-recognition receptors (PRRs) that bind pathogen-associated molecular
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27 patterns (PAMPs) present in viral products. This virus recognition triggers retinoic acid
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29 inducible gene I (RIG-I) signalling that involves interactions with mitochondrial antiviral
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31 signalling proteins (MAVS) which promotes activation of multiple effector molecules and
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33 proinflammatory cytokines. Activation can occur also via toll-like receptor proteins (such
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35 as TLR3) and protein kinase R [1]. HCV expresses proteins that can block innate responses,
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37 at least to a point of allowing a chronic infection to be established in 80% of individuals
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39 following acute HCV infection (for example, NS3 protease cleaves MAVS and TRIF to
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41 inactivate RIG-I, TLR3 and protein kinase R signalling pathways) [1]. Chronicity may
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43 select for HCV mutants capable of continued replication in the presence of multiple
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45 interferon stimulated genes (ISGs). This adaptation may explain the higher effectiveness of
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47 IFN- α based therapies for acute than chronic HCV infections. An activated endogenous
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49 IFN response predicts a poor response to therapies involving IFN- α ([15,16] and references
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51 therein).
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4 Despite IFN being produced in the course of viral infections, it is not known
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6 whether HCV actually replicates under selection to escape products expressed by ISGs
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8 [16]. Viruses evoke the expression of multiple, specific sets of ISGs [17,18]. If HCV
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10 indeed replicates in an environment of ISG expression products, it would be expected that
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12 constellations of mutations may be gradually selected that have increased capacity to
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14 replicate in the IFN-induced antiviral environment. The protracted exposure of HCV to
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16 different types and levels of IFN is expected to have influenced the long-term evolution of
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18 HCV regarding its capacity to respond to therapeutic IFN. Divergent IFN-mediated
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20 evolution may have been reflected in a different vulnerability of HCV genotypes to
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22 standard of care treatment [19]. In this view, exposure to an IFN-based therapy may add a
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24 transient selective pressure related to ones that have been imprinted in the viral genome. If
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26 a level of mutations adequate to survive in the presence of ISGs has been reached during
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28 long-term evolution, new beneficial mutations in response to therapy will occur with lower
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30 probability. In contrast to IFNs, HCV confronts DAAs only during therapy, usually without
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32 a prior history of exposure to the inhibitor. This situation may change if the use of DAAs is
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34 generalized and transmission of DAA-resistant mutants increases the level of circulating
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36 primary resistances.
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45 **Viral fitness and IFN-escape**

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48 An additional difference between resistance to DAAs and to IFN is the effect of
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50 fitness enhancing mutations on the resistance phenotype. The ease of selection of a DAA
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52 resistance mutation depends on the genetic barrier (number of mutations required to attain
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54 the amino acid substitutions needed) and the phenotypic barrier to resistance (or fitness cost
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56 inflicted by the required amino acid substitutions) [20]. A DAA- resistance mutation may
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58 be selected prior to or together with accompanying mutations that compensate for fitness
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4 loss. In the case of IFN, or other stimuli that trigger a pluricomponent antiviral response,
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6 fitness enhancing mutations that do not produce any *bona fide* resistance may suffice for
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8 the virus to survive in the face of an IFN response. Therefore, analyses should be
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10 performed to distinguish IFN resistance mutations from fitness enhancing mutations that
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12 indirectly produce the IFN resistance trait.
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16 Viral quasispecies evolve by replacement of subpopulations of mutant swarms by
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18 others present or arising in the same replicative ensemble [20]. Most HCV subpopulations
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20 that may increase in frequency as a result of mutations that decrease the sensitivity to some
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22 ISG products will nevertheless face the limitation of having to replicate in the presence of
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24 other ISG products. That is, their fitness advantage in such a milieu will be minimal. This
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26 means that unless multiple mutations that would confer resistance to the majority of ISG
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28 products and other innate response elements occur in the HCV genome –unlikely because
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30 they would require accessing remote regions of sequence space [20]– no subpopulation will
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32 reach dominance. Partially selected swarms will still be vulnerable to suppression by the
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34 mutant spectrum that surrounds them [21-26]). If such frequency-enhanced populations
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36 were displaced and remained at memory levels [27] their proportion and duration in the
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38 population would be limited [28-30]. Thus, the environmental complexity confronted by
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40 HCV renders unlikely that specific mutations can be identified as consistently conferring an
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42 IFN-resistant phenotype.
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50 **Host and viral factors in IFN resistance**

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53 Based on the dynamics of viral quasispecies, we hypothesize that the broader the
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55 antiviral response evoked by an agent such as IFN, the more host factor-dependent will the
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57 response be. Signalling pathways and metabolic routes that participate in the IFN-mediated
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59 antiviral state may vary in their activity depending on allele composition. Variation at the
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4 IL-28B locus was the first human genetic polymorphism recognized as influencing the
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6 outcome of standard of care treatment [31]. Polymorphisms in histone deacetylases can
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8 complement the predictive value of IL-28 in the response of chronic HCV to therapy [32].
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10 Other polymorphisms (i.e. KIR2DS [33]) and several host traits (sex, age, ethnic origin,
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12 obesity, insulin resistance, ethanol consumption, HIV-1 infection, degree of liver fibrosis,
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14 etc.) have been associated with the response to IFN [16,34]. Patients whose endogenous
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16 IFN system is activated are poor responders to IFN- α based treatments (reviewed in [16]).
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18 These host influences are interconnected with viral factors which include genotype, viral
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20 load, quasispecies complexity and predominant mutations types in mutant spectra [20,35].
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26 That multiple HCV expression products can contribute to IFN resistance has been
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28 evidenced by the inhibition of antiviral activity of IFN when HCV core, E1, E2 or NS5B
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30 proteins are expressed in *trans* [36,37]. Here we describe the effects of some specific
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32 amino acids. The core protein induces suppressors of cytokine signalling proteins which
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34 block expression of ISGs [38-41]. Substitutions at amino acids 70 and 91 of core confer
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36 resistance to IFN- α , associated with a decrease in IFN-induced phosphorylation of STAT1
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38 and STAT2 and expression of ISGs; the mutants enhanced the expression of suppressors of
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40 cytokine signalling protein 3 and IL-6 [38,42]. Studies with recombinant 1a and 3a HCV
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42 genotypes identified amino acid substitutions at positions 345 and 348 of E1 and 414 of E2
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44 that increased IFN- α resistance to a level that exceeded the fitness effect on viral entry and
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46 release [43]. NS5A includes the interferon sensitivity determining region (ISDR, residues
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48 2209 to 2248, genotype 1) and the interferon and ribavirin resistance determining region
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50 (IRRDR, residues 2334 to 2379, genotype 1) whose composition and number of mutations
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52 have been associated with response to treatment [44-47]. Mutations in these regions were
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selected mainly in non-responder patients, but the mutations were different in each patient. These NS5A regions as well as core positions 70 and 91 may change in the course of treatment and influence its outcome [48,49]. Using HCV replicon cell lines, substitutions in NS3 (S1269Y, K1270R, R1135K), NS4B (Q1737H), NS5A (M2174V, T2319A/N, T2242N, F2256L) and NS5B (A2752V) were associated with IFN resistance [50]. Other analyses have documented additional virus variations in standard of care non-responder patients, some associated with development of hepatocellular carcinoma [51-54].

In a study in which a cell culture-adapted HCV (genotype 2A) preparation was subjected to one hundred virus passages in the absence or presence of increasing doses of IFN- α , amino acid substitutions that conferred IFN- α resistance were distinguished from substitutions that increased viral fitness [55] (Figure 1). Significantly, very few of the substitutions in core, E1, E2, NS3, NS4B, NS5A and NS5B (summarized above) that have been associated with IFN resistance [38,43,48,49,50] are represented in the repertoire selected in HCV replicating in hepatoma cells. Taking the study by Kozuka *et al.* [48] as an example, of the 26 amino acid substitutions in the interferon sensitivity determining region (ISDR) and 45 substitutions in the interferon and ribavirin resistance determining region (IRRDR) of patients subjected to standard of care treatment, none were represented in the study by Perales *et al.* [55]. Although there were four positions at which variations were found in the two studies, the substitutions were different (Figure 2).

Comparison of the mutations described by Perales *et al.* [55] (Figure 1) with those of the study by Serre *et al.* [43] with chimeric HCVs shows some coincidences that may be significant regarding fitness gain or IFN resistance in HCV. In the 1a/JFH1 chimera, NS5A substitution T2076A (numbering according to Serre *et al.* [43]) became also dominant in

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4 HCV passaged one hundred times in the presence of IFN- α in the study by Perales *et al.*
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6 [55] (NS5A substitution T104A in Figure 1). In the 3a/JFH1 chimera, substitutions F345V
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8 (in E1), H837Y (in NS2) and T2076A (in NS5A) (numbering according to Serre *et al.*
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10 [43]) were also found in the study by Perales *et al.* [55]. [I154M/V (in E1), T28A/P (in
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12 NS2), T104A (in NS5A), respectively]. The results of both studies suggest that E1
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14 substitution I154M/V may produce a fitness increase and, consequently, IFN resistance to
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16 HCV. NS2 T28A/P is a potential candidate to confer IFN- α resistance, although it was also
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18 found in low proportion in HCV passaged in absence of IFN- α (see location of these
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20 substitutions in Figure 1).
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27 Again, this dispersion of genetic lesions suggests multiple IFN resistance
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29 mechanisms interwoven with general enhancing fitness effects that impede reaching a
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31 coherent picture that may guide treatment choice regarding inclusion of IFN.
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35 To complicate matters even further, in the prolonged multiplication of HCV in Huh-
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37 7.5 hepatoma cells, virus that had been passaged in the presence of low IFN- α dose and that
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39 was shifted to a higher IFN- α concentration was inhibited to a larger extent than a naive
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41 virus (IFN- α -untreated parental population) subjected directly to the high IFN- α
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43 concentration [55]. This observation indicates that contrary to what is often observed with
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45 standard DAA-like inhibitors with viruses, a history of replication in the presence of IFN- α
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47 did not prepare the virus to confront higher IFN- α doses. Rather, prior replication in the
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49 presence of IFN- α enhanced HCV sensitivity to higher IFN- α doses, by some “priming”
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51 mechanisms. Whether such reverse pre-exposure effects can occur *in vivo* is not known.
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4 Thus, IFN resistance is an extremely complex issue, and the patterns of resistance
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6 development deviate from a direct mutation-competition-selection pattern readily
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8 identifiable with inhibitors that target a defined viral genomic structure or protein.
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10 11 **Concluding remarks** 12 13

14 In the search of HCV mutations that confer resistance specifically to IFN *in vivo*, it
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16 must be considered that resistance to standard of care treatment is not synonymous with
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18 resistance to IFN- α . The presence of ribavirin may alter the intracellular replicative
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20 environment in such a manner that the selected HCV subpopulations that reach a sufficient
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22 fitness level to be detected may not include authentic IFN-specific resistance mutations.
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24 The environment in which IFN evokes selective forces against HCV will be modified when
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26 new inhibitors are incorporated in therapy. Experimental designs in cell culture may help in
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28 distinguishing the favored mutations to yield high HCV fitness in the presence of IFN
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30 alone, ribavirin alone, the mixture of the two or with DAAs. Some studies along these lines
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32 are ongoing. The interest in understanding the molecular determinants of IFN resistance in
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34 HCV is accentuated by the likely connections between the innate and adaptive branches of
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36 the immune response. The resulting immune competence is a key factor in determining
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38 viral clearance when viral load has decreased as a result of pharmacological intervention.
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10 11 12 **Figure Legends**

13 14 **Figure 1.**

15
16 Amino acid replacements found in the consensus sequence of different HCV
17 proteins upon passage of the virus in the absence or presence of IFN- α . The virus used is
18 HCVcc prepared by transfection of Lunet cells with full length transcript from plasmid
19 Jc1FLAG (p7-nsGluc2A) [56]. The preparation of the initial HCVcc and the passage in
20 Huh-7.5 human hepatoma cells were previously described [55]. The upper part gives the
21 amino acid substitutions (single letter amino acid code) found in core, E1, E2, p7, NS2,
22 NS3, NS4A and NS4B; the bottom part gives the substitutions in NS5A and NS5B.
23 Residues were numbered individually for each protein (numbering in the upper black
24 boxes). To facilitate comparisons, the position equivalence with absolute HCV H77
25 numbering is given above the black boxes. The correspondence between the two numbering
26 systems is based on an amino acid sequence alignment using ClustalW, reported in Table
27 S10 of reference [55]. Boxes on the left summarize the passage history of HCVcc (p,
28 passage number; IFN 0.5, 1, 2 or 10, IFN in infectious units/ml included in the culture
29 medium); in some passage series, the information in brackets indicates a number of
30 additional passages in the absence (IFN 0) or presence (IFN 2, IFN 12) of IFN. Blue,
31 yellow, pink and green distinguish the absence or presence of low, medium or high IFN- α
32 concentrations during passage. Two letters at the same position denote a mixture of the two
33 amino acids, and an asterisk indicates that the amino acid was present at a frequency of
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25% or lower. The upper inverted grey triangles indicate amino acid substitutions likely involved in fitness gain. The purple triangles drawn between the two panels highlight amino acid substitutions likely involved in IFN- α resistance. The effect of some of these substitutions are now under study. These assignments are based on the occurrence of substitutions in the absence or presence of IFN- α and their reversion, as explained in [55]. The three polyhedrons shaded in green indicate amino acid substitutions that coincide with those associated with IFN- α resistance in the study by Serre *et al.* [43]. Further experimental details are described in [55]. Figure adapted from [55], with permission from ASM.

Figure 2.

Amino acid replacements found in the consensus sequence of the interferon sensitive determining region (ISDR) and interferon and ribavirin resistance determining region (IRRDR) in HCV subjected to replication in the presence of IFN- α . Sequence alignment (single letter amino acid code) of ISDR (top) and IRRDR (bottom) of HCVcc (genotype 2A) (pink amino acid sequence, numbering of NS5A amino acids according to Perales *et al.* [55]) and HCV genotype 1 (yellow amino acid sequence, numbering of NS5A amino acids according to Kozuka *et al.* [48]). The correspondence between the two numbering systems was based on an amino acid sequence alignment using ClustalW, and reported in Table S10 of reference [55]. Due to the limited sequence identity between the two interferon and ribavirin resistance determining region (IRRDR) sequences, no attempt has been made to optimize this alignment. The amino acids written in red indicate the substitutions identified in HCVcc passaged in the presence of IFN- α ([55] and Figure 1).

Two amino acids separated by a slash indicate a mixture of about 50% in the consensus sequence. The amino acids written in black indicate the substitutions identified in HCV from patients subjected to standard of care treatment [48]. Of note the substitutions reported by Perales *et al.* [55] were detected in some passage series in the presence of IFN- α but not in others (compare Figure 1); in the study by Kozuza *et al.* [48] some substitutions were identified in some patients and time points but not in others.

Figure 1
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Figure 2
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