Characterization of NS5A and NS5B resistance-associated substitutions from genotype 1 HCV infected patients in a Portuguese cohort

Ruben Alexandre Ribeiro Brandão

Dissertação para obtenção do

Grau de Mestre em Microbiologia Médica

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Orientador: Professora Doutora Perpétua Gomes, Instituto Universitário Egas Moniz.
Co-orientador: Professor Doutor Paulo Paixão, Faculdade de Ciências Médicas da Universidade NOVA de Lisboa.

Faculdade de Ciências Médicas da Universidade NOVA de Lisboa; Laboratório de Microbiologia Clinica e Biologia Molecular, Hospital Egas Moniz, CHLO, Lisboa.

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Abstract

Hepatitis C virus (HCV) is considered to be the leading cause of hepatocellular carcinoma (HCC). During the last years, several highly efficacy regimens of direct-acting antivirals (DAAs) with excellent rates of success became available. However, therapeutic failure may occur in up to 10% of treated individuals.

Our aim was to study the profile of NS5 coding region RASs in DAA-naive genotype 1 HCV infected patients, as well as to ascertain an association between treatment failure and the presence of baseline NS5 RASs. A comparison between LiPA and Sanger sequencing genotyping methods was also assessed.

Plasma RNA from 81 DAA-naïve GT1 HCV infected patients was extracted, followed by an in-house nested RT-PCR of the NS5 coding region. PCR products were purified, leading to Sanger population sequencing on the 3130xl ABI Genetic Analyzer. Sequences were aligned using *ChromasPro®* v1.7.6, and analyzed online in hcv.geno2pheno.org.

NS5A RASs were present in 28,4% (23/81) of all GT1 infected patients. The most commonly detected NS5A RAS was Y93C/H with a prevalence of 9,9% (8/81) in all GT1 infected patients. NS5B RASs showed a prevalence of 14,8% (12/81) in all GT1 infected patients, and were only detected in GT1b, being mainly represented by C316N accounting for 40% (10/25). The combined Q30H+Y93H NS5A RASs, were detected at baseline in one HIV/HCV GT1a co-infected patient who later failed a treatment with sofosbuvir/ledipasvir (SOF/LDV) for 12 weeks. An isolated Y93H mutation was also detected at baseline in a relapsing GT1b mono-infected patient. Overall 38,3% (31/81) of all GT1 HCV infected patients presented NS5 RASs at baseline, in which 58% (18/31) were co-infected with HIV/HCV.

The obtained data supports the usefulness of resistance testing prior to treatment initiation, as a statistical significant association was found between treatment failure and the baseline presence of specific NS5 RASs, namely Y93C/H ($p = 0.04$).

Resumo

O vírus da Hepatite C (VHC) é considerado a principal causa de carcinoma hepatocelular. Durante os últimos anos surgiram diversos regimes de antivirais de atuação direta (AADs) com elevada eficácia. Contudo, os insucessos terapêuticos podem ocorrer em até 10% dos doentes tratados.

O nosso objetivo foi estudar as substituições associadas a resistência (SARs) da região codificante NS5 em doentes infetados com VHC de genótipo 1 nunca antes tratados com AADs, assim como averiguar uma associação entre o insucesso terapêutico e a presença de SARs da NS5. Foi igualmente realizada uma comparação entre os métodos de genotipagem por LiPA e sequenciação.

O ARN plasmático de 81 doentes infectados com VHC de GT1 foi extraído, seguido por um *in-house nested RT-PCR* da NS5. Os produtos amplificados foram purificados, levando à sequenciação de Sanger usando o *3130xl ABI Genetic Analyzer*. As sequências foram alinhadas usando o *ChromasPro*® v1.7.6, e analisadas online em *hcv.geno2pheno.org*.

As SARs da NS5A estiveram presentes em 28,4% (23/81) de todos os doentes infetados com VHC de GT1, com a mutação Y93C/H exibindo uma prevalência de 9,9% (8/81). As SARs da NS5B demonstraram uma prevalência de 14,8% (12/81) e foram apenas detetadas no GT1b. As SARs Q30H+Y93H da NS5A estiveram presentes num doente co-infetado com VHC de GT1a e VIH que mais tarde demonstrou um insucesso terapêutico perante um regime de sofosbuvir/ledipasvir durante 12 semanas. Uma mutação Y93H isolada esteve presente num doente recidivo com VHC de GT1b. De modo geral, 38,3% (31/81) de todos os doentes infetados demonstraram SARs da NS5, dos quais 58% (18/31) estavam co-infetados com VHC e VIH.

Os dados obtidos suportam a utilidade dos testes de resistência basais, tendo em conta que foi encontrada uma associação estatisticamente significante entre o insucesso terapêutico e a presença basal de SARs especificas da NS5, nomeadamente a Y93C/H (p=0.04).

Keywords

Hepatitis C virus; Direct-acting antivirals; Resistance-associated substitutions; NS5A; NS5B.

Palavras-chave

Virus da Hepatite C; Antivirais de actuação directa; Substituições associadas a resistência; NS5A; NS5B.

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Abbreviations and acronyms

3D	Ombitasvir/Ritonavir-boosted Paritaprevir + Dasabuvir
ALT	Alanine aminotransferase
ARV	Antiretroviral
ASN	Asunaprevir
BOC	Boceprevir
bp	Base pairs
cDNA	Complementar DNA
CHLO	Centro Hospitalar de Lisboa Ocidental
DAA	Direct-acting antiviral
DCV	Daclatasvir
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DSV	Dasabuvir
EASL	European Association for the Study of the Liver
EBR	Elbasvir
EC_{50}	Half maximal effective concentration
EC_{90}	90% maximal effective concentration
EIA	Enzyme-linked immunosorbent assay
eIF	Eukaryotic initiation factor
EoT	End of Treatment
ER	Endoplasmic reticulum
FW	Forward
GBD	Global Burden of Disease Study
GHDx	Global Health Data Exchange
GLE	Glecaprevir
GT	Genotype
GZR	Grazoprevir
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IFN	Interferon
IHME	Institute for Health Metrics and Evaluation
IHMT	Instituto de Higiene e Medicina Tropical
INNO	Innogenetics
IRES	Internal ribosome entry site
IU	International Units
IVDU	Intravenous drug users
LDL	Low density lipoprotein
LDV	Ledipasvir
LiPA	Line Probe Assay
LOD	Limit of detection
LVP	Lipoviroparticle

min	Minutes
mRNA	Messenger ribonucleic acid
NAT	Nucleic acid test
NGS	Next-generation sequencing
NI	Nucleoside inhibitor
NNI	Non-nucleoside inhibitor
nt	Nucleotide
OBV	Ombitasvir
ODV	Odalasvir
ORF	Open reading frame
PCR	Polymerase chain reaction
pegIFN	Pegylated interferon
PI	Protease inhibitor
PIB	Pibrentasvir
PKR	Protein kinase RNA-activated
PrEP	Pre-exposure prophylaxis
PTV	Paritepravir
PWID	People who inject drugs
QALY	Quality-adjusted life year
RAS	Resistance-associated substitution
RAV	Resistance-associated variant
RBV	Ribavirin
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RNEC	Registo Nacional de Estudos Clinicos
rpm	Rotation per minute
RT-PCR	Reverse transcription polymerase chain reaction
RV	Reverse
RZR	Ruzasvir
sec	Seconds
SMRT	Single Molecule Real-Time
SMV	Simeprevir
SOF	Sofosbuvir
SVR	Sustainable virological response
TE	Treatment-experienced
TN	Treatment-naive
TVR	Telaprevir
UTR	Untranslated region
VEL	Velpatasvir
VLDL	Very low density lipoprotein
VOX	Voxilaprevir
vs.	Versus
WHO	World Health Organization

1. Introduction

1.1. Epidemiology of Hepatitis C virus

Hepatitis C virus (HCV) is considered to be the leading cause of hepatocellular carcinoma (HCC) and further co-morbidities such as cirrhosis and other chronic liver diseases.

A 2013 Global Burden of Disease Study (GBD) described an estimate of 184 million people positive for anti-HCV antibodies of which 130 – 150 million were chronically infected [1], and about 704 000 HCV associated mortalities were reported in the same year [2]. More recent data from the GBD 2017 [3] estimate that about 158 million people are seropositive for HCV, being remarkable that China alone accounts for almost a third of the HCV global prevalence, namely more than 45 million seropositive people (Figure 1.1). The rise of newly and improved HCV screening assays resulting in less false-positive results may have been a determinant factor for this lower estimate. Moreover, the number of HCV associated mortalities has also seen a decrease over the years to about 489 000 reported deaths, of which more than 159 500 were due to HCV related liver cancer [4].

Overall, there is an estimated prevalence of HCV seropositive individuals of about 1,51% in Western Europe [3], and recent data from Portugal carried out on a nationwide population-based cross-sectional study accounting for more than 1 600 Portuguese individuals, has demonstrated an estimated prevalence of 0,54% [5]. Having into account that the adult general Portuguese population is estimated to be about 8 600 000 individuals and the prison population is around 15 000, it would be expected that approximately 48 780 of the total Portuguese population were anti-HCV positive [6]. However, a 2013 report from the Portuguese Department of Prison Services had showed significantly higher prevalences of 15,6% for hepatitis C infection in incarcerated individuals only [6]. Along these lines, there is an underlined low endemicity for hepatitis C infection in the general Portuguese population, in contrast to a very high prevalence in risk groups such as intravenous drug-users and incarcerated individuals. Furthermore, according to the GBD 2017 [3], there is actually an estimated prevalence of more than

194500 people positive for anti-HCV antibodies in Portugal (1,96%), which resulted in 922 HCV associated mortalities back in 2016 [4], consequently overestimating the results of the previously mentioned studies carried out nationwide [5,6].

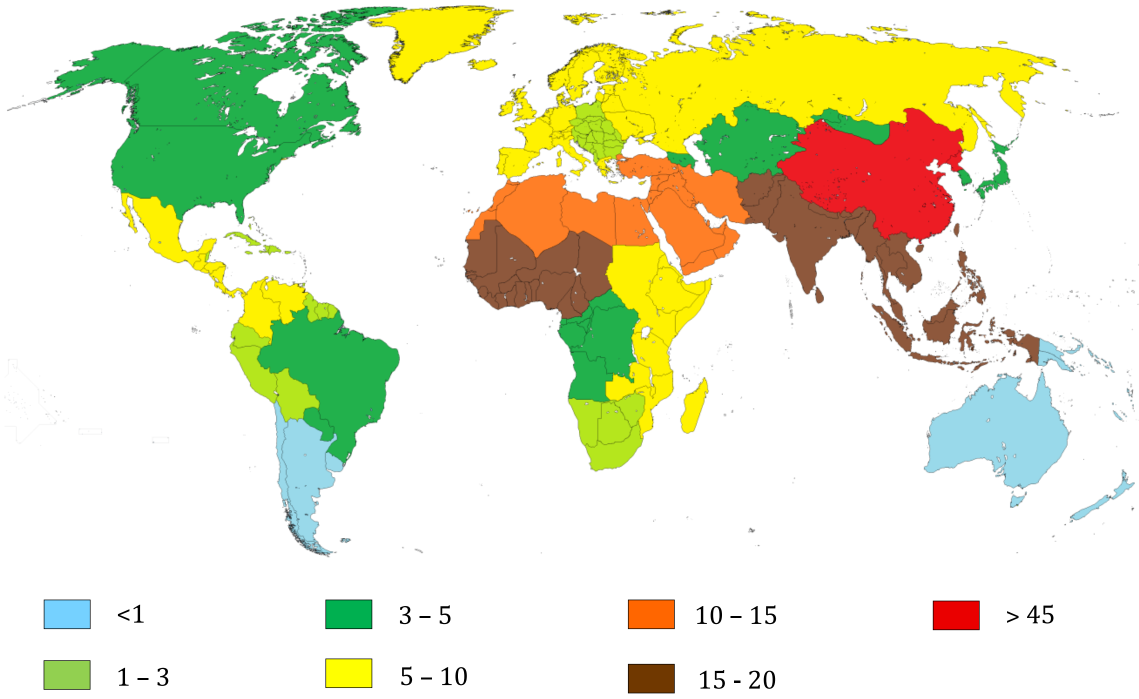


Figure 1.1 Worldwide estimated people with anti-HCV antibodies in 2016 (in millions), in 21 GBD regions. Data obtained from [3,7].

1.2. HCV and the *Flaviviridae* family

1.2.1. Taxonomy and Baltimore classification

Hepatitis C virus of the *Hepacivirus* genus belongs to the *Flaviviridae* family. According to the Baltimore classification, HCV matches the group IV, meaning that is a positive single-stranded RNA virus.

1.2.2. Virus and virion

The RNA genome of HCV is enclosed in an icosahedral capsid surrounded by an envelope with 2 types of glycoproteins, E1 and E2. The genome of HCV bears a length of approximately 9600bp [8] which translates about 3000 amino acids.

The virion presents itself in a spherical form with a diameter of approximately 40 – 80nm in the blood of the infected patients [9]. Moreover, in cell culture systems the size of the virions may also vary within this range [10].

Whilst in the blood of the infected patient, HCV presents itself in a unique form of lipoviroparticle (LVP), seeing that the low density lipoproteins (LDL) and very low density lipoproteins (VLDL) components of this particle are thought to be involved in facilitating virus entry into the cell, as well as in immune system evasion [11].

1.2.3. HCV and *Flaviviridae*

Flaviviridae viruses share some similar characteristics amongst themselves, such as a lipid bilayer with two or more types of envelope glycoproteins, which surrounds a nucleocapsid that encloses the positive single-stranded RNA genome with a length that ranges from 9600bp to approximately 12300bp. Moreover, these viruses are known to have an ORF which translates at least 3000 amino acids, leading to the encoding of structural and non-structural proteins. [12]

1.2.3.1. Structural and pathophysiological differences between HCV and other flaviviruses

While most flaviviruses depend on capping of the 5' region for translation initiation [13], HCV approach resides in the assembly of an internal ribosome entry site (IRES) by folding the 5'UTR into a RNA secondary structure and partially joining it to the domain that encodes for the core region. In this way, it´s possible to create a stable pre-initiation complex by binding the ribosomal subunits with the cellular factors without the need for additional translation initiation factors in the construction of the HCV polyprotein [12,14].

On the opposite side of the genome, HCV displays a less structured and relatively short 3'UTR, when comparing to a highly elaborate structure in the case of other flaviviruses. [12]

1.3. HCV replication cycle

1.3.1. Cell binding and entry

Hepatitis C virus exhibits a restrict tropism as a result of infecting mainly hepatocytes which explains the related hepatic maladies.

The first step in HCV replication cycle resides in cell entry through the binding of envelope glycoproteins E1 and E2 to the glycosaminoglycans cellular receptors [15,16]. Consequently, the low pH inside the endosomal compartment will induce conformational changes in the surface glycoproteins E1 and E2 leading to the fusion of membranes and capside internalization into the cytoplasm via a clathrin mediated endocytosis [17,18].

1.3.2. Genome translation and post-translational processing

The HCV RNA genome located in the cytoplasm after decapsidation is recognized by cellular ribosomes as mRNA, being directly translated into a precursor polyprotein via IRES domains II to IV [19,20]. Thanks to a process of post-translational maturation in the endoplasmic reticulum, this polyprotein is ultimately cleaved by cellular and viral proteases into two structural proteins and eight non-structural proteins.

Translation modulation is facilitated by viral (NS2, NS3, NS4A, NS4B) and host factors involved in the assembly of ribosomes and in the recognition of 5'UTR IRES [21–23]. Moreover, IRES plays an important role in capping independent translation modulation by recruiting viral and cellular proteins, including the initiation factors eIF2 and eEF3 [24,25].

1.3.3. Genome replication mechanism

HCV genome replication occurs once the amount of viral proteins produced is sufficient, and is carried out on the surface of the endoplasmic reticulum (ER) in cytoplasmic viral factories i.e. specific intracellular membrane rearrangements [12,26].

The NS5B viral protein is a RNA-dependent RNA polymerase (RdRp) responsible for viral replication by producing copies of negative-sense single strands of RNA which in turn will serve as templates for the synthesis of genomic RNA strands acting both as mRNA for translation of the polyprotein, synthesis of new intermediates for replication, and as genomes for exiting viral particles [27]. Furthermore, several viral and host proteins such as cyclophilin A and B are known to regulate the synthesis *de novo* of the viral genome by binding the RdRp to the RNA template [28], taking into account that HCV genomic strands are transcribed in an excess of 5x to 10x when compared to the antigenomic strands [12].

1.3.4. Virions assembly and release

The assembly of the progeny virions occurs in the ER and is facilitated by the viral p7 ion channel. The process begins with the relocation of genomic RNA and viral proteins to cytoplasmic organelles designated as lipid droplets, and is regulated by both viral NS5A and host proteins [29]. Subsequently, the NS2 viral protein will allow the viral capsids to acquire an envelope along with the E1 and E2 glycoproteins through budding of the ER membrane [30].

Finally, the new viral particles will undergo on a maturation process via association with lipoproteins, leading to the exocytosis of progeny virions as lipoviroparticles [31].

1.4. HCV transmission routes

Hepatitis C virus is transmitted exclusively between humans typically by blood and/or sexual contact thus demonstrating a narrow specificity for the host. Although the risk of HCV transmission via sexual contact is quite real, it is highly reduced in heterosexual couples without a pre-existent HIV infection [32]. Children born to mothers with HCV infection are also at risk by exhibiting vertical transmission rates of approximately 4 – 8%, which are substantially higher in infants born to HIV co-infected mothers, namely 10,8 – 25% [33,34], and unlike with HBV and HIV, there are no interventions to reduce the risk of vertical HCV transmission during pregnancy [35].

Since HCV was only discovered in 1989 by means of molecular biology tools [36], the majority of cases linked to healthcare until then were due mainly to blood transfusions without a proper pre-screening test and also to procedures like renal dialyze [37,38]. However, injection malpractices by risk groups denominated as people who inject drugs (PWID) have been recently demonstrated to constitute the majority of hepatitis C virus infections since then, namely in developed countries [39].

1.5. Natural course of HCV infection

After exposure to HCV, an incubation period of 2 to 12 weeks leads to an acute infection of which only 10 – 15% of the cases are symptomatic. However, 55 – 85% of the acute cases can progress to a chronic infection, while the remaining 15 – 45% are capable of a spontaneous clearance within 6 months in the absence of any treatment [40].

Failure to treat the chronic infection may initially lead to morbidities such as liver failure and fibrosis, and later to a risk of developing cirrhosis of 15 – 30% in 20 years [41–43]. Consequently, the risk of developing a hepatocellular carcinoma in patients with cirrhosis is about 1 – 7% per year, according to the severity of fibrosis amongst other factors [40].

Recent data suggests that when comparing to non-treated patients, the risk of HCC and other liver morbidities as well as HCV associated mortality are significantly reduced the earlier a sustained virological response (SVR) is achieved [44–46].

The spontaneous clearance of HCV may be associated with host-related factors such as race, weight, the female gender, a young age, and polymorphisms in the IL28B gene. However, the presence of these criteria cannot guarantee with certainty a spontaneous viral clearance in the patient [40].

1.6. HCV genome structure and functions

The HCV genome is comprised of two ORFs flanked by the 5' and 3' UTRs. The first ORF encodes for the HCV polyprotein which is then cleaved into 10 distinct proteins, including 3 structural proteins and 7 non-structural proteins (Table 1.1), whereas the second ORF produces the F protein which is thought to be involved in immune system evasion [47].

1.6.1. Structural proteins

The 5'UTR is of great importance in the capping-independent translation initiation of genomic RNA thanks to the action of a secondary structure with four different stem-loops termed IRES [48]. This region comes very useful in HCV genotyping, seeing that is highly conserved amongst the several genotypes.

The 3'UTR-end is a variable region, set up by a poly U/UC and a 3'X region, and despite being in the opposite side of the genome this region also plays an important role by transferring components from the cellular machinery to the 5'-end, thus potentiating translation [49].

Table 1.1 Hepatitis C virus structural and non-structural proteins.

Hepatitis C virus proteins	Main functions
Structural proteins	
Core	Icosahedral capsid protein
E1	Fusion of membranes and virion internalization
E2	Binding to the cell
Non-structural proteins	
p7	Ion channel formation and assembly of progeny virions
NS2	Viral protease Assembly of progeny virions
NS3	Viral protease Modulation of HCV translation
NS4A	Helicase - role in the viral RNA replication mechanism Co-factor of NS3
NS4B	Modulation of HCV translation
NS5A	Assembly and release of virions Membranous web induction
NS5B	Formation of the replication complex RdRp - replication of the HCV RNA genome

1.6.2. Non-structural proteins

1.6.2.1. NS3/4A protease

The NS3 gene has a length of 1893bp and encodes for the viral protease with 631 amino acids and about 69 kDa (Figure 1.2) [12,50].

NS3 contains a serine protease domain in its N-terminal, while NS4A is a cofactor of NS3 protease activity assuming as the helicase domain of the NS3 multi-functional protein. Overall, the NS3-NS4A protease is essential for the HCV replication cycle, as it catalyzes HCV polyprotein cleavage at the several non-structural proteins junctions, and also exhibits additional properties through its interaction with host cell pathways.

1.6.2.2. NS5A

The NS5A gene has a length of 1344bp and encodes for a phosphorylated zinc-metalloprotein with 448 amino acids and about 56–58 kDa (Figure 1.2) [12,51].

NS5A consists of three domains and does not exhibit any enzymatic activity. However, it plays an important role in the assembly and release of virions (domain III) as well as in viral replication (domains I and II), namely in transcription activation [52,53].

The NS5A complex is also involved in the regulation of cellular pathways by demonstrating several interactions with host proteins, and plays an additional role in immune system evasion, namely in the resistance to IFN-α via binding and blocking of the antiviral effector PKR [54,55].

1.6.2.3. NS5B polimerase

The NS5B gene has a length of 1773bp and encodes for a protein with 591 amino acids and about 68kDa, corresponding to the RdRp (Figure 1.2) [12,56]. The NS5B polymerase belongs to a class of membrane proteins [57,58] and is structurally arranged in a "right hand motif" containing palm and thumb domains [59]. Moreover, it plays an important role in the replication complex formation [60].

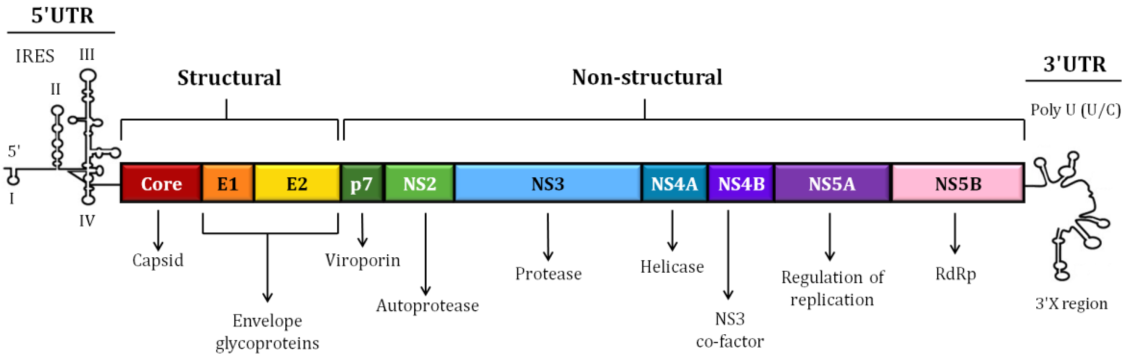


Figure 1.2 Hepatitis C virus genome and polyprotein structure, flanked by the 5' and 3' UTRs consisting of IRES and a poly U (U/C) tract, respectively.

1.7. High genetic variability of HCV and its implications

HCV is highly diversified and demonstrates a geographic distribution being currently divided into 7 genotypes and more than 80 known subtypes. However, current therapeutic regimens are only designed for genotypes 1 – 6 since genotype 7 is of less clinical relevance [61,62]. In this way, each genotype and subtype of HCV is associated with a geographic location, transmission risk, the prevalence and level of genetic diversity [63], as to the baseline resistance-associated substitutions (RASs) towards direct-acting antivirals (DAAs) [64].

The high genetic variability of HCV can first be explained over the NS5B polymerase lack of proofreading associated to a tremendous rate of mutations (10^{-4} – 10^{-5} per copied nucleotide) [44], and to the enormous viral turn-over (10^{10} – 10^{12} virions per day) [65–67] about 100x superior to HIV [68], hence why HCV replicates as a population of quasispecies i.e. a heterogeneous mixture of closely related genomes with a homology superior to 98% which may be present in the same individual [69].

The genetic barrier to resistance is of major importance when dealing with the high genetic variability of HCV in treatment selection, since it is assumed as the number and type of nucleotide changes required to result in amino acid substitution(s) that confer resistance to the antivirals [70], and may vary both within and between the classes of DAAs according to the genotype and subtype of HCV [71,72].

1.7.1. HCV geographic distribution

Genotype 1 is the most prevalent, globally accounting for 83,4 million (46,2%) of all HCV infections, while HCV GT3 constitutes the second most common with 54,3 million (30,1%) cases worldwide, followed by genotypes 2, 4, 6 and 5 accounting for 16,5 million (9,1%), 15 million (8.3%), 9,8 million (5.4%) and 1,4 million (<1%) of the cases, respectively [73]. As such, treatment is immediately recommended after confirming a GT1 HCV infection [41].

It is notable that genotypes 1 and 3 seem to have a worldwide geographic distribution as well as genotype 2 to some extension, while genotype 4 appears to be mainly found in central sub-Saharan Africa and Middle East (Figure 1.3). Genotypes 5 and 6 are restricted to Southern sub-Saharan Africa and Southeast Asia, respectively.

In Portugal, just like at the global level, GT1 is the most prevalent accounting for more than 60% of all HCV infections in Portugal, whereas GT3 occupies the second place with 25% of the infections, followed by GT4 (7%) and GT2 (2%) [74].

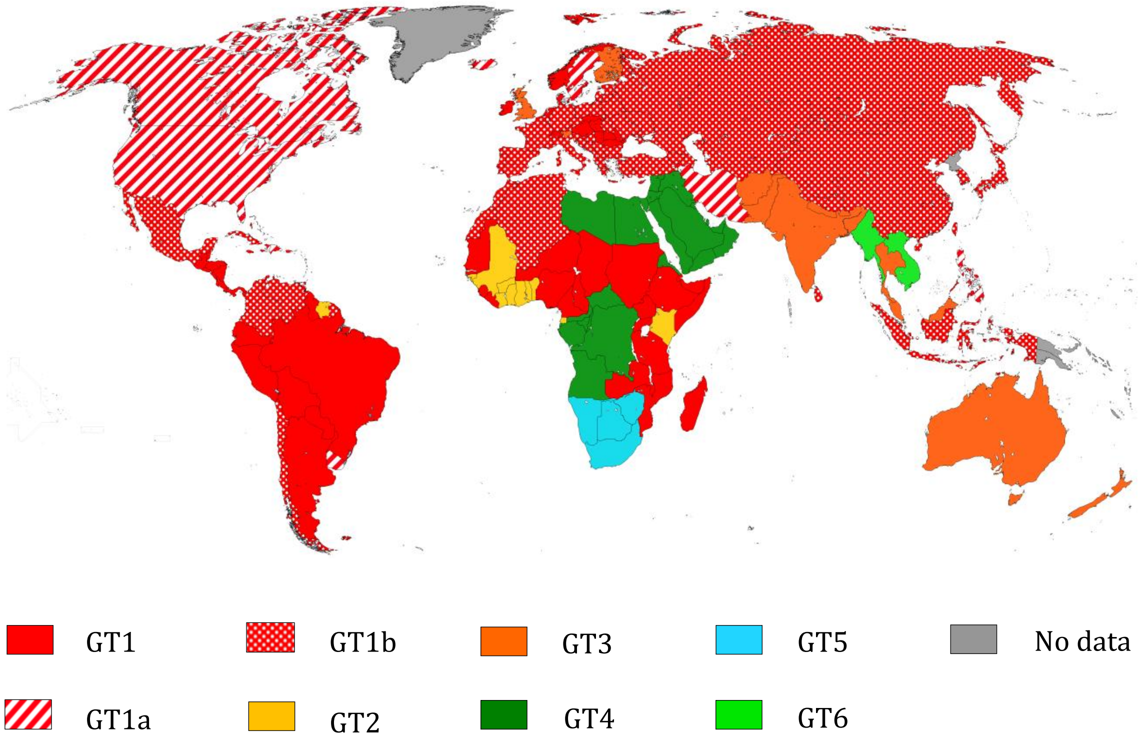


Figure 1.3 Worldwide predominance of HCV genotypes in 21 GBD regions. HCV genotypes 1a and 1b are shown in red just as HCV genotype 1, though additionally differentiated as stripes or dots to indicate the prevalent subtype, respectively. Countries and/or GBD regions in which no data was reported are visualized as grey. Data to construct this map was obtained and adapted out of extensive literature research dating from 2013 to 2017 [73,75–79].

1.8. Screening of HCV infection

Screening for HCV infection should start with a serological test in order to detect anti-HCV antibodies, and if positive is followed by a nucleic acid test (NAT) to search for HCV RNA so confirming the diagnosis of an active infection [41,80]. The NAT is based on a sensitive molecular method, and as the presence of anti-HCV antibodies only demonstrates that there has already been a past exposure to the virus it becomes thus indispensable for the confirmation of the chronic infection, since there are no serological markers that prove that HCV infection is in an acute phase acquired *de novo* [40]. When quantitative HCV RNA tests are not available or accessible, a recommended alternative is the measurement of the HCV core antigen levels by means of an enzyme-linked immunosorbent assay (EIA) [81,82].

The diagnosis of acute HCV infection is performed through an EIA and can only be confirmed with certainty when a seroconversion of the anti-HCV antibodies is achieved. Moreover, the EIA may turn out negative when performed during an early acute infection or when in patients on a profound immunosuppressive state [83].

HCV re-infection is much less common than relapse but not less severe, as it tends to occur essentially in patients at a high risk of infection following spontaneous HCV elimination or after an induced treatment. Re-infection is defined as the reappearance of HCV RNA in the blood at least 6 months after a SVR is achieved, having to demonstrate simultaneously that the new infection is caused by a different strain of HCV. This can be confirmed when the new strain belongs to a different genotype, or if identical, through phylogenetic analysis by checking the genetic distance between the strains in question [40].

1.9. Genotyping HCV: a prognostic tool for treatment selection

HCV genotyping before treatment initiation is currently mandatory to assist physicians in the selection of the most appropriate IFN-free regimen for each specific case [40]. Although some next-generation DAAs already exhibit a pan-genotypic activity, most of the precedent drugs did not demonstrate an equal antiviral activity between all genotypes or even subtypes [84,85], and as such the correct assignment of the HCV genotype is necessary in order to reduce the risk of therapeutic failure [86].

It is well known that HCV genotypes/subtypes play an important role in the response to treatment [87], and several phase III studies [88–91] have demonstrated that intra-genotypic SVR rates in GT1 are generally lower for GT1a than for GT1b according to the selected regimen, subtypes prevalence and to the presence of RASs. However, when mentioning sofosbuvir (SOF) the situation is reversed, seeing that lower SVR rates were observed on treatment-naïve (TN) patients infected with GT1b HCV [92]. The HCV subtyping of GT1a and GT1b should employ a highly accurate test using the 5'UTR with the partial addition of another genomic region, usually the core or the NS5B [40,93].

In the case of mixed infections by different HCV genotypes, only one of them prevails and their impact on potential RASs and SVR rates when using DAAs regimens should be considered [94]. However, these super-infections are rare and occur mainly in intravenous drug users (IVDU) and in patients undergoing hemodialysis or multiple transfusions [95].

1.9.1. HCV genotyping assays

Several commercial assays are currently available to assign the HCV genotype, most of which are oriented to the well characterized and highly conserved 5'UTR. However, since the single use of this genomic region proved to be insufficient for the correct genotyping of all HCV strains [96], the two most frequently used tests are "Abbott Real-time HCV Genotype II" and "INNO-LiPA HCV Genotyping 2.0", which are further directed to the NS5B and core genes, thus providing additional subtype information [97]. Nonetheless, even with the use of increasingly accurate and rigorous genotyping tests, it is common to have indeterminate, unspecific, mixed or invalid cases due to low viral loads, sample inhibitions or even to unknown factors, and under these circumstances genotyping by sequencing can constitute an alternative approach when traditional methods do not work.

1.9.2. HCV genotyping by sequencing the NS5B gene

NS5B sequencing allows an accurate genotyping of HCV, as 53 – 55% of this gene amino acids are conserved amongst all HCV genotypes [98,99]. In addition, a portion of the NS5B gene with 329bp, named Okamoto region (from nt 8282 to 8610 in the H77 reference genome) becomes particularly useful in HCV genotyping for its ability to subtype with a higher level of discrimination, as well as to represent the entire genome of HCV. Moreover, sequencing data obtained from the Okamoto region can lead to the construction of more accurate phylogenetic trees regarding whole-genome and/or polyprotein analysis, when comparing to data obtained from the 5'UTR [100].

Recent studies suggest that the use of the NS5B region may be preferential to the 5'UTR when genotyping by sequencing [101,102]. Moreover, sequencing becomes an added value since it is able to provide additional information on the resistance profile for each patient, and allows the distinction of a relapse from a re-infection through phylogenetic analysis [79,101].

Although NS5B sequencing costs may vary according to each commercial platform and technique, this technique not only allows a very accurate genotyping of HCV but also prevents future therapeutic failures by giving an insight of potential RASs that may be present at baseline prior to treatment initiation.

1.10. Antiviral therapies for HCV infection

1.10.1. Treatment of HCV acute infection

After exposure to HCV, patients are associated with high rates of chronicity, so a treatment should be followed in order to prevent a chronic hepatitis C infection [40].

There is currently no antiviral therapy indicated for post-exposure prophylaxis in the absence of a documented HCV transmission [40]. Along these lines, the ideal estimated starting point for acute hepatitis C treatment is based at the onset of alanine aminotransferase (ALT) elevation, with or without symptoms [103,104], and should use the recommended combination of SOF + LDV for genotypes 1, 4, 5 and 6, or SOF + VEL or SOF + DCV for all genotypes, during a period of at least 8 weeks without RBV [40].

A small number of patients with acute GT1 HCV infection were treated with an IFN-free regimen based on SOF + LDV, exhibiting SVR rates of 93% (13/14) in mono-infected IVDU after 4 weeks of treatment, SVR rates of 77% (20/26) in HIV/HCV co-infected patients after 6 weeks of treatment [105], and SVR rates of 100% in mono-infected non-IVDU after 6 weeks of treatment [106]. As such, patients co-infected with HIV and acute HCV should be treated with the same recommended regimens for HCV acute mono-infection but for an extended period of 12 weeks [40].

1.10.2. Treatment of HCV chronic infection

The primary goal of HCV treatment is to achieve a SVR, which is defined as an undetectable viral load in the blood 12 (SVR12) or 24 (SVR24) weeks after the end of treatment, seeing that the infection is cured in more than 99% of patients who achieve a SVR [40,107].

The secondary goal of HCV treatment is achieved through a SVR and is associated to the prevention and reversion of HCV related hepatic and extrahepatic complications, including hepatic necroinflammation, cirrhosis and fibrosis [40]. Viral clearance following SVR not only is also associated with the normalization of liver enzymes and liver failure reduction, as well as with a decreasing risk of HCC, all-cause mortality and other comorbidities, although to a lesser extent regarding HIV/HCV co-infected patients, but also comes with other more personal and even economical advantages such as an improved quality of life and work productivity [40,108–110].

SVR12 and SVR24 should be evaluated through a sensitive molecular method with a low limit of detection (LOD) ($\leq$15 IU/ml) [40]. However, an undetectable HCV core antigen after the end of treatment (EoT) may also be used as an alternative to HCV RNA screening, when in patients with a detectable core antigen before treatment initiation [111].

1.10.3. Advancement of therapy options

Prior to 2011, the only therapeutic options for HCV chronic infection were based on a combination of pegylated interferon-α (pegIFN-α) with ribavirin (RBV) for 24 – 72 weeks. However, this therapy was associated with severe adverse effects and low SVR rates of 30 – 70% [112,113] ranging between mono-infected and HIV/HCV co-infected patients, according to the IL28B polymorphisms and to the HCV genotype, the last being the most relevant predictor of treatment outcome [114].

Since 2014, IFN-free regimens constitute the best treatment options in chronic hepatitis C infection for TN and treatment-experienced (TE) patients with compensated or decompensated liver disease as a result of the ease of use through all-oral DAAs, increased tolerability showing fewer adverse effects, the lower presence of potential RASs and mainly due to the dramatic increase in viral suppression demonstrating SVR rates above 90% [40,76,88,115–117].

Although DAAs have been shown to be more effective in viral suppression when compared to IFN-based regimens, they are still not 100% effective, namely due to low adherence to treatment and factors related to the progression of liver disease (e.g. high-level fibrosis), as well as to the presence of baseline RASs that may lead to potential therapeutic failures.

1.10.4. Direct-acting antivirals

There are currently approved four classes of DAAs aimed for the non-structural proteins NS3/4A, NS5A and NS5B (Table 1.2). Each class varies in efficacy according to the virological response rates, adverse effects, drug-drug interactions, the genotype of HCV, and to potentially associated RASs [84,118]. As such, the current treatment strategies consist of developing pipeline DAAs with a pan-genotypic activity, in addition to an increasingly potency in order to avoid future relapses.

Table 1.2 Licensed direct-acting antivirals (DAAs) since 2011 and associated sites of action. DAAs respective abbreviations are in parenthesis.

NS3/4A inhibitors	NS5A inhibitors	NS5B nucleotide inhibitors	NS5B non-nucleotide inhibitors
Telaprevir (TVR)	Daclatasvir (DCV)	Sofosbuvir (SOF)	Dasabuvir (DSV)
Boceprevir (BOC)	Ledipasvir (LDV)		
Simeprevir (SMV)	Ombitasvir (OBV)		
Asunaprevir (ASN)	Elbasvir (EBR)		
Paritepravir (PTV)	Velpatasvir (VEL)		
Grazoprevir (GZR)	Odalasvir (ODV)		
Voxilaprevir (VOX)	Pibrentasvir (PIB)		
Glecaprevir (GLE)	Ruzasvir (RZR)		

Since the transmission routes of HIV, HBV and HCV are identical, it's not unusual to see these co-infected patients being treated simultaneously for both infections, however if so it must be done with caution since the large multiplicity of HCV antivirals can lead to potentially complex drug-drug interactions. Along these lines, all patients selected for a therapy with DAAs require a risk assessment of drug-drug interactions prior to treatment initiation [40].

1.10.4.1. NS5A inhibitors

Although the NS5A inhibition mechanism is yet to be fully elucidated, it basically consists on an interaction between the inhibitors and the domain I of the dimer [53].

Present NS5A inhibitors exhibit a high genetic barrier to resistance when compared to the first generation precedents, though not as high as the nucleoside inhibitors (NIs) of the NS5B polymerase [119]. Moreover, pipeline NS5A inhibitors have been increasingly displaying a more comprehensive coverage for all HCV genotypes, as well as higher potencies which in part is related to a synergistic activity with several other HCV inhibitors [81,120].

1.10.4.2. NS5B polymerase nucleoside inhibitors

RdRp is yet another important target to consider in the development of advanced HCV antivirals [12,121]. NIs act via mimicking of the natural substrates which are incorporated into the elongating RNA strand, thus interacting directly with the active site of the polymerase, and ultimately ending the RNA elongation mechanism [102,118].

NIs possess the highest genetic barrier to resistance of all DAA classes, not only because the associated NS5B RASs have a low probability of existing at baseline, but also because these inhibitors require at least three or more nucleotide changes and/or transitions in order to confer a resistance associated mutation [118,122,123]. Several studies have additionally demonstrated the relevant pan-genotypic activity of NIs, mainly due to the highly conserved polymerase catalytic site amongst all HCV genotypes and subtypes [102,124].

1.10.4.2.1. Sofosbuvir

Sofosbuvir was approved in the USA and in Europe back in 2013 and 2014, respectively [125,126], and possesses a pan-genotypic antiviral activity [98] along with a high genetic barrier to resistance [127], which has been fully tested in several clinical trials which demonstrated the absence of RASs through viral breakthrough both in monotherapy and combination regimens with other recommended DAAs [117,128–130], thus proving that the majority of NS5B RASs are indeed of relatively low importance [129,130].

A large phase III study back in 2013 [92], demonstrated the high efficacy of a SOF monotherapy with pegIFN-α + RBV in GT1 infected patients whom never experienced treatment, by exhibiting a SVR rate of 89%. However, as the safety and pharmacokinetics of the metabolites derived from SOF are still being studied, this drug should be used with caution in patients with a severe renal impairment [40].

1.10.4.3. NS5B polymerase non-nucleoside inhibitors

Non-nucleoside inhibitors (NNIs) suppress the NS5B polymerase activity via mechanisms of allosteric inhibition, and can be subclassified according to their allosteric binding sites (Palm 1, Palm 2, Thumb 1, and Thumb 2) [131]. Furthermore, NNIs possess a low genetic barrier to resistance when comparing to NIs [119].

1.10.5. Selection of genotype-dependent therapies

1.10.5.1. Sofosbuvir and ledipasvir combined regimens

The regimen of harvoni (combined fixed dose of SOF and LDV in a single tablet, represented as SOF/LDV) for 12 weeks, with or without ribavirin, is one of the preferred therapies recommended for the treatment of DAA-naïve patients infected with GT1 HCV, with or without compensated cirrhosis (Table 1.3). However, this regimen should be extended to 24 weeks without RBV, when in patients with contraindications or low tolerance for RBV [40].

Due to the high rates of SVR (> 96%) as well as higher adherence rates over the improved safety both on TN and TE patients, a treatment with SOF/LDV ± RBV is therefore recommended for patients infected with HCV of GT1, 4, 5 and 6, with or without cirrhosis [41].

It has been demonstrated that only high risk RASs appear to have an impact on the response to treatment [132], when exposing an HCV infected patient to an IFN-free therapy based in SOF/LDV ± RBV for 12 weeks. Along these lines, optimal SVR rates of 94 – 97% were reported in several clinical trials directed for TN and TE patients infected with GT1 HCV [117,129,130], additionally with no decrease in the SVR rates even when patients had an advanced liver disease status [133].

A combined regimen of SOF + LDV for the treatment of GT1 HCV infection has been proven to be efficient by exhibiting high SVR rates in several clinical trials:

- In the ION-1 clinical trial [129], a 12 weeks regimen with SOF + LDV, with or without RBV, was submitted in TN patients infected with GT1 HCV, leading to SVR12 rates of 97% (211/217) and 99% (211/214), respectively, even though there were later reported two relapses associated with baseline NS5A RASs.

When treatment was extended to 24 weeks, SVR12 rates were of 99% (215/217) and 98% (212/217), with or without RBV, respectively, including the cirrhotic and non-cirrhotic patients for both treatment extensions.

- In the phase III ION-2 clinical trial [117], a 12 weeks regimen of SOF + LDV, with or without RBV, was submitted alternatively in patients previously treated with pegIFN-α ± RBV or a regimen with protease inhibitors (PIs), leading to slightly lower SVR12 rates of 96% (107/111) and 94% (102/109), respectively. When treatment was extended to 24 weeks, the SVR12 rates went up to 99%, with or without RBV, respectively, having increased significantly for both cirrhotic and non-cirrhotic patients.
- For the phase III ION-3 study [130], a SOF + LDV regimen was compared for extensions of 8 vs. 12 weeks in 647 non-cirrhotic TN patients infected with GT1 HCV, leading to SVR12 rates of 93% (201/216) and 94% (202/215) for 8 weeks with or without RBV, respectively, vs. 95% (206/216) for 12 weeks without RBV, proving that although this shorter in duration regimen isn´t so efficient as a 12 weeks extension, it can still be performed with a high success rate in non-cirrhotic TN patients with baseline HCV RNA levels < 6 million IU/ml (as determined by Roche Cobas Taqman HCV assay). Furthermore, it was also reported that the presence of any baseline NS5A RASs was not associated with any relapses.
- In the SOLAR-1 [134] and SOLAR-2 [135] studies, a combined regimen of SOF + LDV with RBV was submitted alternatively for 12 and 24 weeks in patients infected with GT1 and GT4 HCV, whom exhibited an advanced stage of liver disease or recurrence post liver transplantation, demonstrating high rates of SVR12 up to 100% and 94% for GT1 and GT4, respectively.
- In the phase II SIRIUS clinical trial [136], patients with compensated cirrhosis infected with GT1 HCV, whom had previously failed a treatment with PIs, were then treated with a fixed dose of SOF + LDV without RBV for 12 or 24 weeks, achieving SVR12 rates up to 96% (74/77) and 97% (75/77), respectively.
- In the phase IIb ERADICATE study [137], a fixed regimen of SOF + LDV for 12 weeks was submitted in non-cirrhotic TN patients co-infected with HIV and GT1 HCV, leading to SVR12 rates of 97% (36/37) and 100% (13/13), when on or off antiretroviral therapy, respectively.

Table 1.3 Efficacy of current valuable IFN-free treatment regimens approved in Europe since 2013, according to HCV genotype. DAAs based regimens highlighted in green represent optimal efficacies corresponding to SVR rates >90%, whereas the ones marked in orange represent suboptimal efficacies corresponding to SVR rates of 75 – 90%. Combined regimens highlighted in red are currently not approved due to low efficacies <75% or insufficient *in vivo* data. Data adapted and gathered from [40,79] and [138–140], respectively. All DAAs are indicated by their abbreviations, and DAAs combinations commercial designations are in parenthesis.

Combined regimens	GT1	GT2	GT3	GT4	GT5 and 6
2013					
SOF + RBV	Not approved	Suboptimal	Suboptimal	Not approved	Not approved
2014					
SOF + SMV ± RBV	Suboptimal	Not approved	Not approved	Optimal	Not approved
SOF + DCV ± RBV	Optimal	Optimal	Optimal	Optimal	Optimal
SOF/LDV (Harvoni®) ± RBV	Optimal	Not approved	Not approved	Optimal	Optimal
OMB/PTV/Ritonavir (Technivie™ or PrO) ± RBV	Not approved	Not approved	Not approved	Optimal	Not approved
OMB/PTV/Ritonavir + DSV (VIEKIRA XR™ and PAK™, or PrOD or 3D) ± RBV	Optimal	Not approved	Not approved	Not approved	Not approved
2015					
GZR/EBV (Zepatier™) ± RBV	Optimal	Not approved	Not approved	Optimal	Not approved
2016					
SOF/VEL (Epclusa®) ± RBV	Optimal	Optimal	Optimal	Optimal	Optimal
Pipeline*					
SOF/VEL/VOX (Vosevi®)	Optimal	Optimal	Optimal	Optimal	Optimal
GLE/PIB (Mavyret™)	Optimal	Optimal	Optimal	Optimal	Optimal

*DAAs already licensed and approved in Europe, but still in development.

In a recent Portuguese prospective study, 113 cirrhotic GT1 – 4 HCV patients co-infected with HIV (77% accounting for GT1) were treated with SOF/LDV ± RBV or other regimens, achieving SVR12 rates of 93,8%, ultimately resulting in a significant improvement of hepatic function [141].

Another Portuguese study comparing the efficacy of therapies with DAAs between 103 HCV mono-infected and 261 HIV/HCV co-infected patients, with or without cirrhosis, also demonstrated high SVR rates. GT1 was higher in HIV/HCV co-infected patients (52,4% vs. 75,9%, for mono-infected and co-infected patients, respectively), whereas GT3 was higher in HCV mono-infected patients (32% vs. 11,1%, for mono-infected and co-infected patients, respectively), and therapies mostly included SOF + LDV for 73,8% and 87,4% of mono-infected or co-infected patients, respectively, but alternative regimens based in SOF + DCV, pegIFN + SOF or 3D therapy (OMB/PTV/Ritonavir + DSV) were also used, resulting overall in SVR rates of 95,1% vs. 94,6% for mono-infected and co-infected patients, respectively [142].

1.10.5.2. Sofosbuvir and velpatasvir combined regimens

SOF and VEL are currently available in a single tablet fixed-dose combination named epclusa (represented as SOF/VEL). Several studies have already described the high efficiency of epclusa for 12 or 24 weeks in HCV infected patients, with or without cirrhosis, and when comparing to harvoni it is associated to higher SVR rates with the additional advantage of a pan-genotypic activity, thusly being a preferential first-line treatment option recommended by EASL 2016 guidelines for patients infected with genotypes 1 through 6 (Table 1.3) [40].

A joint analysis over the therapeutic efficacy of epclusa-based regimens revealed high SVR rates for all HCV genotypes [143] regardless of the presence of a co-infection with HIV, cirrhosis status, or the patient´s therapeutic history [144]. Thusly, a combined fixed dose of SOF + VEL without RBV for 12 weeks is recommended for the treatment of both TN and TE patients, with or without compensated cirrhosis [40].

The pan-genotypic efficacy of a fixed regimen of SOF + VEL has been demonstrated throughout the ASTRAL clinical trials exhibiting high rates of SVR:

- The combined fixed dose of SOF + VEL was firstly tested on the phase III ASTRAL-1 clinical trial [145] in which 328 of 624 patients infected with GT1 HCV (68% TN, 32% TE, 28% DAA-experienced, and 19% with compensated cirrhosis) were treated for 12 weeks without RBV, achieving SVR12 rates of 98% (206/210) and 99% (117/118) in patients infected with GT1a and GT1b, respectively.
- In the ASTRAL-4 [146] the authors evaluated the efficacy of this regimen for 12 weeks with RBV in 78/87 GT1 HCV infected patients with decompensated

cirrhosis, achieving SVR12 rates of 94% and 100% in patients infected with GT1a and GT1b, respectively.

- The fixed dose of SOF + VEL was later tested in HIV/HCV co-infected patients in the ASTRAL-5 clinical trial [147], on which 78/106 TN and TE patients infected with GT1 HCV, with or without cirrhosis, were treated for 12 weeks without RBV, achieving SVR12 rates of 95% (63/66) and 92% (11/12) in patients infected with GT1a and GT1b, respectively.
- In the ASTRAL-2 and ASTRAL-3 clinical trials [148], the authors compared treatments based on SOF + RBV vs. regimens based on SOF + VEL, leading to SVR12 rates of 94% vs. 99%, and 80% vs. 95 %, in patients infected with GT2 and GT3 HCV, respectively, thus demonstrating the superior efficacy of an epclusa regimen over the precedent monotherapy options.

1.11. Confronting HCV drug resistances

1.11.1. Resistance-associated substitutions

RASs consist of polymorphisms in the target amino acids of the antivirals, leading to a suboptimal therapeutic efficacy with DAAs, which in turn makes the viruses less susceptible to its inhibitory activity [149,150].

RASs can be organized according to a number of factors, either by the associated class and HCV genotype (Table 1.4), the level of reduced susceptibility *in vitro*, the limit of detection for every sequencing method, or even regarding their impact on SVR rates [151].

1.11.2. Origin of RASs in the resistance to DAAs

The presence of RASs associated to a reduced susceptibility to DAAs can be essentially explained by a couple of factors critical to the HCV replication cycle, namely:

- The high turn-over of HCV, exhibiting a replicative potential of $10^{10} – 10^{12}$ virions per day, and additionally a short half-life of only 2 to 3 hours in the peripheral blood [65,66].

- Errors in the viral replication mechanism, involving a high rate of mutations (10^{-5} – 10^{-4} per copied nucleotide) associated to the lack of proofreading activity by the viral RdRp [44,66].

Table 1.4 Clinically relevant NS5 resistance-associated substitutions commonly detected at baseline in GT1 HCV infected patients. The number corresponds to the position on the gene, and the letter preceding the number is the wild-type amino acid, whereas the subsequent letter(s) are the amino acid substitution(s) at that position that are associated with NS5A or NS5B inhibitors resistance. Data gathered through extensive literature search [40,78,98,152–158].

HCV genotype	NS5 coding region amino acid position	
	NS5A	**NS5B**
Genotype 1	M 28 A/G/T/V	L 159 F
	Q 30 E/G/H/K/R	
	L 31 M/V	V 321 A
	P 32 L/S	
	H 58 D	C 316 F/H/N
	Y 93 C/H/N/S	

Most RASs are produced at baseline during the viral cycle, and several *in vitro* studies have estimated that whenever a new wildtype HCV is generated there is a 91% probability of presenting a non-mutated genome, 8,7% of carrying a single substitution, 0,4% of carrying two substitutions, and 0,013% of carrying three substitutions [66]. Moreover, it is conceivable that up to 20% of the viral populations may exhibit these mutations right away from the second day of treatment, which explains an average daily production of $8{,}7\times10^{10}$ and $4{,}2\times10^{9}$ resistance-associated variants (RAVs) with a single or double mutation, respectively [66]. However, RAVs can act differently *in vitro* or *in vivo*, and as such the resistance analysis results should always be interpreted within a clinical context, independently of the consequent theoretical changes to the EC_{50} and EC_{90} when RASs are detected [159].

The emergence of RASs in a treatment based with DAAs is essentially associated with virus-dependent factors, the pharmacological component, and host-dependent factors:

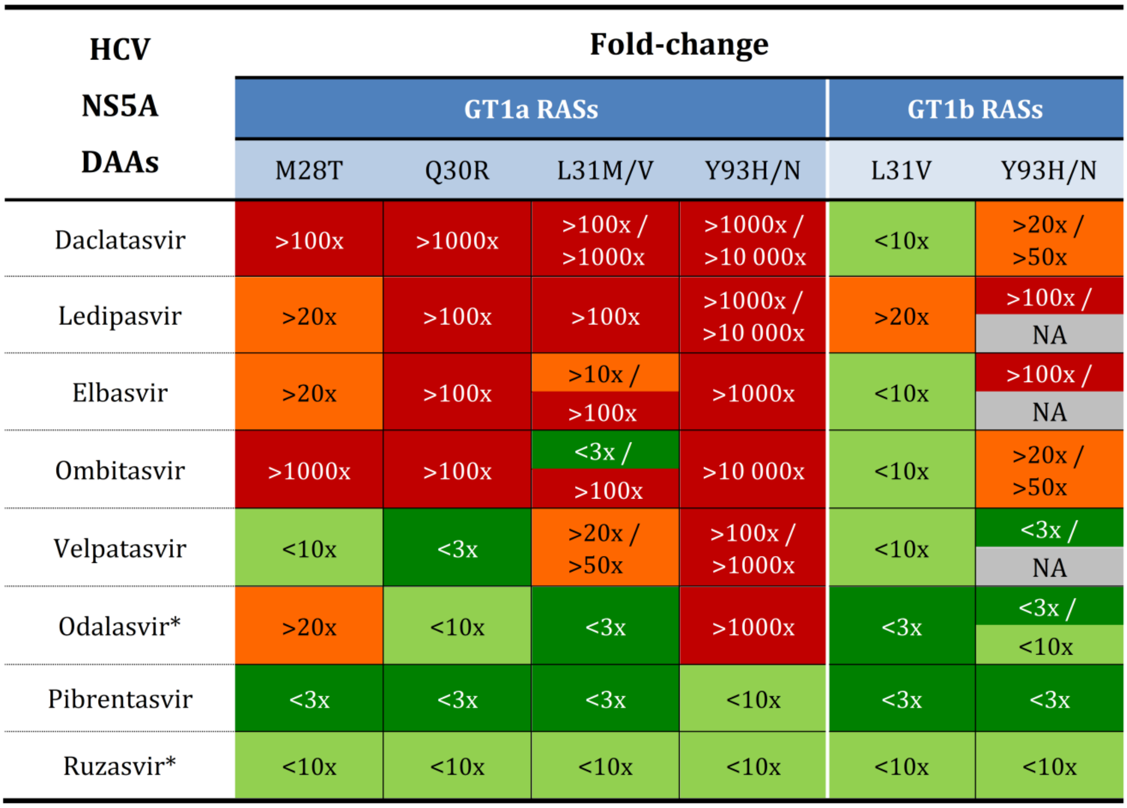
- At the viral level, the frequency and prevalence of RASs are associated mainly with the *in vivo* viral fitness [70], the type of nucleotide changes capable of generating a resistance associated mutation [119], the potential viral breakthrough turning these RAVs into the dominant strains present in the patient [113], the simultaneous occurrence of compensatory or secondary mutations which in turn allows for a more effective replication of the mutant variant [122], and finally to the genotype as well as to the fitness cost since these RAVs normally have a lower replicative capacity than wildtype strains, thus requiring more sensitive methods of detection [70,149,160].
- From a pharmacological point of view, the genetic barrier to resistance of DAAs is perhaps the most important component involved in the presence of RASs, however, the level of exposure to the drug, their potency and pan-genotypic activity are all equally important factors to consider [70,72,123].
- At the host level, the presence of RASs is mainly associated to the immune response, the fibrosis level and hepatic disease progression [149,161], the drug distribution to target cells, $CD8^+$ T cell depletion due to a continuous exposure to the virus [162,163], relapse from a previous treatment [124], and even due to genetic factors such as the male gender and the non-CC IL28B polymorphisms [149,161].

1.11.3. Clinical impact of baseline RASs

Recent studies have described a prevalence of baseline RASs in HCV infected patients that can range from 1 – 80% [164] leading to a decrease in SVR rates between 1 – 50% [164,165], according to the genotype of HCV [124,166], the selected regimen of DAAs [124], and the patient´s therapeutic history, as well as the level of liver disease [165]. Although the prevalence of RASs is variable with a level of resistance depending on relatively few highly specific but high risk mutations for the NS3, NS5A and NS5B regions, their presence is indeed substantial and plays a clear and recognized role in the therapeutic failure [124,154].

It has been shown in the ION clinical trials [117,129,130] that reduced rates of SVR observed after a regimen of SOF + LDV for 12 weeks in patients infected with GT1 HCV are essentially associated with the presence of baseline NS5A RAS, which conferred an *in vitro* high fold resistance (>100) to ledipasvir (Table 1.5) [167].

Table 1.5 Fold-changes in EC_{50} of NS5A inhibitors according to clinically relevant NS5A resistance-associated substitutions in GT1 HCV. Combinations between RASs and DAAs highlighted in red are more likely to result in treatment failures due to the high fold-changes, whereas combinations marked in orange tend to demonstrate intermediate fold-changes but still having an impact on therapy efficacy. RASs highlighted in shades of green represent very low or negative fold-changes associated with resistance to NS5A DAAs and are not likely to have any clinically significant impact. NA, data not available. Data adapted from [79,151].



HCV NS5A DAAs	Fold-change					
	GT1a RASs				GT1b RASs	
	M28T	Q30R	L31M/V	Y93H/N	L31V	Y93H/N
Daclatasvir	>100x	>1000x	>100x / >1000x	>1000x / >10 000x	<10x	>20x / >50x
Ledipasvir	>20x	>100x	>100x	>1000x / >10 000x	>20x	>100x / NA
Elbasvir	>20x	>100x	>10x / >100x	>1000x	<10x	>100x / NA
Ombitasvir	>1000x	>100x	<3x / >100x	>10 000x	<10x	>20x / >50x
Velpatasvir	<10x	<3x	>20x / >50x	>100x / >1000x	<10x	<3x / NA
Odalasvir*	>20x	<10x	<3x	>1000x	<3x	<3x / <10x
Pibrentasvir	<3x	<3x	<3x	<10x	<3x	<3x
Ruzasvir*	<10x	<10x	<10x	<10x	<10x	<10x

* Investigational pipeline direct-acting antiviral

1.11.3.1. Resistance to NS5A inhibitors

NS5A constitutes the most important genomic region considered for the screening of associated clinically relevant RASs, as it displays the highest number of mutations in GT1 HCV [151]. Moreover, the NS5A associated RAVs are capable of persisting for at least one or two years after a therapeutic failure while maintaining the same viral fitness, and in this way exhibiting a negative impact on a retreatment regimen with NS5A inhibitors [89,168–170].

Q30H and Y93H are high level transversion mutations which confer a high replicative fitness in GT1a RAVs [71,123,171], and although the presence of only one of these high level RASs may itself lead to the inhibitory effect of a regimen with NS5A DAAs and consequently to a decrease in SVR rates, the synergistic combination of more than one of these mutations may be associated with a substantial resistance, such as Q30R + Y93H/N which often leads to high *in vitro* EC_{50}-fold resistance (>1000x) to NS5A inhibitors when comparing to the wildtype replicon (Table 1.5) [164,172,173].

1.11.3.2. Resistance to NS5B polymerase inhibitors

Overall, regimens based on NS5B polymerase inhibitors, particularly NIs, tend to exhibit a low prevalence of baseline RASs [174], and the S282T mutation although very relevant in therapeutic failures after a treatment with NIs, is rarely detected at baseline [149]. Along these lines, NS5B RASs tend to occur more easily after a treatment based on NNIs since these drugs bind to the viral polymerase allosteric sites thus presenting a lower genetic barrier to resistance [98].

The S282T mutation confers a high level of resistance to sofosbuvir, however, its natural presence in the daily clinical practice is very rare [149] due to the reduced replicative fitness of the viral variants that possess this mutation, which may also explain why a viral breakthrough for this RAS is rarely detected during a treatment with sofosbuvir [113]. Moreover, even recurrent patients whom present the S282T mutation after the EoT with sofosbuvir, are usually able to quickly eliminate this mutation right after a few weeks thus achieving a complete viral suppression [149,175–177].

1.12. What happens when treatment fails

1.12.1. Management and importance of RASs in treatment failures

RASs are not detected in all recurrent patients since therapeutic failure for a regimen with DAAs is additionally related to other factors associated with a poor response such as the progression of liver disease, the patient´s therapeutic history, the viral load, the efficacy and potency of each DAA, as well as with the genotype of HCV [113]. However, therapeutic failure rates associated with the use of DAAs may range from 5 – 10%, mostly being due to the presence of RASs [161,164,178], which can occur essentially as a post-treatment recurrence with detection rates between 53 – 91% [70,132,179], or occasionally as a viral breakthrough during treatment [161]. Along these lines, RASs importance consequently increases in rescue therapies after virological failure with a suboptimal regimen based on DAAs [164].

The best way to avoid a therapeutic failure associated to the occurrence of RASs is to achieve a complete viral suppression right with the first selected therapeutic regimen, by using a combination of potent DAAs with a high genetic barrier to resistance, a pan-genotypic activity, and preferentially without any cross-resistances [161].

1.12.2. Retreatment guidelines in HCV mono-infected patients

Retreatment should be guided through the patient´s therapeutic history, the experience of the team treating the infected patients, and additionally by the potential response to a new retreatment regimen according to the obtained resistance profile [40], assuming that a resistance test is available to perform which can help physicians to better guide retreatment options and thus reducing substantially the likelihood of another therapeutic failure [180].

According to EASL recommendations on the treatment of hepatitis C [40], patients infected with HCV whom failed a previous IFN-free treatment, should be retreated with a combined regimen of DAAs including a NI with a high genetic barrier to resistance (currently SOF), with the addition of one to three other DAAs, ideally without any cross-resistances considering the previous submitted treatment, for a period of 12 weeks with ribavirin. The retreatment regimen can be extended to a period of 24 weeks with RBV when a high level of hepatic disease progression is verified, or alternatively for 24 weeks without RBV when there is contraindications for its use.

Several studies have demonstrated the efficacy of IFN-free regimens in the retreatment of GT1 HCV infected patients, by exhibiting SVR rates above 97%:

- Sixty-nine HCV infected patients whom failed a prior treatment with SOF + VEL for 12 weeks in previous phase II trials (NCT01858766; NCT01909804; NCT01826981; NCT02202980) were retreated with the same regimen plus RBV for an extended period of 24 weeks, achieving SVR12 rates of 97% (36/37), 93% (13/14) and 78% (14/18) for GT1, 2 and 3, respectively. Moreover, 13% (5/37) of the retreated GT1 infected patients exhibited NS5A RASs at baseline, of which the SVR12 rates were of 100% [181].

- Fifty-one GT1 HCV infected patients whom failed a prior treatment with SOF + RBV with or without IFN-α in previous phase II/III trials [P7977-0221 (NCT01054729); QUANTUM (NCT01435044); PROTON (NCT01188772); ATOMIC (NCT01329978); NEUTRINO (NCT 01641640)] were retreated with a regimen of SOF + LDV plus RBV for 12 weeks, achieving SVR12 rates of 98% (50/51). Moreover, the only patient who didn’t manage to achieve a SVR was a GT3a infected patient previously incorrectly identified as GT1a [182].
- In the QUARTZ-1 trial [183], 22 GT1 infected patients whom failed a prior IFN-free treatment, were retreated with a regimen of OBV/Ritonavir-boosted PTV + DSV + SOF for 12 or 24 weeks, achieving SVR12 rates of 93% (14/15) and 100% (7/7), respectively. Moreover, 82% (18/22) of the retreated patients exhibited baseline RASs for at least one of the four main classes of DAAs.

1.12.3. Retreatment guidelines in co-infected patients

High rates of HIV/HCV co-infection have been reported over the last years due to the similar routes of transmission between both viruses, as well as to existence of risk behavior groups like PWID [41], with an estimate of 2,3 million HIV/HCV co-infected patients worldwide [184].

Since DAAs-based regimens have shown again and again a high virological efficacy, as well as ease of use and tolerability, the indications for an IFN-free treatment of HCV mono-infected patients remain identical to those patients co-infected with HIV, HCV and/or HBV, with or without an indication for transplant. However, HIV/HCV co-infected patients have an additional risk of exhibiting drug-drug interactions and thus may require some treatment changes or dose adjustments when necessary [40].

Although the IFN-free treatment with SOF/VEL is currently highly recommended over its potent pan-genotype activity and high SVR rates, there are still some potential drug-drug interactions to consider when submitting this regimen in HIV/HCV co-infected patients, and in this way should not be given in conjunction with several antiretrovirals (ARVs) such as efavirenz, etravirine and nevirapine, due to possible antagonistic reactions as well as consequent adverse effects on the host [40].

In HCV/HBV co-infected patients, HCV normally leads the activity of chronic hepatitis B since the DNA levels of HBV are frequently low or even undetectable in this co-infection circumstance. As such, patients selected for the treatment of chronic hepatitis C with a DAAs based regimen should also be tested for the HBs antigen as well as to the anti-HBc and anti-HBs antibodies, in order to thoroughly evaluate the replicative state of HBV, and along these lines an HDV infection should be additionally sought [40].

1.12.4. Retreatment guidelines in HCV infected patients with an indication for transplant

The recurrence of hepatitis C after transplantation of an infected organ is universal in the absence of treatment, which can lead to a reduction in the average durability of the transplant and consequently to the patient´s decease. [185]. Along these lines, all HCV mono-infected or HIV/HCV co-infected patients presenting decompensated cirrhosis (Child-Pugh B or C) should be treated with IFN-free regimens based on DAAs, regardless if in a pre-transplantation or post-transplantation indication [40]. This approach is supported by several studies describing the virological efficacy, ease of use and safety of combined regimens of SOF with an NS5A inhibitor in the retreatment of hepatic transplant patients, thus achieving high rates of SVR [186–188], not to mention that these DAAs do not require any dose adjustments of immunosuppressive drugs [40].

HCV recurrence in renal transplanted patients has been systematically associated with several co-morbidities such as an increased rate of cystic fibrosis progression, renal graft failure to function correctly, as well as a reduction in survival rates, particularly in cirrhotic patients. As such, these patients should be retreated with IFN-free regimens following the same recommendations for hepatic transplant recipients [40].

Several studies have demonstrated the high and fast virological efficacy, ease of use and tolerability of an IFN-free retreatment in HCV recurrent renal post-transplant patients, thus achieving high rates of SVR12. Along these lines a regimen based in DAAs is capable of enabling a profound viral suppression in renal transplant recipients [4,111,189,190].

1.12.4.1. Treating HCV infection: before or after transplant?

To date, there is still no agreement on whether HCV infected patients with compensated cirrhosis on the transplant list should be treated for this infection prior to transplantation or immediately after transplantation, as these two approaches have not yet been compared prospectively in random trials using clinical endpoints [40]. While some authors support that treating HCV infected patients selected for a hepatic transplant may improve liver function prior to transplantation and simultaneously prevent a subsequent graft infection, others argue that there are patients who don't have the luxury of waiting any longer for transplantation, claiming that viral suppression is equally achieved with success immediately after transplantation due to the high efficacy and tolerability of current DAAs regimens, thus preventing the graft death and a potential HCV recurrence [91,111,186,191].

1.13. Importance of resistance testing

1.13.1. Management and importance of RASs in treatment failures

Treatment monitoring should include several criteria in order to achieve a profound viral suppression with the least discomfort possible in the patient, such as the SVR rates and efficacy of the regimen, tolerability and potential adverse effects, the ease of use, and drug-drug interactions as well as consequent cross-resistances [40].

The monitoring of treatment efficacy is based on measuring the HCV RNA levels at different time points via a sensitive and accurate test in order to ensure the consistency of results [192,193]. Thusly, in HCV infected patients treated with a DAAs-based regimen, RNA levels or alternatively the core antigen, should be quantified immediately before treatment initiation, at the 2nd and 4th weeks to assess the adherence to treatment (optional), at the end of treatment at weeks 8, 12, 16 or 24 according to the duration of the selected regimen, and finally 12 and/or 24 weeks after the end of therapy in order to evaluate the SVR12 and/or SVR24, respectively [40].

Fatigue, headaches, and nausea are often described as the most commonly observed adverse effects in patients treated with DAAs-based regimens, namely SOF +

LDV, SOF + VEL or SOF + DCV, when comparing to placebo. However, the proportion of patients with adverse effects whom permanently discontinue therapy accounts for less than 1%. In addition, contraindications to the use of ribavirin should always be checked since several side effects are often associated to its inclusion in treatment regimens, comprising a potential mild anemia as well as possible teratogenic and/or embryonic effects [40].

1.13.2. Clinical screening of baseline RASs: preventing therapeutic failures

To date, routine HCV resistance testing prior to treatment is not recommended since access to reliable tests is still limited both financially and logistically, besides there is no consensus on the used techniques, its interpretation and/or notification criteria, and thus would seriously limit access to treatment options which can currently be optimized without the need of an additional resistance profile. Along these lines, baseline resistance testing is not recommended in TN patients, with the exception of SMV based regimens, combined regimens of EBV + GZP in patients infected with GT1a, any regimens submitted in patients infected with GT3 due to its known difficulty in virological responses, and additionally in risk behavior groups. In this way, resistance testing is instead recommended in therapeutic failures following a DAAs-based regimen in order to guide the best retreatment strategy. However, if a baseline resistance test both available, reliable and interpretable is performed (population sequencing or deep sequencing with 15% and 1% cut-off limits, respectively), it should be directed for clinically relevant NS5A RASs (Table 1.4) involved in high level of resistance to NS5A inhibitors, and therefore the selected treatment with DAAs must include RBV and/or be extended in duration should any of these RASs be detected [40,86,177].

1.13.3. Sequencing as a prognostic tool in drug resistance testing

HCV sequencing manages to have a dual functionality as it not only allows for genotype assignment with a greater accuracy and discrimination than current commercial techniques, but also allows for the resistance profile analysis of patients who are about to initiate treatment, and in this way helps physicians to select more appropriate therapeutic options for each patient, thus avoiding potential therapeutic failures thanks to an early detection of high resistance level RASs which could adversely affect viral suppression rates.

The lack of detectability of RAVs is a worrying negative predictor factor which can mislead physicians in the selection of a given treatment, and is often related with the sensitivity of the sequencing technique, the viral load, and the reduced frequency of viral variants with RASs as well as a consequent rapid reversion to the wildtype strains due to the low viral fitness of certain RAVs [70,149,160].

Several NGS platforms like SMRT deep sequencing and Illumina MiSeq are truly sensitive methods which allow for the detection of minority variants at levels below 1%, whereas Sanger sequencing can only succeed to reliably detect variants present above 20 – 30% of the whole population [194–196]. However, insight on the clinical relevance of detecting minority variants at levels below 15 – 20% is still both scarce and debatable, and as such Sanger population sequencing remains the gold standard over its high accuracy and reliability, given that a threshold of 20% appears to be sufficient in the detection of high risk RASs commonly associated to reduced SVR rates in DAAs-based regimens [197].

1.13.4. Resistance testing pre-treatment initiation or post-treatment failure: an endless debate

Current prices of DAAs regimens are very high and variable worldwide, thus most people can´t afford to be treated when government strategies don´t include a total coverage of treatment, which in return represents a risk to public health leading to the potential transmission of the virus, turning further away from the total eradication of HCV. In this way, baseline resistance analysis has been shown to be quite useful in the selection of increasingly specific regimens for each patient, and helps to achieve a complete viral suppression with high SVR rates countering the evolution of the virus in patients with persistent viral loads, as well as preventing future therapeutic failures associated with unnecessary high cost salvage DAAs [149].

Authors supporting baseline resistance tests state that the prevalence of NS5A RASs in GT1 accounts for 6 – 25%, differing not only on HCV genotype but also geographically between countries [155]. These RASs are systematically associated to reduced SVR rates, thus if detected prior to the initiation of treatment, baseline resistance testing would help to prevent potential therapeutic failures by further increasing current SVR rates and averting the emergence of high risk RAVs associated to inadequate regimens of DAAs, being particularly useful in patients whom did not respond to an previous regimen of pegIFN-α ± RBV, with or without an advanced progression of liver disease [98,196]. Furthermore, there is a significant percentage of patients failing the selected treatment due to an incorrect genotype assignment [122], and since HCV genotyping is always required prior to treatment initiation, sequencing would constitute a far more accurate, reliable and discriminatory alternative to commercial genotyping assays, as it would simultaneously allow for a correct subtype assignment within a higher level of confidence, as well as provide an additional baseline resistance profile to assist physicians in guiding more appropriate treatment options based in DAAs specifically directed for the population of RAVs present in the patients, thus preventing future therapeutic failures [101].

On the other side, authors against baseline resistance testing state that natural RASs do not appear to have a significant impact on therapeutic failures or reduced SVR rates to the extent of recommending it in TN patients, seeing that combinations of multiple high level RASs to DAAs are rarely observed in DAA-naïve patients [174], and argue that therapeutic failures are not avoided via resistance testing but rather through the selection of more potent pan-genotypic DAAs by physicians, since the impact of NS5A baseline RASs with a >100 fold-resistance can be reduced by simply increasing the duration of treatment with the inclusion of RBV [157,158]. Moreover, these authors emphasize that therapeutic failures are primarily associated with poor adherence rates, and not so much to baseline RASs, and thus suggest that there should be a greater investment in adherence to therapeutic regimens by the patients, rather than investing in baseline resistance testing.

But in the end, it all comes down to statistics and while some authors argue that with only a 1% rate of relapses, as well as more and more DAAs with a higher potency emerging in the pipeline, baseline resistance testing consequently becomes obsolete, they are actually being very optimistic as it has been demonstrated throughout the years that there is in fact at least a 5−10% rate of therapeutic failures systematically associated to the baseline presence of high risk RASs.

1.14. Objectives and hypotheses

Our main goal was to study the profile of NS5 coding region RASs in DAA-naive genotype 1 HCV infected patients.

Additionally, three separate hypotheses were tested as to ascertain an association between treatment failure and:

a. The non-discriminatory presence of baseline NS5 RASs;
b. The baseline presence of NS5A, NS5B and/or NS5A + NS5B class RASs;
c. The baseline presence of specific NS5 RASs.

A comparison between LiPA and Sanger population sequencing (BLAST and phylogenetic analysis) genotyping methods was also assessed as a way of availing the many uses of resistance testing through sequencing.

2. Material and methods

2.1. Samples

This study was approved by the Ethical Committee for Health of Centro Hospitalar de Lisboa Ocidental (CHLO) with the RNEC Registry Number: 20170700050.

All sampling processing and analysis steps described in this chapter were performed at the Molecular Biology Laboratory of Egas Moniz Hospital, CHLO, Lisbon. A group of 81 clinical samples of plasma from DAA-naïve GT1a and GT1b infected patients selected to initiate treatment with DAAs between 2015 and 2017, were analyzed for baseline NS5 RASs. Eighteen samples corresponded to female patients (22%), while males were the predominant sex accounting for 63 samples (78%) (Table 2.1). The viral load had been previously determined using the Quantitative/Qualitative COBAS® AmpliPrep/TaqMan® HCV Test v2.0, from Roche Molecular Diagnostics (Basel, Switzerland), and all tested samples presented average high HCV RNA loads, ranging from 4,76 $\log_{10}$ to 7,44 $\log_{10}$ (Table 2.1). The HCV genotype had been previously determined using the VERSANT® HCV Genotype 2.0 Assay Line Probe Assay (LiPA), from INNOGENETICS/Siemens Healthineers (Ghent, Belgium). Twenty-five samples corresponded to GT1b (31%), while GT1a was the predominant subtype accounting for 56 HCV infected patients (69%). The IL28B gene polymorphism was additionally inquired in 76 of the 81 patients accounting for 58% (47/81), 28% (23/81) and 8% (6/81) patients with the CT, CC and TT polymorphisms, respectively. Of the 81 patients, 32% were HCV mono-infected (26/81), while 52% (42/81) and 5% (4/81) were co-infected with either HIV or HBV, respectively. Moreover, 11% (9/81) of the patients were simultaneously co-infected with HCV, HIV and HBV. Finally, 3 out of the 81 patients failed a treatment with DAAs (4%), and two patients showed an unknown treatment outcome status (2%) due to lack of adherence to the associated regimen or loss to follow-up evaluation before the SVR12 visit, resulting overall in a SVR rate of 94% (76/81) (Table 2.1).

Table 2.1 Demographic baseline characteristics of the HCV infected patients and treatment outcomes.

	DAA-naive HCV infected patients (n=81)
Mean age, years (range)	49 (23 - 81)
Male sex, n (%)	63 (78%)
Genotype, n (%)	
GT1a	56 (69%)
GT1b	25 (31%)
Monoinfection and co-infection profile, n (%)	
HCV monoinfected	26 (32%)
HIV/HCV	42 (52%)
HCV/HBV	4 (5%)
HIV/HCV/HBV	9 (11%)
Mean $\log_{10}$ HCV RNA, IU/mL (range)	6,22 (4,8 - 7,4)
HCV RNA, IU/mL, n (%)	
<2 million	43 (53%)
2-6 million	30 (37%)
>6 million	8 (10%)
IL28B, n (%)	
CC	23 (28%)
CT	47 (58%)
TT	6 (8%)
Unknown	5 (6%)
Outcome after treatment with DAAs, n (%)	
SVR12 or SVR24	76 (94%)
Treatment failure	3 (4%)
Unknown	2 (2%)

2.2. HCV RNA extraction

HCV nucleic acids were extracted from 500µl of plasma previously conserved at -80ºC, ultimately eluting 45µl of RNA product using the bioMérieux's automated NucliSENS® EasyMAG® system v2.0 (with silica) (Boseind, Boxtel, The Netherlands), according to the manufacturer´s protocol.

2.3. PCR and sequencing primers design

In order to cover the NS5A and NS5B coding regions, each amplification reaction used two primers, one forward and one reverse, encompassing a length of approximately 4800bp and 3700bp for the RT-PCR (outer PCR) and nested PCR (inner PCR), respectively (Supplementary Table 1). The in-house PCRs and sequencing protocols, including the primers for cDNA synthesis, nested PCR and Sanger sequencing were originally designed by Rute Marcelino (MSc, Medical Microbiology Department, IHMT, Lisbon). ClustalX v2.0 (http://www.clustal.org/clustal2/) and GENEDOC v2.7 (http://www.psc.edu/biomed/genedoc/) softwares were used for the alignment and search of HCV genome consensus regions in reference sequences downloaded from the Los Alamos HCV database (https://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html), respectively. Oligonucleotides of 15 – 20bp were designed based after some of the consensus regions and tested upon standard compatibility features regarding GC content and melting temperature (Tm) using the OligoAnalyzer v3.1 online tool from INTEGRATED DNA TECHNOLOGIES (https://eu.idtdna.com/calc/analyzer). Degenerate primers were used for PCR and sequencing reactions in order to increase the detection ambiguity and reliability of a more diverse set of quasispecies variants. A total set of 10 primers (Supplementary Table 1), two of which being subtype specific, were selected to cover the NS5 coding region of GT1 HCV (Figure 2.1), in this way presenting the best results in the Sanger sequencing protocol optimization. However, since the 3'-end of the NS5B gene is a poly-U (U/C) region highly composed of hairpins and secondary structures which obstruct the annealing process, the associated FW6 sequencing primer (Supplementary Table 1) was in this way unable to completely hybridize during the sequencing reactions, and three important amino acid positions (A553, G554, and S556) associated with resistance to dasabuvir were consequently left uncovered. Moreover, 12 additionally tested back-up sequencing primers were saved for later use if necessary (Supplementary Table 1). All primers were synthesized by Invitrogen/ALFAGENE (Carcavelos, Portugal), with the exception of RV9 which was manufactured by TIB MOLBIOL (Eresburgstraße, Berlin, Germany).

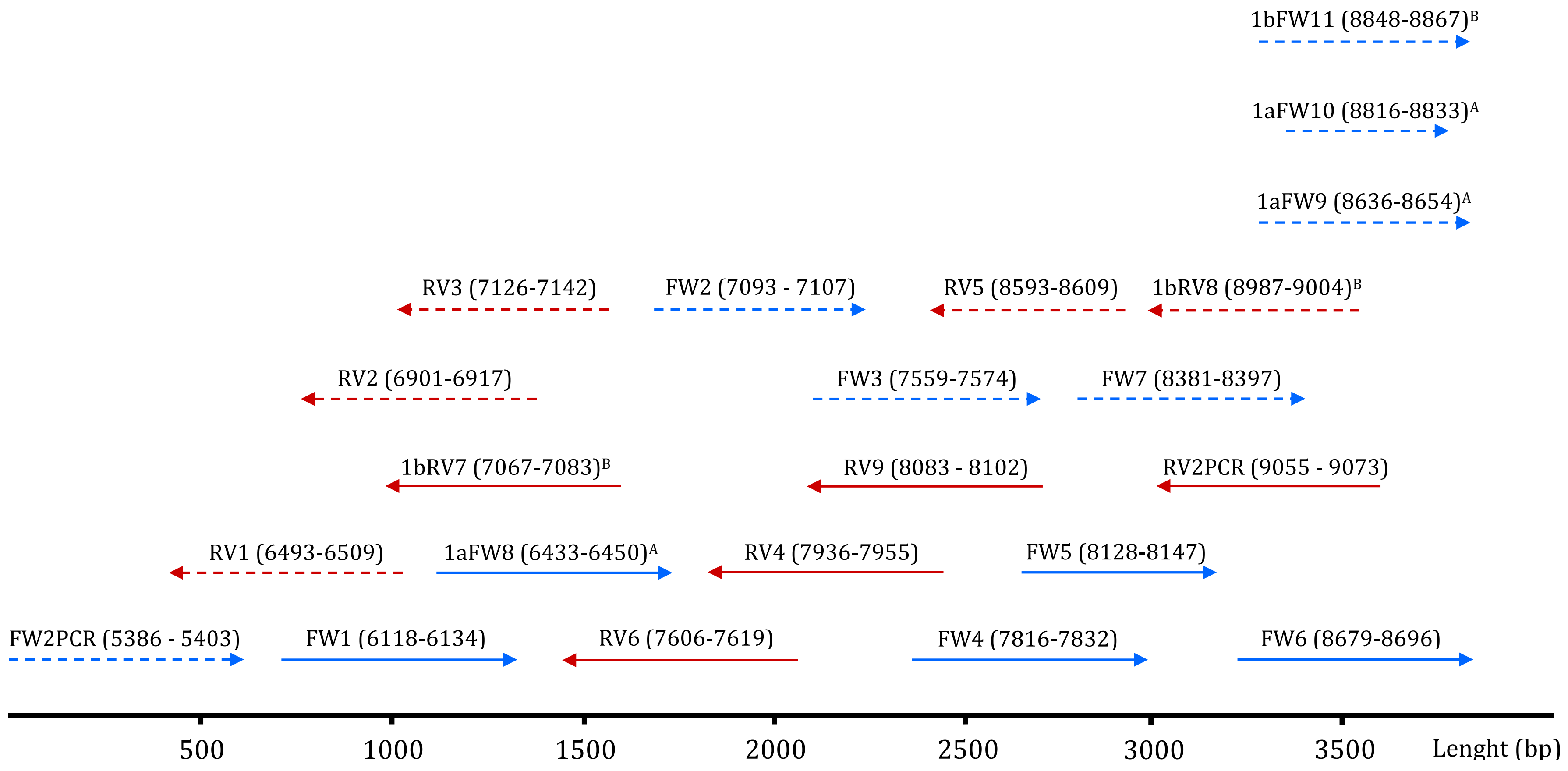


Figure 2.1 Sequencing primers considered to cover the entire NS5 coding region of HCV. Primers in red are anti-sense and primers in blue are sense. Continuous lines correspond to the main primers used to cover the NS5 region, whereas dashed lines represent back-up primers to use if necessary. Numbers in parenthesis represent the location (bp) in the HCV genome using the reference H77.

[A] Primers for GT1a only.

[B] Primers for GT1b only.

2.4. cDNA-synthesis and nested PCR

A reverse transcription in-house PCR was immediately performed after RNA extraction to guarantee the viability of the HCV nucleic acids and the success of the amplification process as well as the quality of HCV sequences and consequently of the sequencing chromatogram, since the genomic structure of RNA tends to be very unstable and continuous sampling frosting and defrosting should be avoided. Along these lines, HCV RNA was reverse transcribed using the OneStep RT-PCR Kit from QIAGEN, and this first round of amplification (outer PCR) was performed under the following final conditions (Table 2.2): OneStep RT-PCR Buffer (1X), 2,5µl of OneStep RT-PCR Enzyme Mix (Supplementary Table 2), dNTPs Mix (400µM each), 0,5µM sense primer FW1, 0,5µM anti-sense primer RV1, 10U/reaction of protector RNase inhibitor (Supplementary Table 2), 10µl of template RNA, and RNase-Free water (Supplementary Table 2) to make up a final reaction volume of 50µl. Afterwards, the remaining extracted RNA templates were conserved at -80ºC.

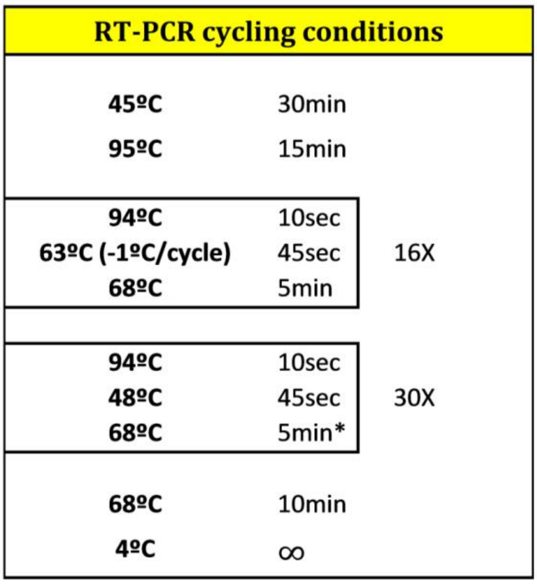
Table 2.2 RT-PCR (outer PCR) mix reagents and associated volumes calculated accordingly to the reaction concentrations (final concentrations), using the Qiagen One Step RT-PCR Kit. Blank spaces represent expendable or unobtainable information.

Qiagen One Step RT-PCR Kit		**Mix volumes for a total of 50µl/reaction (40µl of mix + 10µl of template RNA)**
Mix reagents	**Final concentration**	
QIAGEN OneStep RT-PCR Buffer (5X)	1X	10µl
dNTP mix (10mM each)	400µM each	2µl
Primer FW1PCR (10µM stock solution)*	0,5µM	2,5µl
Primer RV1PCR (10µM stock solution)*	0,5µM	2,5µl
QIAGEN OneStep RT-PCR Enzyme Mix (contains Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq® DNA Polymerase)	-	2,5µl
Protector RNase Inhibitor (2000U)	10U/reaction	0,25µl
RNase-free water	-	20,25µl

* Sold separately from the Qiagen One Step RT-PCR Kit.

LifeECO thermal cycler by Hangzhou BIOER Technology or GeneAmp® PCR System 9700 by Applied Biosystems were used for the amplification process, and cycling conditions were as follows (Table 2.3): a reverse transcription step at 45ºC for 30min, a HotStarTaq polymerase activation step at 95ºC for 15min to initiate PCR, a touchdown PCR step of 16 cycles of 10sec at 94ºC (denaturation step), 45sec at 63ºC with -1ºC/cycle (annealing step), and 5min at 68ºC (extension step), a final PCR step of 30 cycles of 10sec at 94ºC, 45sec at 48ºC, and 5min at 68ºC with a time increment of +3sec/cycle, and a final elongation step of 10min at 68ºC. The reaction was then cooled down to 4ºC, and RT-PCR products were stored at -20ºC or immediately used in the nested PCR step.

Table 2.3 RT-PCR (outer PCR) cycling conditions.



RT-PCR cycling conditions		
45ºC	30min	
95ºC	15min	
94ºC	10sec	16X
63ºC (-1ºC/cycle)	45sec	
68ºC	5min	
94ºC	10sec	30X
48ºC	45sec	
68ºC	5min*	
68ºC	10min	
4ºC	∞	

* Increments of +3sec/cycle.

Two microliters of the RT-PCR reaction product were amplified in a second in-house nested PCR divided in two mixes as recommended by the Expand High Fidelity PCR System kit (Roche), under the following conditions (Table 2.4): Mix 1 [dNTPs mix (200µM each), 0,3µM sense primer FW2, 0,3µM anti-sense primer RV2, and RNase-Free water to make up a mix volume of 23µl] (Supplementary Table 2) and Mix 2 [Expand High Fidelity Buffer (1X) without $MgCl_2$, 0,4% DMSO, 3mM $MgCl_2$, 3U/reaction of Expand High Fidelity Enzyme mix, and RNase-Free water to make up a mix volume of 25µl] (Supplementary Table 2). Finally, 2µl of template cDNA were mixed with 23µl of Mix 1, following the addition of 25µl of Mix 2 to make a final reaction volume of 50µl for each sample.

Table 2.4 Nested PCR (inner PCR) mix reagents and associated volumes calculated accordingly to the reaction concentrations (final concentrations), using the Expand High Fidelity PCR System from Roche. Blank spaces represent expendable or unobtainable information.

Expand High Fidelity PCR System, Roche			**Mix volumes for a total of 50µl/reaction [(23µl of mix A + 2µl of template cDNA) + 25 µl of mix B]**
		Final concentration	
Mix A reagents	dNTP mix (25mM each), BIOLINE*	200uM each	0,4µl
	Primer FW2PCR (10µM stock solution)*	0,3µM	1,5µl
	Primer RV2PCR (10µM stock solution)*	0,3µM	1,5µl
	LiChrosolv® water for chromatography, Merck*	-	19,6µl
Mix B reagents	Expand High Fidelity Buffer without $MgCl_2$ (10X)	1X	5µl
	$MgCl_2$ (25 mM stock solution)	3mM	6µl
	DMSO (100%), Sigma-Aldrich*	0,4%	0,2µl
	Expand High Fidelity Enzyme mix (500U in 2x75µl pack size) (contains Taq DNA polymerase and Tgo DNA polymerase)	≈ 3U/reaction	0,87µl
	LiChrosolv® water for chromatography, Merck*	-	12,93µl

* Sold separately from the Expand High Fidelity PCR System.

LifeECO thermal cycler (Hangzhou BIOER Technology) or GeneAmp® PCR System 9700 (Applied Biosystems) were used for the amplification process, and cycling conditions were as follows (Table 2.5): a denaturation step at 95ºC for 3min, a touchdown PCR step of 16 cycles of 15sec at 94ºC, 30sec at 60ºC with -1ºC/cycle, and 3min at 68ºC, a final PCR step of 30 cycles of 15sec at 94ºC, 30sec at 45ºC, and 3min at 68ºC with a time increment of +5sec/cycle, and a final elongation step of 7min at 68ºC. The reaction was then cooled down to 4ºC, and nested PCR products were stored at -20ºC.

Table 2.5 Nested PCR (inner PCR) cycling conditions.

Nested PCR cycling conditions		
95ºC	3min	
94ºC **60ºC (-1ºC/cycle)** **68ºC**	15sec 30sec 3min	16X
94ºC **45ºC** **68ºC**	15sec 30sec 3min*	30X
68ºC	7min	
4ºC	∞	

* Increments of +5sec/cycle.

2.5. Gel electrophoresis and purification of PCR products

Analysis of the PCR products was done via agarose gel electrophoresis. Seven microliters of PCR product were mixed with 3µl of DNA loading buffer and then run on a 1% agarose gel incorporated with RedSafe^{TM} Nucleic Acid Staining Solution (Supplementary Table 2), at 100V. Five microliters of 1 kb Plus DNA Ladder (Supplementary Table 2) were adjacently run with the PCR products to ascertain the length of the associated fragments, namely 4800bp and 3700bp for the RT-PCR and nested PCR, respectively. When positive, the nested PCR products were purified using the PCR Cleanup Kit protocol from Abbott Molecular Inc. by adding the respective ExoSAP cleanup reagent (Supplementary Table 2) to the nested PCR product in a proportion of 1:4, under the following cycling conditions (Table 2.6): 30min at 37ºC followed by 15min at 80ºC.

Table 2.6 cDNA purification cycling conditions.

cDNA purification cycling conditions	
37ºC	30min
80ºC	15min
4ºC	∞

The reaction was then cooled down to 4ºC, and dilutions from 1:2 to 1:10 of the purified PCR products were followed if necessary, accordingly to the intensity of the electrophoresis bands i.e. the more intense the band the higher the dilution. Purified nested PCR products were stored at -20ºC.

2.6. Sequencing and resistance profile analysis

Sanger population sequencing (15% cut-off value) on 3130xl ABI PRISM Genetic Analyzer by HITACHI/ThermoFisher Scientific (Tokyo, Japan) was performed for the purified nested PCR products, using a BigDye™ Terminator v3.1 Cycle Sequencing kit (Supplementary Table 2) and a total of 9 primers per GT1a or GT1b in order to cover the entire NS5 coding region. The sequencing process was separated in two sets of mix reactions per primer, one for genomic regions of difficult hybridization by the designed sequencing primers (Reaction Mix A) and other for regions of easy hybridization (Reaction Mix B), and performed under the following final concentration conditions: Reaction Mix A [BigDye™ Sequencing Buffer (0,75X), 2µl of BigDye™ Terminator 3.1 Reaction Mix, 1µl of primers (5µM stock solution) RV4, FW6 and RV9 separately per reaction, 1µl of purified template cDNA, and RNase-Free water to make up a reaction volume of 20µl] (Table 2.7) and Reaction Mix B [BigDye™ Sequencing Buffer (0,75X), 1µl of BigDye™ Terminator 3.1 Reaction Mix, 1µl of primers (5µM stock solution) RV2PCR, FW1, FW4, FW5, RV6, 1bRV7 and 1aFW8, separately per reaction, 1µl of purified template cDNA, and RNase-Free water to make up a reaction volume of 10µl] (Table 2.8). GeneAmp® PCR System 9700 (Applied Biosystems) was used for the sequencing reaction, and cycling conditions were as follows (Table 2.9): a denaturation step at 96ºC for 5min, 35 cycles of 5sec at 94ºC, 10sec at 50ºC, and 4min at 60ºC, followed by a cool down to 4ºC. Back-up sequencing primers, were also available to use accordingly to the Reaction Mix A, if the main primers were not successful in producing a complete sequence (Supplementary Table 1).

Table 2.7 Sanger population sequencing reaction mix A reagents and associated volumes calculated accordingly to the reaction concentrations (final concentrations), adapted for 3130xl ABI PRISM Genetic Analyzer. Blank spaces represent expendable or unobtainable information.

Sequencing Reaction A		**Mix volumes per primer for a total of 20µl/reaction (19µl of mix + 1µl of purified template cDNA)**
Mix reagents	**Final concentration**	
BigDye™ Terminator v3.1 Cycle Sequencing Reaction Mix, Applied Biosystems	-	2µl
BigDye® Terminator v1.1, v3.1 Sequencing Buffer (5X), Applied Biosystems	0,75X	3µl
Primer* (5µM stock solution)	0,25µM	1µl
LiChrosolv® water for chromatography, Merck	-	13µl

* RV4, FW6 and RV9, separately per reaction. If needed, apply same reaction conditions to back-up primers: FW2PCR, RV1, FW2, RV2, FW3, RV3, RV5, FW7, 1bRV8, 1aFW9, 1aFW10 and 1bFW11.

Table 2.8 Sanger population sequencing reaction mix B reagents and associated volumes calculated accordingly to the reaction concentrations (final concentrations), adapted for 3130xl ABI PRISM Genetic Analyzer. Blank spaces represent expendable or unobtainable information.

Sequencing Reaction B		**Mix volumes per primer for a total of 10µl/reaction (9µl of mix + 1µl of purified template cDNA)**
Mix reagents	**Final concentration**	
BigDye™ Terminator v3.1 Cycle Sequencing Reaction Mix, Applied Biosystems	-	1µl
BigDye® Terminator v1.1, v3.1 Sequencing Buffer (5X), Applied Biosystems	0,75X	1,5µl
Primer* (5µM stock solution)	0,5µM	1µl
LiChrosolv® water for chromatography, Merck	-	5,5µl

* RV2PCR, FW1, FW4, FW5, RV6, 1bRV7 and 1aFW8, separately per reaction.

Table 2.9 Sanger population sequencing cycling conditions for reaction mixes A and B, adapted for 3130xl ABI PRISM Genetic Analyzer.

Sequencing cycling conditions		
96ºC	5min	
94ºC	5sec	35X
50ºC	10sec	
60ºC	4min	
4ºC	∞	

A purification of the sequencing reaction products was needed before running on the sequencing equipment, and started by distributing 52µl of sodium acetate buffer diluted in 100% ethanol per MicroAmp® reaction plate well (Supplementary Table 2), followed by a strong centrifugation at 3300rpm for 20 minutes. The reaction plate was then inverted and softly centrifuged at 900rpm for one minute to remove excess waste. Subsequently, 150µl of 70% ethanol were distributed per reaction well, followed by a strong centrifugation at 3300rpm for 5 minutes, and the reaction plate was again inverted and softly centrifuged at 900rpm for one minute. The reaction plate was left to dry for 2 minutes, and 20µl of formamide were added per MicroAmp® reaction plate well setting up 16-capillary complete runs. Lastly, sequencing reaction plates were run on the 3130xl ABI PRISM Genetic Analyzer and nucleotide sequence chromatograms for each sample were obtained.

The primers nucleotide sequences for each sample were joined in a single contig and edited using *ChromasPro®* software v1.7.6. (Technelysium Pty Ltd). Finally, complete nucleotide sequences were converted to FASTA format and analyzed online in Geno2pheno [HCV] v0.92 (http://hcv.geno2pheno.org/), providing the baseline resistance analysis profile for each patient. All sequences obtained in this study, were submitted to the REGA HCV database from KU LEUVEN.

2.7. Phylogenetic analysis for HCV genotyping

A reference alignment of 195 Okamoto region consensus sequences ranging from genotypes 1 to 7, specific for the purpose of HCV genotyping, were downloaded from the Los Alamos HCV sequence alignments database (https://hcv.lanl.gov/content/sequence/NEWALIGN/align.html). Those were aligned together with the 81 consensus sequences obtained by Sanger sequencing as described in this study, using ClustalX software v2.0. The obtained alignment was edited using GENEDOC software v2.7, and phylogenetic trees were constructed using MEGA v6 software (http://www.megasoftware.net/). Neighbor-Joining and Maximum-Likelihood statistical methods were used for tree building, both following the Kimura 2-parameter substitution model and Gamma distributed rates among sites. Additionally, NJ/BioNJ tree inference option was selected for the maximum-likelihood algorithm. Ten thousand bootstrap replicates were performed for both algorithms in order to evaluate the robustness of the tree. The resulting phylogenetic trees were visualized and esthetically edited using FigTree software v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

2.8. Statistical analysis

Statistical analysis was carried out using IBM® SPSS® Statistics v19 (SPSS Inc., Chicago, Illinois, USA) and Microsoft Excel. The association between the presence of NS5 coding region RASs and continuous variables was analyzed using the Mann–Whitney U or Kruskal-Wallis H nonparametric tests, whereas categorical variables were analyzed through a qui-square test, with p-values below $\alpha=0.05$ indicating statistical significance.

3. Results and discussion

3.1. Comparison between HCV genotyping methods

The resistance profile analysis via sequencing conveniently provides a quick way of HCV genotyping through an online nucleotide BLAST. In this way, a thorough comparison between LiPA and Sanger population sequencing (BLAST and phylogenetic analysis) genotyping methods was conceived as an additional way of exploring the many uses that sequencing is capable of providing, as well as to validate the best method undertaken.

Overall, the subtype assignment of all 81 HCV sequences obtained in this study revealed a total of seven discrepancies (Table 3.1), of which six were due to incomplete, ambiguous or inconclusive LiPA genotyping and one due to rather wrong genotype attribution by LiPA.

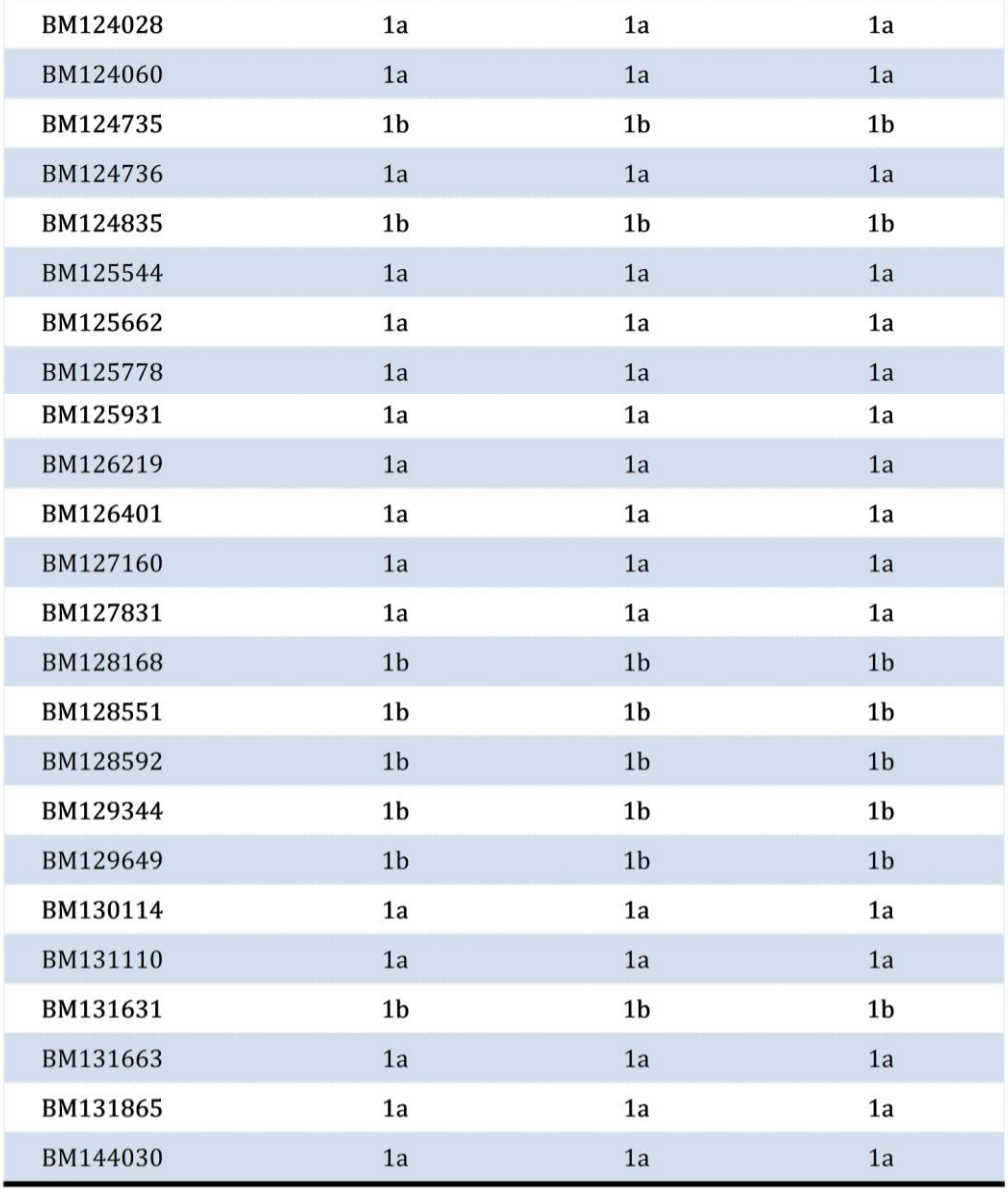
Phylogenetic analysis of the obtained GT1 HCV sequences using the Okamoto region was successful for subtype assignment, being supported by bootstrap values well above 70% when based on the Neighbor-Joining method following the Kimura 2-parameter substitution model, namely 91% and 90% for GT1a and GT1b clades (Figure 3.1), respectively. These bootstrap values are further supported by a second more rigorous phylogenetic tree based on the Maximum-Likelihood method following the Kimura 2-parameter substitution model, namely 84% and 89% for GT1a and GT1b clades (Figure 3.2), respectively, still much confidently higher than 70%. Furthermore, although bootstrap values for genotype 1 attribution *per se,* were slightly under 70% for both phylogenetic trees (69% and 65% for Neighbor-Joining and Maximum-Likelihood methods, respectively), all 81 GT1 HCV sequences were indubitably genotyped and subtyped, seeing that the previously described GT1a and GT1b clades bootstrap values were much higher than 70% for both phylogenetic analysis methods.

Finally, even though there were no genotype attribution discrepancies in any of the relapsing patients on this study, an incorrect genotype assignment by the current commercial genotyping assays stills remains a significant factor in the treatment failure of HCV infected patients [122].

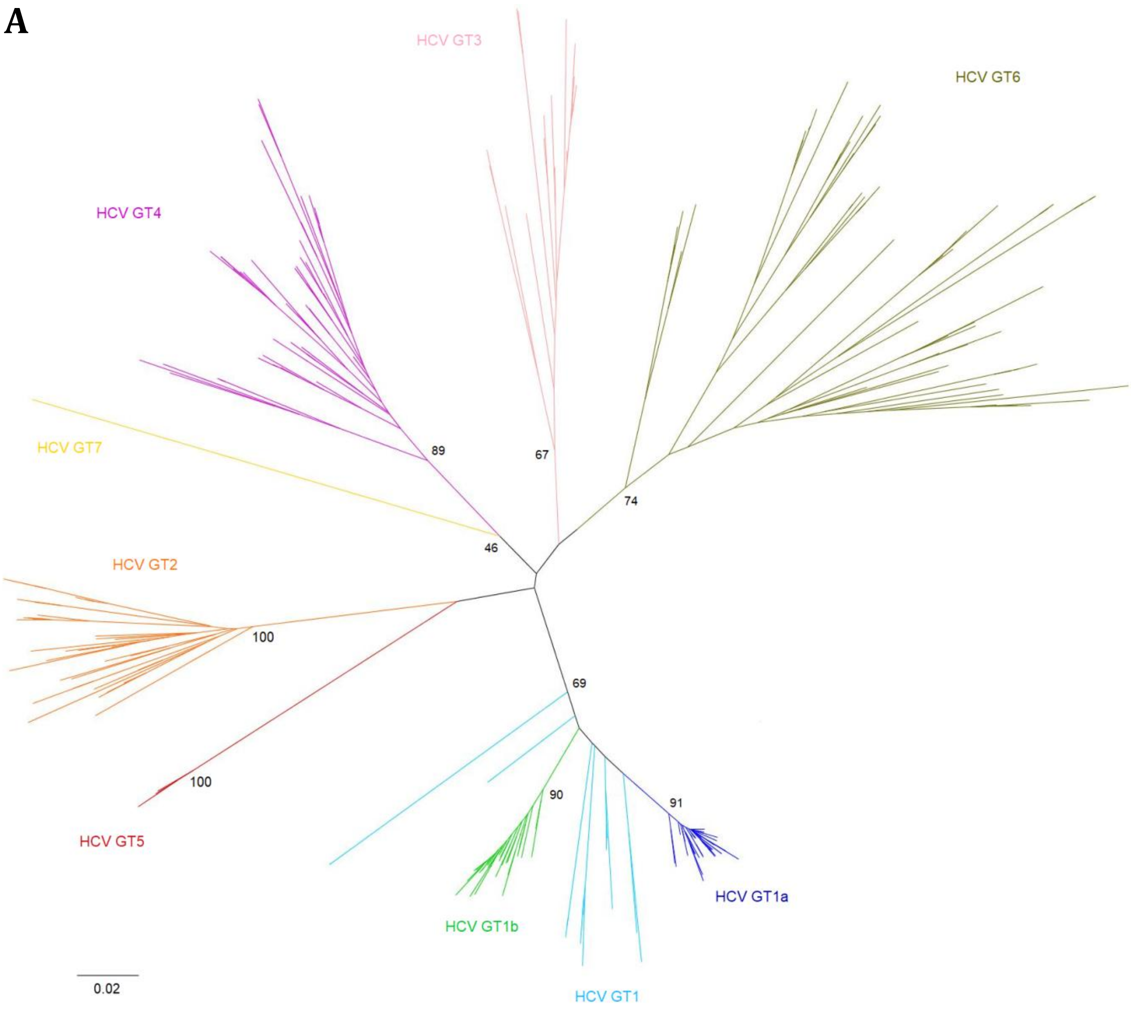
Table 3.1 Genotype assignment of all 81 HCV infected patients. Genotype attribution discrepancies between Inno-LiPA genotyping and Sanger sequencing are highlighted in yellow. In sequencing genotyping, the obtained HCV sequences were submitted to hcv.geno2pheno.org/ for both resistance and genotyping profiling, following a nucleotide BLAST (blast.ncbi.nlm.nih.gov/) to ascertain a resemblance between the query sequences and the NCBI database DNA sequences. A posterior phylogenetic analysis based on the Neighbor-Joining and Maximum-Likelihod methods was conducted on the obtained sequences, for a subtype assignment with higher accuracy.

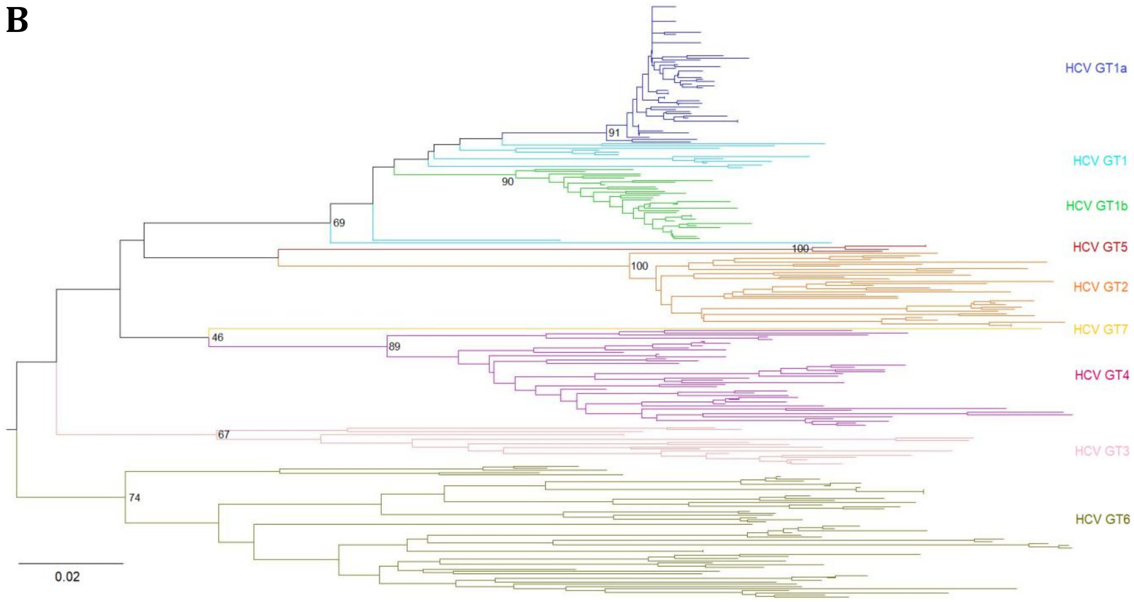
Clinical Sample	HCV Genotyping Methods		
	Inno-LiPA	Geno2pheno/ BLASTN	Phylogenetic analysis
BM102985	1a	1a	1a
BM107537	1a	1a	1a
BM108200	1a	1a	1a
BM108338	1a	1a	1a
BM109872	1a	1a	1a
BM110182	1b	1b	1b
BM110257	1a	1a	1a
BM110270	1a	1a	1a
BM110368	1a	1a	1a
BM110779	1a	1a	1a
BM111766	1a	1a	1a
BM111865	1b	1b	1b
BM111884	1a	1a	1a
BM112750	1b	1b	1b
BM113509	1a	1a	1a
BM113563	1a	1a	1a
BM113639	1b	1b	1b
BM113642	**1b, not excluding GT6**	**1b**	**1b**
BM113894	1a	1a	1a
BM113968	1a	1a	1a
BM114356	1b	1b	1b
BM114606	1a	1a	1a
BM114771	1a	1a	1a

BM114784	**Inconclusive**	**1a**	**1a**
BM114835	**No information on database**	**1b**	**1b**
BM114989	1a	1a	1a
BM114999	1a	1a	1a
BM115059	1a	1a	1a
BM115485	1b	1b	1b
BM115537	1b	1b	1b
BM115669	1a	1a	1a
BM115778	**1a + 1b**	**1b**	**1b**
BM116166	1b	1b	1b
BM116188	1a	1a	1a
BM116276	1a	1a	1a
BM116604	1a	1a	1a
BM116828	1a	1a	1a
BM117188	1a	1a	1a
BM117192	1b	1b	1b
BM117203	1a	1a	1a
BM117204	**1b, not excluding GT6**	**1b**	**1b**
BM117272	1a	1a	1a
BM118084	1b	1b	1b
BM118129	**1b**	**1a**	**1a**
BM118658	1a	1a	1a
BM118662	1b	1b	1b
BM120956	1a	1a	1a
BM121559	**No information on database**	**1b**	**1b**
BM122802	1a	1a	1a
BM123017	1a	1a	1a
BM123018	1a	1a	1a
BM123019	1a	1a	1a
BM123020	1a	1a	1a
BM123445	1a	1a	1a
BM123631	1a	1a	1a
BM123728	1a	1a	1a
BM123806	1b	1b	1b



BM124028	1a	1a	1a
BM124060	1a	1a	1a
BM124735	1b	1b	1b
BM124736	1a	1a	1a
BM124835	1b	1b	1b
BM125544	1a	1a	1a
BM125662	1a	1a	1a
BM125778	1a	1a	1a
BM125931	1a	1a	1a
BM126219	1a	1a	1a
BM126401	1a	1a	1a
BM127160	1a	1a	1a
BM127831	1a	1a	1a
BM128168	1b	1b	1b
BM128551	1b	1b	1b
BM128592	1b	1b	1b
BM129344	1b	1b	1b
BM129649	1b	1b	1b
BM130114	1a	1a	1a
BM131110	1a	1a	1a
BM131631	1b	1b	1b
BM131663	1a	1a	1a
BM131865	1a	1a	1a
BM144030	1a	1a	1a


A
HCV GT3
HCV GT6
HCV GT4
HCV GT7
89
67
74
46
HCV GT2
100
100
69
90
91
HCV GT5
HCV GT1a
HCV GT1b
HCV GT1
0.02


B
HCV GT1a
91
HCV GT1
90
HCV GT1b
69
100
HCV GT5
100
HCV GT2
46
HCV GT7
89
HCV GT4
67
HCV GT3
74
HCV GT6
0.02

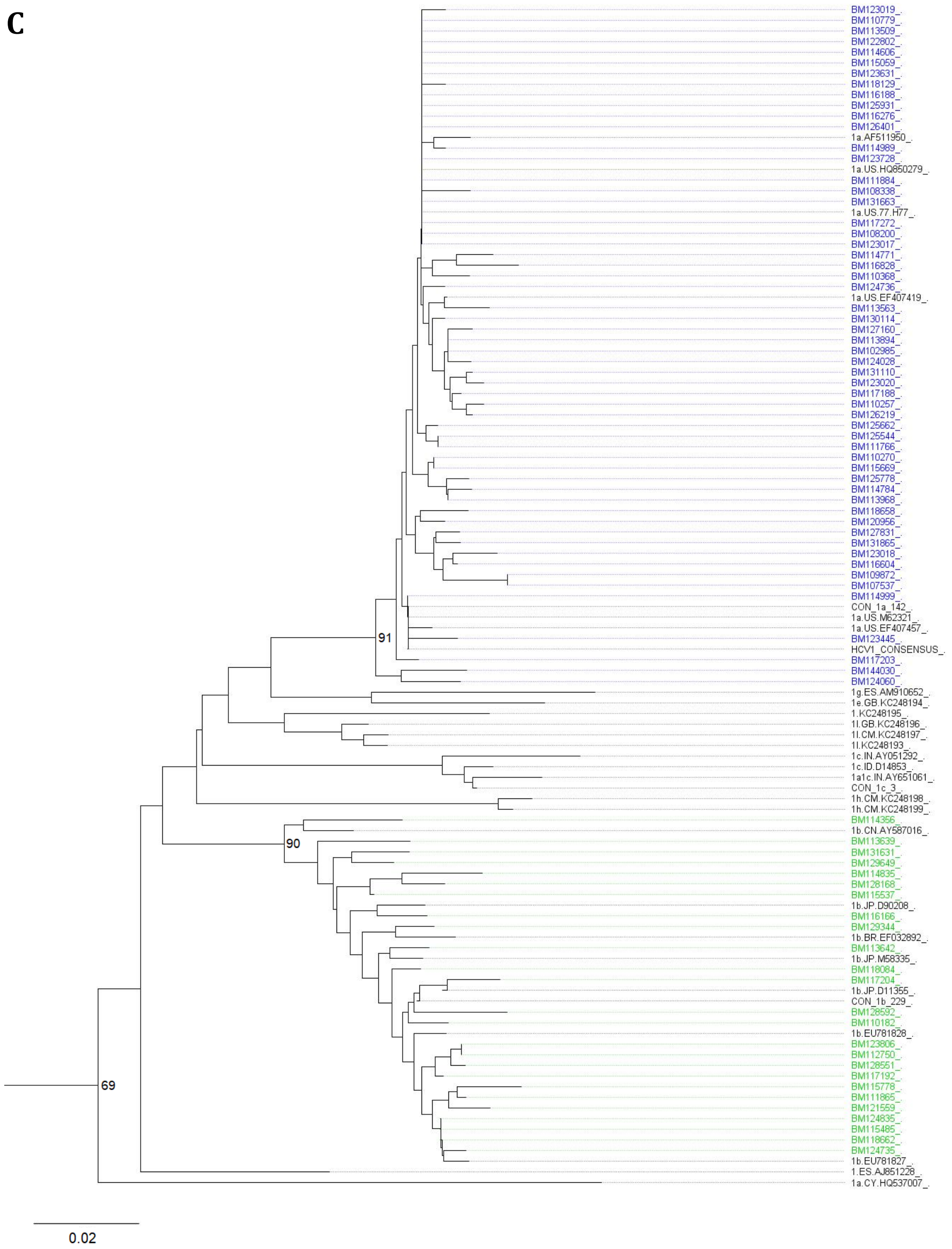
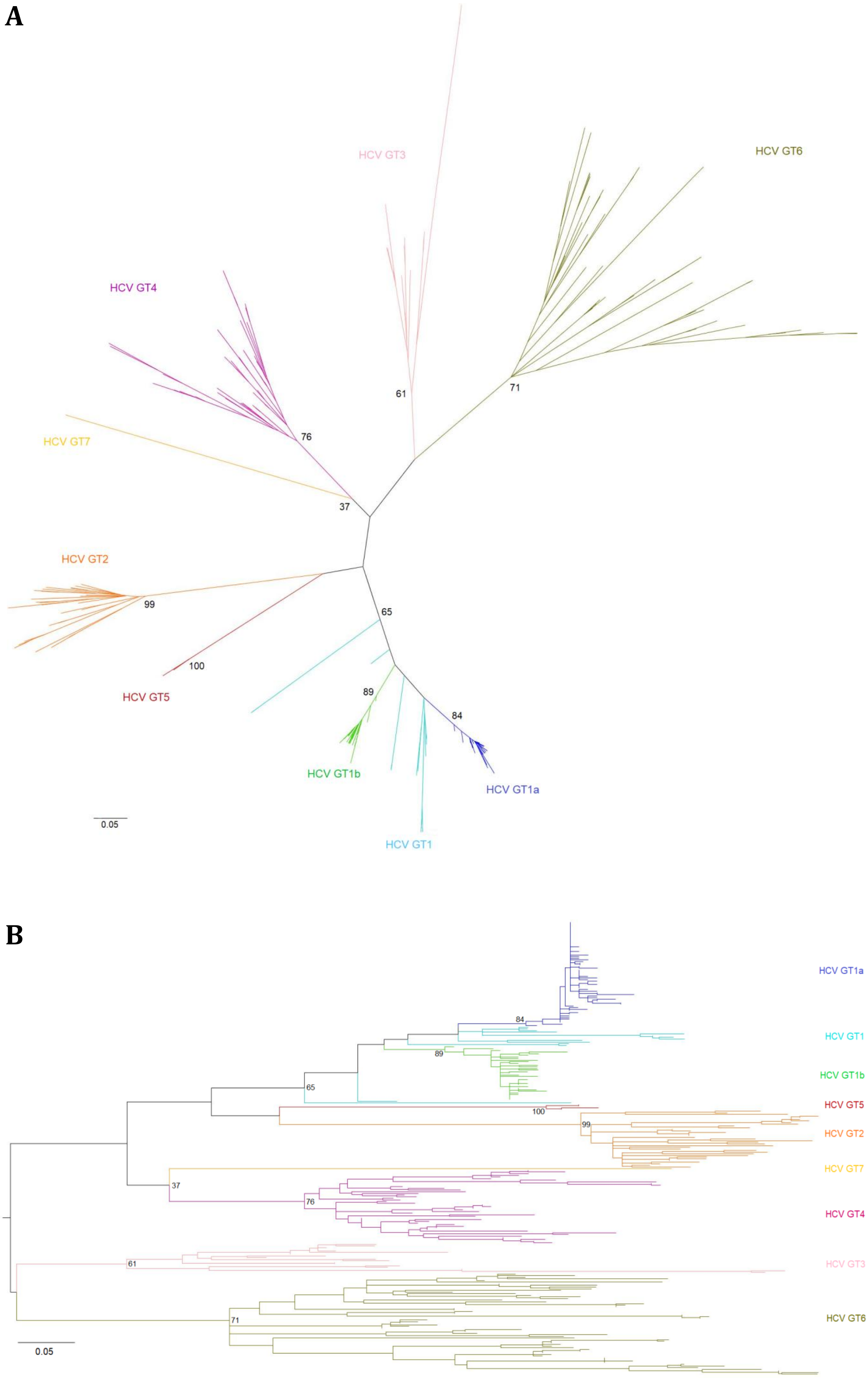


Figure 3.1 Phylogenetic consensus trees of the obtained GT1 HCV sequences, based on the Neighbor-Joining method using MEGA v6: **A**, Radial layout tree view of all HCV genotypes; **B**, Rectangular layout tree view of all HCV genotypes; **C**, Genotype 1 clade with GT1a and GT1b obtained sequences marked in blue and green, respectively. Ten thousand bootstrap replicates were performed to evaluate the robustness of the topology, and main clades bootstrap values are indicated beside of each node. The genetic distance scale bar is at the bottom of each figure, indicating the number of nucleotide substitutions per site along each lineage.


A
HCV GT3
HCV GT6
HCV GT4
61
71
76
HCV GT7
37
HCV GT2
99
65
100
HCV GT5
89
84
HCV GT1b
HCV GT1a
0.05
HCV GT1
B
HCV GT1a
84
HCV GT1
89
HCV GT1b
65
HCV GT5
100
99
HCV GT2
HCV GT7
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HCV GT3
71
HCV GT6
0.05

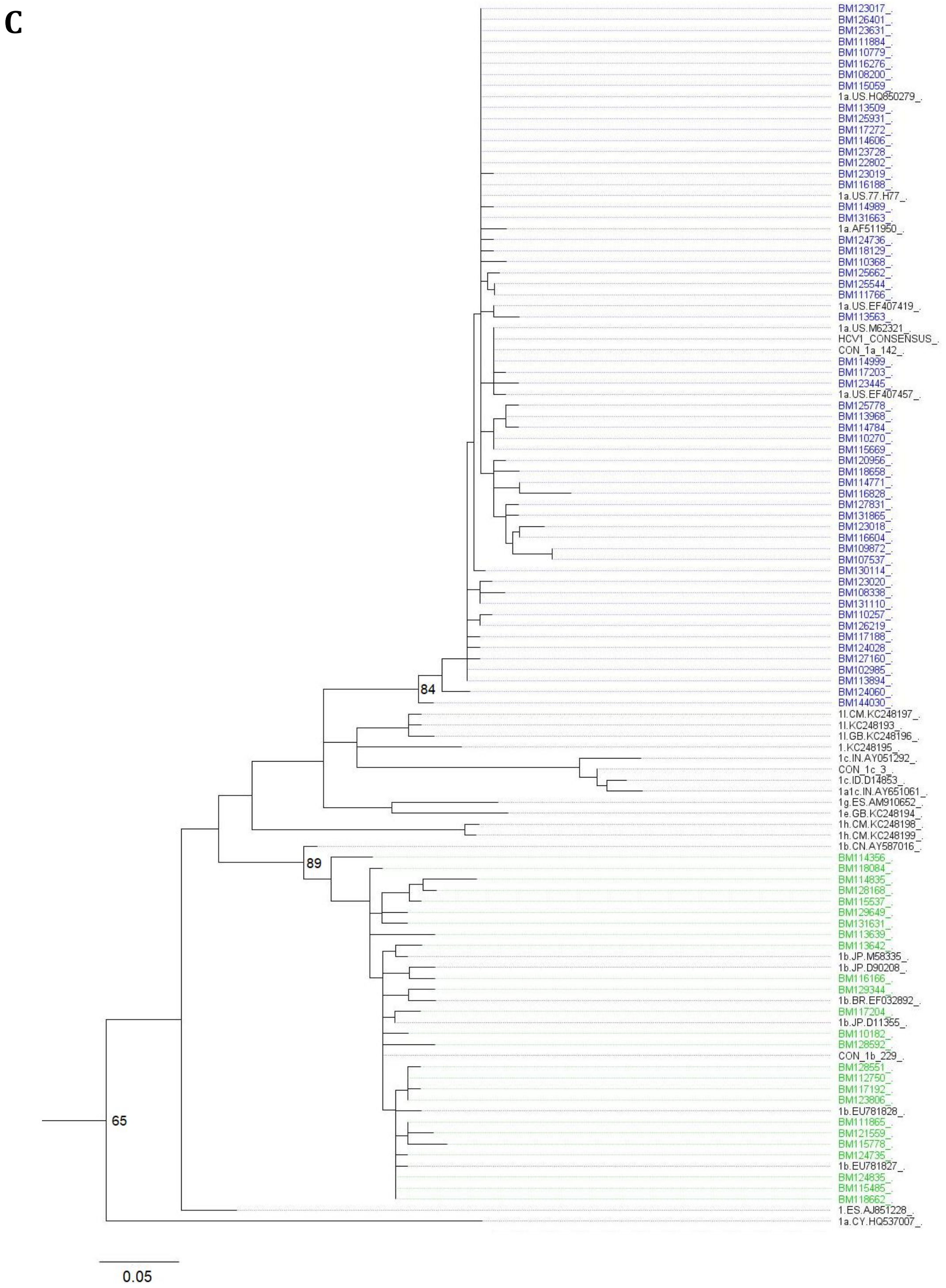

Figure 3.2 Phylogenetic consensus trees of the obtained GT1 HCV sequences, based on the Maximum-Likelihood method using MEGA v6: **A**, Radial layout tree view of all HCV genotypes; **B**, Rectangular layout tree view of all HCV genotypes; **C**, Genotype 1 clade with GT1a and GT1b obtained sequences marked in blue and green, respectively. Ten thousand bootstrap replicates were performed to evaluate the robustness of the topology, and main clades bootstrap values are indicated beside of each node. The genetic distance scale bar is at the bottom of each figure, indicating the number of nucleotide substitutions per site along each lineage.

3.2. Statistical analysis

Using a confidence level of 95%, statistical analysis indicated that treatment failure was not significantly associated with either the class of NS5 RASs ($p = 0.549$) or the mere non-discriminatory presence of baseline NS5 RASs ($p = 0.232$). However, a very strong statistical significant association was found between treatment failure and the presence of specific baseline NS5 RASs, namely Y93C/H ($p = 0.04$, with a Phi and Cramer´s V strength of association effect size of 0.376).

An additional association between the presence of baseline NS5 RASs and several extra variables such as the viral load right before treatment initiation, age, sex, the IL28B polymorphism, co-infection status and HCV genotype, was parallelly sought over as to find a connection to other relevant variables common in the clinical practice of HCV infection besides treatment failure. Overall, few significant associations were found between the presence of NS5 RASs and any of these variables, nonetheless HCV genotype stood out by revealing a statistical significant association to the non-discriminatory presence of baseline NS5 RASs ($p = 0.001$), namely with GT1b being the strongest predictor of this association, as well as to the class of NS5 RASs ($p=0.000$), namely with GT1b and NS5B RASs being the strongest predictors of association. Moreover, HCV genotype also exhibited a significant association to the presence of specific baseline NS5 RASs, namely A92E/T ($p = 0.007$), L159F ($p = 0.000$), C316N ($p=0.000$), and C451I/Y ($p=0.007$), being GT1b ever the strongest predictor of these associations. Finally, the IL28B polymorphism revealed a statistical significant association to the non-discriminatory presence of baseline NS5 RASs ($p=0.041$), namely with IL28B CC polymorphism being the strongest predictor of association, as well as to the presence of specific baseline NS5 RASs, namely H58C/P/S ($p = 0.02$) and the major Y93C/H mutation ($p = 0.026$), with the IL28B TT and CC polymorphisms being the strongest predictors of association, respectively.

3.3. Resistance profile analysis

3.3.1. Prevalence of NS5 RASs and treatment outcome

Amongst all genotype 1 infected patients, 38,3% (31/81) presented NS5 RASs at baseline, with NS5A class RASs showing the highest prevalence, followed by NS5B RASs and the combined NS5A + NS5B RASs, namely 23,5% (19/81), 9,9% (8/81) and 4,9% (4/81), respectively (Figure 3.3). However, the prevalence of NS5A and NS5B RASs would rise up to 28,4% (23/81) and 14,8% (12/81), respectively, when not considering separately the dual-class RASs. In a study from 2016 analyzing data from phase II/III trials of 2108 GT1 infected patients in order to evaluate the effects of baseline NS3, NS5A and NS5B RASs on the response to a SOF/LDV based regimen, up to 16% (338/2108) and 2,5% (43/1692) of patients had detectable NS5A and NS5B RASs, respectively [158].

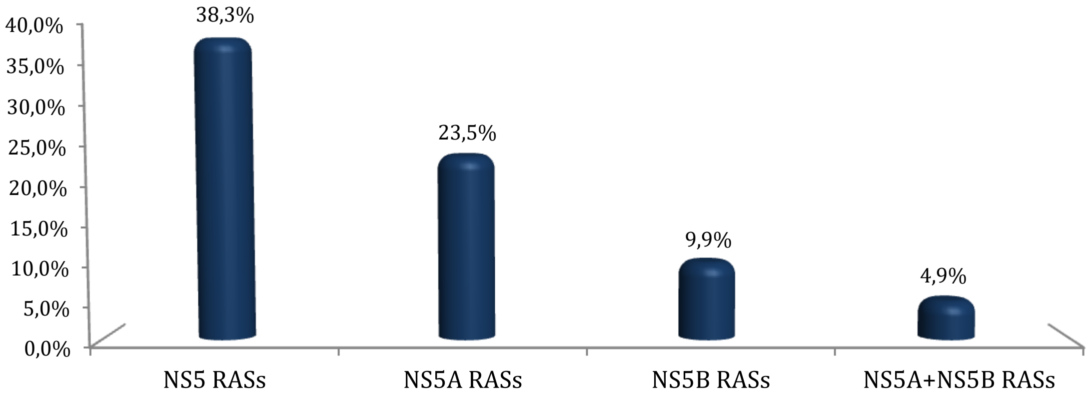

Figure 3.3 Baseline prevalence of NS5 RASs in all GT1 HCV infected patients. Substitution analyses were conducted on Sanger sequencing data with a 15% cut-off limit. Dual-class RASs are assigned as NS5A + NS5B RASs.

About 27% (15/56) of the GT1a infected patients presented NS5 RASs, although only NS5A class, comparing to a much higher percentage of NS5 RASs in the GT1b infected patients accounting for 64% (16/25), with NS5B RASs prevailing at 32% (8/25) followed by NS5A and dual-class RASs both at 16% (4/25) (Figure 3.4). However, from another point of view, the GT1b patients showed a higher prevalence of NS5A RASs than for GT1a, namely 32% (8/25), when not considering separately the dual-class RASs. In two studies comparing the effect of baseline NS5A RASs on a SOF/LDV based regimen [198,199], the authors equally found a higher prevalence of baseline RASs for GT1b than for

GT1a, namely 25,2% (52/206) and 17,6% (25/142) vs. 13,2% (40/303) and 7,1% (12/170), for each case study respectively, but still insignificant on SVR rates, except for treatment-experienced patients with decompensated cirrhosis infected with GT1a HCV in which these RASs conferred a high fold resistance (>100x).

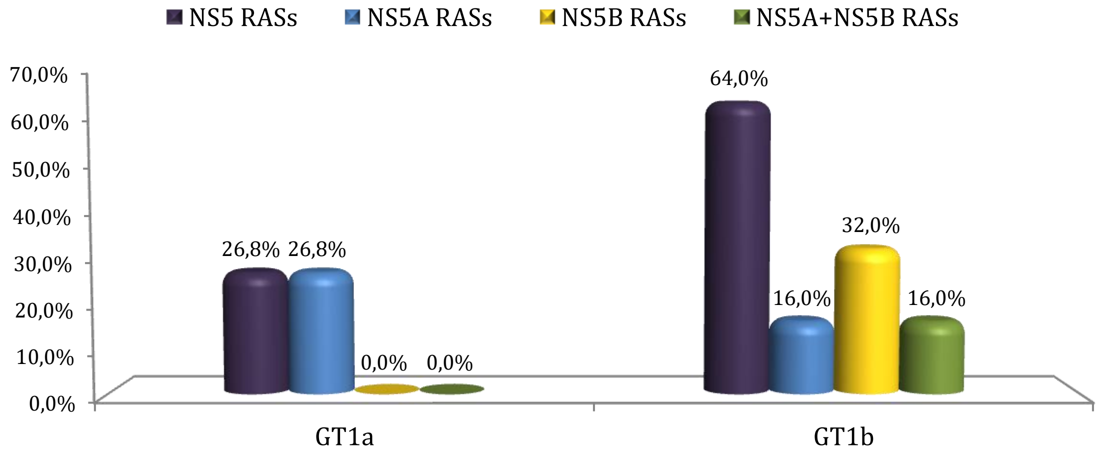

Figure 3.4 Percentage of HCV infected patients with NS5 RASs, according to HCV subtype. Substitution analyses were conducted on Sanger sequencing data with a 15% cut-off limit. Dual-class RASs are assigned as NS5A + NS5B RASs.

NS5A constitutes the most important genomic region considered for the screening of associated RASs. As such, 19,8% (16/81) of all 81 GT1 infected patients showed at least one RAS that conferred more than 100-fold resistance to ledipasvir (Q30H/R, L31M, and/or Y93C/H), namely 21,4% (12/56) and 16% (4/25) of GT1a and GT1b infected patients, respectively, and have also been described in several clinical trials as the most frequently encountered clinically relevant baseline NS5A RASs in GT1 HCV [40,76,98,152,154–156]. Along these lines, all regimens based in NS5A inhibitors should include a NI with a high genetic barrier to resistance, currently sofosbuvir, since this combination has been shown to elicit a profound viral suppression [200,201] as well as higher SVR rates when treatment duration is extended to 24 weeks with or without ribavirin. This data is further supported from deep sequencing analysis of baseline NS5A RASs in 276 GT1 infected patients, which showed a prevalence of 21,4% (59/276) of high level NS5A RASs conferring resistance to ledipasvir [202], though a contemporary study described a much lower prevalence, as 11,5% (102/887) of treatment-naive GT1 infected patients had at least one RAS that conferred >100-fold resistance to ledipasvir [158].

Dual-class RASs were only observed in 4 GT1b infected patients, whereas combined intra-class RASs were detected in 4 GT1a patients concerning the NS5A gene (Q30H + Y93H), and then in one and six GT1b patients for the NS5A (A92T + Y93H) and NS5B (L159F + C316N and L159F + C316N + C451Y) regions, respectively (Figure 3.5).

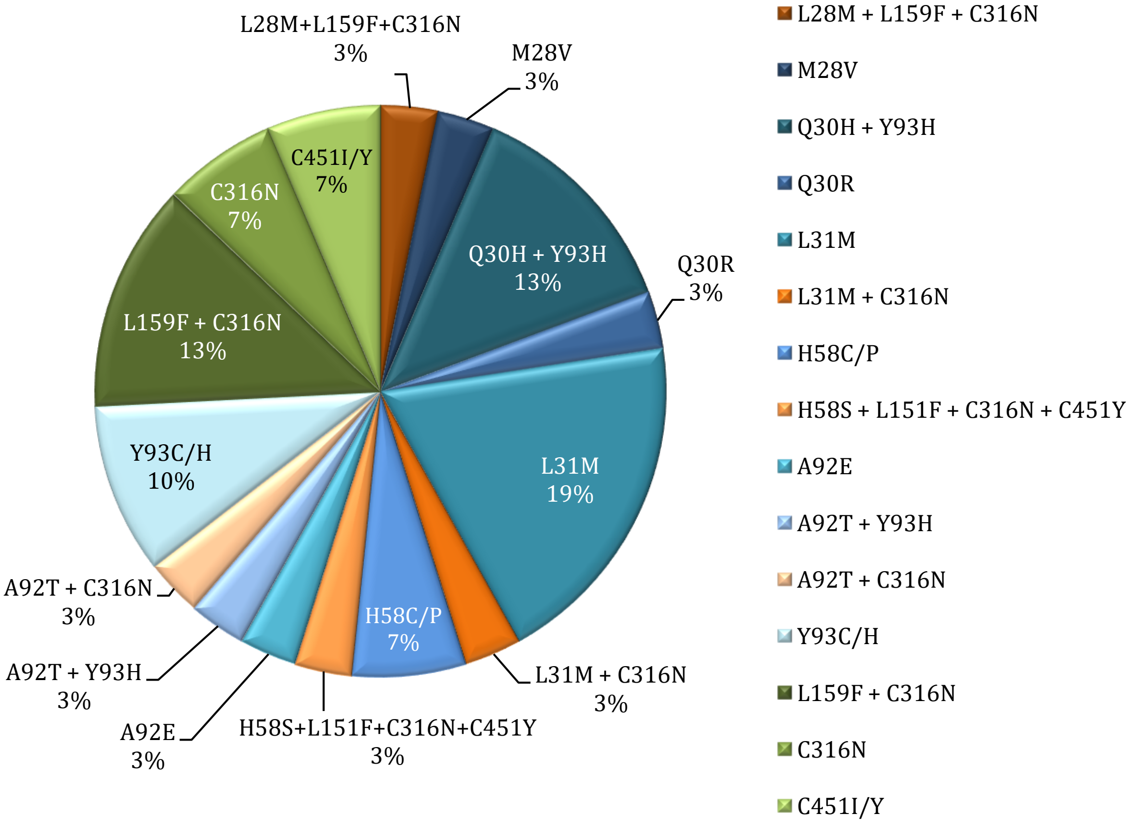

Figure 3.5 Baseline prevalence of detected NS5 RASs combinations within GT1 infected patients with RASs. NS5A, NS5B and dual-class RASs are viewed in variations of blue, green and orange, respectively. Substitution analyses were conducted on Sanger sequencing data with a 15% cut-off limit.

Baseline resistance testing of the NS5 coding region showed that the efficacy of SOF and NS5A inhibitors, mainly LDV, was not compromised in most patients whom showed a natural presence of RASs before treatment initiation, since NS5 RASs were present in 38,2% of patients who achieved a SVR, with 22,4% (17/76), 10,5% (8/76), and 5,3% (4/76) corresponding to NS5A, NS5B and dual-class RASs, respectively (Figure 3.6). Nonetheless, 66,7% (2/3) of the relapsing patients presented NS5A class only RASs, as the combined Q30H + Y93H mutations, causing high level resistance to all NS5A inhibitors, were detected at baseline in one HIV/HCV GT1a co-infected patient who later failed a treatment with SOF/LDV for 12 weeks. However, another relapsing HIV/HCV GT1a co-infected patient whom also failed the same therapeutic settings previously described, showed no NS5 RASs at baseline. Furthermore, an isolated Y93H mutation was also detected at baseline in one GT1b mono-infected patient experiencing virological relapse.

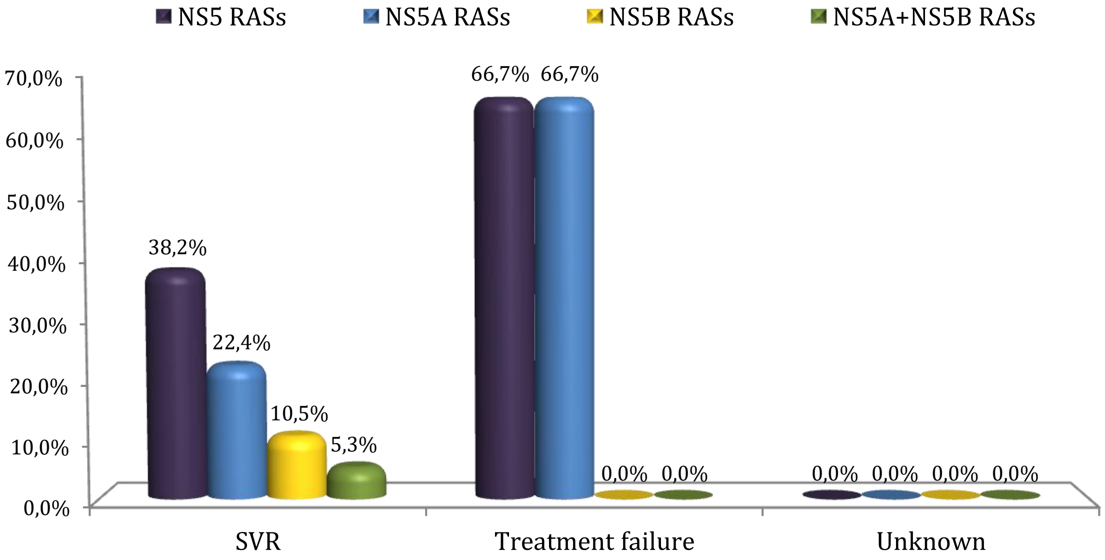

Figure 3.6 Percentage of HCV infected patients with NS5 RASs, according to treatment outcome status. Substitution analyses were conducted on Sanger sequencing data with a 15% cut-off limit. Dual-class RASs are assigned as NS5A + NS5B RASs.

Even though the Y93H mutation showed a significant effect on treatment outcome (p=0.04) being detected as a dominant substitution for the two patients who did not achieve SVR12 with NS5A RASs, most patients with at least one NS5A RAS conferring >100-fold resistance to ledipasvir still achieved SVR12. This data is further corroborated from a recent study in which the reduction in SVR rates of 2108 GT1 infected patients who received a regimen of SOF + LDV, appeared to be driven predominantly by patients with NS5A RASs, namely with Y93H being detected as a dominant substitution, although the general presence of baseline NS5A and NS5B RASs had showed no significant impact on treatment outcome, with SVR rates greater than 91% [158]. Moreover, a deep sequencing analysis of baseline NS5A RASs in 276 and 32 patients infected with GT1 and GT4 HCV, respectively, showed that all patients who failed to achieve SVR12 presented at least one NS5A RAS (M28T, Q30H/K/R, L31M/V and/or Y93C/H) [202].

Finally, no RASs were detected in the two GT1a infected patients with an unknown treatment outcome.

Overall, 93,5% (29/31) of patients with baseline NS5 RASs achieved SVR12, of which 54,8% (17/31), 25,8% (8/31) and 12,9% (4/31) corresponded to NS5A, NS5B and NS5A + NS5B class RASs, respectively, comparing to only two patients (6,5%) whom experienced virological failure by carrying NS5A RASs (Figure 3.7).

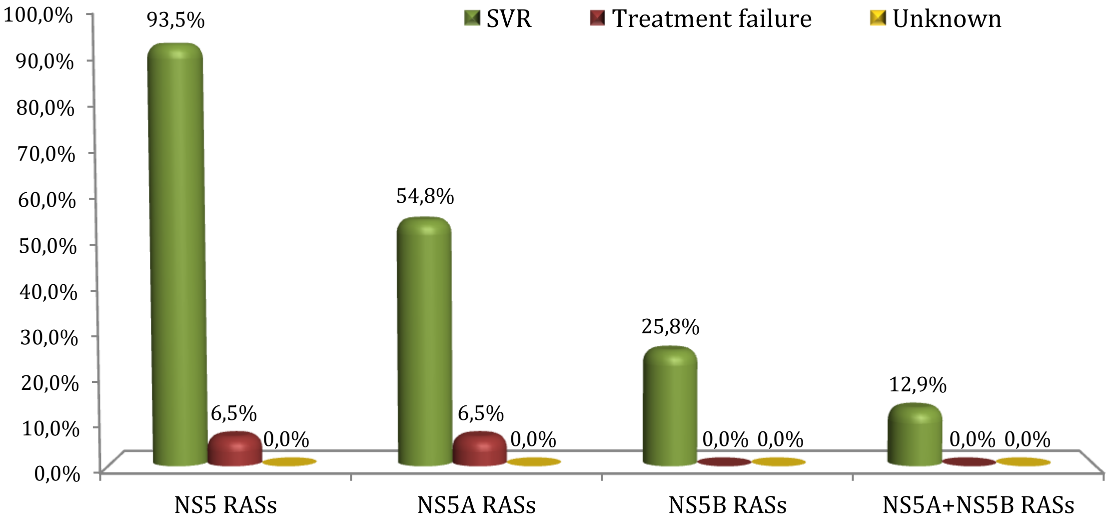

Figure 3.7 Baseline prevalence of NS5 RASs in GT1 HCV infected patients, according to treatment outcome status. Substitution analyses were conducted on Sanger sequencing data with a 15% cut-off limit. Dual-class RASs are assigned as NS5A + NS5B RASs.

3.3.2. Prevalence of NS5 RASs and environmental factors

The prevalence of NS5 RASs was amazingly balanced between the male and female sex, namely 38,1% (24/63) vs. 38,9% (7/18), respectively (Figure 3.8). Regarding the IL28B gene polymorphism, patients exhibiting the IL28B CC polymorphism showed the highest prevalence of NS5 RASs, followed by the IL28B TT and CT polymorphisms, namely 60,9% (14/23), 50% (3/6) and 27,7% (13/47), respectively (Figure 3.9). Furthermore, up to 20% of patients with an unknown IL28B polymorphism presented NS5 RASs, of which all corresponded to NS5B class RASs.

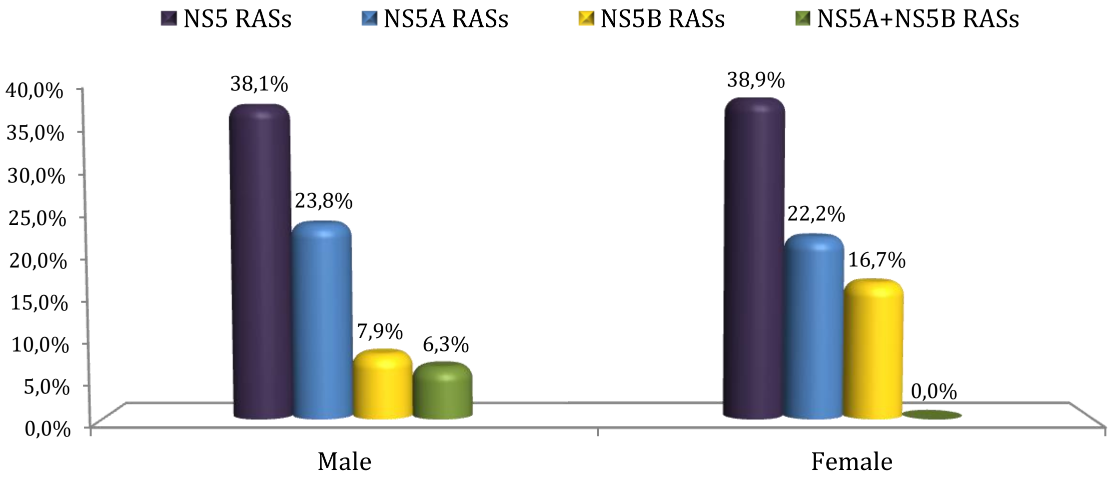

Figure 3.8 Percentage of HCV infected patients with NS5 RASs, according to sex. Substitution analyses were conducted on Sanger sequencing data with a 15% cut-off limit. Dual-class RASs are assigned as NS5A + NS5B RASs.

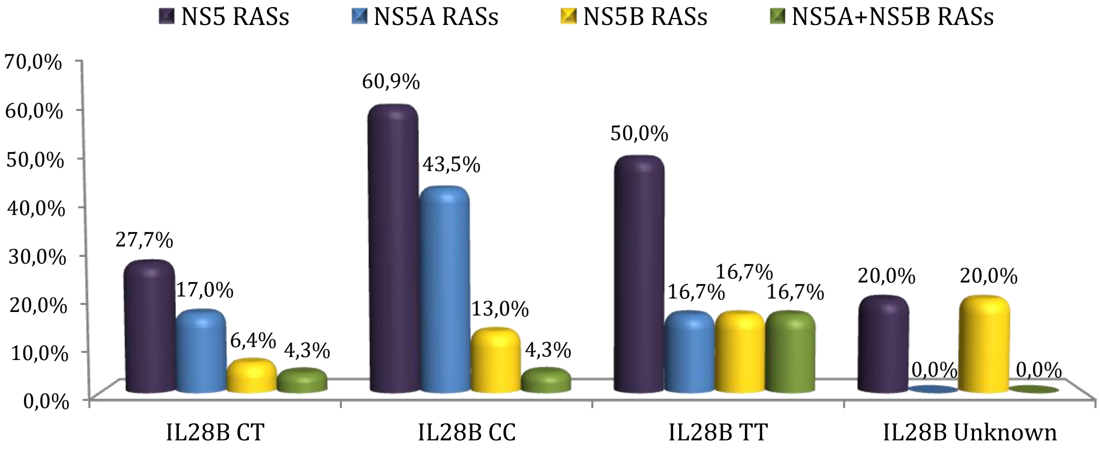

Figure 3.9 Percentage of HCV infected patients with NS5 RASs, according to the IL28B gene polymorphism. Substitution analyses were conducted on Sanger sequencing data with a 15% cut-off limit. Dual-class RASs are assigned as NS5A + NS5B RASs.

According to the monoinfection and co-infection status, HCV mono-infected patients showed the highest prevalence of NS5 RASs, followed by HCV/HIV/HBV co-infected, HCV/HIV co-infected and HCV/HBV co-infected patients, namely 46,2% (12/26), 44,4% (4/9), 33,3% (14/42) and 25% (1/4), respectively (Figure 3.10). Additionally, of the 31 GT1 HCV infected patients whom presented NS5 RASs at baseline, about 58% (18/31) were co-infected with HIV/HCV when disregarding HBV co-infections.

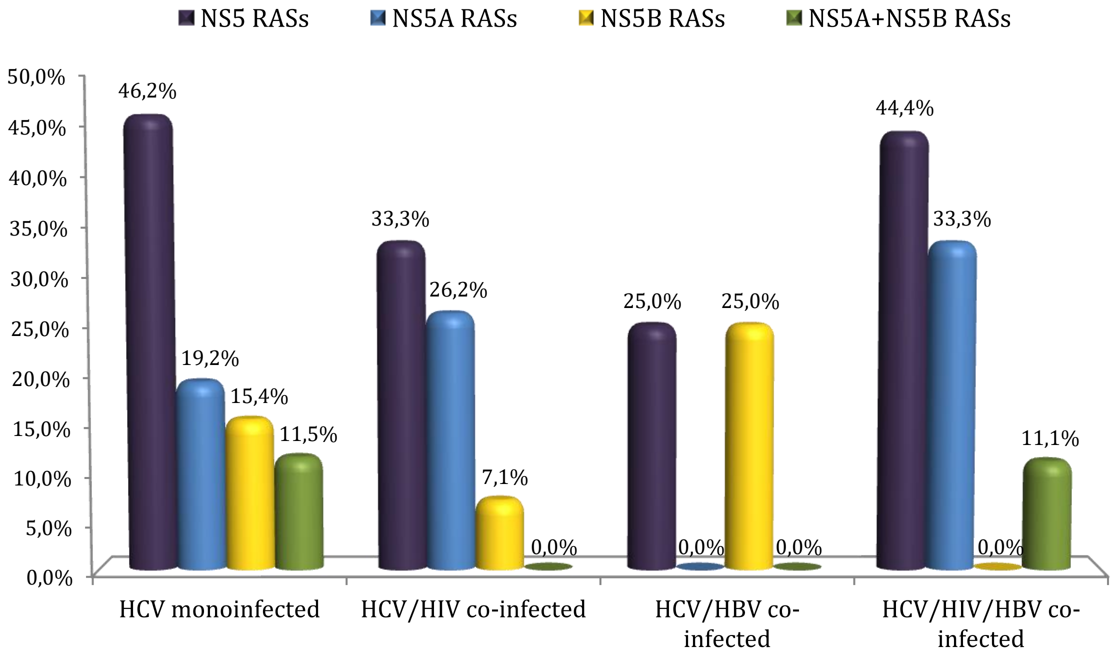

Figure 3.10 Percentage of HCV infected patients with NS5 RASs, according to the monoinfection and co-infection profiles. Substitution analyses were conducted on Sanger sequencing data with a 15% cut-off limit. Dual-class RASs are assigned as NS5A + NS5B RASs.

3.3.3. Prevalence of NS5A specific RASs

Major NS5A RASs were detected in 23,2% (13/56) of GT1a infected patients (M28V, Q30H/R, L31M and/or Y93C/H) and in 16% (4/25) of GT1b infected patients (L31M and Y93H), although minor NS5A RASs were also detected in 3,6% (2/56) of GT1a patients (H58C/P) and in 20% (5/25) of GT1b patients (L28M, H58S and A92E/T).

The most commonly detected NS5A RAS was Y93C/H with a baseline prevalence of 9,9% (8/81) in all GT1 infected patients, followed by L31M and Q30H/R with a prevalence of 8,6% (7/81) and 6,2% (5/81), respectively (Figure 3.11). Moreover, Y93C/H showed a higher prevalence in GT1b patients than in GT1a, namely 12% (3/25) vs. 8,9% (5/56), respectively. The baseline Y93C/H mutation largely accounted for 25,8% (8/31) of all patients carrying RASs, followed by L31M and Q30H/R, with 22,6% (7/31) and 16,1% (5/31), respectively (Figure 3.12). A 2015 analysis of more than 3000 GT1 NS5A sequences has demonstrated a higher baseline prevalence of the Y93C/H/N mutation, especially in GT1b, being mostly found in South Korea (15,3%) followed by Japan (13,9%) and Spain (13,6%), whereas in GT1a the baseline Q30E/H/R mutation was described as the most commonly detected, with the highest prevalence being found in

Italy (4,9%) followed by France (4,8%) and New Zealand (3,9%) [155]. Furthermore, an analysis from the same year of NS5A sequences in 132 Japanese GT1b HCV infected patients, established that baseline NS5A RASs are common in treatment-naive patients infected with GT1b, demonstrating an even higher prevalence of the Y93H mutation, namely 25% (32/132) [195].

3.3.4. Prevalence of NS5B specific RASs

NS5B RASs accounted for 14,8% (12/81) of GT1 HCV infected patients when not considering separately the dual-class RASs, and were only detected in GT1b patients, all of which achieved SVR12. Furthermore, these were mainly represented by the C316N mutation, accounting for 40% (10/25) of GT1b infected patients, and showing the highest prevalence amongst all patients with baseline NS5 RASs, namely 32,3% (10/31) (Figure 3.12). The major S282T mutation, causing high level resistance to sofosbuvir was never detected, however, the baseline L159F mutation which confers a reduced susceptibility to this drug, was detected in 24% (6/25) of GT1b infected patients, and in 19,4% (6/31) of all patients carrying NS5 RASs. Along these lines, patients treated with regimens based on NS5B polymerase inhibitors, particularly NIs, tend to exhibit a low prevalence of baseline RASs [174], but when detected the most frequently encountered appear to be L159F, C316N/Y, L320F, V321A, M414T, and S556G [76,98,102,203–205].

Dasabuvir on the other hand possesses a low genetic barrier to resistance, as the associated C316N mutation tends to achieve a substantial baseline prevalence of 10 – 36% in GT1b infected patients [98,206,207]. Moreover, an analysis of more than 7800 NS5A and NS5B sequences from HCV infected patients across 22 countries showed prevalences of up to 34% for the L159F mutation in GT1b infected patients [155]. Finally, several studies analyzing the natural presence of NS5B RASs equally refute an associated significant impact on SVR12 rates [152,208], which further strengthens the obtained data.

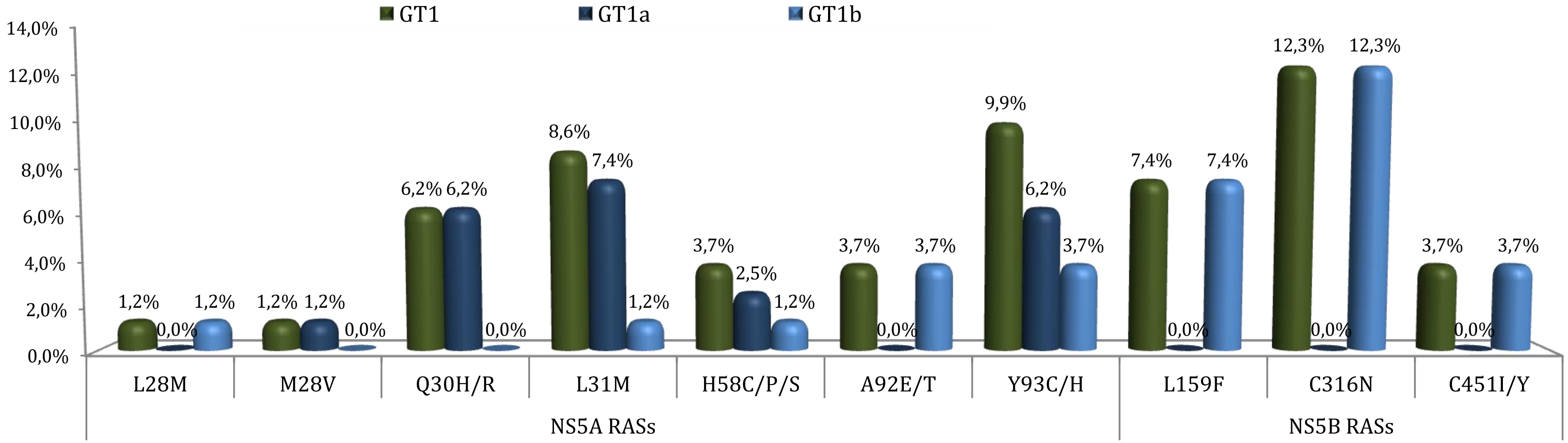

Figure 3.11 Baseline prevalence of specific NS5 RASs in all 81 HCV infected patients by subtype. Substitution analyses were conducted on Sanger sequencing data with a 15% cut-off limit. Dual-class RASs are assigned as NS5A + NS5B RASs.

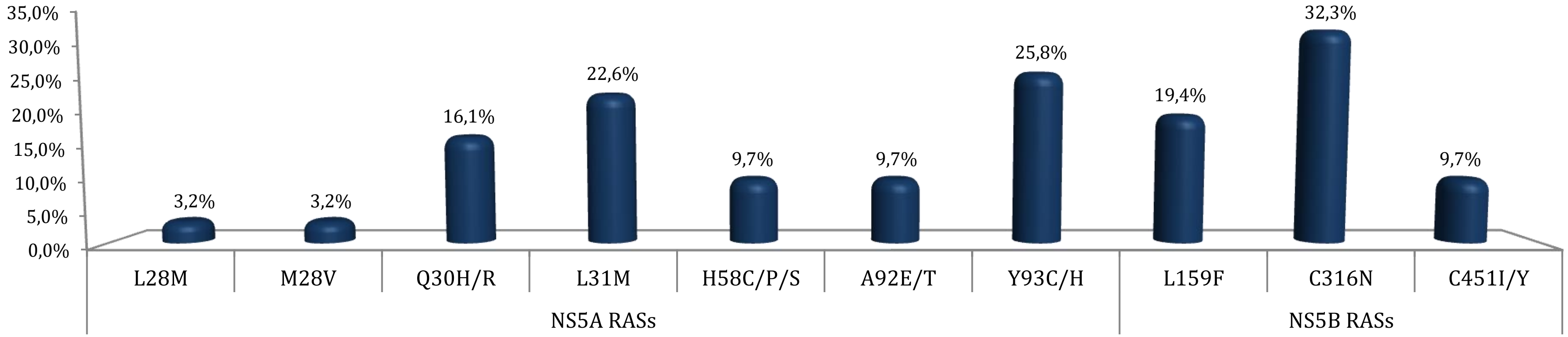

Figure 3.12 Baseline prevalence of specific NS5 RASs within GT1 infected patients with RASs. Substitution analyses were conducted on Sanger sequencing data with a 15% cut-off limit.

4. Conclusions

According to the WHO Global baselines and coverage targets [209], there is an estimated 90% reduction of HCV and HBV infections and related deaths up to 2030. However, HCV treatment coverage worldwide is still below 10%, emphasizing the need of considerable HCV and HBV treatment scale-up to reach the WHO HCV and HBV treatment coverage targets prior to 2030 [209]. In this way, the strategies for HCV elimination ought to be fully complied and mainly focused on a Test & Treat routine in which rapid point-of-care testing should be progressively more implemented in the clinical practice, as well as on Prevention & Awareness services specially directed to major risk groups. Additionally, research should be promoted for the development of a HCV vaccine as well as to the implementation of a HCV prophylaxis directed in particular for risk groups such as PWID just like what was recently successfully done with PrEP (Pre-Exposure Prophylaxis) for HIV [210–213], as these strategies would be an asset in achieving the treatment coverage targets up to 2030, thus becoming a step closer to HCV global elimination in populations of both HCV mono-infected and co-infected patients with HIV and/or HBV.

HCV sequencing is constantly improving and becoming more widely available. In summary, baseline sequencing can help in the selection of an optimal therapy, especially in difficult-to-treat patients, and phylogenetic analysis came as great aid to this study over its rigorous methodology of genotyping in comparison to commercially available assays like LiPA, which consequently corrected several misclassified samples. In addition, phylogenetic analysis comes quite useful in the clinical practice as it can be used to distinguish relapses from re-infections, as well as to find out how transmission networks work in a more epidemiological setting.

Failure to completely sequence the NS5B region was explained by virtue of the 3'-end of the HCV genome being a poly-U (U/C) region highly composed of hairpins and secondary structures, which consequently left uncovered three important amino acid positions (A553, G554, and S556) associated with resistance to dasabuvir. Consequently, a potential solution could reside in improving both RT-PCR and nested PCR primers in order to replicate a relatively longer fragment that contains all NS5A and NS5B amino acid positions associated to resistance. Moreover, the use of NGS technology could also constitute a solution to this problem since some platforms can bypass the use of sequencing primers, resulting in a potentially cheaper resistance testing and a much more revealing methodology in terms of mutations due to the 1% cut-off limit.

The obtained data supports the usefulness of resistance testing prior to treatment initiation, thus preventing relapses associated to the presence of baseline RASs, as a statistical significant association was found between treatment failure and the baseline presence of specific NS5 RASs, namely Y93C/H ($p = 0.04$). With this in mind, an interesting recommendation to take into account would be to implement a protocol of baseline resistance testing directed only for the clinically relevant Y93 amino acid position of the NS5A gene, since all other NS5 RASs, excluding the rare S282T mutation which was not detected, do not seem to have a significant impact on treatment outcome when on sofosbuvir plus NS5A inhibitors based regimens. However, this reduced sampling can constitute a limiting factor, since it may underestimate the statistical analysis results, and lead to overall higher RASs rates when comparing to other previous studies [78,135,152,155,157,158,174,195,214].

In conclusion, baseline NS5 RASs seem to have minimal effects on patient responses to sofosbuvir plus NS5A inhibitors (LDV, DCV, VEL) based therapies, having been well established through cumulative research data that when baseline NS5A RASs in particular do have effects, they could be largely over-come by extending treatment duration and/or through treatment intensification with the addition of RBV, thus drastically reducing the impact of NS5A baseline RASs [145,149,208,215,216]. Additionally, the cost-effectiveness of baseline resistance testing was accessed in a recent paper describing that the inquiry of baseline NS5A RASs prior to a treatment with EBR/GZR is indeed a cost-effective measure resulting in more QALYs (quality-adjusted life years) among GT1a treatment-naive or treatment-experienced patients, when comparing to a treatment with EBR/GZR without baseline resistance testing, and LDV/SOF or 3D based regimens, thusly being another factor in favor of baseline resistance testing prior to treatment initiation [217].

On a final note, the importance of total adherence to the treatment should always be encouraged and insisted upon in an elucidative way by physicians in order to prevent potential relapses in patients, since lack of adherence to treatment is perhaps the main cause of RASs related treatment failure.

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6. Supplementary material

Supplementary Table 1 Primers for RT-PCR (outer PCR), nested PCR (inner PCR) and sequencing of HCV NS5 coding region.

Primer name	Purpose	Direction	Primer sequence (5'- 3')	Location (reference H77) (bp)	GC content	Average Tm
FW1PCR	RT-PCR NS5A-NS5B region	Sense	GTCGTCACBAGY ACCTGG	5311 - 5328	62%	56ºC
RV1PCR	RT-PCR NS5A-NS5B region	Antisense	GAGACASGCTGT GATAWATG	9297 - 9316	45%	51ºC
FW2PCR	Nested PCR NS5A-NS5B region / Sequencing NS5A-NS5B region –Back-up option	Sense	GTGGTCATHGTR GGYAGG	5386 - 5403	57%	53ºC
RV2PCR	Nested PCR NS5A-NS5B region / Sequencing NS5A-NS5B region	Antisense	CCCTATTGATYT CACCTGG	9055 - 9073	50%	51ºC
FW1	Sequencing NS5A-NS5B region	Sense	GCHGTGCARTGG ATGAA	6118 - 6134	52%	53ºC
RV1	Sequencing NS5A-NS5B region –Back-up option	Antisense	CCRTTYYTGACA TGTCC	6493 - 6509	50%	49ºC
FW2	Sequencing NS5A-NS5B region –Back-up option	Sense	TGGMGRCARGA GATG	7093 - 7107	57%	49ºC

RV2	Sequencing NS5A-NS5B region –Back-up option	Antisense	GGRTCRGTRAGC ATGGA	6901 - 6917	56%	53ºC
FW3	Sequencing NS5A-NS5B region –Back-up option	Sense	TCYTCYATGCCC CCYCT	7559 - 7574	62%	57ºC
RV3	Sequencing NS5A-NS5B region –Back-up option	Antisense	TTRTTYTCYGAC TCMAC	7126 - 7142	41%	46ºC
FW4	Sequencing NS5A-NS5B region	Sense	AARGTCACHTTT GACAG	7816 - 7832	40%	46ºC
RV4	Sequencing NS5A-NS5B region	Antisense	GCBGARTGYGGG GGCGTCAG	7936 - 7955	73%	65ºC
FW5	Sequencing NS5A-NS5B region	Sense	AAGCCAGCTCGY CTYATCGT	8128 - 8147	55%	59ºC
RV5	Sequencing NS5A-NS5B region –Back-up option	Antisense	ACGAGCATBGTG CAGTC	8593 - 8609	60%	55ºC
FW6	Sequencing NS5A-NS5B region	Sense	CTTCACGGAGGC TATGAC	8679 - 8696	56%	52ºC
RV6	Sequencing NS5A-NS5B region	Antisense	GACCADGAYCCG TCRCT	7606 - 7619	61%	55ºC

FW7	Sequencing NS5A-NS5B region –Back-up option	Sense	TYTACCARTGYTGTGAC	8381 - 8397	44%	48ºC
1bRV7	Sequencing GT1b NS5A-NS5B region	Antisense	CATCTCCTGCCGCCACA	7067 - 7083	65%	58ºC
1aFW8	Sequencing GT1a NS5A-NS5B region	Sense	ACACTCGCTGCCVCTGTG	6433 - 6450	65%	60ºC
1bRV8	Sequencing GT1b NS5A-NS5B region –Back-up option	Antisense	AGGTCAAGTGGCTCAATGGA	8987 - 9004	50%	56ºC
1aFW9	Sequencing GT1a NS5A-NS5B region –Back-up option	Sense	CTTCACGGAGGCTATGACC	8636 - 8654	58%	55ºC
RV9	Sequencing NS5A-NS5B region	Antisense	ACGATRAGRCGAGCTGGCTT	8083 - 8102	55%	59ºC
1aFW10	Sequencing GT1a NS5A-NS5B region –Back-up option	Sense	GACAGCAAGACACACTCC	8816 - 8833	56%	53ºC
1bFW11	Sequencing GT1b NS5A-NS5B region –Back-up option	Sense	AACTCCTGGCTAGGCAACAT	8848 - 8867	50%	56ºC

Supplementary Table 2 List of materials and reagents used along the sampling processing, from the HCV RNA extraction to the sequencing final step.

Materials and reagents	HCV RNA extraction	RT-PCR	Nested PCR	Gel electrophoresis	PCR products purification	Sequencing
Micro Tubes 1,5ml, SARSTEDT	X	X	X			X
MicroAmp™ Reaction Tubes with Caps 0,2 ml, ThermoScientific		X	X		X	X
96-Well PCR base and cover		X	X	X	X	X
Single Channel F1 FinnPipettes (P5000, P1000, P200, P100, P50, P20 and/or P10), ThermoScientific	X	X	X	X	X	X
TIPOR-ML Multichannel pipette (P200), Orange Scientific						X
Biosphere Filter Tips 1250µl, SARSTEDT	X	X	X			X
Finntips Filter Sterile (10µl, 20µl, 100µl, 200µl, and/or 5ml), ThermoScientific	X	X	X	X	X	X
LiChrosolv® water for chromatography, Merck		X	X			X
NucliSENS® easyMAG® Disposable Immuno Standard Modules	X					
BIOHIT Multichannel pipette e1200, BioMérieux	X					
SafetySpace Filter Tips Sterile (1200µl), SARTORIUS	X					

NucliSENS® easyMAG® Extraction Buffers 1, 2 and 3	X					
NucliSENS® easyMAG® Lysis Buffer	X					
NucliSENS® easyMAG® Magnetic Silica	X					
OneStep RT-PCR Kit, QIAGEN		X				
Expand High Fidelity PCR System, Roche			X			
DMSO (100%), Sigma-Aldrich			X			
Certified™ Molecular Biology Agarose, Bio-Rad				X		
TBE buffer (10X pH 8,3 tris-borate-EDTA buffer), Merck				X		
GeneRuler 1 kb Plus DNA Ladder, ThermoScientific				X		
Agarose Gel Loading Buffer, Abbott				X		
RedsafeTM Nucleic Acid Staining Solution (20 000x), iNtRON Biotechnology				X		
MicroAmp 8-Tube Strip 0,2µl, Applied Biosystems				X		
PCR Cleanup Kit, Abbott					X	
BigDye™ Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems						X

BigDye® Terminator v1.1, v3.1 Sequencing Buffer (5X), Applied Biosystems						**X**
Sodium acetate buffer solution [3M pH 5,2±0,1 (25ºC)], SIGMA-ALDRICH						**X**
Ethanol (99,9% V/V), Proclinica						**X**
3130 POP-6™ Performance Optimized Polymer, Applied Biosystems						**X**
Buffer (10X) with EDTA, Applied Biosystems						**X**
HiDi™ Formamide, Applied Biosystems						**X**
MicroAmp® Optical 96-Well Reaction Plates with MicroAmp® Full Plate Covers, Abbott						**X**
COSTAR® Reagent Reservoir 50ml, Corning Incorporated						**X**
Tubes (15ml and 50ml), SARSTEDT						**X**