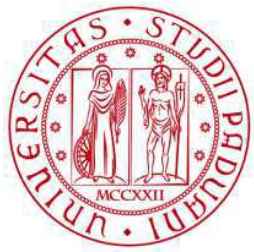

University of Padova

Department of Pharmaceutical and Pharmacological Sciences

DOCTORAL COURSE IN PHARMACOLOGICAL SCIENCES

CURRICULUM PHARMACOLOGY, TOXICOLOGY AND THERAPY

XXIX CYCLE

Development and validation of a high-throughput system for the genotyping of Hepatitis C Virus

Thesis realized with the financial support of Centro Ricerche Scientifiche Dott. Dino Paladin

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ABSTRACT

The subject of this dissertation is the work I carried out at the Università degli Studi di Padova in collaboration with the CRS (*Centro Ricerche Scientifiche*) Dott. Dino Paladin, where I conducted part of the project. The CRS Dott. Dino Paladin is a center specialized in biotechnology research and that collaborates with AB ANALITICA srl for the commercialization of the developed diagnostic CE IVD devices.

My PhD project is focused on the development of a standardized and performing procedure for the Hepatitis C Virus (HCV) genotyping. HCV is a small, enveloped, positive sense single-strand RNA virus belonging to *Flaviviridae* family and it is the etiologic agent of the so called post-transfusion non-A, non-B hepatitis. The World Health Organization (WHO) evaluates more than 130-150 million of persons worldwide that are chronically HCV infected, and the morbidity and mortality attributable to HCV infection continues to increase. Although the research aims to formulate a pangenotypic anti-viral regimen, nowadays the HCV genotype information is still required to choose the best anti-viral treatment for the patient. Because of the high demand for this type of analysis, we decided to design and develop a high-throughput process for the HCV genotyping, starting from the clinical sample up to the genotype result.

In the first part of my PhD work, I dealt with the development of an automated process for the extraction of pathogen nucleic acids. In particular, we developed the GENEQUALITY X120 Pathogen kit to use with the automated platform GENEQUALITY X120 (AB ANALITICA srl). This kit revealed extraction efficiency for both pathogen DNA and RNA and therefore it has been chosen for the HCV RNA extractions.

In the second part of my study, I focused on the performance evaluation of the AMPLIQUALITY HCV TYPE PLUS device, which is a kit based on a Reverse Line Blot (RLB) technique intended for the genotyping of HCV. The results revealed genotyping and subtyping efficiency. The developed kits are currently CE IVD marked and commercialized.

ABBREVIATIONS AND ACRONYMS

AIFA	Agenzia Italiana del Farmaco
AISF	Associazione Italiana per lo Studio del Fegato
ALT	Alanine Transaminase
ASV	Asuneprevir
BOC	Boceprevir
cLIA	ChemiLuminescent ImmunoAssay
CMIA	Chemoluminscent Microparticle ImmunoAssay
DAA	Direc Antiviral Agent
DCV	Daclatasvir
DDI	Drug-Drug Interaction
DSV	Dasabuvir
EBR	Elbasvir
EIA	Enzyme ImmunoAssay
EMA	European Medicines Agency
ER	Endoplasmic Reticulum
eRVR	Extended Rapid Virologic Response
ETR	End-of-Treatment Response
EVR	Early Virologic Response
FDA	Food and Drug Administation
GZR	Grazoprevir
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCC	HepatoCellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HVR	Hypervariable Region
IC	Internal Control
IFN-α	Interferon α
IRES	Internal Ribosome Entry Site
LDV	Ledipasvir
LT	Liver-Transplantation
MEIA	Microparticle Enzyme ImmunoAssay
NAT	Nucleic Acids Test
NI	Nucleotide Inhibitor
NNI	Non-Nucleotide Inhibitor
OMV	Ombitasvir
ORF	Open Reading Frame

PCR	Polymerase Chain Reaction
PEG	PolyEthylene Glycol
PI	Protease Inhibitor
PTV	Paritaprevir
RAV	Resistance Associated Variant
RBV	Ribavirin
RIBA	Recombinant ImmunoBlot Assay
RLB	Reverse Line Blot
RVR	Rapid Virologic Response
SIM	Simeprevir
SOF	Sofosbuvir
SVR	Sustained Virologic Response
TB	Tuberculosis
TMA	Transcription Mediated Amplification
TVR	Telaprevir
Ve	Elution Volume
VPV	Velpatasvir
Vs	Sample Volume
WHO	World of Health Organization
5'-3' UTR	5'-3'-Untranslated Region

1. INTRODUCTION: THE IMPORTANCE OF HCV GENOTYPING

1.1. PAVING THE WAY FOR PERSONALIZED MEDICINE

DEFINING PERSONALIZED MEDICINE

'It's far more important to know what person the disease has than what disease the person has.' Hippocrates [1]

The concept of personalized medicine dates back many hundreds of years. It was not until the 19th century, however, that developments in chemistry, histochemistry and microscopy allowed scientists to begin to understand the underlying causes of diseases. In this period, Sir William Osler (1849-1919) recognized that:

'variability is the law of life, and as no two faces are the same, no two bodies are alike, and no two individuals react alike, and behave alike under the abnormal conditions we know as disease' [2].

Moreover, a few decades later, it was introduced for the first time the concept of '*factors of risk*' with The Framingham Study, where it was studied a population composed of individuals with a higher risk then other of developing a specific disease [3].

To date, several advances in the scientific knowledge led an important breakthrough: as a result of the Human Genome Project [4,5] and of the HapMap Project [6,7], it was sequenced and mapped the entire human genome. In addition, great strides have been made in mapping the molecular pathways. All these findings have driven the rise of a new predictive science, called personalized medicine.

Personalized medicine is a broad and rapidly advancing field of health care for which each person has unique clinical, genetic, genomic and environmental information. The main goal of personalized medicine is to optimize medical care and outcomes for each individual, including prevention strategies, medication types, treatments and dosages, which differ from person to person.

The interindividual variability regards the drug response too. All patients do not respond to the same medicine in the same way. In the past, the differences in the risk-benefit ratio between patients taking the same drug was attributed to non genetic factors such as age, sex, nutritional states, general medical condition and lifestyle. Today, in addition

to these factors, the differences in patient genetic make-up have been recognized to play an important role in the individual response to drug.

In conclusion, we are witnessing a transition from a 'trial-and-error medicine', for which doctors prescribed drugs empirically changing the dosage or trying another one if the original was inefficient, to a new era of individualized medicine, for which doctors have the tools to predict the factor risk of the patient for a disease and eventually its response to therapies.

HCV AND PERSONILEZED MEDICINE

Regarding the infectious diseases, an important aspect to consider before treating the patient is the physiology of the etiologic agent. The great ability of microorganisms to evolve constantly to survive to the external environment is due to their high genetic variability, which exists at several different levels. In fact, a unique virus species branches out in genotypes, which are divided also in a wide range of subtypes. The substantial genetic divergence that distinguishes these viral subgroups entails differences in their protein set, for example in their membrane glycoproteins or non-structural proteins. To date, these viral proteins are the major targets of the antiviral drugs.

Although the antiviral drugs have become increasingly more selective over time, they may be more, less or not effective depending on the genotype of the virus. This phenomenon is called antiviral drug resistance and, as abovementioned, it depends on the protein set hence on the genetic make-up of the specific viral subgroup.

Hepatitis C virus (HCV), the subject of my dissertation, is a representative example of what is described in this paragraph. In 2015, AIFA (*Agenzia Italiana del Farmaco*) in collaboration with AISF (*Associazione Italiana per lo Studio del Fegato*) introduced a new algorithm for the therapy of chronic hepatitis C [8]. The AIFA-AISF algorithm shows the optimal therapeutic options for the use of several anti-HCV drugs, considering also the genotype of HCV.

In summary, personalized medicine considers not only the conditions of the individual patient, but also, in case of infective diseases, the genetic make-up of the etiologic agent. Indeed, an understanding as comprehensive as possible about patient's information, general medical conditions, genetic markers and disease is necessary to optimize his therapy, prescribing him the right drug at the right dose at the right time.

1.2. INTRODUCING THE HEPATITIS C VIRUS

1.2.1. HISTORY AND TAXONOMY

Evidence of the existence of Hepatitis C virus (HCV) dates back about to 40 years old, when it was known that neither Hepatitis A virus (HAV), nor Hepatitis B virus (HBV) were the etiologic agent of the 65% of post-transfusion hepatitis. Several studies [9,10] identified HCV as the responsible of these post-transfusion non-A, non-B hepatitis and its genome was cloned for the first time in 1989 [11].

HCV is a small, enveloped, positive sense single-strand RNA virus, member of *Hepacivirus* genus, belonging to *Flaviviridae* family. The comparative analysis among HCV nucleotide sequences, recovered from infected individuals from different geographical regions, revealed the presence of at least seven major genetic groups (1-7), in other words genotypes, and a several number of subtypes (67 confirmed and 20 provisional) [12]. (Fig 1).

Regarding HCV global geographic distribution (Fig. 2), the genotype 1 is estimated to account 83.4 million (46.2%) of infected cases, with over one-third located in East Asia. The genotype 3 is the next most common and there are about 54.3 million (30.1%) cases globally, approximately three-quarters of which occur in south Asia; the remaining are located in parts of Scandinavia. Genotypes 2, 4, and 6 are responsible for the majority of the remaining cases of HCV worldwide, with an estimated 16.5 million (9.1%), 15.0 million (8.3%), and 9.8 million (5.4%) cases, respectively. East Asia accounts for the greatest numbers of genotype 2 and genotype 6 HCV cases, while North Africa and the Middle East have the largest number of genotype 4 cases. Genotype 5 is responsible for the fewest HCV cases globally (1.4 million, <1% of all HCV cases), the great majority of which occurs in Southern and Eastern sub-Saharan Africa. Finally, genotype 6 is present at the highest frequencies in East and Southeast Asia, but is the dominant genotype in only one country, Laos; it is also prevalent in neighboring Vietnam [12].To date, only one genotype 7 infection has been reported; it was isolated in Canada from a Central African immigrant [15].

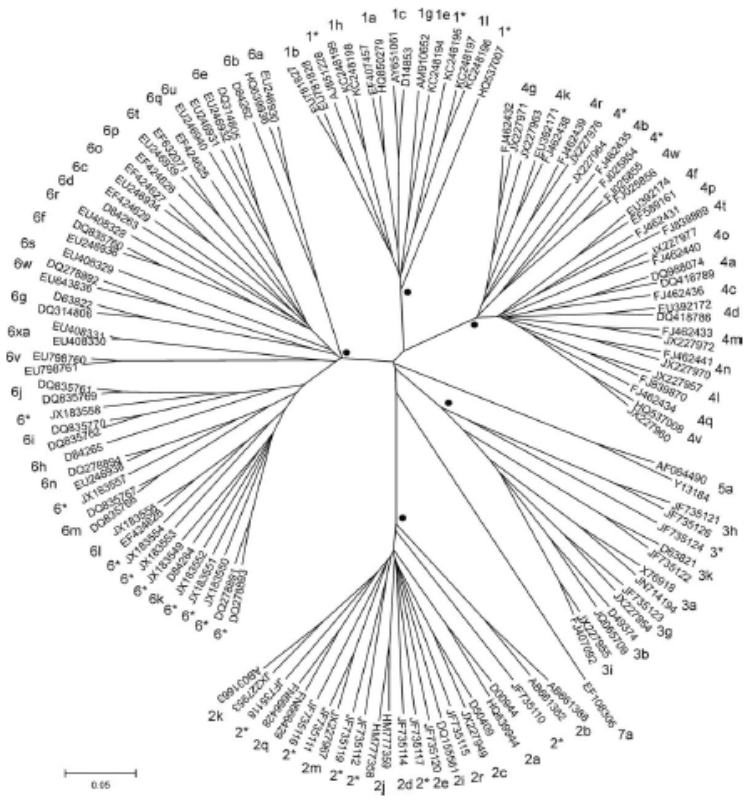


Fig 1. Phylogenetic tree of 129 representative complete coding region sequences. Up to two representatives of each confirmed genotype/subtype were aligned and a neighbor joining tree constructed using maximum composite likelihood nucleotide distances between coding regions using MEGA5.Sequences were chosen to illustrate the maximum diversity within a subtype. Tips are labeled by accession number and subtype (*unassigned subtype). For genotypes 1, 2, 3, 4, and 6, the lowest common branch shared by all subtypes and supported by 100% of bootstrap replicates (n= 1,000) is indicated by● [14]

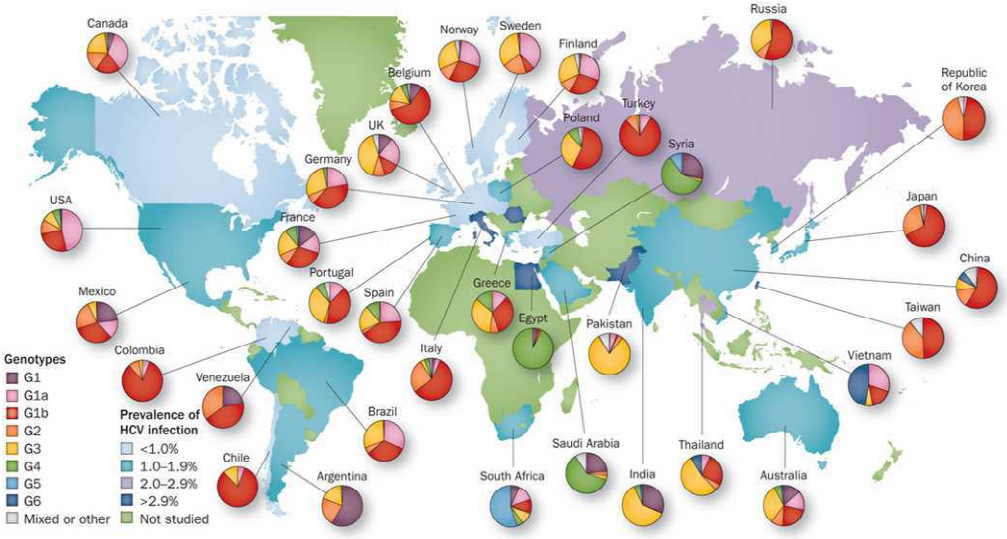


Fig 2. Valuation of the prevalence of HCV infection worldwide and global geographic distribution of HCV genotypes. Data could be underestimated inasmuch HCV infection may remain asymptomatic also for many years [16].

1.2.2 GENOME AND VIRAL STRUCTURE

HCV consists in a virion of 50-55nm of diameter, composed of a double-layer lipid envelope to which the glycoprotein E1 and E2 are anchored. This envelope surrounds a nucleocapsid of 30-35 diameter made up of multiple copies of a core protein, within which the viral genome is conserved [17] (Fig. 3).

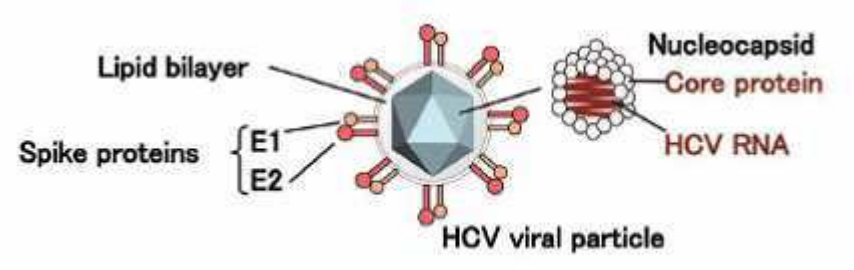


Fig. 3. Structure of a HCV virion.

HCV is a positive single-stranded RNA virus, which genome consists in approximately 9400 nucleotides in length. The genome contains a single ORF (Open Reading Frame), which is translated into a polyprotein of 3000 aminoacids. This polyprotein is subsequently processed by viral and host proteases into three structural proteins (core, E1 and E2), seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5 and NS5B) and an additional protein ARF (Alternate Reading Frame) derived from a -2/+1 ribosomal frameshift, which probably occurs at, or near to, codon 11 of the core protein sequence [18]. The genes encoding the structural proteins are located at 5' of the ORF and the genes encoding the non-structural proteins follow them downstream.

The ORF is flanked by the 5'- and 3'-untranslated regions (5'-UTR/NCR and 3'-UTR/NCR), which regulates the viral replication. The 5′-UTR is highly conserved among different HCV isolates and it is composed by four highly ordered domains numbered from I to IV. Domains II, III and IV constitute the IRES that is essential for cap-independent translation of the viral RNA. Domain I is essential for HCV RNA replication, too [19]. The 3′-UTR consists in three functional regions: a short variable region (about 40 nucleotides), a poly (U/UC) tract with an average length from 30 to 80 nucleotides, and an almost invariant 98 nucleotides RNA element, designated the X-tail. The conserved elements in the 3′-UTR, including a minimal poly (U) tract of about 25 bases, are essential for the viral replication [20]. Besides the 5′- and 3′-UTRs, an essential cis-acting replication element (CRE) was identified in the sequence that encodes the C-terminal region of NS5B [21].

The genetic organization and the polyprotein processing of hepatitis C virus (HCV) are shown in the figure 4 (Fig. 4).

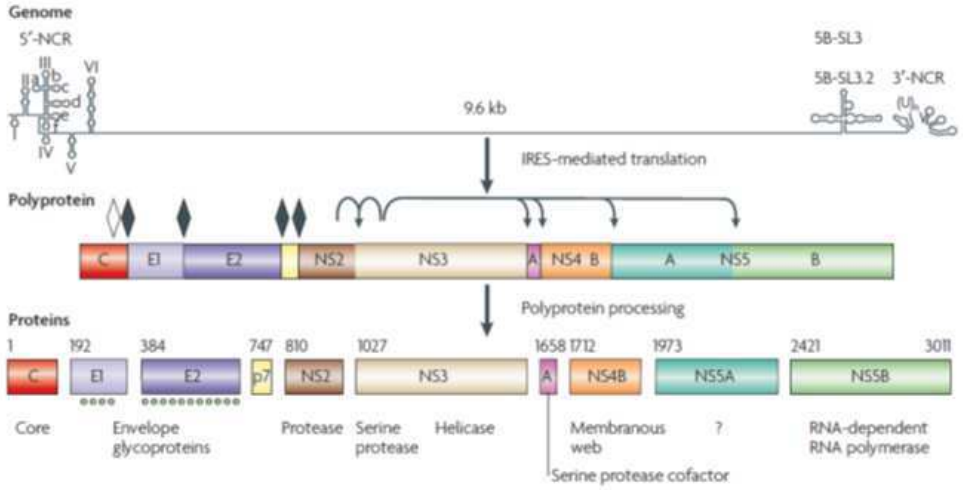


Fig. 4. The 9.6-kb positive-strand RNA genome is schematically depicted at the top. Simplified RNA secondary structures in the 5′- and 3′-UTR and the core gene are shown. Internal ribosome entry site (IRES)-mediated translation yields a polyprotein precursor that is processed into the mature structural and non-structural proteins. Amino-acid numbers are shown above each protein (HCV H strain; genotype 1a; GenBank accession number AF009606). Solid diamonds denote cleavage sites of the HCV polyprotein precursor by the endoplasmic reticulum signal peptidase. The open diamond indicates further C-terminal processing of the core protein by signal peptide peptidase. Arrows indicate cleavages by the HCV NS2–3 and NS3–4A proteases. Dots in E1 and E2 indicate the glycosylation of the envelope proteins [20].

FUNCTION OF STRUCTURAL PROTEINS

The CORE protein forms the viral nucleocapside and, moreover, it takes part into the cell-signaling regulation as well as into viral assembly and RNA transduction [22,23]. An internal signal sequence located between the CORE and E1 sequences targets the nascent polypeptide to the endoplasmic reticulum (ER) membrane for translocation of the E1 ectodomain into the ER lumen, where the cleavage of the signal sequence by signal peptidase yields an immature 191 aminoacids CORE. Further C-terminal processing yields the mature 21-kDa protein of 173–179 aminoacids. The N-terminal hydrophilic domain (D1) of the mature CORE contains a high proportion of basic aminoacid residues and has been implicated both in RNA binding and homo-oligomerization. The CORE is a α-helical protein that is found on membranes of the ER and on the surface of lipid droplets. The association with lipid droplets, which is mediated by the central, relatively hydrophobic domain (D2), may have a role during viral replication and/or virion morphogenesis [24].

The envelope proteins E1 and E2 are glycosylated and form a non-covalent complex, which is the building block for the viral envelope. HCV glycoprotein maturation and folding is a complex process that involves the ER chaperone machinery and depends on

disulphide bond formation as well as glycosylation process. The transmembrane domains of E1 and E2, located at their C-terminal, are involved in heterodimerization and they have ER retention properties. Each of them contains a hydrophobic patch that functions as an internal signal peptide for the downstream E2 and p7 proteins [25]. Comparative analysis among E1 and E2 nucleotide sequences show a high hypervariability in particular in two regions of E2 (HVR1 and HVR2). These HVRs play an important role in the viral life cycle and in the viral evasion from the immune system.

FUNCTION OF NON-STRUCTURAL PROTEINS

The p7protein is a 63 aminoacids polypeptide that is often incompletely cleaved from the E2. The p7 is not required for RNA replication in vitro but is essential for productive infection in vivo. It has been suggested that belongs to the viroporin family and that could have an important role in viral particle maturation and release [26].
The NS2 protein is an auto-protease (cysteine-protease) which intervenes in the initial phases of the viral assembly and morphogenesis, probably interacting with glycoproteins E1 and E2, and with NS3/NS4A. Moreover, it interacts with other cell proteins, as CIDE-B, to inhibit apoptosis of the host cell [27].

The NS3 protein is a multifunctional enzyme, with an N-terminal protease activity, including a catalytic serine, and a C-terminal helicase and NTPase activities. These protease and helicase domains are independent structural units connected by a linker region. The NS3 acts in complex with the NS4A polypeptide, which is a non-covalently bound cofactor, that enhance the protease activity. NS3/4A takes part into the processing of the viral polyprotein [28].

The NS4B protein is a relatively poorly characterized 27-kDa protein, whose function is to induce the formation of the membranous network, a specific membrane alteration that serves as a scaffold for the HCV replication complex [29].

The NS5A protein is a multifunctional phosphoprotein that can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms. It has an important role in the HCV life cycle and probably modulates the efficiency of HCV RNA replication; in fact, the NS5A would attach the viral RNA onto intracellular membranes and coordinate its different fates during HCV replication [30].

The NS5B protein is the viral RNA-dependent RNA polymerase (RdRp) and it is the key enzyme responsible for HCV replication. The NS5B utilizes the HCV RNA genome as a template for RNA synthesis without a need of a primer. Initiation of RNA synthesis is slow and accompanied by frequent dissociation events. Formation of the first phosphodiester bond stabilizes the complex, which then passes to the elongation phase. These functionally distinct complexes are associated to distinct conformations and subdomains of the enzyme, such as ‘thumb’, ‘fingers’ and ‘palm’, which are the major target for the antiviral intervention. Moreover, this enzyme lacks of a

proofreading function; this feature, together with the high replicative activity, accounts for genetic variability of HCV [31].

1.2.3 LIFE CYCLE

Hepatocytes are the main target cells of HCV, but infection of B cells, dendritic cells and other cell types has also been reported. Different types of receptors and molecules mediate the entry of the virus into hepatocytes, such as CD81, a tetraspanin protein found on the surface of many cell types, the LDL receptor (LDLR), the scavenger receptor class B type I (SR-BI), claudin-1 (CLDN1), occludin (OCLN)and others, but their role remains still not fully elucidated [32,33,34].

Virus internalization depends on clathrin-mediated endocytosis and the acid pH of the endosome induces the fusion between the viral envelope and the endosomal membrane, culminating in the release of the viral RNA into the cytoplasm of the host cell. At this point, the translation of the viral proteins starts.

The positive single-stranded RNA of HCV is translated directly without additional passages. The viral IRES consists in domains II, III and IV of the 5′-UTR, together with the first 24–40 nucleotides of the CORE-coding region. The 40S ribosomal subunit recognizes and attaches the viral IRES to form a 48S complex in association with the eukaryotic translation initiation factor3 (eIF3) and the ternary complex (eIF2•Met-tRNAi•GTP) [20]. This pre-initial complex summons the 60S ribosomal subunit to activate the translation process.

Translation of the HCV ORF yields a polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases into the mature structural and non-structural proteins. The structural proteins and the p7 polypeptide are processed by the ER signal peptidase whereas the non-structural proteins are processed by two viral proteases, the NS2 and the NS3 [20].

After the translation process, the viral RNA replication happens. Starting from the viral single-stranded RNA, NS5B provides to the development of a double stranded filament, which is successively separated by NS3, achieving the RNA antisense, which functions as template for the synthesis of the new RNA molecules. When all viral components are ready, they are assembled at the ER to arrange complete viral particles.

Although their role is not well understood, the membranes and the lipid droplets (LD) play a key role in the viral life cycle. Several functions have been suggested: physical support and organization of the RNA replication complex, compartmentalization and local concentration of viral products, attachment of the viral RNA during unwinding,

provision of lipid constituents important for replication and protection of the viral RNA from double-strand RNA-mediated host defenses or RNA interference [20].

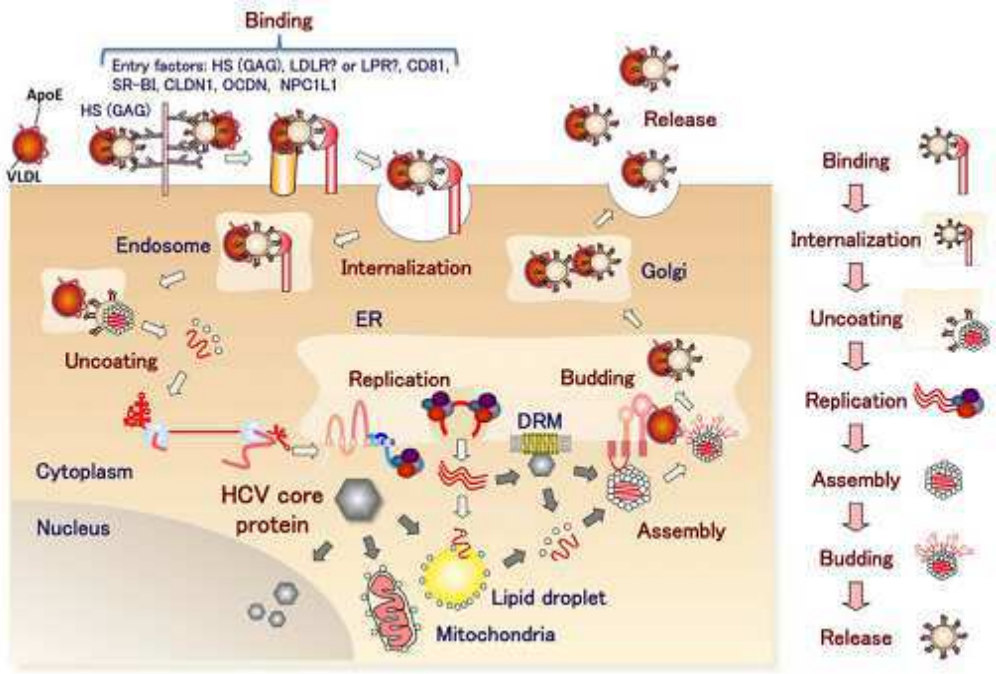


Fig. 5. The HCV life cycle can be summarized in the following steps: 1. Entry into the host cell; 2. Uncoating of the viral RNA; 3. Translation and polyprotein processing; 4. Viral RNA replication; 5. Viral particles assembly; 6. Release of the new virions.

1.2.4 CLNICAL INFECTIONS

EPIDEMIOLOGY

Globally, the morbidity and mortality attributable to HCV infection continues to increase. According to estimates from the Global Burden of Disease study, the number of deaths due to hepatitis C was 333.000 in 1990, 499.000 in 2010 and 704.000 in 2013 [35,36]. The increase in number of deaths reflects the high incidence of hepatitis C during the mid-twentieth century, which is thought to have increased dramatically starting in the 1940s due to the expanded use of parenteral procedure and injection drug use [37]. The incidence declined in the 1990s following the discovery of the HCV, resulting in the introduction of screening of blood HCV, improvements in infection control and safer injection practices. Despite the declining incidence, a large number of persons who were infected 30-60 years ago are now dying from HCV-related complications.

More recent analyses of the global prevalence of HCV indicate that there may be fewer persons living with HCV infection than previously estimated. In 2013, a systematic review concluded that there were 184 million persons with a history of HCV infection (presence of anti-HCV antibody) [38]. Of those, an estimated 130-150 million may be chronically infected (HCV-RNA positive). A more recent systematic review that excludes older studies estimates 115 million persons are anti-HCV antibody positive and 80 million have chronic infection (Appendix 1) [39]. This lower estimate may be explained by a declining incidence as well as improved diagnostic serological test for HCV, resulting in fewer false-positive results.

If correct, this lower burden of disease would be lower than previously thought; nevertheless, the number of people needing treatment remains high. There are also improved estimations of the prevalence of HCV in Africa [40].

NATURAL HISTORY

HCV is primarily transmitted through percutaneous exposure to blood. Its infection is strongly associated with health inequity: in fact, it is most commonly caused by unsafe injection practices and procedures such as renal dialysis and unscreened blood transfusions. Data show that over 16 billion injections are administered yearly worldwide and 40% of these are considered to be unsafe (mainly in sub-Saharan Africa and Asia)[43]. Secondly, in middle- and high-income countries, most HCV infections occur among people who use unsterile equipment to inject drugs and contaminated drug solutions, in effect of the estimated 16 million people in 148 countries who

actively inject drugs, 10 million have serological evidence of an HCV infection [44]. Other modes of transmission of HCV include cosmetic procedure (such as tattooing and body piercing), scarification, circumcision and sexual transmission, which is more common in HIV-positive persons, particularly in men who have unprotected sex with men [45]. Finally, the risk of transmission of HCV from an infected mother to her child is about 4-8%[46].

HCV causes both acute and chronic hepatitis. The incubation of HCV before developing the acute infection is about 6-12 weeks. The acute infection is usually asymptomatic, and it is rarely associated with life-threatening diseases. About 25-45% of infected persons spontaneously clear the virus within six months of the infection without any treatment. Almost all the remaining 55-86% of persons will develop chronic HCV infection. Left untreated, chronic HCV infection can cause liver cirrhosis, liver failure and HCC (HepatoCellular Carcinoma) (Fig. 6). Of those with chronic HCV infection, the risk of developing cirrhosis is about 20%, instead the risk of HCC in persons with cirrhosis is approximately 2-4% per year [47]. These risks, however, can vary, depending upon certain patient characteristics or behaviours, as excess in alcohol consumption, HBV/HIV co-infections or immunosuppression [48], conditions that accelerate progression of liver disease in infected persons.

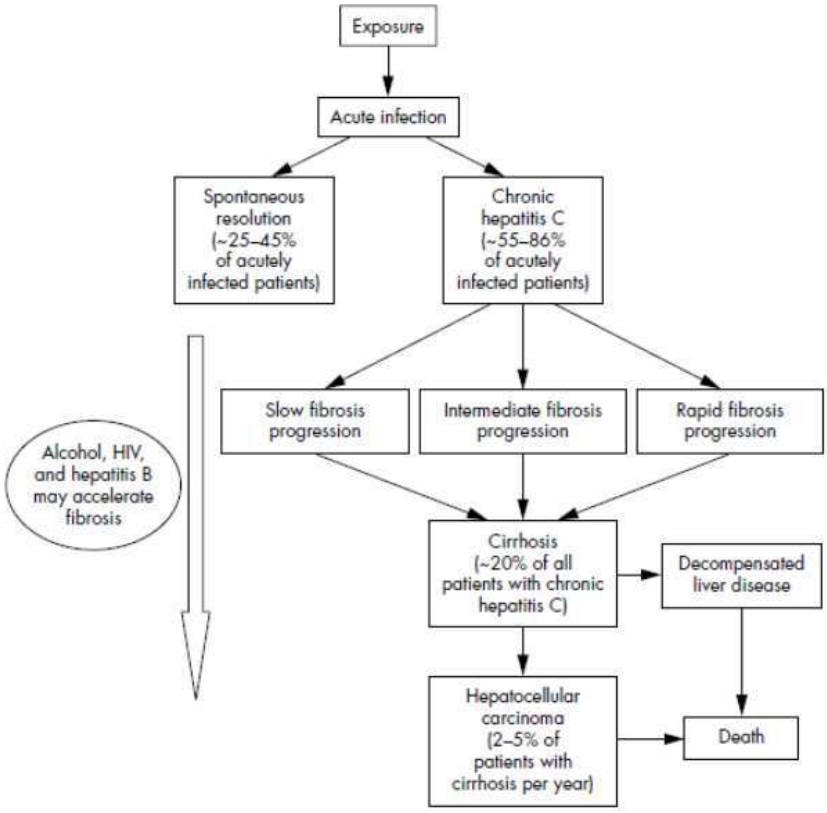


Fig. 6. Natural history of HCV infection [47].

The persistent inflammation of the liver due to chronic HCV infection involves a progressing disease in which the liver tissue is replaced with a fibrotic one. The stage of liver fibrosis can be estimated with the liver biopsy-scoring system METAVIR: a score 0-2 indicates a mild fibrosis without a compromised functionality, whereas a score 3-4 reveals more severe conditions in which the liver parenchyma shows several fibrotic septa, compromised functionality and impaired blood flow, a disease called cirrhosis. Other non-invasive and patented tests for the assessment of liver fibrosis are now available and they are based on blood indices and imaging modalities (Table 2).

Test	Components	Requirements	Cost
APRI	AST, platelets	Simple serum and haematology tests	+
FIB-4	Age, AST, ALT, platelets	Simple serum and haematology tests	+
FibroTest	gGT, haptoglobin, bilirubin, A1 apolipoprotein, α2-macroglobulin	Specialized tests. Testing at designated laboratories	++
FibroScan®	Transient elastography	Dedicated equipment	+++

ALT: alanine aminotransferase; APRI: aminotransferase/platelet ratio index; AST: aspartate aminotransferase; gGT: gamma glutamyl transpeptidase

Table 2. Selected non-invasive tests to assess liver fibrosis [51].

Cirrhosis can be classified as compensated or decompensated. Possible clinical manifestations of compensated cirrhosis comprise loss of weight and appetite, hepatomegaly or/and splenomegaly, high level of serum bilirubin and hyperalbuminaemia, although many patients may be symptomless until compensated cirrhosis becomes decompensated. Decompensated cirrhosis is defined as a status with one of the following symptoms: jaundice, ascites and encephalopathy [48]. For assessing the degree of liver disease , The Child-Pugh Score [50] is used. It consists in a system to classify the patients as Class A, B or C based on clinical and laboratory criteria (Table 3a). Those with class C have the most severe liver disease.

Points	1	2	3
Encephalopathy	None	Minimal (grade 1 or 2)	Advanced (grade 3 or 4)
Ascites	Absent	Controlled	Refractory
Total bilirubin (μmol/L) (mg/dL)	<34 (<2)	34–51 (2–3)	>51 (>3)
Albumin (g/dL)	>3.5	2.8–3.5	<2.8
Prothrombin time (seconds) or PT-INR	<4 or <1.7	4–6 or 1.7–2.3	>6 or >2.3

PT-INR; prothrombin time international normalized ratio
Child–Pugh Class A: 5–6 points
Child–Pugh Class B: 7–9 points
Child–Pugh Class C: 10–15 points

Table 3a. Child–Turcotte–Pugh score (Child–Pugh score) [51].

As with any cause of hepatocellular injury, chronic viral infection leads to the compensatory proliferation of hepatocytes. This regenerative process is aided and abetted by a plethora of growth factors, cytokines, chemokines and other bioactive substances that are produced by activated immune cells and they promote cell survival, tissue remodeling and angiogenesis. The activated immune cells also produce other mediators, such as oxygen species, that are genotoxic and mutagenic. One key molecular step seems to be activation of the NF-κB pathway in hepatocytes in response to the mediators derived from the activated immune cells. Activation of the NF-κB pathway within hepatocytes blocks apoptosis, allowing the dividing hepatocytes to incur genotoxic stress and to accumulate mutations. This is the dominant mechanism in the pathogenesis of viral-induced HCC. Moreover, HCV also contains proteins within its genome that may directly promote the development of cancer, for example, the CORE may have a direct effect on tumorigenesis, possibly by activating a variety of growth-promoting signal transduction pathways. HCC is a primary malignancy of the liver andIt is now the third leading cause of cancer deaths worldwide, with over 600.000 new cases per year.

Decompensated cirrhosis and HCC are the main cause of liver transplantation, without which the possibility to survive are drastically reduced (survival of 5 years is reduced to 50%) [52].

In addition, disease associated with HCV infection is not confined to the liver. In fact, extrahepatic manifestations of HCV include cryoglobulinaemia, glomerulonephritis, thyroiditis and Sjögren syndrome, insulin resistance, type 2 diabetes and skin disorders such as porphyria cutaneatarda and lichen planus. Persons with chronic HCV infection are more likely to develop cognitive dysfunction, fatigue and depression [53]. These outcomes may be associated with replication of the virus in the brain; however, the causal link between these manifestations and chronic HCV infection is not certain [54].

COINFECTIONS

Because of shared routes of transmission, persons infected with HCV may have simultaneously an infection of another pathogen. The main known coinfection is HIV/HCV. Certain groups, in particular people who inject drugs, have high rates of this coinfection; however there are no reliable estimates of the global prevalence. One analysis indicates that 2,3 million persons may be coinfected globally, while an analysis from Africa estimated that 5,7% of persons with HIV were coinfected with HCV [40,41].

Secondly, in countries as Asia, sub-Sahara Africa and South America the coinfection of Hepatitis B virus (HBV) and HCV is commonly observed, where up to 25% of HCV infected persons may have HBV.Finally, it is worth elucidating also that groups at high

risk of HCV infection are also at high risk of infection with tuberculosis (TB) [42], as TB is endemic in many countries where blood products are not screened routinely. In particular, people who inject drugs are more at risk of developing TB, regardless also of their HIV status and among them, two out of three will have anti-HCV antibodies.

1.3. THE IMPORTANCE OF HCV GENETIC VARIABILITY

1.3.1 CLASSIFICATION: GENOTYPE, SUBTYPE AND QUASISPECIES

As abovementioned, the high error rate of RNA-dependent RNA polymerase, which lacks proofreading activity, and the pressure by the host system, have driven the evolution of HCV into at least 7 viral variants, which diverge each other for the genotype, and more than 80 subtypes (Figure 1, paragraph 1.2). The 7 HCV genotype groups show a difference each other of a 30-35% of nucleotide sites, otherwise for every genotype the RNA sequence homology of the subtypes is about 75-80% (Table 3b). This major genetic variability is concentrated in the sites encoding membrane protein, as E1 and E2 glycoprotein, to guarantee viral spread among different hosts, whereas coding sequences of CORE protein and of non-structural proteins, as NS3, are highly conserved. Interestingly, 5' UTR site shows the least variable sequence and this is due to its importance for the viral replication [13]. Moreover, the high mutation rate of HCV generates the production of a large number of different but closely related viral variants during the infection, usually referred to as quasispecies. The fittest of these newly generated variants are continuously selected in the environment in which the virus replicates, on the basis of their own replication capacities and numerous selection pressures, particularly the host immune responses. At a given time point during infection, the HCV quasispecies distribution reflects the balance among continuous generation of new variants, the need to conserve essential viral functions, and positive selection pressures exerted by the replicative environment.

Terminology	Definition	% Nucleotide similarity[a]
Genotype	Genetic heterogeneity among different HCV isolates	65.7–68.9
Subtype	Closely related isolates within each of the major genotypes	76.9–80.1
Quasispecies	Complex of genetic variants within individual isolates	90.8–99

Table 3b. Terminology commonly used in studies related to HCV genomic heterogeneity. [a]% Nucleotide similarity refers to the nucleotide sequence identities of the full-length sequences of the HCV genome [105].

1.3.2 CLINICAL IMPLICATIONS

The enormous genetic HCV diversity together with some host factors has made it difficult to control viral dispersal. Originally, the extent of genetic heterogeneity of HCV was not fully appreciated. However, the breadth of the genetic heterogeneity of HCV is great, and this has important implications in diagnosis, pathogenesis, treatment and vaccine development. For example, the genetic heterogeneity of the HCV quasispecies acts as an epidemiologic marker since it entails the evasion of the host immune system, making the infection persistent. Furthermore, the genetic heterogeneity may influence the sensitivity and specificity of the serologic and virologic assays for the detection of HCV (see paragraph 1.5).

Secondly, several studies show that the HCV genotype-subtype plays a role in the liver disease progression acting together with other cofactors such as viral load, alchol intake and length of time of HCV infection. In particular, in patients with chronic HCV, infection with genotype 1b is reportedly associated with a more severe liver disease and a more aggressive course than is infection with other HCV genotypes [106, 107]. Moreover, liver transplantation recipients with HCV genotype 1 have the highest risk of advanced fibrosis and lowest sustained virologic response rate [108].

In addition, the HCV genotype-subtype is one of the main prognostic factors for the choice of the best fitted antiviral therapy for the infected patient. As detailed in paragraph 1.4 (Treatment of HCV infection), the HCV genotype was a strong indicator for an ineffective therapy: for example, genotype 1 infection was poorly treated with IFNα therapy. With the introduction of the new Direct Antiviral Drugs (DAAs), the idea of a unique treatment for all genotype was born: in fact, these new drugs aim at having an antiviral action against more genotypes as possible, also acting in combination each other. Despite the good premises, this pangenotipyc regimen is not completely reached yet, resulting in a better response of the DAAs in respect of determined genotypes than others. Consequently in the clinical routine the HCV genotype information, together with other host conditions (alchol intake, HCV coinfections, renal and liver function, drug-drug interactions, Figure 7a) is still require to determine the best antiviral treatment.

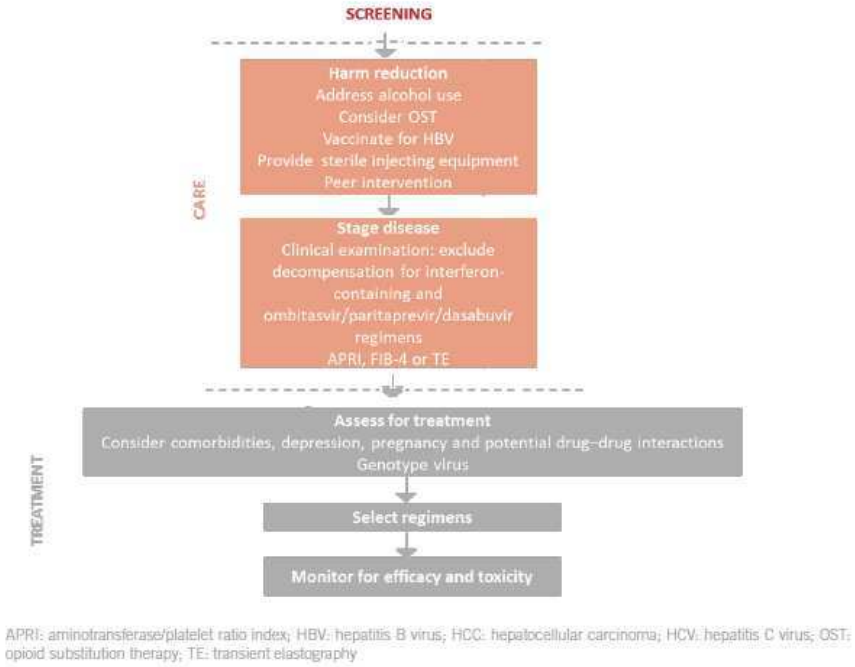


Fig. 7a. Adapted from WHO guidelines 2016; patient treatment pathway [51].

Finally, there are other aspects regarding the virus that may influence the response to the therapy: point mutations in different regions of the viral genome may determine the insurgence of resistance to the antiviral treatment and hence influence negatively the therapeutic outcome. This genetic barrier, defined as the number and nature of nucleotide changes required to confer resistance, is called also RAVs (Resistance Associated Variants). The most important RAVs is Gln80Lys variant (Q80K), which is highly fit and found in genotype 1a (argument deepened in the following paragraphs). Furthermore, data indicate that baseline RAVs are more important as a regimen is stressed, for example, when therapy is shortened or more difficult populations are treated, and suggest that longer duration of therapy might overcome the influence of baseline RAVs [70].

1.4. TREATMENT OF HCV INFECTION

1.4.1 INTRODUCTION: THE IFNα ERA AND THE NEW GENERATIONS OF DIRECT-ACTING ANTIVIRAL DRUGS

In the decades following the discovery of HCV, the treatment of infected persons became possible. In particular, the purpose of the therapy is to eradicate the virus in the patients with chronic infection, in order to prevent cirrhosis and its complications and to improve their quality of life.

The first treatment for HCV was based on a monotherapy of interferon-alpha (IFNα), which derives from the cytokine released by host cells in the presence of a pathogen. When administered by subcutaneous injection, it inhibits the replication of HCV and it modulates the immune response against liver infected cells [56]. Hereafter, Ribavirin (RBV) was added to use in combination with IFNα, in order to increase the cure rates. The RBV is a synthetic structural analogue of the guanosine, which explicates an antiviral activity through different mechanisms of action not yet completely understood. For example, it has been proposed that RBN modulates the Th1/Th2 lymphocyte balance, it inhibits the IMPDH (Inosine Monophosphate Deydrogenase Enzyme), or it has a direct inhibitory effect on the viral NS5B, it may introduce lethal mutagenesis on the HCV RNNA or finally it impairs the translation via eIF4E inhibition [103]. More recently, because of the short half-life of the IFNα after parenteral injection, new formulations of IFNα were developed: PEG-IFNα2a, which is IFNα2a conjugated to a branched a 40kD PEG molecule, and PEG-IFNα2b, which is IFNα2b conjugated to a linear 12kD PEG molecule. These PEG-formulations act similar to the standard IFNα but they present slower adsorption, lower enzymatic degradation and slower clearance. A systematic review was conducted to assess the efficacy of PEG-IFNα/RBV versus IFNα/RBV in treatment-naïve adults and children with chronic HCV infection. Outcomes assessed were SVR, decompensated liver disease, HCC, all-cause mortality, adverse events and quality of life. The available evidence indicated that the use of PEG-IFNα/RBV was more effective at achieving SVR compared with the standard IFNα/RBV [98]. For these reasons, the dual therapy of PEG-IFNα/RBV has been identified for several years as the gold standard therapy [57]. Although PEG-IFNα/RBVshowed good performances, it was anyway efficient for the 80% of patients with genotype 2-3 infection but for fewer than 50% of patients with genotype 1 infection. Moreover, it was associated to severe side effects affecting the central nervous system (such as headache, neuropathy and depression), the growth and the development of children (slowdown) andthe cardiovascular system. Furthermore, acute hypersensitivity reactions have been observed (urticarial and bronchoconstriction), as well as thyroid abnormalities (both hypo- and hyper-) or ophthalmic disorders (retinal hemorrhage or

occlusion of retinal vessels) and more commonly pyrexia, mental disorders and hypotension due to fluid depletion. Finally, it was verified that a low efficiency of the interferon-based therapy could be due to a high presence at baseline of ISG proteins (Interferon Stimulated Gene), which was found more commonly in patients with genotype 1 and 4 infection [102].Further, the presence of anti-IFN antibodies developed during the treatment may interfere with regimen efficacy.

A dramatic improvement in HCV therapy have followed the introduction of oral medicines that directly inhibites the replication cycle of HCV. Their development was achievable as a result of the greater comprehension of the HCV fisiopathology (Fig. 7b). These medicines, called Direct-Acting antiviral (DAAs), are shorter in treatment duration, they entail fewer side effects and they lead to higher SVRs rather than the PEG-IFNα/RBV therapy.

The first-generation marketed DAAs were coadministered with PEG-IFNα and RBV but they were effective only in treating patients infected with HCV genotype 1, moreover they might cause several severe side effects, in particular among persons with advanced liver disease [56]. The second-generation DAAs have higher rates of SVRs, they are safer and they can be used in combinations each other, obviating the need for PEG-IFNα and RBV. These combinations have demonstrated a good efficacy in general, although cure rates among certain patient subgroups remains lower [59].

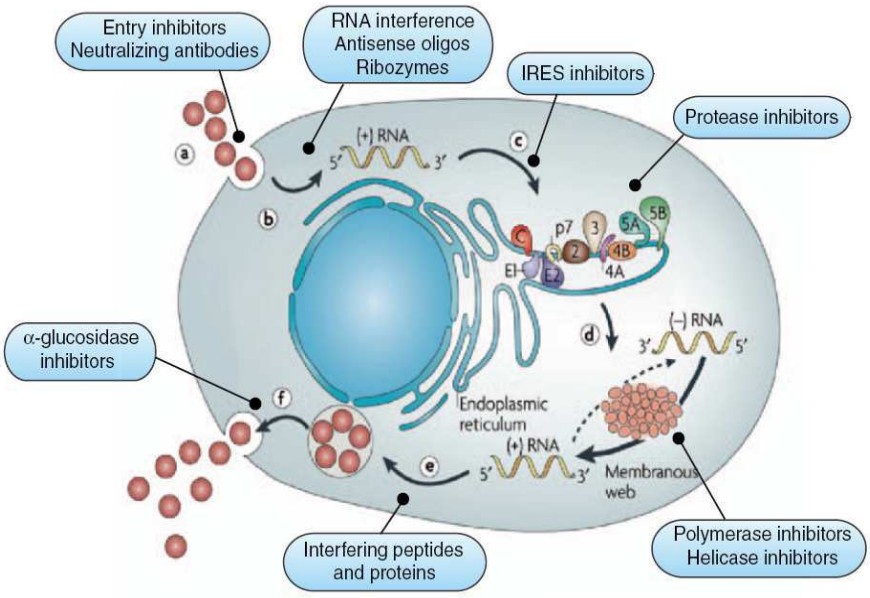


Fig. 7b. HCV life cycle and possible molecular targets for the antiviral therapy [58]

1.4.2 DIRECT ANTIVIRAL AGENTS: AN IMPORTANT BREAKTHROUGH

1.4.2.1 CLASSIFICATION AND MECHANISMS OF ACTION

Understanding the mechanisms of action of the DAAs as well as related issues, including the molecular basis for resistance, helps to guide drug development and clinical use. Several reviews have described how insights into the viral life cycle have facilitated the development of DAAs [63,64]. To date, inhibition of HCV replication has focused on three major viral targets: the NS3/4A, the NS5B and the NS5A.

Protease inhibitors (PIs): The HCV NS2 and NS3 are both proteases with well-characterized enzymatic activities, making them logical targets for pharmaceutical intervention strategies. In spite of efforts to develop inhibitors of NS2 and NS3, only compounds that target NS3 have advanced in clinical use. *Ciluprevir* (BILN-2061) was the first HCV PIs which advanced in clinical trials [65] but it was stopped because of its severe toxic effects. Although the failure, these studies validated NS3 as a therapeutic target. Furthermore, available crystal structure of NS3 in complex with candidate compounds showed that most interactions are mediated via the protease domain, whereas weak contacts happens with the helicase region [66]. *Boceprevir (BOC)* and *Telaprevir (TVR)* are an example of drugs that react with the catalytic site of the protease domain and they were approved in 2011, representing the first class of DAAs successfully used in the clinic. Although these first-generation PIs stand for a major advance in the treatment of HCV, they have several limitations, including narrow genotype specificity and a low barrier to resistance. Moreover, there are several possible mutations (RAVs) in the NS3, which lead amino acid substitutions conferring drug resistance (Table 5). Other compounds, which belong to the second wave of the first-generation PIs, include *Simemprevir (SIM)*, *Paritaprevir (PTV)* and *Asuneprevir (ASV),* but they have similar limitations of the first wave. Conversely, the second generation of PIs, comprising *Grazoprevir (GZR),* aim at a more pangenotypic activities that reduce viral escape through common RAVs of the first-generation PIs [67].

What is more, studies show that PIs have an additional indirect effect on HCV: PIs therapy restores TLR3 and DDX58 signalling, leading to endogenous IFN production and its associated antiviral activity [68].

Generation			Active against HCV genotype	Genetic barriers			Resistant association variants			
1st, 1st-wave	VX-950	Telaprevir	1a/1b, 2	Low	V36A/M		R155K/T/Q	A156S/D/T/V		
1st, 1st-wave		Boceprevir	1a/1b, 2	Low	V36A/M		R155K/T/Q	A156S/D/T/V		
1st, 2nd-wave	BI 201335	Faldaprevir	1a/1b, 2	Moderate			R155K/T/Q		D168A/V/T/H	V170A/T
1st, 2nd-wave	TMC-435	Simeprevir	1, 2, and 4–6	Moderate		Q80R/K			D168A/V/T/H	
1st, 2nd-wave	MK-7009	Vaniprevir	1a/1b	Moderate			R155K/T/Q	A156S/D/T/V	D168A/V/T/H	
1st, 2nd-wave	BMS-650032	Asunaprevir	1, 4	Moderate		Q80R/K	R155K/T/Q		D168A/V/T/H	
1st, 2nd-wave	ABT-450	Paritaprevir	1	Moderate			R155K/T/Q		D168A/V/T/H	
2nd	GS-9857		1–4				R155K/T/Q	A156S/D/T/V	D168A/V/T/H	
2nd	ACH-1625	Sovaprevir								
2nd	MK-5172	Grazoprevir	1a/1b, 2, and 4–6	High			R155K/T/Q	A156S/D/T/V	D168A/V/T/H	

Table 5. Profile of PIs [67].

NS5B inhibitors: Clinically relevant NS5B inhibitors can be classified into non-nucleotide inhibitors (NNIs) and nucleotide inhibitors (NIs) that act at distinct stages of RNA synthesis [69].

NNis interfere through a non-competitive mechanism with the conformational dynamics of NS5B at its transition from initiation to elongation process. In particular, different classes of NNIs can bind to distinct regions in the 'thumb' subdomain ('thumb' sites I and II). 'Thumb' site I inhibitors comprise compounds with a benzimidazole or indole scaffolds, such as *Beclabuvir*, whereas 'thumb' site II inhibitors have thiophene-based structures, such as GS-9669. Unfortunately, compared to NIs, NNIs have limitations in their antiviral effectiveness (67). In addition to these classes of inhibitors, there are other compounds that interfere with the 'palm' subdomains, which is a hydrophobic area in close proximity of the active site. These 'palm' site inhibitors might affect RNA synthesis through interference with nucleotide incorporation at the level of initiation [70]. Usually, these inhibitors comprise a benzothiadiazine scaffold, such as *Dasabuvir (DSV)*. Comparatively to PIs, NNIs have different RAVs (Table 6).

NIs interfere through a competitive mechanism with the incoming nucleoside triphosphate for the binding and the incorporation process. They are usually administrated as prodrugs that require metabolic activation to the triphosphate form that is eventually accommodated at the nucleotide-binding site of NS5B (70). The main NIs is *Sofosbuvir (SOF),* which is a phosphoramidateprodrug that is ultimately activated inanuridine analogue. Compared to NNIs, NIs commonly show pangenotypic activity with a higher barrier to resistance (Table 6).

		Binding site	Active against HCV genotype	Genetic barriers	Resistant association variants					
Nucleotide										
GS-7977	Sofosbuvir		1a, 1b, and 2–6	High	S282T					
Nonnucleoside										
BMS-791325	Beclabuvir	Site I		Moderate	A421V	P495S/Q/L/A/T				
ABT-333	Dasabuvir	Site III		Moderate	C316Y/N	S368T	M414T/I/V/L	Y448C/H	G554D/S	S556G D559G
GS9669		Site II		Moderate	L419S	R422K	M423T/I/V/T	I482L/V/T	A486/V/I/T/M	V494A
MK-3682				Moderate						

Table 6. Profile of NS5B inhibitors [67].

NS5A inhibitors: NS5A inhibitors show exceptional potencies due to the involvement of this protein at various stages in the viral life cycle, particularly in the replication and the assembly. Probably, the most important passage is the inhibition of the formation of the virus-induced membranous web that provides the site for the viral replication [70]. The first NS5A has been *Daclatasvir (DCV)*, which is a symmetric dimeric molecule that may compete with viral RNA such that a dynamic equilibrium occurs between NS5A dimers that bind either RNA or the drug [71]. *Ledispavir (LDV)* and *Ombitasvir (OMV)* are related compounds also approved for clinical use [72]. *Elbasvir (EBR)* and *Velpatasvir (VPV)* are NS5A inhibitors of a second generation and they show potent broad genotype antiviral coverage than the first generation (Table 7).

Generation			Active against HCV genotype	Genetic barriers	Resistant association variants			
1st	BMS-790052	Daclatasvir	1b > 2a > 1a	Moderate			L31F/M/V	Y93C/H/N
1st	GS-5885	Ledipasvir	1a, 1b	Moderate			L31F/M/V	Y93C/H/N
1st	ABT-267	Ombitasvir	1 > 2–6	Moderate	M28T	Q30E/R		Y93C/H/N
Broad activity	MK-8742	Elbasvir	1–4	Unavailable	M28T	Q30L/R	L31<	Y93H/N
2nd	GS-5816	Velpatasvir	1–6	Unavailable				
2nd	ACH-3102		1–5	High				Y93H

Table 7. Profile of NS5A inhibitors [67].

1.4.2.2 CONTRAINDICATIONS: DRUG-DRUG INTERACTION

The emergence of data regarding the use of DAAs has resulted in a broader understanding of the complex DDIs. DDIs entail that drugs have to be used in combination with extremely caution which results in a dose adjustment, altered timing of administration, additional monitoring or in a contraindicated administration.

Some DAAs as *SIM*, *PTV*, *DCV*, ZEPATIER regimen, and in part also *OMB* and *DSV* are extensively metabolized by the cytochrome (CYP) 3A4 system. Therefore, substances that are moderate or strong inducers or inhibitors of CYP3A4 are not recommended as these may led to significant respectively lower or higher exposure of the DAAs. These substances may include anticonvulsants (such as carbamazepine, oxcarazepine, phenobarbital and phenytoin), antibiotics (such as erythromycin, clarithromycin and telithromycin), antimycobacterials and systemically administered antifungals (such as rifampin and fluconazole respectively) and a great number of antiretroviral drugs (such as HIV protease inhibitors). Moreover, dose adjustments are needed with some antiarrhytmics, warfarin, calcium channel blockers, HMG Co-A reductase inhibitors and sedative/anxiolitics. Other CYP involved in the metabolism of the DAAs are CYP2C8 and CYP1A2-1B3: CYP2C8 is the main metabolizer of *DSV* and CYP1A2-1B3 is engaged minimally with *SIM*.

In DAAs pathways, moreover, the transport proteins are interested. Some DAAs such as *SOF*, *LDV*, *DVC*, *PTV* and *DSV* are transported by P-gp (P-glycoprotein) and BCRP (Breast

Cancer Resistant Protein), and therefore any co-administered drugs that are potent P-gp and BCRP inducers will decrease DAAs plasma concentration, leading to a reduced therapeutic effect. Whereas co-administration with drugs that inhibit P-gp and BCRP may increase the exposure of DAAs though clinical consequences are unlikely. Some P-gp substrates are digoxin and dabigatran, cyclosporines and more over. Another important transporter is OAT1B1/B3 whose substrates are *SIM*, *DCV* and *PTV.*

Finally, other significant known DDIs concern *SOF*: if co-administrated with Amiodarone in combination with another DAA may result in serious symptomatic bradycardia. The mechanism for this DDI is poorly understood and it reported to have caused one fatality due to cardiac arrest [94]. Moreover, *OMB*, *PTV* and *DSV* may have numerous DDIs in combination with Ritonavir.

In conclusion, a comprehensive DDI programme should be consulted prior to treatment initiation [93]. The regulatory guidance of both EMA and FDA and other guidelines for example of EASL supply specific details on DDIs fot he anti-HCV therapy. Moreover, the WHO suggests to consult the University of Liverpool webpage on hepatitis drug interactions (http://www.hep-druginteractions.org/) [51].

1.4.2.3 CLINICAL DATA

VIROLOGICAL RESPONSE

To better understand the clinical data about the DAAs regimens, it is important to define the so-called virological response. The viral kinetics represents an important parameter to monitor the response to the therapy over time. In fact, the HCV RNA decline during the treatment is highly associated to the achievement of a good therapeutic response. HCV RNA is measured at specific times before, during and after the treatment: in particular at baseline, at weeks 4 and 12, at the end of the treatment (week 24 or 48 depending on the duration of the therapy) and then 24weeks after the end of the treatment (that is at week 48 or 72) (Fig. 8).

Fig. 8. Times of HCV RNA detection.

Each specific time of HCV RNA detection coincides with a parameter called virologic response, which is critical to determine the efficacy of the therapy (Fig. 9). In particular,

at week 4 and 12 Rapid Virologic Response (RVR) and extended RVR (eRVR) or Early Virologic Response (EVR) are respectively evaluated, whereas at the end of the treatment End-of-Treatment Response (ETR) is estimated and finally 24 weeks later Sustained Virologic Response (SVR) is determined.

Overall, the gold standard criterion indicating a successful therapy is the achieving of the SVR. SVR can be detected also 12 weeks (SVR12) after the treatment. It is known that the infection is cured in more than 99% of patients who achieve an SVR [93], in fact,a systematic review shows as in analyses of SVR durability, the incidence of late relapse is extremelylow (1%) [61]. Moreover, histologic regression of both necroinflammation and fibrosis has been demonstrated in paired liverbiopsy samples in SVR-achieving patients, who have significantly fewer liver-related complications, less hepatocellular carcinoma, and fewer liver-related deaths than non-responders or those untreated [61].

On the other end, the absence of EVR is predictive of a failed treatment: in fact the 99% of patients who do not achieve EVR, they do not even accomplish SVR [62].

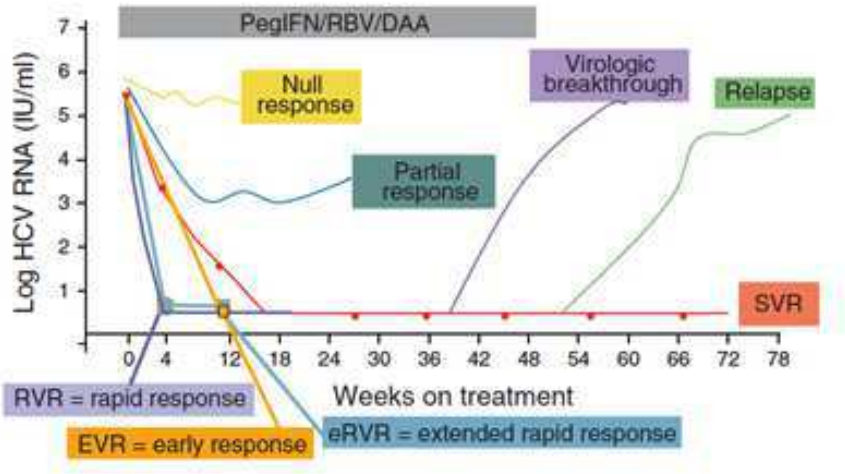


Fig. 9. Rapid virologic response (RVR): undetectable hepatitis C virus (HCV) RNA at week 4. Extended RVR (eRVR): HCV RNA <10-15 IU/ml at weeks 4 and 12. Early virologic response (EVR): ≥ 2 log 10 reduction from baseline HCV RNA, but virus remains detectable (partial EVR) or is undetectable (complete EVR) at week 12. Early responders: HCV RNA <10-15 IU/ml at week 8. Partial response: ≥ 2 log 10 reduction from baseline HCV RNA at week 12, but virus remains detectable through week 24 or treatment end. Breakthrough: undetectable HCV RNA during treatment followed by appearance of HCV RNA, despite continued treatment. End-of-treatment response (ETR): undetectable HCV RNA at the end of treatment. Sustained virologic response (SVR): undetectable HCV RNA at 24 weeks after treatment completion. Relapse: undetectable viremia during treatment and/or at the end of treatment, but subsequent viremia following treatment cessation. Non-response: detectable circulating HCV RNA throughout treatment. Null-response: <2 log 10 reduction from baseline HCV RNA during the treatment è [60].

IFNα-BASED DAAs REGIMENS

DAAs regimens have been approved for anti-HCV therapy starting from 2011, when the first-generation of PIs, *TVR* and *BOC*, were approved in combination with PEG-IFNα/RBV [www.fda.gov]. Although this triple therapy represented a step forward compared to the conventional IFNα therapy, it was associated to severe adverse effects. In fact, currently the treatment guidelines do not recommend it [51]. Although with the introduction of DAAs the treatment for HCV infection is moving away from the use of interferon, PEG-IFNα/RBV is still recommended in some cases; in fact, it is used in combination with some of the second wave of first generation PIs, such as *SIM, SOF* and *DCV*. The phase III of QUEST-1/2 trials shows that *SIM* plus PEG-IFNα/RBV for 12 weeks followed by PEG-IFNα/RBV for 12 or 36 weeks according to criteria for response-guided therapy results in SVR of 80 to 81% of treatment-naïve patients with genotype 1 infection [74,75]. However, a new issue arose: the importance of baseline RAVs: the Q80K viral polymorphism, which is estimated to be present in the 48% of untreated patients with genotype 1a infection [76], reduces the rates of SVR similar to those treated with PEG-IFNα/RBV alone (~58%). Finally, clinical trials point out that the rate of SVR in triple therapy including *SIM* is 83% in previously untreated patients with genotype 4 [77].

The most potent IFNα-based DAA regimen appears to be the triple therapy with *SOF*. In the phase III of NEUTRINO trial, a 12-week regimen of *SOF* plus PEG-IFNα/RBV results in a SVR of 92% in previously untreated patients with genotype 1a, 82% in those with genotype 1b, 96% in those with genotype 4 and moreover one patient with genotype 5 and all six patients with genotype six show SVR [78]. Recently, data from the BOSON study have suggested that this triple therapy may have an active role also in the treatment of cirrhotic patients with genotype 2-3 infection [79].

Similarly, *DCV* has been investigated in IFNα-based regimens for patients with genotype 1 and 4 infection. The phase IIb of COMMAND-1 trial indicates that *DCV* plus PEG-IFNα/RBV for 12 weeks followed by PEG-IFNα/RBV for 36 weeks yields a SVR in 55% of previously untreated patients with genotype 1a, 77% of those with genotype 1b and 100% of those with genotype 4 [80]. Besides, the recent addition of *ASV* to this regimen increases the SVR rates to over 90% in cirrhotic patients with genotype 1 and 4 infection [81].

Notwithstanding, the IFNα-based regimens are no longer recommended in the guidelines, at least as first-line therapy for treatment-naïve patients, because they are inferior to IFNα-Free oral DAAs combinations in term of both safety and tolerability profiles.

IFNα-FREE DAAs REGIMENS

The era of IFN-Free DAAs regimens promises a revolutionary change in the therapeutic options for those in most need of HCV eradication. These regimens are well tolerated, with low rates of serious adverse events or discontinuation of therapy [82]. The first IFNα-Free regimen to achieve a SVR comprised the combination of *ASV* and *DCV* for 24 weeks [83]. Data of IFNα-Free DAAs treatments regard predominantly patients with genotypes 1, 2 and 3, whereas there are limited data for genotypes 4 and 6. A systematic review summarizes the significant trials of IFNα-free DAA regimens according to genotype 1-4 [84] (Tables 1 and 2 of Appendix 2).

To sum up, several combinations of DAAs with different mechanisms of action and levels of genetic barrier are proved. Moreover, the inclusion of RBV and Ritonavir, a PI for anti-HIV therapy, has also evaluated. Clinical trials confirm that the addition of RBV to the drug regimen betters the number of patients who reach a SVR [85]. This aspect may due to the importance of RBV for reducing the incidence of and delaying the emergence of genetic barrier; however, the mechanisms underlying its effects remain unclear [86]. Secondly, it appears from trials that often the increase in the duration of the treatment (from 12 weeks to 24 weeks) may improve SVRs [87,88]. Another important point is that naïve patients, in other words patients who have not already received a treatment before, are usually more responsive than patients with an already treatment-experience. Moreover, patients with genotype 3 infection draw less benefit from these regimens than others infected with other genotypes [84].

Finally, a small number of pangenotypic regimens have been studied in the decompensated-cirrhotic population, with the majority of data arising from the pre-Liver-Transplantation (LT) setting. For these patients awaiting LT, the primary aim of the therapy is to prevent recurrent HCV infection of the new liver, which is associated with reduced graft and patient survival [89]. A systematic review summarized these data [84] (Table 3 in Appendix 2).

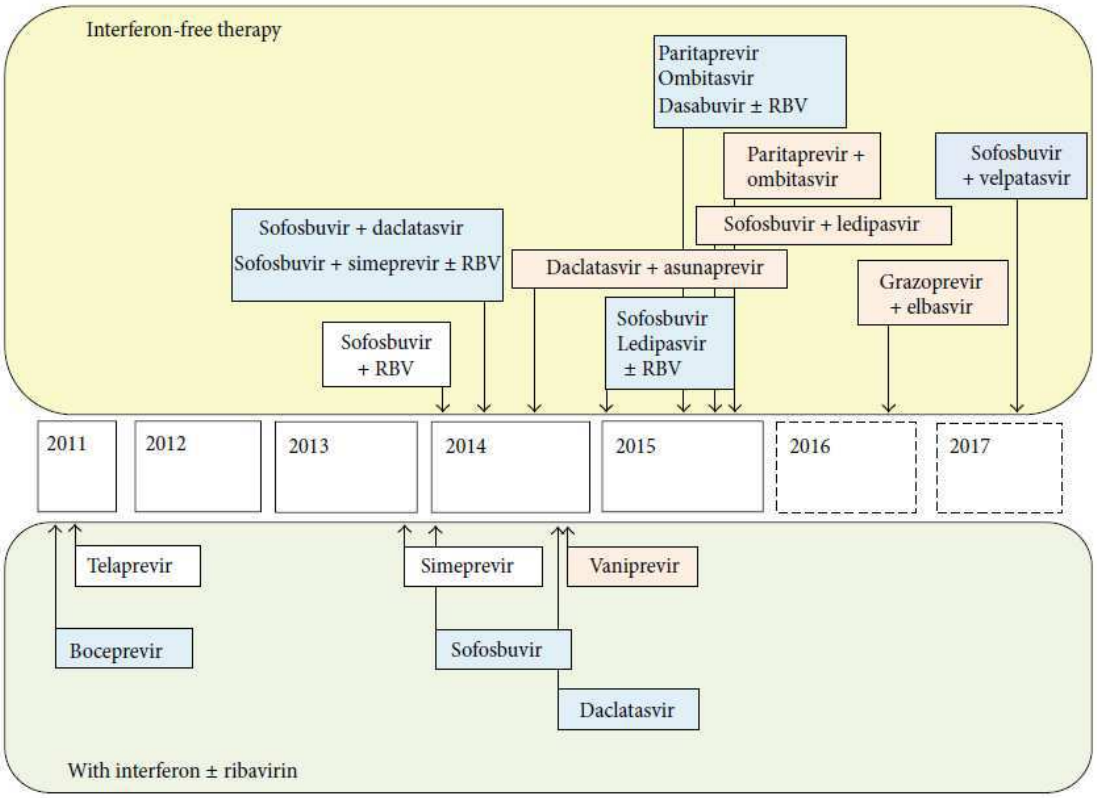


Fig. 11. Treatment of HCV with DAAs that are approved or that are going to be approved [67].

MONITORING FOR ADVERSE EVENTS

Monitoring guidance for the detection of adverse events to HCV treatment has largely been based on the experience PEG-IFNα/RBV therapy. Newer DAAs regimens are much better tolerated by patients, as they have fewer adverse events and thus less need for early discontinuation of therapy. Therefore the frequency of routine laboratory monitoring may be reduced; however, there remains the need for laboratory monitoring in patient with cirrhosis or renal impairment, HIV coinfection, potential DDIs and who are treated in combination with PEG-IFNα/RBV. The WHO supplies a summary framework for the monitoring of the adverse events in these patients [51].

Despite the main side effects of DAAs regimens may comprise fatigue, headache, nausea and sometimes anemia, some cases of severe adverse events were observed: studies on NIs demonstrate that if NIs are incorporated by host RNA or DNA polymerases, severe toxicity might ensue, aspect that led the development of many promising agents in this class being halted [72]. Moreover, possible hepato-toxicity have emerged in patients with decompensated cirrhosis treated with *SOF* [91]. Similar data are obtained in a study in which seven of 160 patients treated with *SOF* plus *LDV* and Ribavirin died during therapy in the pre-LT or post-LT setting: subtle hepatic and/or systemic toxicity due to DAAs regimen cannot be excluded as cause of death [92]. Finally, it has been recognized also that *SOF* might cause cardiotoxic effects.

1.4.2.4 STATE OF ART

Advances in drug development and the availability of a wide range of clinical trials are revolutionizing HCV treatment. Each year the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approve several DAAs regimens. The approved DAAs, by both FDA and EMA, to date are listed in Table 7b.

COMPOUNDS	PRODUCT	PRESENTATION	POSOLOGY
PEG-IFNα2a	PEGASYS	Solution for injection containing 180, 135 or 90 µg of PEG-IFNα2a	Once weekly subcutaneous injection of 180 µg (or less if dose reduction needed)
PEG-IFNα2b	INTRON	Solution for injection containing 50 µg per 0.5 ml of PEG-IFNα2b	Once weekly subcutaneous injection of 1.5 µg/kg (or less if dose reduction needed)
RIBAVIRIN	REBETOL	Capsules containing 200 mg of RBV	Two capsules in the morning and 3 in the evening if body weight <75 kg or Three capsules in the morning and 3 in the evening if body weight ≥75 kg
SOFOSBUVIR	SOVALDI	Tablets containing 400mg of SOF	One tablet once daily (morning)
SIMEPREVIR	OLYSIO	Capsules containing 150mg of SIM	One capsule once daily (morning)
DACLATASVIR	DAKLINZA	Tablets containing 30 or 60 mg of DCV	One tablet once daily (morning)
SOFOSBUVIR/LEDIPASVIR	HARVONI	Tablets containing 400 mg of SOF and 90 mg of LDV	One tablet once daily (morning)
PARITAPREVIR/OMBITASVIR /RITONAVIR	TECHNIVIE	Tablets containing 75 mg of PTV, 12.5 mg of OMV and 50 mg of Ritonavir	Two tablets once daily (morning)
DASABUVIR	EXVIERA	Tablets containing 250mg of DSV	One tablet twice daily (morning and evening)
GRAZOPREVIR/ELBASVIR	ZEPATIER	Tablets containing 50mg of EBR and 100mg of GZR	One tablet once daily
SOFOSBUVIR/VELPATASVIR	EPCLUSA	Tablets containing 400mg of SOF and 100mg of VPV	One tablet once daily

Table 7b. Approved anti-HCV drugs in 2016.

In addition to these, the VIEKIRA PAK is approved and it consists in the combination of TECHNIVIE with EXVIERA.

Because of the great availability of DAAs and their use in combinations, several guidelines are prepared, which serve as a guide for persons managing hepatitis treatment programmes and clinicians. These guidelines are defined by a chosen panel of experts and they supply evidences and recommendations for the screening, the care and the treatment of persons with HCV infection. The recommendations are formulated using a grading system that consists usually in an adaptation of the Grading of Recommendations Assessment, Development and Evaluation (GRADE) method. This is a method that provides guidance and tools to define research questions, to develop an analytical framework, to conduct systematic reviews, to assess the overall quality of the evidence and to determine the direction and strength of the recommendations [51].

In Appendix 3 (Table 5 and 6), the treatment recommendations written by the European Association for the Study of the Liver (EASL) are reported. These recommendations elucidate the regimens and the durations of anti-HCV treatment to be used according to the HCV genotype and the clinical outcome of the patient. The

DAAs approved in 2016 (ZEPATIER and EPCLUSA) are not integrated yet in the recommendations, and their indications of use are listed below (Table 8).

Patient Population	Treatment	Duration
Genotype 1a: Treatment-naïve or PegIFN/RBV-experienced* without baseline NS5A polymorphisms†	ZEPATIER	12 weeks
Genotype 1a: Treatment-naïve or PegIFN/RBV-experienced* with baseline NS5A polymorphisms†	ZEPATIER + ribavirin	16 weeks
Genotype 1b: Treatment-naïve or PegIFN/RBV-experienced*	ZEPATIER	12 weeks
Genotype 1a or 1b: PegIFN/RBV/PI-experienced‡	ZEPATIER + ribavirin	12 weeks
Genotype 4: Treatment-naïve	ZEPATIER	12 weeks
Genotype 4: PegIFN/RBV-experienced*	ZEPATIER + ribavirin	16 weeks

*Peginterferon alfa + ribavirin.
†Polymorphisms at amino acid positions 28, 30, 31, or 93.
‡Peginterferon alfa + ribavirin + HCV NS3/4A protease inhibitor.

Patient Population	Recommended Treatment Regimen
Patients without cirrhosis and patients with compensated cirrhosis (Child-Pugh A)	EPCLUSA for 12 weeks
Patients with decompensated cirrhosis (Child-Pugh B and C)	EPCLUSA + ribavirin for 12 weeks

Table 8. Approved anti-HCV drugs in 2016 Zepatier and Epclusa. Epclusa s indicated for the treatment of adult patients with chronic HCV genotype 1, 2, 3, 4, 5, or 6 infection (Prescribing Information Taken by FDA).

Finally, other guidelines are available formulated by the World Health Organization (WHO) [51], the *Associazione Italiana per lo Studio del Fegato* (AISF) [95], the American Association for the Study of Liver Disease (AASLD) in collaboration with the Infection Disease Society of America (IDSA) [96], and the US Department of Veterans Affair [97], and others.

1.5. DIAGNOSIS OF HCV INFECTION

Today, the laboratory diagnosis of HCV infection is extremely reliable thanks to available assays capable of identifying persons infected with a sensitivity and a sensibility near to 100%. The diagnosis of HCV infection is essentially based on the positivity of anti-HCV antibodies and on the increase of transaminases (in particular ALT). Considering that the onset of the disease is usually asymptomatic, the identification of the infection is usually casual or following screening test for anti-HCV antibodies (for surgeries, subjects at risk, blood donations, etc.) or also in case of a casual finding of hypertransaminasemia.

The diagnostic assays for the diagnosis and management of HCV infection include two classes: indirect assays that evaluate the antibody response to viral antigen (anti-HCV, RIBA) and direct assays that detect the presence in serum of the viral genome (qualitative or quantitative HCV-RNA) or of a viral antigen (such as core antigen). Over the past 15 years, the diagnostic assays underwent considerable progresses: the availability of more sensitive and accurate methods and the introduction of automated equipment in clinical diagnostic, allow to perform multiple tests in a short time and to support high workloads to confront with the routine need of the laboratory.

1.5.1 INDIRECT ASSAYS

Serologic assays detect HCV specific total antibodies (IgM and IgG) and they are used to screen and diagnose HCV exposure. Anti-HCV antibodies are revealed thanks to a capture antigen (usually recombinant) which is specific of viral regions and hence the binding antigen-antibody can be detected through different techniques. Over times, serologic assays evolved from a first to a third generation of tests depending on the number and the type of capture antigens involved. In 90s, the first generation assays used as the capture antigen the recombinant C100-3 (from NS4 viral protein), whereas the second generation assays detected antibodies against CORE (recombinant capture antigen C22-3) and NS3 and NS4 (capture antigen C200). To date, the third generation assays have reached a specificity and a sensibility of more than 99%. These tests (anti-HCV 3.0) are capable to identify antibodies against CORE (capture antigen C22-3), NS3 and NS4 (capture antigens C33-C, C100-3, C200) and, for more, NS5 regions.

Serologic assays can be used as screening assays or confirmatory methods.

SCREENING ASSAYS

The screening assays are based on the detection of anti-HCV antibodies through a simple enzyme immunoassay (EIA) and they are entailed in multiple uses. For example, they are used for studies of prevalence and incidence of HCV infection in the general population or for the screening of categories of subjects at risk (such as people who inject drugs, hemophiliacs, people undergoing dialysis or transplantation etc). In EIAs, the binding antigen-antibody is indicated through a colorimetric reaction but to date, new versions of signal detection based on fluorescence and chemiluminescence (cLIA: ChemiLuminescent ImmunoAssay) techniques are available. Moreover, new immobilization methods for the capture antigens are available: MEIA (Microparticle Enzyme ImmunoAssay) and CMIA (Chemoluminscent Microparticle ImmunoAssay). The screening assays produce quantitative results and the criteria for interpreting them are generally based on the calculation of a value cut-off/ratio as recommended by the company that produced and marketed the tests. The table 9 elucidates some of the screening assays currently on the market.

Despite the good performances of the actually used third generation EIAs, even among a population with a low prevalence of infection, a specificity of 99% does not guarantee that there may be false positives. In immunocompetent persons with a prevalence of anti-HCV antibodies less than 10% (such as blood donors, military, general population, health professionals or subjects afferent to a clinic for sexually transmitted diseases), the rate of false positive results can also reach the 35%; whereas in immunocompromised subjects (for example persons undergoing dialysis) it can arrive at 15%. For this reason, a positive result in a screening assay should be verified through a supplementary confirmation test with high specificity (confirmatory methods).

PRODUCT/COMPANY	TECHNIQUE	SPECIFICITY	SENSITIVITY
PRISM, Abbott	ChLIA*	99,73%	100%
Imx, Abbott	MEIA*	99,92%	100%
Architect, Abbott	CMIA*	99,6%	99,1%
Axsym, Abbott	EIA*	99,6-99,84%	100%
Vitro, Ortho	ChLIA*	99,7-99,8%	100%
HCV, Ortho	EIA	99,9%	99,9-100%
Innotest, Innogenetics	EIA	99,8%	100%
Monolisa, BioRad	EIA	99,8%	100%
Access, BioRad	ChLIA*	99,2-99,85%	100%

Table 9. Example of screening assays. The specificity and the sensitivity values refer to the brochure of the kit.

CONFIRMATORY ASSAYS

The confirmatory assays are tests of third generation based on the principle of immunoblot on strip (RIBAs, Recombinant ImmunoBlot Assays) and they are used to confirm the presence of HCV specific antibodies for individuals who have tested positive by the screening assays. The results of these RIBA are interpreted as positive, undetermined or negative depending on the number of bands present on the strip (parameter recommended by the company that produced the test). A positive result is explained as positive for HCV infection even if it is not possible to distinguish between an active and a resolved infection, hence a HCV-RNA assay should be performed. A negative result is interpreted as negative for HCV infection and it indicates a false positive from the screening assay. Rarely, negative results for anti-HCV both in screening assays and confirmatory assays were observed in cases of acute infection with late seroconversion to anti-HCV antibodies or in cases of HCV infection resolved with an antibody titer below the detection limit of the assays. Occasionally, immunocompromised subjects resulted both EIA and RIBA negative are positive for HCV-RNA [99]. Finally, an undetermined result reveals a screening assay not estimable. In particular, it indicates a false positive screening assay, with a probability of 1-2% in blood donor, <5% in the general population, 4-5% in health workers and 10% in people undergoing dialysis [99]. In the clinical practice, a subject with an undetermined RIBA should be rechecked after at least one month and in case of another undetermined result, HCV RNA assays should be done. Moreover, undetermined results from confirmatory assays have been observed in subjects with a recent infection in seroconversion phase or occasionally in persons with a chronic HCV infection [99]. The table 10 elucidates some of the confirmatory assays currently on the market.

PRODUCT/COMPANY	TECHNIQUE	SPECIFICITY	SENSITIVITY
INNO-LIA, Innogenetics	Immunoblot	92,2-94,3%	100%
RIBA, Ortho	Immunoblot	98,8%	100%

Table 10. Example of confirmatory assays. The specificity and the sensitivity values refer to the brochure of the kit.

1.5.2 DIRECT ASSAYS

The detection of viral components is needed to diagnose an active HCV infection. Direct methods include molecular HCV assays and quantification of HCV Core antigen.

MOLECULAR ASSAYS

The molecular assays or NAT tests (Nucleic Acids Testing) determine the presence of the viral nucleic acids (HCV-RNA) in serum or plasma samples and they are used to confirm the diagnosis of active HCV infection in subjects with the positive serological-confirmatory assays. An active HCV infection can be early revealed because the HCV-RNA can be detected before specific antibodies become appreciable (within 1-3 weeks after exposure). Moreover, NAT tests are required for confirm a chronic HCV infection by the presence of both anti-HCV antibodies and HCV-RNA over 6 months after the exposure [100].

The highly conserved 5'UTR region is the target of choice for the HCV genome detection. The research of HCV-RNA can be carried out with both qualitative and quantitative assays. These assays are based on different techniques, such as reverse polymerase chain reaction (RT-PCR), transcription-mediated amplification (TMA), branched DNA assays (bDNA) and more recently the amplification in real time of the viral HCV-RNA (Real Time PCR). The choice to use a qualitative or a quantitative assay depends on the clinical context. For example, during the anti-HCV treatment, the evaluation of the SVR requires a qualitative test, whereas before starting the therapy and at 12 weeks for the evaluation of EVR a quantitative assay is necessary [99]. To date, Real Time PCR assays allow, in the same session, to determine the presence and contemporaneously to quantify the HCV-RNA (in a dynamic range from 30 to 10^8 UI/ml) and it becomes the reference method for the quantification of HCVRNA levels in clinical practice according to European and American guidelines. The table 11 lists some of the confirmatory assays currently used in clinical practice.

Regarding the interpretation of the results, a negative HCV-RNA result is not fully representative of a resolved infection indeed the viral replication during an infection is usually intermittent: a negative HCV-RNA result explains a resolved infection in the 15-25% of subjects who become infected in adulthood an in the 40-45% of children and youth population [99]. For this reason, the evaluation of HCV-RNA over time is recommended to have more decisive data.

In addition, starting from 2002 in Italy, NAT tests for the screening of blood donors have been introduced, aiming at minimize the risk of HCV transmission through blood transfusions.

PRODUCT/COMPANY	TECHNIQUE	ASSAY	ANALYTICAL SENSITIVITY (Probit analysis 95%)	RANGE
COBAS Ampliscreen, Roche	PCR	Qualitative	43 UI/ml (Plasma samples); 56 UI/ml (Serum samples)	-
Amplicor, Roche	PCR	Qualitative	13 UI/ml (Plasma samples); 8 UI/ml (Serum samples)	-
Versant, Bayer-GenProbe	TMA	Qualitative	5,3 UI/ml	-
Amplicor Monitor, Roche	PCR	Quantitative	-	600-850.000 UI/ml
COBAS TaqMan, Roche	Real Time PCR	Quantitative	-	$30\text{-}2x10^{8}$ UI/ml
Versant bDNA, Bayer	bDNA	Quantitative	-	$3.200\text{-}40x10^{6}$ copie/ml
COBAS Ampliscreen, Roche	PCR	Screening	41,9 UI/ml single sample; 28,8 UI/ml minipool	-
Procleix Ultrio, Chiron	TMA	Screening	1,9 UI/ml	-

Table 11. Example of NAT assays. The analytical sensitivity and range values refer to the brochure of the kit.

HCV CORE ANTIGEN DETECTION ASSAYS

The HCV Core antigen detection and quantification is based on the detection of the CORE antigen in the serum of the patients and its levels are significantly related to those of HCV-RNA [101]. HCV Core assays could be used in alternative of the HCV-RNA assays for three different conditions: to distinguish active from resolved HCV infections; to identify HCV infection in the antibody window period; to identify HCV infection in seronegative individuals at high risk for HCV infection, such as patients undergoing dialysis.

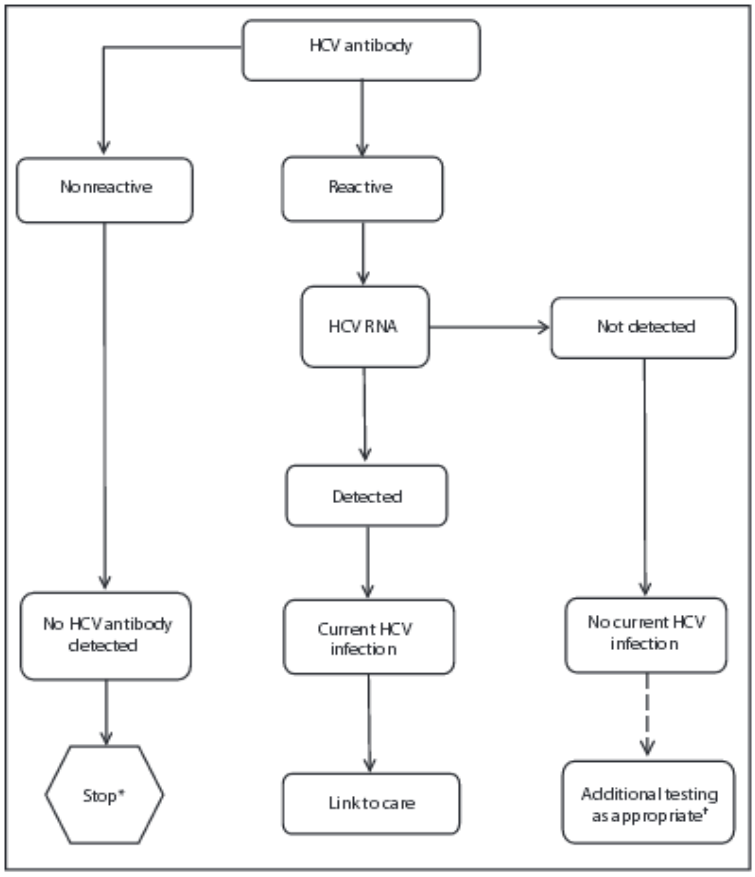


Fig. 12. Scheme of the recommended testing sequence for identifying current HCV infection [96]. * For persons who might have been exposed to HCV within the past 6 months, testing for HCV RNA or follow-up testing for HCV antibody should be performed. For persons who are immunocompromised, testing for HCV RNA should be performed; † To differentiate past, resolved HCV infection from biologic false positivity for HCV antibody, testing with another HCV antibody assay can be considered. Repeat HCV RNA testing if the person tested is suspected to have had HCV exposure within the past 6 months or has clinical evidence of HCV disease, or if there is concern regarding the handling or storage of the test specimen.

1.5.3 GENOTYPING AND SUBTYPING ASSAYS

Although DAAs therapy aims at a pangenotypic regimen, to date the genotype information is necessary before starting the anti-viral treatment; in fact, the current recommendations show the DAAs regimen to be adopted depending on the HCV genotype. Moreover, for the same reason, also the subtype of the virus is important to know. The gold standard for HCV genotyping is the genome sequencing of the NS5B region and subsequent phylogenetic analysis; however, this in-house method is restricted to reference centers. Therefore, other techniques have become available and actually there are many commercial assays for HCV genotyping based on them (Table 12). These techniques comprise indirect and direct assays that consist in the detection of the HCV genotypes respectively with genotype specific antibodies and competitive enzyme immunoassays or with direct analysis of specific regions of the viral genome through methods as Real Time PCR, RLB (Reverse Line Blot) or Fragment Analysis.

Over time, the region of choice for the direct genotyping assays has been the highly conserved 5'UTR indeed this region allows a well differentiation between several HCV genotypes (1 to 7a). Recently it has been recognized that 5'UTR is not sufficient to allow the differentiation of genotype 1 from 6 and a good discrimination between subtypes 1a and 1b. In fact, genotype 1b shares an identical 5'UTR sequence with genotypes 6 variants (subtypes c to l), furthermore the discover of the existence of clades within the genotype 1a allow the recognition of the misdiagnosis between clade I AND II (genotype 1a) and genotype 1b (Fig. 13) [104]. For these reasons, the guidelines recommends to use assays that analyze a second viral region in combination with the 5'UTR. The two regions most frequently chosen for this combined analysis are the NS5b and the CORE sequences because they are informative to discriminate subtypes and genotypes 1 to 6, but also sufficiently conserved to allow primers annealing.

PRODUCT/COMPANY	TECHNIQUE	N OF ANALYZED SEQUENCES	GENOTYPES/SUBTYPES	Notes
VERSANT HCV Genotype 2.0 Assay (LiPA),SIEMENS	PCR + Reverse-hybridization	Two: 5'UTR, CORE	1-6 genotypes and 15 subtypes (Including 1a-1b and 6c-l).	Differentiation of subtypes 1a vs.1b showing more than 96% concordance with NS5B sequencing
Gen-C 2.0 (NUCLEAR LASER)	PCR + Reverse-hybridization	Two: 5'UTR, CORE	1-7 genotypes and subtypes (including 1a-1b and 6f-g-m-q)	Accuracy in differentiation of subtypes 1a vs.1b of 100%

Table. 12. Example of genotyping and subtyping assays. The notes refer to the brochure of the kit.

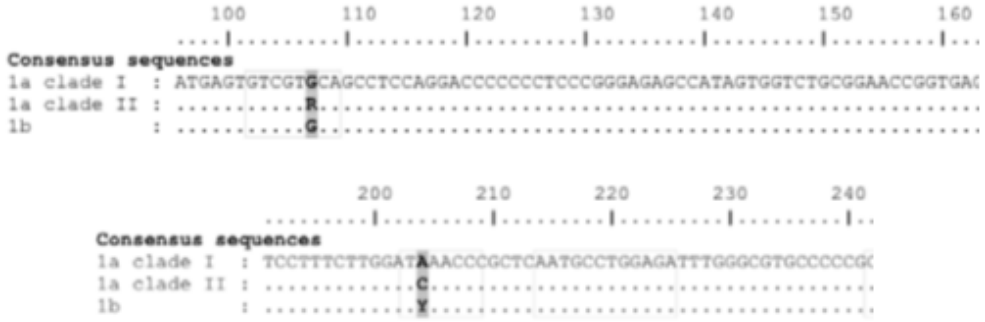


Fig. 13. Alignment of the 5'UTR consensus sequences of subtype 1a clade I and II and subtype 1b. At position 107 subtype 1a clade I exhibit a G, as subtype 1b, leading to misdiagnosis. Moreover, also the usual presence of a C at position 204 in subtype 1a clade II can entail misdiagnosis with subtype 1b, where C and T are the most frequent nucleotides in the same position [104].

Finally, although commercially genotyping assays reached over time higher specificity and sensitivity, routine genotyping methods, however, may cause wrong, inaccurate, or incomplete assignment in up to 10% of cases, due to indeterminate results, mixed infections, wrong subtyping, and even wrong genotyping. In these cases, the samples should be retested using either an alternative commercial assay or the reference method (NS5b sequencing), increasing the costs of the analyses.

1.5.4 RAVs DETECTION

Although the presence of different RAVs, currently guidelines consider only the Q80K mutation testing, which, as abovementioned, is a NS3 polymorphism that reduces the efficacy of the treatment with *SIM*. The presence of Q80Kis associated to the genotype 1a, and therefore patients with genotype 1a infection need to be tested for the Q80K prior to prescribe *SIM* and to consider an alternative therapy if it is detected.

2. AIM OF THE STUDY

As the HCV genotype is a crucial information for the clinical routine, we decided to develop a high-throughput system able to supply accurate data about the genotype and the subtype of the virus and at the same time to satisfy the requests of the diagnostic laboratories. In detail, this work is focused on the design and the validation of a standardized and performing process starting from the clinical sample up to the genotype and subtype result. Because of the high demand for this type of analysis, we chose to develop modular workcells as possible automated, in order to make the process fast and simple for the user. As shown in figure 14, these modules consist in:

1) Automated extraction of viral nucleic acid (HCV RNA) starting from the clinical sample;
2) One step reaction for the retrotranscription and amplification (RT-PCR) of the HCV-RNA;
3) Genotyping of the PCR product.

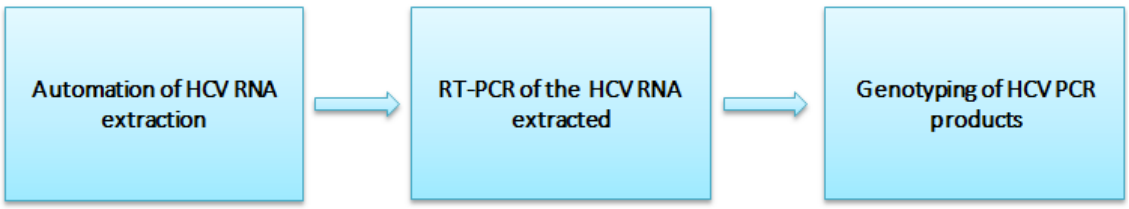


Fig. 14. Scheme of the modular workcells for the genotyping analysis of HCV.

In the first part of this project I focused on the design and development of an automated kit for the extraction of pathogen nucleic acids. In the second part, I worked on the evaluation of the HCV genotyping with the AMPLIQUALITY HCV TYPE PLUS device.

3. PART I: AUTOMATED EXTRACTION KIT DEVELOPMENT

In the first part of this project I focused on the design and development of an automated kit for the extraction of pathogen nucleic acids.

3.1 MATERIALS AND METHODS

3.1.1 GENEQUALITY X120

The automated extraction kit has been designed to be used with the GENEQUALITY X120 platform, which is a liquid handler developed by AB ANALITICA (Fig.15). This workstation has been chosen because it offers an innovative technology for higher process reliability, performance, and flexibility. The main peculiarity stands in its air displacement pipetting, which guarantees high pipetting accuracy and precision, and in the CO-RE system (Compressed O-Ring Exspansion) that attaches disposable tips using a highly robust lock-and-key style mechanism, in order to eliminate mechanical stress and minimizing the production of aerosols. Moreover, it supplies a series of commands such as anti-droplet and aspiration-dispense monitoring controls. The deck of the instrument has been configured with two shakers, one of which able to heat, a magnet, a drain for the liquid and solid wastes, and a set of carriers for introducing samples, reagents and tips. This entire system lends itself to numerous applications, among which the possibility to develop a high-throughput extraction process.

Fig. 15. Automated platform GENEQUALITY X120.

3.1.2 EXTRACTION PROTOCOL DESIGN

A nucleic acids extraction is a process for the purification of DNA/RNA from samples using a combination of physical and chemical methods. Although different purification techniques exist, there are some common steps. The first passage consists in the mechanical and chemical breaking of the cell membranes (cell lysis) for the release of the nucleic acids. Usually, the cell lysis occurs under continuous thermo-shaking in a reagent mainly composed of detergents and surfactants. In addition, a protease is used to digest the membrane proteins. After the lysis, the exposed DNA/RNA is purified from the other components by different procedures:

1) Ethanol precipitation: since the nucleic acid is insoluble in these alcohols, it aggregates together, giving a pellet upon centrifugation. The precipitation is improved by increasing of ionic strength, usually by adding sodium acetate;
2) Phenol-chloroform extraction: the phenol denatures proteins that after centrifugation stay in the organic phase while the aqueous phase containing the nucleic acid mixed with the chloroform that removes phenol residues from solution;
3) Adsorption on a solid support: the nucleic acid is bound on a solid phase depending on the pH and the salt concentration of the binding buffer. The solid phase is commonly composed of silica materials that show a good affinity for the DNA/RNA. To enhance this affinity, functional groups can be added. The solid phase can be arranged in mini-column or magnetic beads.

For the development of the automated extraction kit we have chosen the adsorption technique because it shows major efficiency than the others in the pathogen nucleic acids extraction. In particular we opted for the magnetic beads since they have more affinity than the mini-column and they are the best format to be adapted to the GENEQUALITY X120 platform. Once the binding step occurs, the magnetic beads are washed several times in order to remove possible contaminants and at the end of the process, the DNA/RNA is eluted from the solid phase to a specific buffer.

Several commercial extraction reagents have been tested in order to choose the best performing combination that fitted our purpose. The commercial names of these reagents are not reported, in protection of industrial secrecy.

In addition to these reagents, we thought to provide the extraction kit of internal controls (ICs), in order to monitor the presence of inhibitors or other factors that may cause false-negative results. The internal controls are in fact used as indicator of a good nucleic acid extraction and quality of sample. An IC should be easy to produce and to standardize and additionally, It should be stable, noninfectious, and suitable for different assays. In particular, we opted for exogenous ICs that are added to the sample

before starting the extraction protocol. We decided to supply the kit with an internal control for the monitoring of the DNA extraction (IC DNA) and another control for the RNA (IC RNA). As regard the IC DNA, a linear plasmid of β-globin has been chosen, because it has already been studied in the company, showing good stability and extraction performances. Concerning the IC RNA, an E. coli phage has been selected, since it is resistant to RNase degradation, even at high storage temperatures and it is sufficiently stable for the routine use. The chosen ICs offer the possibility once extracted to be downstream evaluated with the pathogen target by different analysis techniques (Real time PCR and Reverse Line Blot).

Finally, a carrier has been added to the extraction kit, which favors the pathogen nucleic acids extraction, by co-precipitation.

3.1.3 PACKAGING DESIGN

A crucial point in the development of the extraction kit has been the selection of the packaging to be use in combination with the platform GENEQUALITY X120. Different parameters has been evaluated , such as the type of plastics and containers to use, the reagents volumes to include and how to seal the components. Several options have been tested in order to choose the most stable packaging and to obtain no degradation and evaporation of reagents during time.

3.1.4 NUCLEIC ACIDS EXTRACTION WITH BIOROBOT EZ

The CE IVD instrument BioRobot EZ1 (Qiagen) has been used as reference method for the evaluation of our extraction kit. The BioRobot EZ1 DSP System used with the EZ1 DSP Virus kit (Qiagen) enables automated purification of viral nucleic acids starting from different type of samples. The extractions have been performed following the manufacturer's instructions, and adding our Internal Controls to monitoring the extraction efficiency (not provided by the EZ1 kit).

3.1.5 NUCLEIC ACIDS EXTRACTION WITH NUCLISENSE

A second CE IVD instrument, the NucliSENS®easyMag®, has been used as reference method for the evaluation of our extraction kit. It is an automated system for total nucleic acid extraction from different sample types and volumes.

3.1.6 SAMPLES

The samples involved in the study are composed by synthetic samples, clinical specimens and samples derived from International EQA (External Quality Assessment).

The synthetic samples are obtained spiking the pathogen or its DNA/RNA into synthetic matrices or transport buffers, such as AcroMetrix™ EDTA Plasma Dilution Matrix (Thermo Fisher Scientific) or PBS 1X (Sigma Aldrich). The pathogens used for the spiked samples are:

1) The 1st WHO International Standard for Human Cytomegalovirus (CMV) (WHO International Laboratory for Biological Standards, UK). The preparation contains the HCMV Merlin strain in a universal buffer comprising Tris-HCland human serum albumin and found negative for HBsAg, anti-HIV and HCV RNA;
2) The 1st WHO International Standard for Epstein-Barr Virus (EBV) (WHO International Laboratory for Biological Standards, UK). The preparation contains the EBV B95-8 strain in a universal buffer comprising Tris-HCl, human serum albumin and trehaloseand found negative for HBsAg, anti-HIV and HCV RNA;
3) Plasmids (pls) pEX-A2 (Eurofin Genomics) containing a sequence of CMV.

For mimics negative samples the matrices have been used without adding any pathogens.

A total of 381 clinical specimens has been included in this study. A detailed list is reported in table 13.

N° of samples	Matrix	Pathogen positivity
47	Whole Blood	CMV, EBV, HHV6
53	Whole Blood	Negative for CMV and EBV
48	Plasma/Serum	CMV, EBV, HHV8, ENTERO
29	Plasma/Serum	Negative for CMV and EBV
13	BAL/CSF	ADV, EBV, CMV, ENTERO, HHV6, HSV1-2
10	BAL/CSF	Negative for CMV and EBV
81	Urine/Cervical swabs	STI
50	Cervical swabs	HPV
50	Cervical swabs	Negative for HPV

Table. 13. List of clinical samples extracted in the study. CMV: Human CitomegaloVirus; EBV: Epstein-Barr Virus; HHV6: Human Herpesvirus 6; HHV8: Human Herpesvirus 8; ENTERO; Enterovirus; AVD: Adenovirus; HSV1-2: Herpesvirus 1-2; STI: Sexual Transmitted Infections; HPV: Human Papillomavirus. BAL; BronchoAlveolar Lavage; CSF: Cerebral Spinal Fluid.

In order to evaluate the proficiency of our automated kit, some quality panels have been used. The QCMD (Quality Control for Molecular Diagnotics) analyzed in the study are reported in table 14.

Pathogen	QCMD
ENTERO	2015_CHALLENGE 1 e 2; 2016 CHALLENG 1
ADV	2015-2016_CHALLENGE 1-2
CMV	2015-2016_CHALLENGE 1-2
EBV	2015-2016_CHALLENGE 1-2
JCV	2015-2016_CHALLENGE 1-2
BKV	2015-2016_CHALLENGE 1-2
VZV	2015-2016_CHALLENGE 1-2
HSV	2015-2016_CHALLENGE 1-2
Parvo B19	2015-2016_CHALLENGE 1-2

Table. 14. List of QCMD extracted in the study. CMV: Human CitomegaloVirus; EBV: Epstein-Barr Virus; JCV: John Cunningham Virus; BKV: Poliomavirus BK; ENTERO; Enterovirus; AVD: Adenovirus; VZV: Varicella Zooster Virus; HSV: Herpesvirus1-2; Parvo B19: Parvovirus B19.

3.1.7 REAL TIME PCR KIT FOR PATHOGEN QUANTITATIVE DETECTION

The real time PCR analysis has been chosen to evaluate the extraction efficiency. We selected this method because is fast and simply, and the analysis allows to verify different important parameters:

1) The quality of the extract, which can be verified by the amplification plot: the presence of contaminants in fact inhibits the amplification curve;
2) The extraction efficiency of the Internal controls, which is verified by their Ct values;
3) The extraction efficiency of the pathogen, which is verified by its quantification in LOG10 copies/ml, where copies/ml is: (copies/rx) x Ve x (1000/Vs). Copies/rx stands for copies per reaction; Ve for the Elution Volume; Vs for the starting volume of the processed sample;
4) The extraction repeatability: The extraction efficiency in a same session is analyzed by the Coefficient of Variation (CV), that is evaluated according to the following calculation:

$$\frac{\text{Ct SD}}{\text{Mean Ct}} \times 100$$

Ct SD: Standard deviation of the mean Ct of the extracts of the session; Mean Ct: The mean of the Ct of the extracts processed in the same session.

For the real time PCR analysis molecular diagnostic kits provided by AB ANALITICA for the detection of infectious diseases have been used. In details they are reported in table 15:

Kit	Detected Pathogen
REALQUALITY RQ-CMV	Human Citomegalovirus
REALQUALITY RS-EBV	Epstein-Barr Virus
REALQUALITY RS-HSV 1	Herpesvirus 1
REALQUALITY RS-HSV 2	Herpesvirus 2
REALQUALITY RS-HHV 6	Human Herpesvirus 6
REALQUALITY RS-HHV 8	Human Herpesvirus 8
REALQUALITY RS-ADENO	Adenovirus
REALQUALITY RS-JCV	John Cunningham Virus
REALQUALITY RS-BKV	Poliomavirus BK
REALQUALITY RQ-ENTERO	Enterovirus
REALQUALITY RS-VZV	Varicella Zooster Virus
REALQUALITY RS-PARVO B19	Parvovirus B19
REALQUALITY RS-CHLAM T	Sexual Transmitted Infections
REALQUALITY RQ-STI	Sexual Transmitted Infections
REALQUALITY RQ-HPV HR Multiplex	Human Papillomavirus

Table. 15. List of molecular diagnostic kit used in the study for the detection of various pathogens.

In addition to the pathogen, this kits allow to detect the internal controls used for the extractions.

The experiment of the study have been conducted on the real time PCR BioRad DX instrument (BioRad).

3.2 RESULTS

3.2.1 OPTIMIZATION OF EXTRACTION PROTOCOL

In the first part of my Ph.D project, I developed a prototype for a new automated extraction kit to be used with the workstation GENEQUALITY X120. Several solutions have been tested to obtain an efficient packaging of the kit. Finally, the extraction reagents have been included into two sealed cartridges and a series of tubes as shown in figure 16.

Fig. 16. GENEQUALITY X120 Pathogen kit.

The device has been called GENEQUALITY X120 Pathogen kit and it allows to extract up to 96 samples.

Several conditions have been evaluated for the design of the automated process. To sum up, once loaded in the workstation the samples to be, the automated extraction kit and the necessary plastics (such as tips), the system can start without any other actions by the operator. The extraction process occurs in a work plate where the samples, the internal controls and the extraction reagents are dispensed. For each extraction step, the work plate is moved through different position of the workstation, from the thermo-shakers to the magnet. Finally the eluates are recovered in a storage plate without the magnetic beads. To simplify the application of this platform, an easy-to-use interface for the user have been developed, which serves to set the extraction conditions, among which: N° of sample to be extracted, Volume of sample to process (Vs), Elution Volume (Ve).

Finally, several parameters have been tested to obtain an efficient automated extraction method.

3.2.1.1 EVALUATION OF EXTRACTS QUALITY

Different experiment tests have been done to evaluate the quality of the extracts obtained with our automated extraction kit. The results show that the real time PCR plots present good amplification curves of the extracted internal controls (IC DNA and IC RNA), indicating the absence of possible extraction contaminants (protein, ethanol…) in the eluates.

3.2.1.2 EVALUATION OF OPTIMAL STARTING VOLUMES

For this evaluation we decided to verify the efficiency of the our automated extraction method starting from 200 µl and 400 µl of sample. For this experiment, eight aliquots of human plasma have been extracted with the GENEQUALITY X120 Pathogen kit in the same session. In parallel an operator with the same reagents has performed the extraction manually. Two aliquots have been also extracted with a reference CE IVD instrument. The extractions have been performed processing 200 µl and 400 µl of sample and eluting in 90 µl. The results are shown in table 16.

IC DNA					
	Manual extractions		Automated extractions		BioRobot EZ1
	Mean Ct	CV	Mean Ct	CV	Mean Ct
Vs 200ul	29,78	0,75%	30,08	0,73%	30,7
Vs 400ul	29,73	1,26%	30,24	1,33%	30,41

IC RNA					
	Manual extractions		Automated extractions		BioRobot EZ1
	Mean Ct	CV	Mean Ct	CV	Mean Ct
Vs 200ul	30,56	0,38%	29,97	0,46%	30,76
Vs 400ul	31,01	1,44%	29,95	1,01%	31,41

Table 16. Mean Ct and CV values for the controls IC DNA and IC RNA extracted in manual, in automation and with the BioRobot EZ1 starting from 200 µl and 400 µl (Vs). The CV has not been calculated for the two extracts of the BioRobot EZ1.

The Mean Ct values relative to the GENEQUALITY X120 Pathogen kit results (Automated extractions) are comparable to those relative to the manual kit (Manual extractions) and to the reference method (BioRobot EZ1). These data indicates that the extraction efficiency for both DNA (IC DNA) and RNA (IC RNA) of our method is equivalent to the references, starting from both 200 µl and 400 µl of sample. Moreover, the CV values

show that the automated process has a good repeatability, comparable to the manual extraction.

3.2.1.3 EVALUATION OF OPTIMAL ELUTION VOLUMES

For this evaluation we decided to consider three different elution volumes: 90 µl, 120 µl, 180 µl. For this experiment, eight aliquots of human plasma spiked with WHO CMV (final concentration of 2500 UI/ml) have been extracted with the GENEQUALITY X120 Pathogen kit in three consecutive sessions, varying the elution volume. The experiment has been performed starting from both 200 µl and 400 µl of sample. Two aliquots have been also extracted with a reference CE IVD instrument. The results are shown in table 17.

Vs 200ul							
	Target WHO CMV			IC DNA		IC RNA	
	Mean Ct	CV	Mean LOG copies/ml	Mean Ct	CV	Mean Ct	CV
Ve 90ul	35,31	1,48%	3,9	31,38	1,06%	30,53	0,91%
Ve 120ul	35,64	2,07%	3,94	31,56	1,02%	30,63	1,20%
Ve 180ul	36,61	1,40%	3,84	31,68	0,92%	30,85	1,05%
BioRobot EZ1	35,47	-	3,87	32,46	-	30,62	-

Vs 400ul							
	Target WHO CMV			IC DNA		IC RNA	
	Mean Ct	CV	Mean LOG copies/ml	Mean Ct	CV	Ct	CV
Ve 90ul	35,54	1,37%	3,6	31,94	1,20%	31,17	1,92%
Ve 120ul	35,58	2,11%	3,71	31,89	0,75%	30,9	0,88%
Ve 180ul	36,24	1,07%	3,69	31,76	0,43%	30,7	1,24%
BioRobot EZ1	34,91	-	3,78	31,45	-	31,2	-

Table 17. Mean Ct and CV values for the target WHO CMV, the controls IC DNA and RNA extracted with different elution volumes (90 µl, 120 µl, 180 µl Ve) and with the reference method BioRobot EZ1, starting from 200 µl and 400 µl of sample (Vs). The quantification of the target WHO CMV is also reported as a mean value among the extracts values (Mean LOG copies/ml). The CV has not been calculated for the two extracts of the BioRobot EZ1.

As the table shows, the quantification of the target is comparable for all elution volumes, both starting from 200 µl and 400 µl of sample. Moreover, the DNA and RNA extraction efficiency of our automated kit is confirmed by the CV values of the target and the internal controls and by their Ct comparability with the BioRobot EZ1.

3.2.1.4 EVALUATION OF OPTIMAL CARRIER CONCENTRATION

The previous experiments have been conducted with a carrier concentration of 18 µg per sample. With the following experiment we have tried to decrease the concentration of carrier in order to verify that the extraction efficiency is maintained even with lower carrier concentrations. For this experiment, eight aliquots of human plasma spiked with WHO EBV (final concentration of 2500 UI/ml) have been extracted with the GENEQUALITY X120 Pathogen kit in three consecutive sessions, varying the carrier concentration: 18 µg per sample, 9 µg per sample and 3 µg per sample. Two aliquots have been also extracted with a reference CE IVD instrument. The extractions have been performed processing 400 µl of sample and eluting in 90 µl. The results are reported in table 18.

	Target WHO EBV			IC RNA	
	Mean Ct	CV	Mean LOG copies/ml	Mean Ct	CV
18ug per sample	34,64	1,28%	3,73	31,24	0,94%
9ug per sample	34,03	1,33%	3,81	31,15	1,28%
3ug per sample	34,14	1,44%	3,77	31,52	1,10%
BioRobot EZ1	34,28	-	3,74	30,46	-

Table 18. Mean Ct and CV values for the target WHO EBV and the control IC RNA extracted with different concentration of carrier (18 µg per sample, 9 µg per sample and 3 µg per sample) and with the reference method BioRobot EZ1. The quantification of the target WHO EBV is also reported as a mean value among the extracts values (Mean LOG copies/ml). The CV has not been calculated for the two extracts of the BioRobot EZ1.

The values obtained reveal that the quantification of the target is comparable among the extraction with the different carrier concentrations, indicating that decreasing the carrier quantity the DNA extraction efficiency is not lost. Similar results are obtained for the RNA extraction, considering the Mean Ct of the IC-RNA. Moreover, the DNA and RNA extraction efficiency of our automated kit is confirmed by the CV values of the target and the internal controls and by their Ct comparability with the BioRobot EZ1. In conclusion, the carrier concentration of the GENEQUALITY X120 Pathogen kit has been switched from 18 µg to 3 µg per sample.

3.2.1.5 EVALUATION OF THE ABSENCE OF CROSS-CONTAMINATION

The absence of cross-contaminations during a specific process is a mandatory requirement because if cross-contamination occurs, it is the first cause of false positive results. The following experiment has been done to verify the absence of cross-contaminations in our automated process. For this evaluation, twenty-four aliquots of CMV pls (final concentration of 10^4 copies/µl in PBS 1X and twenty-four aliquots of only PBS 1X have been extracted in the same session with the GENEQUALITY X120 Pathogen kit. In order to increase the possibility of cross-contaminations among the wells of the work plate, the samples to be extracted have been arranged in the platform in such a way to be checkerboard dispensed (Fig. 17). The extraction have been performed processing 400 µl of sample and eluting in 90 µl.

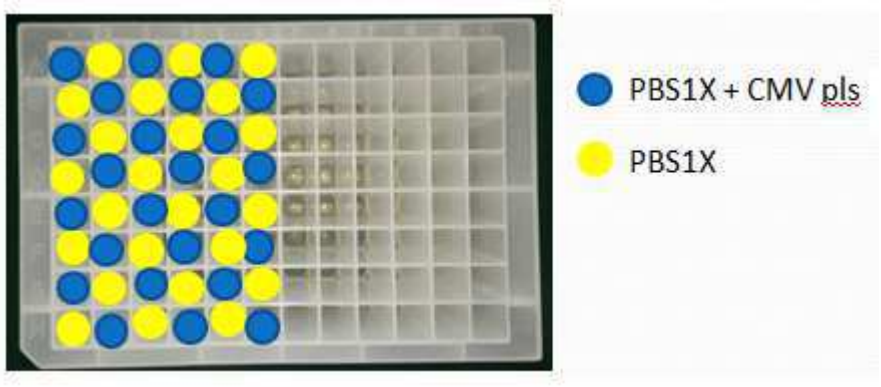


Fig. 17. Checkerboard dispensation of the sample in the work plates.

In the first experiment, more than 50% of the negative extracts were positive for the viral target (false positive), indicating that the cross-contamination phenomenon occurred. Considering the steps of the protocol, the binding may have been most crucial for cross-contamination: in this step the liquid volume in the work plate wells is greater than in the other and then a liquid transfer among the wells may occur during the shaking. Therefore, different shaking speed of the binding have been tested to set the better one to avoid the cross-contaminations (Table 19).

Experiment	Shaking Speed (rpm) Binding Step	False Postive
1	1200	16/24
2	900	13/24
3	750	0/24

Table 19. Number of false positive obtained on 24 negative samples, changing the shaking speed of the binding step (1200-900-750 rpm).

As the data reveal, the 750 rpm option is the best to avoid cross-contaminations. To confirm this result, other extractions with this condition has been performed (Table 20).

Experiment	Shaking Speed (rpm) Binding Step	False Postive
1	750	0/24
2		0/24
3		0/24

Table 20. Number of false positive obtained on 24 negative samples, with the 750 rpm shaking speed in three different extractions.

In order to verify that the extraction efficiency has not been lost by decreasing the shaking speed of the binding step, the positive samples (PBS 1X + CMV pls) extracted with GENEQUALITY X120 Pathogen kit in the three experiments with the 750 rpm, have been compared with the two aliquots of the same positive sample extracted with a reference CE IVD device in the same conditions. The results are shown in the table 21.

	Target CMV pls			IC DNA	
	Mean Ct	CV	Mean LOG copies/ml	Mean Ct	CV
Experiment 1	23,03	0,95%	7,04	32,43	1,65%
BioRobot EZ1	22,65	-	7,15	32,16	-
	Mean Ct	CV	Mean LOG copies/ml	Mean Ct	
Experiment 2	23,03	0,69%	7,06	35,59	1,26%
BioRobot EZ1	22,73	-	7,16	34,80	-
	Mean Ct	CV	Mean LOG copies/ml	Mean Ct	
Experiment 3	23,04	0,78%	7,13	33,40	0,86%
BioRobot EZ1	22,88	-	7,18	32,65	-

Table 21. Mean Ct and CV values for the target and the control IC DNA. The quantification of the target CMV pls is also reported as a mean value among the extracts values (Mean LOG copies/ml). The values are grouped by the three experiments performed with the shaking speed 750 rpm for the binding step (Experimet 1-2.3). For each experiment the values relative to the extraction with the reference method BioRobot EZ1 are reported, expect for the CV.

As the data of the table reveals, the values obtained in the three experiments with our automation process are comparable to those relative to respective BioRobot EZ1. Moreover, the extraction efficiency is confirmed by the CV values of the target and internal control (IC DNA).

In conclusion, the shaking speed setted for our automated extraction protocol has been switched from 1200 to 750 rpm.

3.2.2 VALIDATION OF GENEQUALITY X120 PATHOGEN KIT PROTOTYPE

After achieving the prototype standardization of the automated extraction kit, a phase of the project dedicated to the evaluation of its diagnostic performance has been started and the following parameters has been investigated.

3.2.2.1 REPEATIBILITY AND REPRODUCIBILITY EVALUATION

The repeatability and the reproducibility have been investigated by Intra- and Inter-Assay experiments.

The intra-assay experiment verifies the repeatability of the process within the same extraction session. For this evaluation, forty-eight aliquots of human plasma spiked with WHO EBV (final concentration of 2500 UI/ml) have been extracted in the same session. The experiment have been performed processing both 200 µl and 400 µl of sample and eluting in 90 µl. The results are reported in table 22.

Intra-Assay experiment							
	Target WHO EBV			IC DNA		IC RNA	
	Mean Ct	CV	Mean LOG copies/ml	Mean Ct	CV	Mean Ct	CV
Vs 200ul	33,98	1,34%	4,03	31,31	1,76%	30,42	1,35%
Vs 400ul	34,70	1,36%	3,71	31,99	1,44%	31,44	1,18%

Table 22. Mean Ct and CV values for the target and the controls IC DNA and IC RNA extracted with the developed automated process, starting from 200 µl and 400 µl of sample (Vs). The quantification of the target WHO EBV is also reported as a mean value among the extracts values (Mean LOG copies/ml).

The intra-assay repeatability is indicated by the CV values for the different targets. As shown, the intra-assay repeatability of the developed automated process is always <2% for the different DNA and RNA target (WHO EBV, IC DNA and IC RNA), starting from both 200 µl and 400 µl of sample.

The inter-assay experiment verifies the reproducibility of the process among different extraction sessions. For this evaluation, sixteen aliquots of human plasma spiked with WHO EBV (final concentration of 2500 UI/ml) have been extracted in three consecutive sessions, changing the batches of reagents in order to increase the variability. The experiment have been performed processing both 200 µl and 400 µl of sample and eluting in 90 µl. The results are shown in table 23.

Vs 200ul							
	Target WHO EBV			IC DNA		IC RNA	
	Mean Ct	CV	Mean LOG copies/ml	Mean Ct	CV	Mean Ct	CV
Extraction 1	33,92	1,58%	3,98	30,71	1,27%	29,66	1,18%
Extraction 2	33,72	1,79%	4,04	30,65	0,82%	29,51	0,73%
Extraction 3	33,89	0,96%	3,99	30,70	0,76%	30,28	1,08%

Vs 400ul							
	Target WHO EBV			IC DNA		IC RNA	
	Mean Ct	CV	Mean LOG copies/ml	Mean Ct	CV	Mean Ct	CV
Extraction 1	33,69	0,98%	3,99	30,56	1,21%	31,10	1,72%
Extraction 2	33,86	1,45%	3,95	30,88	1,17%	31,47	2,62%
Extraction 3	34,19	1,25%	3,85	31,07	1,31%	31,30	1,86%

Table 23. Mean Ct and CV values for the target and the controls IC and IC-RNA relative to the three different extraction (Extraction 1-2-3) with the developed automated method, starting from 200 µl and 400 µl of the sample (Vs). The quantification of the target WHO EBV is also reported as a mean value among the extracts values (Mean LOG copies/ml).

The Inter-Assay reproducibility has been evaluated calculating the CV among the Mean Ct of the three extractions (Table 24).

	Inter-assay reproducibility		
	Target WHO EBV	IC DNA	IC RNA
Vs 200ul	0,25%	0,09%	1,12%
Vs 400ul	0,60%	0,69%	0,48%

Table 24. The table shows the obtained inter-assay reproducibility for the 3 extractions performed starting from 200 ul and 400 ul OF SAMPLE (Vs).

The data reveal that the Inter-Assay Reproducibility is always <1,5% for both DNA and RNA extractions (WHO EBV, IC DNA, IC RNA) and starting from the different volume samples.

3.2.2.2 ANALYTICAL SENSITIVITY EVALUATION

The experiment evaluating the analytical sensitivity or detection limit investigates the minimum viral load that developed automated process is able to extract with 95% of confidence. For the evaluation, two samples with serial dilutions respectively of WHO CMV and WHO EBV have been prepared in human plasma to obtain these concentrations:

1) 250 UI/ml;
2) 200 UI/ml;
3) 125 UI/ml;
4) 62,5 UI/ml;

5) 50 UI/ml.

Eight aliquots for each concentration have been extracted in the same session. The extractions have been performed processing 400 ul of sample and eluting in 90 ul. The extracts have been tested in triplicate by real time PCR. The results have been analyzed by Probit with a confidence of 95% (Fig. 18).

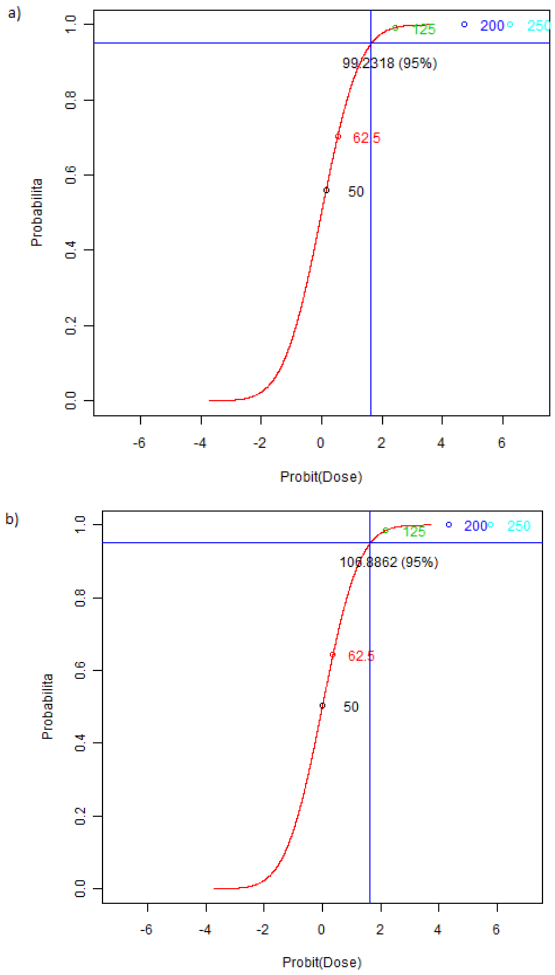


Fig. 18. Analysis Probit for the WHO EBV (a) and WHO CMV (b) targets in plasma samples.

The results indicate that the GENEQUALITY X120 Pathogen kit has a probability of 95% to detect a viral load of 99,2318 UI/ml for the WHO EBV and of 106,8862 UI/ml for the WHO CMV.

3.2.2.3 LINEAR RANGE EVALUATION

The linear range is the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample. For the evaluation, two samples with serial dilutions respectively of WHO CMV and WHO EBV have been prepared in human plasma to obtain these concentrations:

1) 5 x 10^5 UI/ml;
2) 5 x 10^4 UI/ml;
3) 5 x 10^3 UI/ml;
4) 5 x 10^2 UI/ml;
5) 2,5 x 10^2 UI/ml;
6) 1,25 x 10^2 UI/ml.

Six aliquots for each concentration have been extracted in the same session, processing 400 µl of sample and eluting in 90 µl. The results are shown in figures 19 and 20:

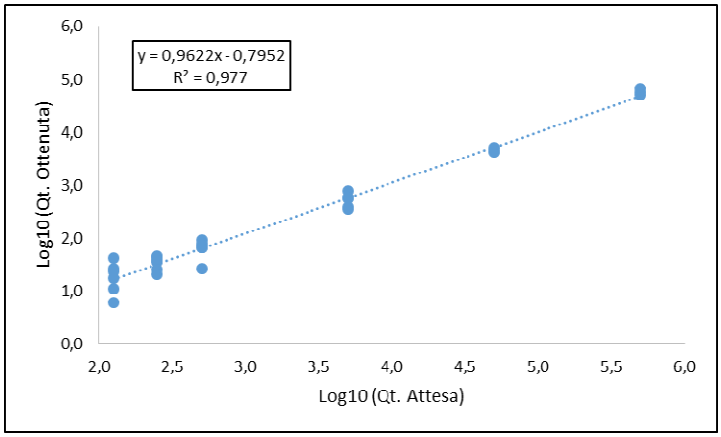


Fig. 19. Graphic representation of the linearity range experiment obtained for the WHO EBV target.

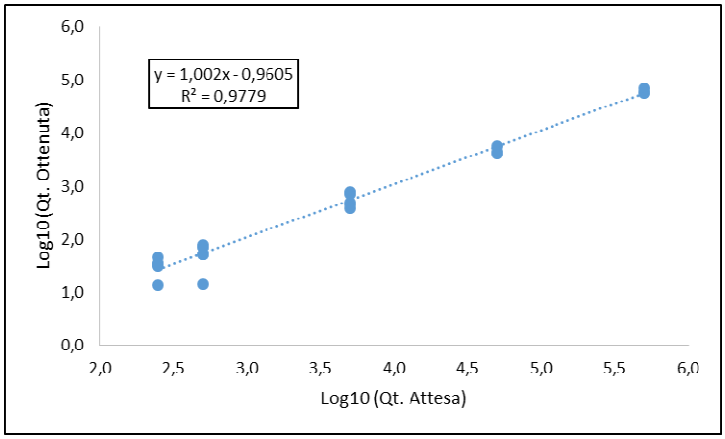


Fig. 20. Graphic representation of the linearity range experiment obtained for the WHO CMV target.

The data show that the GENEQUALITY X120 pathogen kit allows to extract in a linear way in a range of 125-5 x 10^5 UI/ml for the WHO EBV and of 250-5 x 10^5UI/ml for the WHO CMV target.

3.2.2.4 EXTRACTION EFFICIENCY EVALUATION

At least 300 clinical samples have been extracted with the GENEQUALITY X120 Pathogen kit to test its extraction efficiency. The clinical samples analyzed were positive for different targets with different viral load. Different types of clinical specimens have been included (table 13).

N° of samples	Matrix	Pathogen positivity
47	Whole Blood	CMV, EBV, HHV6
53	Whole Blood	Negative for CMV and EBV
48	Plasma/Serum	CMV, EBV, HHV8, ENTERO
29	Plasma/Serum	Negative for CMV and EBV
13	BAL/CSF	ADV, EBV, CMV, ENTERO, HHV6, HSV1-2
10	BAL/CSF	Negative for CMV and EBV
81	Urine/Cervical swabs	STI
50	Cervical swabs	HPV
50	Cervical swabs	Negative for HPV

Table. 13. List of clinical samples included in the study. CMV: Human CitomegaloVirus; EBV: Epstein-Barr Virus; HHV6: Human Herpesvirus 6; HHV8: Human Herpesvirus 8; ENTERO; Enterovirus; AVD: Adenovirus; HSV1-2: Herpesvirus 1-2; STI: Sexual Transmitted Infections; HPV: Human Papillomavirus. BAL; BronchoAlveolar Lavage; CSF: Cerebral Spinal Fluid.

The same samples have been extracted with reference CE IVD devices (BioRobot EZ1, NucliSENS EasyMag). The results indicate that in >90% of cases the GENEQUALITY X120 Pathogen kit shows comparable performances to the NucliSENS EasyMag device. In the remaining 10%, the positive results obtained with the NucliSENS has been lost with the GENEQUALITY X120 Pathogen kit but resulted negative also with the BioRobot EZ1, indicating that probably these clinical samples have been degraded due to repeated thawing cycles.

In order to increase the number of specimens investigated, also QCMD samples have been extracted with the developed automated process (Table 14).

Pathogen	QCMD
ENTERO	2015_CHALLENGE 1 e 2; 2016 CHALLENG 1
ADV	2015-2016_CHALLENGE 1-2
CMV	2015-2016_CHALLENGE 1-2
EBV	2015-2016_CHALLENGE 1-2
JCV	2015-2016_CHALLENGE 1-2
BKV	2015-2016_CHALLENGE 1-2
VZV	2015-2016_CHALLENGE 1-2
HSV	2015-2016_CHALLENGE 1-2
Parvo B19	2015-2016_CHALLENGE 1-2

Table. 14. List of QCMD extracted in the study. CMV: Human CitomegaloVirus; EBV: Epstein-Barr Virus; JCV: John Cunningham Virus; BKV: Poliomavirus BK; ENTERO; Enterovirus; AVD: Adenovirus; VZV: Varicella Zooster Virus; HSV: Herpesvirus1-2; Parvo B19: Parvovirus B19.

The same samples have been extracted with a reference CE IVD devices (BioRobot EZ1). The study reveals that the extraction performance of the GENEQUALITY X120 Pathogen kit are comparable to those of the reference method. Moreover, the quantification of the targets are inner the range declared by the QCMD, indicating a good extraction efficiency of our process.

4. PART II: EVALUATION OF HCV GENOTYPING WITH AMPLIQUALITY HCV TYPE PLUS DEVICE

The second part of my PhD project, I focused on the performance evaluation of the AMLIQUALITY HCV TYPE PLUS kit, intended for the genotyping of HCV.

4.1 MATERIALS AND METHODS

4.1.1 AMPLIQUALITY HCV TYPE PLUS KIT

The AMPLIQUALITY HCV TYPE PLUS device is a kit based on the Reverse Line Blot technique (RLB) for the determination of the genotypes 1-7 of HCV. The AMPLIQUALITY HCV TYPE PLUS kit provides the necessary equipment for the assay starting from the HCV RNA extracted. The HCV RNA undergoes a multiplex RT-PCR (Retrotranscription and PCR in a single step) for the 5'UTR and CORE regions with biotinylated primers. The reaction mix includes also a dUTP/Cod UNG system that is active at room temperature and removes any contamination of previous reactions. The PCR products are then hybridized to oligonucleotide probes that are spotted on a laminated nitrocellulose membrane and recognize specific sequences of the 5'UTR and CORE regions. The hybridization process is followed by a series of washes to eliminate the non-specific binding. Finally, a colorimetric reaction (streptavidine-alkaline phosphatase conjugated plus substrate) underlies with brownish bands the positions in which the hybridization occurred. At the end of the assay the strips show different band patterns that allow the identification of the genotype and in most cases the subtypes of the virus. The kit provides also an Internal Control RNA (IC-RNA) for the efficiency monitoring of the extraction process and the RT-PCR. This control consists in the MS2 phage of *E. Coli*, which is stable and absence from the clinical samples. Moreover, a DNA positive control representative of a genotype 1a sequence is supplied for the monitoring efficiency of the genotyping process.

The Figure 21 shows an example of strip of the kit AMPLIQUALITY HCV TYPE PLUS. It includes:

A. Two red marker lines facilitate the correct position of the strips on the reading card or interpretation sheet;

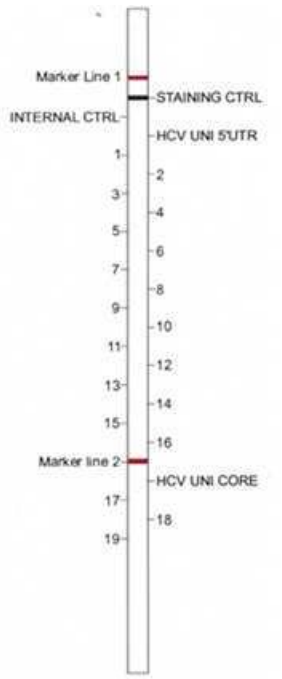


Fig. 21. Strip Layout

B. STAINING CTRL: It is the staining control band that allows the user to assess the staining reaction (substrate conversion);
C. INTERNAL CTRL: It recognizes the internal control (IC-RNA), thus permitting the assessment of the quality of HCV RNA extract, which provides indications concerning successful completion of the extraction and RT-PCR to avoid false negative results;
D. HCV UNI 5'UTR: it shows the presence of a PCR product from the 5'UTR;
E. HCV UNI CORE: It shows the presence of a PCR product from the CORE region, thid band has to be considered in case that the genotype-specific bands for the 5'UTR indicate that the sample is positive for genotype HCV 1;
F. Sixteen bands (position 1 to 16) containing oligonucleotide probes that recognize sequences of the 5'UTR in a genotype-dependent manner;
G. Three bands (position 17 to 19) containing oligonucleotide probes that recognize sequences of the CORE in a genotype-dependent manner.

At the end of the assay, the interpretation of the band patterns is based on specific interpretative tables that are shown in the Appendix 4. The interpretation tables comprises two sections: Part A, dedicated to the identification of genotype 6 and subtypes 1a and 1 b using the information from both the 5'UTR and the CORE regions, and Part B, dedicated to the identification of genotypes 2, 3, 4, 5 and 7 according to the information from the 5'UTR. If the strip shows any of the 5'UTR band patterns that correspond to the patterns described in table A (HCV genotyping 1 or 6), the user proceeds to the analysis of the CORE region band patterns and will base the genotyping solely on this analysis. If there are no bands for the CORE region or the results for the CORE region are inconclusive, genotype 1 and 6 cannot be reliably distinguished and identification of HCV genotype 1 has to be based on the information provided by the 5'UTR.

To simply the use of these tables, an interpretative software has been developed and called HCV TYPE PLUS Strip Reader (Fig. 22).

Using a straightforward point and click interface, the user inputs the results from the strip into the interpretation software. The software evaluates the data and produces a report.

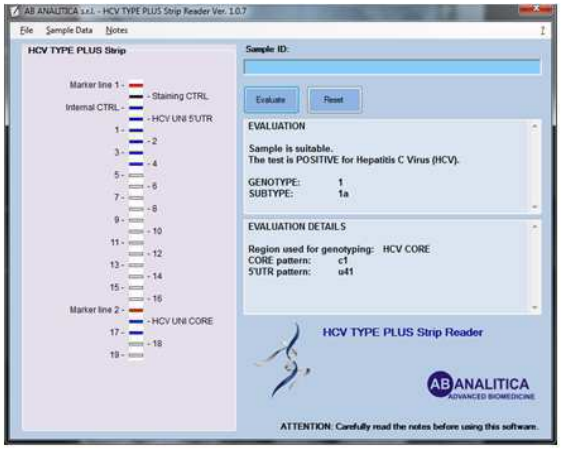


Fig. 22. Mask shown by HSCV TYPE PLUS Strip reader.

In the first window on the right, the following data are shown:

1) If the strip is OK: presence of staining control and IC-RNA signal;
2) Genotyping results;
3) Subtyping result (if appropriate).

In the second window other details of the evaluation are shown:

1) The region information used for the interpretation: 5'UTR, CORE or both;
2) The alphanumeric code corresponding to the matching pattern in the interpretation tables.

4.1.2 AUTOBLOT

The validation studies of AMPLIQUALITY HCV TYPE PLUS have been conducted with the Autoblot 3000H instrument (MedTec). The AutoBlot 3000H fully automates the Line blot assays. Following the manual addition of test samples, the AutoBlot incubates, washes, and performs subsequent reagent additions as defined by the operator during the programming phase. It permits easy setup with walk-away performance, sounding an alarm when the test is complete. The 3000H model has a heated platform, magnetic stirrer, and heated bottle plate for hybridizations and stringent washes that require heat. The unit is fully programmable from the front panel and stores up to ten protocols within the unit, allowing for full customization of blot assays for dispense, incubation,

and aspiration. The AutoBlot dispenses and aspirates up to twenty strips in 90 seconds. The experiments of this study have been conducted according to the following protocol (Table 24).

STEP	REAGENTS	INCUBATION	TIME OF INCUBATION
1) Denaturation of amplicon	Amplicon + Denaturation Solution, containing Sodium hydroxide	Room temperature	5'
2) Hybridization	Hybridization solution, containing Sodium citrate, Sodium chloride, Sodium dodecyl sulfate and preservatives	50°C Moderate shaking	1 h
3) Stringent wash	Stringent wash solution, containing Sodium citrate, Sodium chloride, Sodium dodecyl sulfate, and preservatives	50°C Moderate shaking	2'
5) Incubation with Streptavidine AP-Conjugated	Streptavidine AP-Conjugated diluted in stringent wash solution	50°C Moderate shaking	15'
6) Wash	Rinse solution, containing magnesium chloride, sodium chloride, Trizma and detergents.	Room temperature Moderate shaking	2'
7) Wash	Rinse solution, containing magnesium chloride, sodium chloride, Trizma and detergents.	Room temperature Moderate shaking	2'
8) Colorimetric reaction	NBT/BCIP solution, containing NBT, BCIP, Tris buffer and $MgSO_4$	Room temperature Moderate shaking AT DARK LIGHT	10'
9) Blocking of reaction	Blocking solution, containing Citric acid	Room temperature Moderate shaking	2'
10) Final wash	Distilled water	Room temperature Moderate shaking	2'

Table 24. Scheme of the protocol for the visualization on strip

4.1.3 GENEQUALITY X120 PATHOGEN KIT

The samples analyzed for the evaluation study of the AMPLIQUALITY HCV TYPE PLUS kit have been extracted with the GENEQUALITY X120 Pathogen kit, developed in the first part of this project. In particular, the extraction have been performed processing 400 µl of sample and eluting in 90 µl.

4.1.4 SEQUENCING NS5B REGION

The NS5b sequencing is still the gold standard method for HCV genotyping. In order to confirm the results of RLB genotyping, the NS5b sequencing have been performed. The RT-PCR was made with specific primers for NS5b region, previously reported by Sandres-Sauné et al [105]; the same enzyme mix utilized for our HCV genotyping test has been used, but with a different thermal profile, that fitted to melting temperature

of these primers. The table 25 summarizes the thermal profile used for the NS5b amplification.

Hold	Temperature	Time	Number of cycles
Reverse transcription	48°C	30 minutes	1
HotStart	95°C	10 minutes	1
Denaturation	95°C	30 seconds	40
Annealing	52°C	60 seconds	
Extension	60°C	60 seconds	
Storage	10°C	∞	1

Table 25. Thermal profile of NS5b sequencing.

The amplified products have been purified by using ExoSap-IT® (Affymetrix), according to the manufacturer's protocol. Reaction of sequencing was performed by BMR Genomics, with the BigDyes terminator cycle sequencing kit v3.1 (Applied Biosystems). The sequencing reactions were run on ABI 3730XL (Applied Biosystems) and ABI 3100 (Applied Biosystems) and were analyzed with FinchTV software v1.4 (Geospiza). The resulted sequences are then analyzed using the program BLAST (Basic Local Alignment Search Tool) available at NCBI (National Center for Biotechnology Information) to obtain the corresponding genotype.

4.1.5 ANALYSIS OF HCV RNA EXTRACTS WITH THE ARTUS HCV RG RT-PCR Kit

The clinical samples used in the evaluation study have been previously tested with the Artus HCV RG RT-PCR Kit (Qiagen) to confirm the absence or presence of HCV RNA. This analysis have also provided the viral load of HCV in the positive samples.

4.1.6 SAMPLES

The samples included in the study are synthetic samples, clinical specimens and samples derived from International EQA (External Quality Assessment).

The synthetic samples are obtained spiking the virus or the synthetic nucleic acid into the matrix, such as AcroMetrix™ EDTA Plasma Dilution Matrix (Thermo Fisher Scientific). In particular are used:

1) The 4th WHO International Standard for Hepatitis C Virus (HCV) (WHO International Laboratory for Biological Standards, UK). The standard consists of genotype 1a HCV antibody negative, HCV RNA-positive plasma, diluted in pooled

human plasma and found negative for HIV-1 RNA, HBV DNA, HAV RNA and parvovirus B19 DNA;

2) Plasmids (pls) pEX-A2 (Eurofin Genomics) containing the 5'UTR and the CORE regions of the subtypes HCV 6c, 6d, 6e, 6l and 6n and the genotype 7a.

The clinical specimens analyzed in the study are reported in table 26:

N° of samples	Matrix	HCV
264	Plasma/serum	Positive
155	Plasma/serum	Negative

Table 26. List of sample used in the evaluation study.

In order to evaluate the proficiency of our genotyping kit, also some quality panels have been used, such as the HCV Acrometrix control (Thermo Fisher Scientific)

4.1.7 INTERFERING SUBSTANCES

In order to test the possible interfering substances, we used the Acrometrix Inhibition Panel (Thermo Scientific) and in addition the hemoglobin (Table 27).

Interfering Substance	Concentration
EDTA	30 mM
Heparin	40 USP/mL
Lipid	1420 mg/dL
Bilirubin	32 mg/dL
Haemoglobin	2 g/L

Table 27. List of substances and their concentration that have been evaluated as possible interfering substances.

4.2 RESULTS

The experiments for the evaluation study of the AMPLIQUALITY HCV TYPE PLUS kit have been carried out according to the UNI EN 13612 and as defined by the CTS (Common Technical Specification) for *in vitro* diagnostic medical devices (Common Decision of the 30 November 2009). In particular, the following parameters have been investigated.

4.2.1 REPEATIBILITY AND REPRODUCIBILITY EVALUATION

The repeatability and the reproducibility of the AMPLIQUALITY HCV TYPE PLUS device have been investigated by Intra- and Inter-Assays.

The intra-assay experiment investigates the repeatability of the result using the same reagents and instruments. For this evaluation, three positive HCV RNA extracts with different viral load and below 100.000 UI/ml have been tested ten times by the same operator on the same day and using the same batch of reagents. The results are shown in table 28.

Sample	Viral Load	Expected Genotype	Valid Results
1	71377 UI/mL	HCV 2	10/10
2	55 105 UI/mL	HCV 1b	10/10
3	38697 UI/mL	HCV 1b	9/10

Table 28. Viral load of the three sample tested, their expected genotype and the obtained results.

The data of the table reveal that for 29 of 30 cases the AMPLIQUALITY HCV TYPE PLUS kit has detected the expected genotype (valid result). The only non valid result has reveal a genotype 1 without subtyping the 1b. These data indicate that the intra-assay repeatability for the kit in validation is >96%.

The inter-assay experiment verifies the reproducibility of the result among different sessions, varying factors that could contribute to the inaccuracy of the method. For this evaluation, three positive HCV RNA extracts with different viral load have been tested four times by different operators and varying the thermal cycler. The results are reported in table 29.

Sample	Viral Load	Expected Genotype	Operator	Thermal Cycler	Valid Results
1	160074 UI/mL	HCV 1b	Operator 1	ABI 2720	4/4
			Operator 2	My Cycler Biorad	4/4
2	176732 UI/mL	HCV 1b	Operator 1	ABI 2720	4/4
			Operator 2	My Cycler Biorad	3/4
3	633425 UI/mL	HCV 2	Operator 1	ABI 2720	4/4
			Operator 2	My Cycler Biorad	4/4

Table 29. The table shows the viral load of the three sample tested, their expected genotype, the operator (1-2) and the thermo cyclers used, and the obtained valid results with the kit in validation.

The data reveal that for the Operator 1 and the thermo cycler ABI 2720 (Applied Biosystem) the AMPLIQUALITY HCV TYPE PLUS kit has detected the expected genotype (valid result) in all the tests carried out, whereas for the Operator 2 and the thermo cycler My Cycler (BioRad) in 11 of 12 cases. The non valid result has reveal a genotype 1 without subtyping the 1b.

4.2.2 ANALYTICAL SPECIFICITY EVALUATION

The experiment evaluating the analytical specificity verifies the ability of the AMPLIQUALITY HCV TYPE PLUS kit to detect the target without any cross-reactivity. This cross-reactivity has been considered and analyzed *in silico* during the design and selection of primer and probes of the kit. The cross-reactivity has been verified also *in vitro*, testing samples negative for HCV RNA and positive for other pathogens (Table 30).

TYPE OF SAMPLE	VIRAL LOAD OR DNA CONCENTRATION	N° OF SAMPLES	AMPLIQUALITY HCV TYP PLUS RESULT
DNA of Epstein-Barr virus (EBV)	1.2×10^3 copies/mL	2	Negative
	1.8×10^3 copies/mL		
DNA of Herpes simplex virus 1 (HSV-1)	43×10^6 copies/mL	2	Negative
	150×10^6 copies/mL		
DNA of Herpes simplex virus 2 (HSV-2)	43×10^6 copies/mL	2	Negative
	2×10^6 copies/mL		
DNA of Cytomegalovirus (CMV)	2×10^4 copies/mL	2	Negative
	26×10^3 copies/mL		
DNA of Human herpesvirus 6B (HHV-6B)	1.5×10^5 copies/mL	2	Negative
	500 copies/mL		
DNA of Human herpesvirus 8 (HHV-8)	6.5×10^4 copies/mL	2	Negative
	2.3×10^4 copies/mL		
DNA of Varicella-zoster virus (VZV)	100 copies/mL	2	Negative
	100 copies/mL		
DNA of BK human polyomavirus	2.66×10^7 copies/mL	2	Negative
	2,500 copies/rx		
DNA of Human papilloma virus 16 (HPV-16)	Data not available	1	Negative
DNA of Human papilloma virus 18 (HPV-18)	Data not available	1	Negative
DNA of *Neisseria gonorrhoeae*	35×10^3 copies/mL	2	Negative
	4×10^3 copies/mL		
DNA of *Chlamydia trachomatis*	18×10^4 copies/mL	2	Negative
	20×10^4 copie/mL		
Human genomic DNA	54 ng/uL	1	Negative

Table 30. List of the pathogens tested for the *in vitro* cross-reactivity experiment. The viral load results obtained with the AMPLIQUALITY HCV TYPE PLUS kit are also reported.

As data show, all the samples tested with AMPLIQUALITY HCV TYPE PLUS has resulted negative, confirming the absence of cross-reactivity.

4.2.3 ANALYTICAL SENSITIVITY EVALUATION

The experiment evaluating the analytical sensitivity investigates the lower viral load of HCV detectable with 85% of confidence (detection limit). For this evaluation, serial dilutions of the WHO HCV have been prepared in human plasma to obtain these concentrations:

1) 26.000 UI/ml;
2) 13.000 UI/ml;
3) 6.500 UI/ml;
4) 3.250 UI/ml;
5) 1.625 UI/ml;
6) 812.5 UI/ml

Five aliquots for each concentration have been extracted. The obtained eluates have been collected in a tube to form a single RNA extract solution for each dilution. Then, the HCV RNA extracts have been tested in three separate PCR sessions and in six replicates per session. The results have evaluated by Probit with a confidence interval of 95% (Fig. 23).

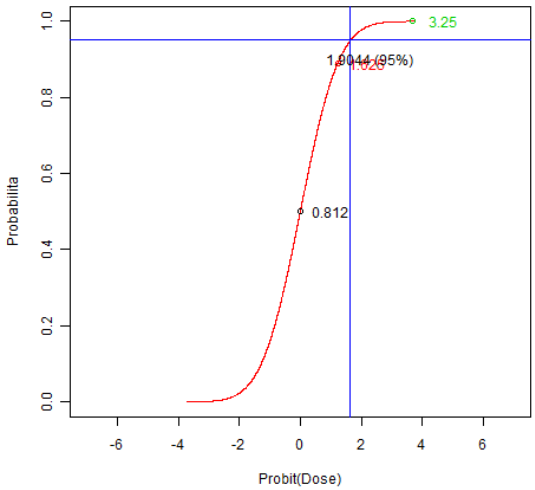


Fig. 23. Result of the probit analysis for determination of the detection limit of the kit AMPLIQUALITY HCV TYPE PLUS

The calculated detection limit of the AMPLIQAULITY HCV TYPE PLUS is 1.9044 UI/ml.

4.2.4 DIAGNOSTIC SPECIFICITY EVALUATION

The experiment for the evaluation of the diagnostic specificity of AMPLIQUALITY HCV TYPE PLUS kit investigates the ability of the kit to detect the absence of genotype in known HCV RNA negative clinical samples. For this evaluation, 155 samples have been extracted and analyzed with our genotyping kit. The results show that the presence of the control bands and the absence of the genotyping bands are confirmed in all the 155 strips tested. Therefore, the diagnostic specificity of the AMPLIQUALITY HCV TYPE PLUS device resulted 100% (Fig. 24).

$$\frac{TN}{TN + FP} \times 100 \longrightarrow \frac{155}{155 + 0} \times 100$$

Fig. 24. Formula for the determination of the diagnostic specificity. TN (True Negative): N° of samples analyzed with our genotyping kit and resulted correctly negative; FP (False Positive): N° of samples analyzed with our genotyping kit but resulted positive.

4.2.5 DIAGNOSTIC SENSITIVITY EVALUATION

The experiment for the evaluation of the diagnostic sensitivity of AMPLIQUALITY HCV TYPE PLUS kit investigates the ability of the kit to detect the expected genotype. For this study, 264 clinical samples with different viral load have been analyzed (Fig. 25).

Number of genotypes included in the study	
Genotype 1	142
Genotype 2	67
Genotype 3	24
Genotype 4	25
Genotype 5	5
Genotype 6	1
Total samples	264

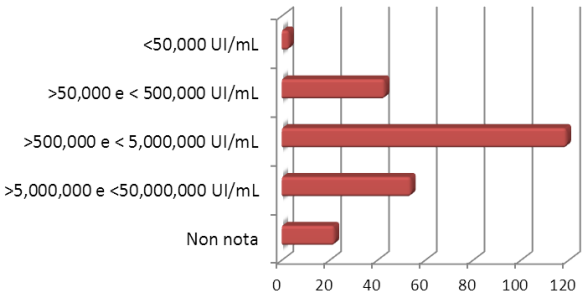


Fig. 25. List of clinical samples analyzed in the study, grouped by genotype and viral load.

The genotyping resulted are reported in table 31.

AMPLIQUALITY HCV TYPE PLUS RESULT	Number of samples
Genotype 1	5
Genotype 1, subype a	32
Genotype 1, subtype b	104
Genotypes 1 e 4	1
Genotype 2	45
Genotype 2, subtypes 2a/2c	21
Genotype 2, subtype 2b	1
Genotype 3	5
Genotype 3, subtype 3a	18
Genotype 3, subtype 3c	1
Genotype 4	5
Genotype 4, subtypes 4a/b/c/d/f	8
Genotype 4 subtype 4d	12
Genotype 5, subtype 5a	5
Genotype 6	1

Table 31. Summary of the genotyping results obtained using the kit AMPLIQUALITY HCV TYPE PLUS.

To verify the genotyping efficiency of our kit, the same extracts have been also sequenced for the NS5b region. This comparison has been possible only for 246 extracts, because for 18 extracts we obtained a failure of sequencing. The comparison results are shown in table 32.

		AMPLIQUALITY HCV TYPE PLUS RESULT										
	Genotype	1	2	3	4	5	6	1/4	1a	1b	Total	
Reference Method Result	1											Reference Method Result
	2		65								65	
	3			22							22	
	4				21						21	
	5					5					5	
	6						1				1	
	1/4							1			1	
	1a	1							31		32	
	1b	1								98	99	
	Total	2	65	22	21	5	1	1	31	98	246	
		AMPLIQUALITY HCV TYPE PLUS RESULT										

Table 32. Summary of the genotyping results obtained using the kit AMPLIQUALITY HCV TYPE PLUS and the sequencing of the NS5b (reference method).

As shown by the data, the genotypes resulted are comparable to those obtained by the sequencing. Regarding the subtyping of 1a and 1b, the AMPLIQUALITY HCV TYPE PLUS kit has detected the correct subtype in 129 cases on 131. In two cases the AMPLIQUALITY HCV TYPE PULS has detected a genotype 1 without subtyping them respectively in 1a and 1b.

In order to test also rare genotypes, synthetic plasmids for the subtypes 6c, 6d, 6e, 6l and 6n and the genotype 7a have been analyzed. They have not been extracted but added at a concentration of 10^4copies/µl to the RT-PCR reaction. The obtained strip are shown in figure 26.

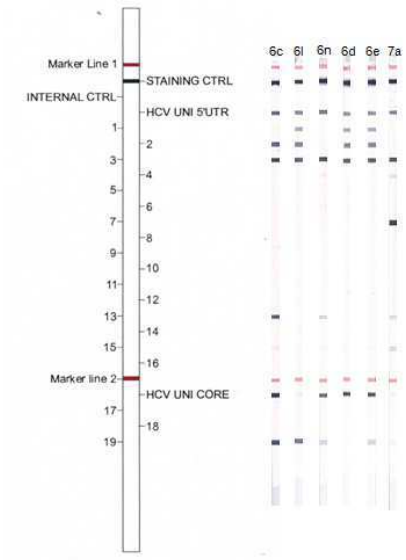


Fig 26. Genotyping results obtained using the kit AMPLIQUALITY HCV TYPE PLUS for the genotype 6 and 7.

The AMPLIQUALITY HCV TYPE PLUS is able to correctly detect the genotype 7a, and the subtypes of the 6, except for the subtype 6d that is detected as genotype 1.

4.2.6 INTERFERING SUBSTANCES EVALUATION

The experiment for the evaluation of possible interfering substances verifies the ability of the AMPLIQUALITY HCV TYPE PLUS kit to detect the correct genotype in presence of substances that may alter the result. For this evaluation, endogenous and exogenous substances have been tested (table 27).

Interfering Substance	Concentration
EDTA	30 mM
Heparin	40 USP/mL
Lipid	1420 mg/dL
Bilirubin	32 mg/dL
Haemoglobin	2 g/L

Table 27. List of substances and their concentration that have been evaluated as possible interfering substances.

For each interfering substance, two plasma samples have been prepared with a concentration of $2x10^5$ UI/ml of the HCV Acrometrix control plus the addition of the interfering substance at the final concentration shown in table 27. Two positive plasma samples without any substances have been tested as control. The prepared samples have been extracted and ten replicates of each extract have been tested for the genotype with our kit. The number of replicates have been chosen according to the Maximum Likelihood method, considering a significance level of 0,05. For the interpretation of results, a binomial distribution has been used:

-H_1: the interfering substance alters the resulted genotype;
-H_0: the interfering substance do not alter the resulted genotype.

Two or less non valid genotypes (different from the expected) have been accepted as a kit variability and in this case the H_0 was accepted. On the contrary, for more than two non valid genotypes the H_0 was rejected in favor of the H_1.

As the table 34 reports, only the heparin resulted to interfere with the assay of the kit in validation. In fact, the heparin is known to be a potent inhibitor of the PCR polymerase.

SUBSTANCE	N. OF INVALID RESULTS	STATISTICAL RESULT	INTERFERENCE
EDTA	0/20	H_0 accepted (C.I. 95%)	NO
HEPARIN	10/20	H_0 rejected (C.I. 95%)	YES
		H_1 accepted	
LIPID	0/19	H_0 accepted (C.I. 95%)	NO
BILIRUBIN	0/20	H_0 accepted (C.I. 95%)	NO
HAEMOGLOBIN	0/20	H_0 accepted (C.I. 95%)	NO

Table 34. Summary of the results of the interfering substances experiment.

5. DISCUSSION

Although the incidence of HCV infection declined in the 1990s with the introduction of improvements in infection control and safer injection practices, a large number of persons worldwide results still HCV positive. Nowadays, the research aims to formulate a pangenotypic regimen based on the new DAAs combination therapies, but nevertheless the HCV genotype information is still required to choose the best anti-viral treatment for the patient. Because of the high demand for this type of analysis and the request of standardized procedures by the specialized laboratories, we decided to develop a high-throughput system for the HCV genotyping. The goal of our project is developing a process starting from the clinical sample up to the genotype result, which includes the HCV RNA extraction, the RT PCR and the genotyping (Fig 14).

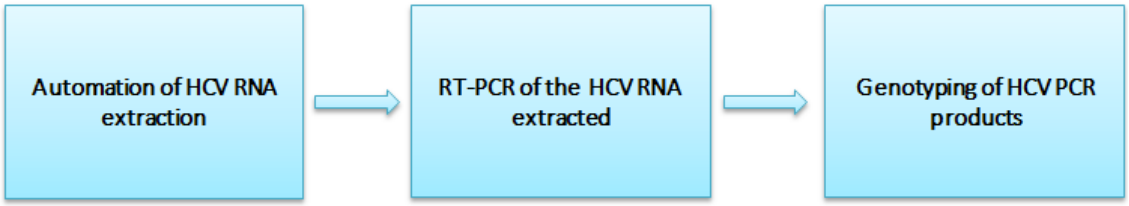


Fig. 14. Scheme of the modular workcells for the genotyping analysis of HCV.

In the first part of my PhD project, I dealt with the development of a kit for the extraction of pathogen nucleic acids. To make the process as possible high-throughput, we decided to design this kit for its use with the platform GENEQUALITY X120 (AB ABANLITICA). The choice of the extraction reagents and the automated format has been a crucial point for the prototype development. At the end we obtained a prototype of GENEQUALITY X120 Pathogen kit. This kit consists in two sealed cartridges and five tubes that include reagents ready to use for the automated extraction of pathogen DNA and RNA, based on the magnetic beads technology. To make reliable the extraction process, we provided the kit with two types of Internal Control (IC), which assure the monitoring of the DNA and RNA extractions (IC DNA and IC RNA respectively), indicating the potential presence of inhibitors or other factors that may cause false-negative results. Moreover, a carrier has been added to favor the pathogen nucleic acids purification.

The prototype showed good performances, confirmed by the quality of the DNA and RNA extracts verified by real time PCR. In addition, it revealed to be versatile, displaying extraction efficiency with different combination of sample and elution volumes. In

particular, in these work to be competitive and to satisfy the customers' requests, we have tested 200µl and 400µl as sample volumes to process and 90µl, 120µl, and 180µl as elution volumes.
Another critical point in the development of the automated extraction process has concerned the evaluation of cross-contaminations. The first set protocol revealed presence of cross-contamination among the wells of the work plate because of a too high shaking speed during the binding step. Decreasing the shaking speed we have solved the problem, preserving however the extraction efficiency.
The GENEQUALITY X120 pathogen kit validation study showed repeatability and reproducibility of the automated extraction process and moreover a high sensitivity of pathogen nucleic acids purification. In addition, the extraction efficiency have been evaluated analyzing more than 400 samples, regarding different matrices and DNA or RNA pathogen targets. The results were comparable with the reference methods.
In addition, the GENEQUALITY X120 platform has been supplied with barcode readers in order to guarantee the traceability of the entire process, from the sample to the extract.
Finally, since the requirements for the *in vitro* diagnostic medical device have been complied, the GENEQUALITY X120 Pathogen kit is currently CE IVD mark and commercialized.

In the second part of my PhD work, I focused on the performance evaluation of the HCV genotyping assay. The HCV genotype is one of the factor influencing the disease progression and still the major predictor of the SVRs in the DAAs therapies. Thus, the HCV genotyping is essential to apply the most appropriate therapeutic regimen. Since the HCV genotypes differ at 31-34% of nucleotide positions of complete genomic sequencing and the subtypes about 20%, the detection of the correct HCV genotype/subtype requests high specific assays. The gold standard for HCV genotyping is the NS5B sequencing and subsequent phylogenetic analysis, but generally this type of assay is used by reference centers, which design homemade protocols, based on their specific required. Therefore, other types of assays, based on Real-Time PCR or RLB technologies, are commercially available. The major part of them target the viral 5'UTR, because it is the most conserved one. This region is able to well differentiate the virus at the genotype level, except for genotype 1 and 6 (subtypes c to l). Moreover, it cannot lead to the subtyping genotype 1, because the HCV subtype 1a segregates in two distinct clades, which are termed 1a clade I and 1a clade II. The 5'UTR sequence of 1a clade I is identical to that of subtype 1b. Recent study shows that using the sole 5'UTR as target for genotyping, there is a mistyping approximately of 25% and 10% of HCV subtype 1a and 1b strains, respectively [104]. Therefore, it is evident that the analysis of the sole 5'UTR is not sufficient to allow a good determination of HCV genotype/subtype and that evaluation of another region is necessary.

In this study, the AMPLIQUALITY HCV TYPE PLUS kit performances have been evaluated, according to the to the UNI EN 13612 and as defined by the CTS (Common Technical Specification) for in vitro diagnostic medical devices (Common Decision of the 30 November 2009). This kit has previously designed in the company in order to well differentiate genotype 1 to 6 and subtypes 1a and 1b thanks to the presence of the CORE region in addition to the 5'UTR. Our assay showed a diagnostic specificity of 100% and a diagnostic sensitivity of 100% at genotype level and >98% at subtype level.
A crucial point has been the recovery of samples positive for the rare genotypes, such as 6 and 7. To solve the problem, we decided to use synthetic constructs containing the 5'UTR and the CORE regions of the genotype 7a and of some subtypes of the 6.
The good subtyping results of our test are important to avoid the genotype/subtype misdiagnosis related to the analysis of the sole 5'UTR. In fact, this kind of mistyping could affect the management of the therapeutic regimen, in term of both DAAs combination to administered or duration of therapies, as shown by the EASL treatment recommendations [93].
In addition, the AMPLIQUALITY HCV TYPE PLUS kit is based on a RLB technique, which revealed to be a simple and fast method and exhibits a high sensitivity and specificity. The same detailed genotyping information could be achieved with Real-Time technologies, but is necessary performing multiple amplification reactions for each sample. Instead, RLB allows the acquisition of a lot of sequence information in a single run. RLB is also the ideal technology for identification of co-infections by different HCV strains. NS5b sequencing can give a definite result for genotype but evaluation of more HCV isolates presence in the same sample is very difficult.
Finally, since the requirements of the CTS have been complied, the AMPLIQUALITY HCV TYPE PLUS is currently CE IVD mark and commercialized.
Moreover, the use of AMPLIQUALITY HCV TYPE PLUS kit showed good performances starting from HCV RNA extracted with the GENEQUALITY X120 Pathogen kit. An important parameter which have assured the continuity of the process is the use of the same IC RNA by both the developed kit.

In conclusion, the combination of the GENEQUALITY X120 Pathogen kit and the AMPLIQUALITY HCV TYPE PLUS guarantees a standardized and performing process for the HCV genotyping (Fig. 27). The high-throughput of this system is guarantee by the use of automated platform for both the extraction and the genotyping procedures (respectively GENEQUALITY X120 and AUTOBLOT). Moreover, the following experiments will aim to the use of the platform GENEQUALITY X120 for the PCR setup of the HCV RNA extracted, in order to avoid the introduction of possible errors done by the operator and to increase the high-throughput performance of the process.

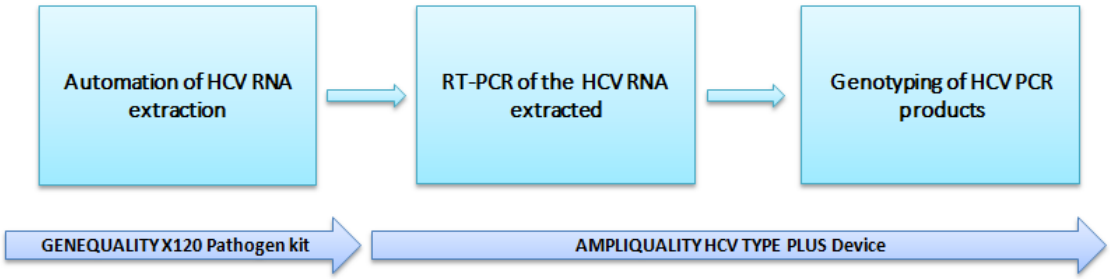


Fig. 27. Scheme of the modular workcells for the genotyping analysis of HCV and the developed kits used for this analysis.

REFERENCES

[1] Abrahams E. et al.
The History of Personalized Medicine. In E. Gordon and S. Koslow (Eds).
Integrative Neuroscience and Personalized Medicine. (3-16). New York: OxfordUniversity Press. 2010

[2] Ginsburg GS. et al.
Genomic and personalized medicine: foundations and applications.
Translational Research. 2009; 154(6):277-287

[3] Kannel W. et sl.
Factors of risk in the development of coronary heart disease-six year follow-up experience. The Framingham Study.
Ann Intern Med. 1961; 55:33-50

[4] Lander ES., Linton LM., Birren B. et al.
International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome.
Nature. 2001; 409:860-921

[5] Venter JC, Adams MD, Myers EW et al.
The sequence of the human genome.
Science. 2001; 291:1304-1351

[6] The International HapMap Consortium.
A haplotype map of the human genome.
Nature. 2005; 437:1299-1320

[7] The International HapMap Consortium.
A second generation human haplotype map over 3.1 million SNPs.
Nature. 2007; 449:851-862

[8] Notice n° 739 from 'Pillole dal Mondo'
Section of AIFA (Agenzia Italiana del Farmaco)
www.agenziafarmaco.gov.it

[9] Alter HJ, Purcell RH, et al.
Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis.
N Engl J Med. 1989 Nov 30; 321(22):1494-500.

[10] Choo Q.L., Weiner A.J.et al.
Hepatitis C virus: the major causative agent of viral non-A, non-B hepatitis
Med Bull. 1990; 46(2):423-41

[11] Choo Q.L., Kuo G. et al.
Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science. 1989; 244(4902):359-62*

[12] Messina J.P., Humprhreys I. et al.
Global Distribution and Prevalence of Hepatitis C Virus Genotypes.
Hepatol. 2015; 61(1):77-87

[13] Simmonds P.
Genetic diversity and evolution of hepatitis C virus--15 years on.
J Gen Virol. 2004; 85(11):3173-88

[14] Smith D.B., Bukh J. et al.
Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource.
Hepatol. 2014; 59(1):318-27

[15] Murphy D.G., Willems B. et al.
Use of sequence analysis of the NS5B region for routine genotyping of hepatitis C virus with reference to C/E1 and 5' untranslated region sequences.
J Clin Microbiol. 2007; 45(4):1102-12

[16] Hajarizadeh B., Grebely J. et al.
Epidemiology and natural history of HCV infection.
Nat Rev Gastroenterol Hepatol. 2013; 10(9):553-62

[17]Heller T., Saito S. et al.
An in vitro model of hepatitis C virion production.
Proc Nat Acad Sci U S A. 2005; 102(7):2579-83

[18]Branch A.D., Stump D.D. et al.
The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others.
Semin Liver Dis. 2005; 25(1):105-17

[19]Wang C., Sarnow P. et al.
Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism.
J Virol. 1993; 67(6):3338-44

[20] Moradpour D., Penin F. et al.
Replication of hepatitis C virus.
Nat Rev Microbiol. 2007;5(6):453-63

[21] You S., Stump D.D. et al.
A cis-acting replication element in the sequence encoding the NS5B RNA-dependent RNA polymerase is required for hepatitis C virus RNA replication.
J Virol. 2004; 78(3):1352-66

[22] Santolini E., Migliaccio G. et al.
Biosynthesis and biochemical properties of the hepatitis C virus core protein.
J Virol. 1994; 68(6):3631-41.

[23] Tellinghuisen T.L., Rice C.M. et al.
Interaction between hepatitis C virus proteins and host cell factors.
CurrOpinMicrobiol. 2002; 5(4):419-27

[24] Boulant S., Montserret R. et al.
Structural determinants that target the hepatitis C virus core protein to lipid droplets.
J Biol Chem. 2006;281(31):22236-47

[25] Dubuisson J., Penin F. et al.
Interaction of hepatitis C virus proteins with host cell membranes and lipids.
Trends Cell Biol. 2002;12(11):517-23

[26] Sakai A., Claire M.S. et al.
The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences.
ProcNatlAcadSci U S A. 2003; 100(20):11646-51

[27] Lindenbach B.D., Rice C.M. et al.
Unravelling hepatitis C virus replication from genome to function.
Nature. 2005;436(7053):933-8

[28] Wölk B., Sansonno D. et al.
Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines.
J Virol. 2000;74(5):2293-304

[29] Egger D., Wölk B. et al.
Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex.*J Virol. 2002; 76(12):5974-84*

[30] Appel N., Pietschmann T. et al.
Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. *J Virol. 2005; 79(5):3187-94*

[31] Moradpour D., Brass V. et al.
Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication.
J Virol. 2004; 78(23):13278-84

[32] Agnello V., Abel G. et al.
Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor.
ProcNatlAcadSci U S A. 1999; 96(22):12766-71

[33] Scarselli E., Ansuini H. et al.
The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus.
EMBO J. 2002; 21(19):5017-25

[34] Evans M.J., von Hahn T. et al.
Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry.
Nature. 2007; 446(7137):801-5

[35] Lozano R., Naghavi M. et al.
Global and regional mortality from 235 causes of death for 20 age groups in 1990 and

2010: a systematic analysis for the Global Burden of Disease Study 2010.
Lancet. 2012; 380(9859):2095–128

[36] GBD Mortality and Causes of Death Collaborators.
Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the GlobalBurden of Disease Study 2013.
Lancet. 2015; 385(9963):117–71

[37] Magiorkinis G., Magiorkinis E. et al.The global spread of hepatitis C virus 1a and 1b: a phylodynamic and phylogeographic analysis.
PLoS Med. 2009; 6(12):e1000198

[38] MohdHanafiah K., Groeger J. et al.Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence.
Hepatology. 2013; 57(4):1333–42

[39] Gower E., Estes C. et al.
Global epidemiology and genotype distribution of the hepatitis C virus infection.
J Hepatol. 2014; 61(1 Suppl):S45–57

[40] Rao V.B., Johari N. et al.
Hepatitis C seroprevalence and HIV co-infection in sub-Saharan Africa: a systematic review and meta-analysis.
Lancet Infect Dis. 2015; 15(7):819–24

[41] Platt L., Easterbrook P. et al.
Prevalence and burden of HCV co-infection in people living with HIV: a global systematic review and meta-analysis.
Lancet Infect Dis. published online 24 February 2016; DOI: http://dx.doi.org/10.1016/S1473-3099(15)00485-5 (http://www.thelancet.com/pdfs/journals/laninf/PIIS1473-3099%2815%2900485-5.pdf, accessed 21 March 2016)

[42] Lin S.Y., Chen T.C. et al.
Incidence rates of tuberculosis in chronic hepatitis C infected patients with or without interferon based therapy: a population-based cohort study in Taiwan.
BMC Infect Dis. 2014; 14:705

[43] World Health Organization (WHO).
WHO guidelines on the use of safety-engineered syringes for intramuscular, intradermal and subcutaneous injections in health-care settings.
2015 (http://www.who.int/injection_safety/global-campaign/injection-safety_guidline.pd

[44] Nelson P.K., Mathers B.M. et al.
Global epidemiology of hepatitis B and hepatitis C in people who inject drugs: results of systematic review.
Lancet. 2011; 378(9791):571–83

[45] Danta M., Brown D. et al.
Recent epidemic of acute hepatitis C virus in HIV-positive men who have sex with

men linked to high-risk sexual behaviours.
AIDS. 2007; 21(8):983–91

[46] Thomas D.L., Villano S.A. et al.
Perinatal transmission of hepatitis C virus from human immunodeficiency virus type 1-infectedmothers.
Transmission Study. J Infect Dis. 1998; 177(6):1480–8

[47] Lo Re III V., Kostman J.R.
Management of chronic hepatitis C.
Postgrad Med J. 2005; 81:376–382

[48] Freeman A.J., Law M.G. et al.
Predicting progression to cirrhosis in chronic hepatitis C virus infection.
J Viral Hepat. 2003;10(4): 285–93

[49] Bedossa P., Poynard T.An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group.
Hepatol. 1996; 24(2):289–93

[50] Durand F., Valla D.Assessment of prognosis of cirrhosis.
Semin Liver Dis. 2008; 28(1):110–22

[51] World Health Organization (WHO).Guidelines for the screening, care and treatment of persons with chronic hepatitis C infection.
2016 (http://www.who.int/hepatitis/publications/hepatitis-c-guidelines-2016/en/)

[52] Alberti A., Chemello L. et al.
Natural history of hepatitis C.
J Hepatology 1999;31 suppl 1: 17-24

[53] Fletcher N.F., McKeating J.A.
Hepatitis C virus and the brain.
J Viral Hepat. 2012;19(5):301–6.

[54] Forton D.M., Karayiannis P. et al.Identification of unique hepatitis C virus quasispecies in the central nervous system and comparative analysis of internal translational efficiency of brain, liver, and serum variants.
J Virol. 2004;78(10):5170–83

[55] Crespo J,. Lozano J.L,. et al.
Prevalence and significance of hepatitis-C viremia in chronic active hepatitis-B.
Am J Gastroenterol. 1994; 89(8):1147–51

[56] Parekh P.J., Shiffman M.L. et al.
The role of interferon in the new era of hepatitis C treatments.
Expert Rev Gastroenterol Hepatol. 2014; 8(6):649–56

[57] Hayashi N., Takehara T.
Antiviral therapy for chronic hepatitis C: past, present, and future.
J Gastroenterol. 2006; 41(1):17-2

[58] Pockros PJ.
New direct-acting antivirals in the development for hepatitis C virus infection.
Therap Adv Gastroenterol. 2010; 3(3):191-202

[59] Schinazi R., Halfon P. et al.
HCV direct-acting antiviral agents: the best interferon-free combinations.
Liver Int. 2014; 34 (Suppl 1):69–78

[60] Yee S.H., Chang F.M. et al.
Update on the Management and Treatment of Hepatitis C Virus Infection: Recommendations from the Department of Veterans Affairs Hepatitis C Resource Center Program and the National Hepatitis C Program Office.
Am J Gastroenterol. 2012; 101:2360-78

[61] Pearlman B.R., Traub N.
Sustained Virologic Response to Antiviral Therapy for Chronic Hepatitis C Virus Infection: A Cure and So Much More.
CID; 2011; 52:889-900

[62] Davis G.L., Wong J.B. et al.
Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C.
Hepatology. 2003 Sep;38(3):645-52

[63] Bartenschlager R., Lohmann V. et al.
The molecular and structural basis of advanced antiviral therapy for hepatitis C virus infection. *Nat. Rev. Microbiol. 2013; 11:482–96*

[64] Scheel T. K., Rice C. M.
Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. *Nat. Med. 2013; 19:837–49*

[65] Lamarre D. et al.
An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature. 2003; 426:186–9*

[66] Schiering, N. et al.
A macrocyclic HCV NS3/4A protease inhibitor interacts with protease and helicase residues in the complex with its full-length target.
Proc. Natl Acad. Sci. 2011; 108:21052–56

[67] Vermehren J. Sarrazin C.
The role of resistance in HCV treatment.
Clin Gastroenterol. 2012; 26, no. 4, pp. 487–503, 2012

[68] Liang Y. et al.
Antiviral suppression versus restoration of RIG-I signaling by hepatitis C protease and polymerase inhibitors
Gastroenterol. 2008; 135:1710–8

[69] Sofia M.J., Chang, W. et al.
Nucleoside, nucleotide, and non-nucleoside inhibitors of hepatitis C virus NS5B

RNA‐dependent RNA-polymerase.
J. Med. Chem. 2012; 55:2481–531

[70] Götte M., Feld J.J.
Direct-acting antiviral agents for hepatitis C: structural and mechanistic insights.
NATURE REVIEWS|GASTROENTEROLOGY & HEPATOLOGY
doi:10.1038/nrgastro.2016.60 Published online 5 May 2016

[71] Ascher D.B. et al.
Potent hepatitis inhibitors bind directly to NS5A and reduce its affinity for RNA.
Sci Rep. 2014; 4:4765

[72] Feld J.J.
Interferon-free strategies with a nucleoside/nucleotide analogue.
Semin Liver Dis. 2014; 34:37-46

[73] Sarrazin C. et al.
Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir.
Gastroenterol. 2007; 132:1767–77

[74] Jacobson I.M., Dore G.D. et al.
Simeprevir with pegylated interferon alfa 2a plus ribavirin in treatment-naïve patients with chronic hepatitis C virus genotype 1 infection (QUEST-1): a phase 3, randomised, double-blind, placebo controlled trial.
The Lancet. 2014; 384 (9941):403–13

[75] Manns M., Marcellin P. et al.
Simeprevir with pegylated interferon alfa 2a or 2b plus ribavirin in treatment naive patients with chronic hepatitis C virus genotype 1 infection (QUEST-2): a randomised, double-blind, placebo controlled phase 3 trial
The Lancet. 2014; 384(9941):414–26

[76] Nguyen L.T. et al.
Baseline prevalence and emergence of protease inhibitor resistance mutations following treatment in chronic HCV genotype‐1‐infected individuals.
Antivir. Ther. 2014: 20;865–9

[77] Moreno C.,Hezode C. et al.
Efficacy and safety of simeprevir with PegIFN/ribavirin in na¨ıve or experienced patients infected with chronic HCV genotype 4.
J Hepatol. 2015; 62(5):1047–55

[78] Lawitz E., Mangia A. et al.
Sofosbuvir for previously untreated chronic hepatitis C infection.
N Engl J Med. 2013; 368(20):1878-87

[79] Foster G.R., Pianko S. et al.
Efficacy of sofosbuvir plus ribavirin with or without peginterferon-alfa in patients with hepatitis C virus genotype 3infection and treatment-experienced patients with cirrhosis and hepatitis C virus 2 infection: a randomized study.
Gut. 2015; 64:948-56

[80] H´ezode C., Hirschfield G.M. et al.
Daclatasvir plus peginterferon alfa and ribavirin for treatment-naïve chronic hepatitis C genotype 1 or 4 infection: a randomized study.
Gut. 2015; 64:948–56

[81] Jensen D., Sherman K. et al.
Daclatasvir and asunaprevir plus peginterferon alfa-2a and ribavirin in patients with HCV genotype 1 or 4 infection: Phase 3 HALLMARKQUAD results.
Open Forum Infect Dis. 2014; 1(1): S234

[82] Welzel T.M., Dultz G. et al.
Interferon-free antiviral combination therapies without nucleosidic polymerase inhibitors.
J Hepatol. 2014; 61(1S): S98–107

[83] Lok A.S. et al.
Preliminary study of two antiviral agents for hepatitis C genotype 1.
N Engl J Med. 2012; 366:216–224

[84] Majumdar A.,Kitson M.T. et al.
Systematic review: current concepts and challenges for the direct-acting antiviral era in hepatitis C cirrhosis.
Aliment Pharmacol Ther. 2016; 43:1276–92

[85] Afdhal N., Zeuzem S. et al.
Ledipasvir and sofosbuvir for untreated HCV genotype 1 infection.
N Engl J Med 2014; 370: 1889–98

[86] Feld J.J.
Is there a role for ribavirin in the era of hepatitis C virus direct-acting antivirals?
Gastroenterol. 2012; 142:1356-59

[87] Afdhal N., Reddy K.R. et al.
Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection.
N Engl J Med 2014; 370:1483–93

[88] Lawitz E., Sulkowski M.S. et al.
Simeprevir plus sofosbuvir, withmor without ribavirin, to treat chronic infection with hepatitis C virus genotype 1 in non-responders to pegylated interferon and ribavirin and treatment-naive patients: the COSMOS randomised study.
Lancet 2014; 384:1756–65

[89] Gambato M., Lens S. et al.
Treatment options in patients with decompensated cirrhosis, pre- and post-transplantation.
J Hepatol 2014; 61(1S): S120–31

[90] McKenzie R. et al.
Hepatic failure and lactic acidosis due to fialuridine (FIAU), an investigational nucleoside analogue for chronic hepatitis B.
N Engl J Med. 1995; 333:1099–105

[91] Welker M.W. et al.
Lactic acidosis in patients with hepatitis C virus cirrhosis and combined ribavirin/ sofosbuvir treatment.
J Hepatol. 2016; 64:790–799

[92] Charlton M. et al.
Ledipasvir and sofosbuvir plus ribavirin for treatment of HCV infection in patients with advanced liver disease.
Gastroenterol. 2015; 149:649–659

[93] EASL (European Association for Study of Liver).
EASL recommendations on treatment of hepatitis C 2015.
J Hepatol 2015; 63:199–236.

[94] FDA (Food and Drug Administration).
FDA hepatitis update—important safety information: Harvoni and Sovaldi, 2015.
Available at: http://content.govdelivery.com/accounts/USFDA/bulletins/f97c71 (accessed 2 July 2015)

[95] AISF (Associazione Italiana per lo Studio del Fegato).
Documento di indirizzo dell'Associazione Italiana per lo Studio del Fegato per l'uso razionale di antivirali diretti di seconda generazione nelle categorie di pazienti affetti da epatite C cronica ammesse alla rimborsabilità in Italia.
Available at: http://www.webaisf.org/pubblicazioni/documento-aisf-hcv-2016.aspx (accessed May 2016)

[96] AASLD (American Association for the Study of Liver Desase) – IDSA (Infection Desease Society of America).
Recommendations for Testing, Managing, and Treating Hepatitis C.
Available at: http://www.hcvguidelines.org *(accessed April 2016)*

[97] VA (U.S Department of Veterans Affair).
Chronic Hepatitis C Virus (HCV) Infection: Treatment Considerations from the Department of Veterans Affairs National Hepatitis C Resource Center Program and the National Viral Hepatitis Program in the Office of Patient Care Services.
Available at: http://www.hepatitis.va.gov/provider/guidelines/hcv-treatment-considerations.asp (accessed March 2016)

[98] World Health Organization (WHO).
PICO 6: Treatment - Pegylated interferon plus ribavirin versus standard interferon plus ribavirin for chronic hepatitis C infection: a meta-analytical systematic review
Appendix 3: Evidence summaries. Available at:
http://www.who.int/hepatitis/publications/hepatitis-c-guidelines/en/ (accessed April 2014)

[99] Documenti di revisione della letteratura preparati dagli esperti.
Conferenza di Consenso "Lo screening per infezione da HCV negli adulti in Italia"
Available at: www.snlg-iss.it/cms/files/revisione_cc_epatite_c.doc

[100] **European Association for the Study of the Liver.**
EASL Clinical Practice Guidelines: management of hepatitis C virus infection.
J Hepatol. 2011; 55(2):245-64

[101] Descamps V., Op de Beeck A. et al.
Strong correlation between liver and serum levels of hepatitis C virus core antigen and RNA in chronically infected patients.
J Clin Microbiol. 2012; 50(2):465-80

[102] Sarasin-Filipowicz M., Oakeley E.J. et al.
Interferon signaling and treatment outcome in chronic hepatitis C.
Proc Natl Acad Sci U S A. 2008; 105(19):7034-9

[103] Feld J.J., Hoofnagle J.H.
Mechanism of action of interferon and ribavirin in treatment of hepatitis C.
Nature. 2005; 436(7053):967-72

[104] Chevaliez S., Bouvier-Alias M. et al.
Hepatitis C virus (HCV) genotype 1 subtype identification in new HCV drug development and future clinical practice.
PLoS One. 2009; 4(12):e8209

[105] Nizar N Zein.
Clinical Significance of Hepatitis C Virus Genotypes.
Clinical Microbiology Reviews. 2000; 13(2):223-35

[106] Nousbaum J.B., Pol S. et al.
Hepatitis C virus type 1b (II) infection in France and Italy.
Ann. Intern. Med. 1995; 122:161-8

[107] Silini E., Bono F. et al.
Differential distribution of hepatitis C virus genotypes in patients with and without liver function abnormalities.
Hepatology. 1995; 21:285-90

[108] Campos-Varela I., Lai J.C. et al.
Hepatitis C genotype influences post-liver transplant outcomes.
Transplatation. 2015; 99(4):83-40

APPENDICES

Appendix 1

Regions	Anti-HCV prevalence (CI)[a]	Viraemic HCV prevalence (CI)[a]	Viraemic rate	2013 population (millions)	Anti-HCV infected (millions)	Viraemic HCV infected (millions)
Asia Pacific, high income	1.1% (0.5–1.7%)	0.8% (0.4–1.2%)	74%	182	2.0 (0.9–3.0)	1.5 (0.6–2.2)
Asia, Central	5.4% (3.5–6.8%)	2.3% (1.5–3.0%)	43%	84	4.5 (2.9–5.7)	1.9 (1.3–2.5)
Asia, East	1.2% (0.4–1.8%)	0.7% (0.3–1.1%)	60%	1434	16.6 (6.3–25.3)	10.0 (3.9–15.1)
Asia, South	1.1% (0.7–1.5%)	0.9% (0.5–1.2%)	81%	1650	18.8 (11.3–24.5)	15.2 (8.9–19.8)
Asia, Southeast	1.0% (0.8–1.8%)	0.7% (0.5–1.1%)	63%	635	6.6 (5.3–11.3)	4.2 (3.4–7.2)
Australasia	1.4% (1.0–1.5%)	1.0% (0.8–1.1%)	75%	28	0.4 (0.3–0.4)	0.3 (0.2–0.3)
Caribbean	0.8% (0.2–1.3%)	0.6% (0.1–0.9%)	70%	39	0.3 (0.1–0.5)	0.2 (0.0–0.4)
Europe, Central	1.3% (1.1–1.6%)	1.0% (0.9–1.2%)	80%	119	1.5 (1.3–1.9)	1.2 (1.1–1.5)
Europe, Eastern	3.3% (1.6–4.5%)	2.3% (1.1–3.0%)	69%	207	6.8 (3.4–9.3)	4.7 (2.4–6.3)
Europe, Western	0.9% (0.7–1.5%)	0.6% (0.5–1.0%)	70%	425	3.7 (3.0–6.3)	2.6 (2.1–4.4)
Latin America, Andean	0.9% (0.4–1.3%)	0.6% (0.3–0.9%)	70%	57	0.5 (0.2–0.7)	0.4 (0.2–0.5)
Latin America, Central	1.0% (0.8–1.4%)	0.8% (0.6–1.1%)	75%	246	2.6 (1.9–3.5)	1.9 (1.4–2.6)
Latin America, Southern	1.2% (0.5%–2.1%)	0.9% (0.4%–1.6%)	79%	62	0.8 (0.3–1.3)	0.6 (0.2–1.0)
Latin America, Tropical	1.2% (0.9–1.2%)	1.0% (0.7–1.0%)	80%	207	2.5 (1.9–2.6)	2.0 (1.5–2.1)
North Africa/ Middle East	3.1% (2.5–3.9%)	2.1% (1.7–2.6%)	66%	469	14.6 (11.9–18.2)	9.7 (7.8–12.1)
North America, high income	1.0% (1.0–1.9%)	0.8% (0.7–1.4%)	76%	355	3.7 (3.4–6.7)	2.8 (2.6–5.0)
Oceania	0.1% (0.1–0.6%)	0.1% (0.1–0.4%)	69%	10	0.0 (0.0–0.1)	0.0 (0.0–0.0)
Sub-Saharan Africa, Central	4.2% (2.4–9.2%)	2.6% (1.5–5.5%)	61%	100	4.3 (2.4–9.2)	2.6 (1.5–5.5)
Sub-Saharan Africa, East	1.0% (0.6–3.1%)	0.6% (0.4–2.0%)	62%	385	3.9 (2.4–12.1)	2.4 (1.6–7.9)
Sub-Saharan Africa, Southern	1.3% (0.8–2.5%)	0.9% (0.6–1.7%)	69%	75	1.0 (0.6–1.9)	0.7 (0.4–1.3)
Sub-Saharan Africa, West	5.3% (2.9–9.1%)	4.1% (2.3–6.7%)	77%	367	19.3 (10.5–33.3)	14.9 (8.5–24.6)
Other	1.9% (1.0–3.4%)	1.3% (0.7–2.4%)	69%	27	0.5 (0.3–0.9)	0.4 (0.2–0.7)
Total	**1.6% (1.3–2.1%)**	**1.1% (0.9–1.4%)**	**70%**	**7162**	**114.9 (91.9–148.7)**	**80.2 (64.4–102.9)**

[a] Presence of antibody indicating exposure to HCV
[b] Presence of RNA indicating chronic HCV infection

Estimated prevalence of HCV infection by Global Burden of Disease regions [39].

Appendix 2

Table 1 | Key interferon-free trials for patients with HCV genotype 1 or 4 cirrhosis

Study	Trial phase	Cirrhotic patients enrolled *n* (%)	Genotype	Prior treatment	Drug regimen	Duration (weeks)	SVR12
ION-1[73]	3	136 (16)	1	Naïve	SOF/LDV	12	94% (32/34)
					SOF/LDV	24	94% (31/33)
					SOF/LDV+RBV	12	100% (33/33)
					SOF/LDV+RBV	24	100% (36/36)
ION-2[74]	3	88 (20)	1	Treatment experienced	SOF/LDV	12	86% (19/22)
					SOF/LDV	24	100% (22/22)
					SOF/LDV+RBV	12	82% (18/22)
					SOF/LDV+RBV	24	100% (22/22)
SIRIUS[76]	2	155 (100)	1	PI failure	SOF/LDV+RBV	12	96% (74/77)
					SOF/LDV	24	97% (75/77)
Lawitz *et al.*[79]	2, 3 pooled data	19 (46)	1a	SOF/LDV failure	SOF/LDV	24	74% (14/19)
Pianko *et al.*[98]	2	17 (30)	1, (3)*	PI failure	SOF/VPV†	12	100% (7/7)
					SOF/VPV+RBV†	12	90% (9/10)
Gane *et al.*[100]	2	20 (27)	1	Naïve	SOF/VPV+GS-9857	6	87% (13/15)
				Experienced	SOF/VPV+GS-9857	6	60% (3/5)
COSMOS[64]	2b	41 (25)	1	Naïve or null responders	SIM+SOF	12	86% (6/7)
					SIM+SOF	24	100% (10/10)
					SIM+SOF+RBV	12	91% (10/11)
					SIM+SOF+RBV	24	100% (13/13)
OPTIMIST-2[65]	3	103 (100)	1	Naïve	SIM+SOF	12	88% (44/50)
				Treatment experienced	SIM+SOF	12	79% (42/53)
Pearlman *et al.*[86]	3	82 (100)	1a	Naïve or null responders	SIM+SOF	12	93% (54/58)
					IFN+RBV+SOF	12	75% (18/24)
TURQUOISE-II[80]	3	380 (100)	1	Naïve	PTV/R/OMV+ DSV+RBV	12	92% (191/208)
					PTV/R/OMV+ DSV+RBV	24	96% (165/172)
TURQUOISE-III[81]	3b	60 (100)	1b	Naïve	PTV/R/OMV+DSV	12	100% (27/27)
				IFN experienced	PTV/R/OMV+DSV	12	100% (33/33)
GIFT-1[83]		42 (12)	1b	Naïve	PTV/R/OMV	12	91% (38/42)
HALLMARK-DUAL[87]	3	223 (30)	1b	Naïve	DCV+ASV	24	91% (29/32)
				IFN nonresponder	DCV+ASV	24	87% (55/63)
				IFN ineligible/ intolerant	DCV+ASV	24	79% (88/111)
HALLMARK-NIPPON[89]	3	22 (10)	1b	IFN nonresponder	DCV+ASV	24	91% (10/11)
				IFN ineligible/ intolerant	DCV+ASV	24	91% (10/11)
C-WORTHY[90]	2	123 (100)	1	Naïve	GZR/EBR	12	97% (28/29)
					GZR/EBR	18	94% (29/31)
					GZR/EBR+RBV	12	90% (28/31)
					GZR/EBR+RBV	18	97% (31/32)
C-EDGE TN[91]	3	70 (22)	1‡, 4, 6	Naïve	GZR/EBR	12	97% (68/70)
C-EDGE TE [92]	3	120 (33)	1‡, 4, 6	IFN experienced	GZR/EBR	12	89% (33/37)
					GZR/EBR	16	92% (35/38)
					GZR/EBR+RBV	12	89% (31/35)
					GZR/EBR+RBV	16	100% (37/37)
C-SALVAGE[93, 94]	2	34 (43)	1	PI failures	GZR/EBR+RBV	12	94% (32/34)§
C-SWIFT [97]	3	53 (37)	1,(3)*	Naïve	GZR/EBR+SOF	6	80% (16/20)
					GZR/EBR+SOF	8	94% (17/18)

Table 1 | (Continued)

Study	Trial phase	Cirrhotic patients enrolled *n* (%)	Genotype	Prior treatment	Drug regimen	Duration (weeks)	SVR12
ASTRAL-1 [99]	3	121 (19)	1, (2, 4, 5, 6)	Naïve and treatment experienced	SOF/VPV	12	99% (72/73)
Ruane *et al.*[103]	2	14 (23)	4	Naïve	SOF+RBV	12	33% (1/3)
					SOF+RBV	24	100% (3/3)
				Treatment experienced	SOF+RBV	12	50% (2/4)
					SOF+RBV	24	100% (4/4)
Doss *et al.* [104]	2	18 (17)	4	Naïve	SOF+RBV	12	67% (2/3)
					SOF+RBV	24	100% (3/3)
				Treatment experienced	SOF+RBV	12	60% (3/5)
					SOF+RBV	24	67% (4/6)

SVR12, sustained virological response at 12 weeks post end-of-treatment; SOF, sofosbuvir; LDV, lepidasvir; RBV, ribavirin; SIM, simprevir; PTV, paritaprevir; R, ritonavir; OMV, ombitasvir; DSV, dasabuvir; IFN, pegylated interferon; DCV, daclatasvir; ASV, asunaprevir; GZR, grazoprevir; EBR, elbasvir; VPV, velpatasvir; PI, protease inhibitor.

* Genotype 3 data listed in Table 2

† 100 mg VPV

‡ >90% genotype 1

§ SVR24 (SVR at 24 weeks post end-of-treatment)

Summary of the significant trials of IFNα-free DAA regimens according to genotype 1-4 [84].

Appendix 2

Table 2 | Key interferon-free trials for patients with HCV genotype 2 or 3 cirrhosis

Study	Trial Phase	Cirrhotic patients enrolled *n* (%)	Genotype	Prior treatment	Drug regimen	Duration (weeks)	SVR12 (%)
FISSION[66]	3	100 (20)	2	Naïve	SOF+RBV	12	91% (10/11)
					IFN+RBV	24	62% (8/13)
			3	Naïve	SOF+RBV	12	34% (13/38)
					IFN+RBV	24	30% (11/37)
BOSON[69]	3	219 (37)	2	Treatment experienced	SOF+RBV	16	87% (13/15)
					SOF+RBV	24	100% (17/17)
					SOF+IFN	12	94% (15/16)
			3	Naïve or treatment experienced	SOF+RBV	16	51% (29/57)
					SOF+RBV	24	79% (44/56)
					SOF+IFN	12	88% (51/58)
POSITRON[105]	3	31 (15)	2	IFN ineligible, intolerant or unwilling	SOF+RBV	12	94% (16/17)
			3		SOF+RBV	12	21% (3/14)
FUSION[105]	3	68 (34)	2	IFN non-responders	SOF+RBV	12	60% (6/10)
					SOF+RBV	16	78% (7/9)
			3	IFN non-responders	SOF+RBV	12	19% (5/26)
					SOF+RBV	16	61% (14/23)
VALENCE[106]	3	72 (22)	2	Naïve	SOF+RBV	12	100% (2/2)
				IFN non-responders	SOF+RBV	12	78% (7/9)
			3	Naïve	SOF+RBV	24	92% (12/13)
				IFN non-responders	SOF+RBV	24	62% (29/47)
ASTRAL-1[99]	3	121 (19)	(1)*, 2, (4, 5, 6)	Naïve and treatment experienced	SOF/VPV	12	100% (10/10)
ASTRAL-2[107]	3	38 (14)	2	Naïve and treatment experienced	SOF/VPV	12	100% (19/19)
					SOF+RBV	12	99% (18/19)
ASTRAL-3[107]	3	163 (30)	3	Naïve and treatment experienced	SOF/VPV	12	91% (73/80)
					SOF+RBV	12	66% (55/83)
Pianko *et al.*[98]	2	52 (50)	(1)*, 3	Treatment experienced	SOF/VPV†+RBV	12	96% (25/26)
					SOF/VPV†	12	88% (23/26)
ALLY-3[108]	3	32 (21)	3	Naïve	SOF+DCV	12	73% (16/22)
				Treatment experienced	SOF+DCV	12	63% (5/8)
ALLY-3+	3	36 (72)	3	Naïve and treatment experienced	SOF+DCV+RBV	12	83% (15/18)
					SOF+DCV+RBV	16	89% (16/18)
C-SWIFT[97]	3	53 (37)	(1)*,3	Naïve	GZR/EBR+SOF	12	91% (10/11)

SVR12, sustained virological response at 12 weeks post end-of-treatment; SOF, sofosbuvir; RBV, ribavirin; IFN, interferon; GZR, grazoprevir; EBR, elbasvir; VPV, velpatasvir.

* Genotype 1 data listed in Table 1.

† 100 mg VPV.

Summary of the significant trials of IFNα-free DAA regimens according to genotype 2-3 [84].

Appendix 2

Table 3 | Significant interferon-free trials in patients with decompensated HCV cirrhosis

Study	Genotype	Patient characteristics	Drug regimen	Duration (weeks)	SVR12 (%)
ALLY-1*[113]	1,2,3,4,6	Compensated/decompensated pre-transplant	SOF+DCV+RBV	12	CTP A 92% (11/12) CTP B 94% (30/32) CTP C 56% (9/16)
SOLAR-1[115] (Cohort A)	1, 4	Decompensated pre-transplant	SOF/LDV+RBV	12	CTP B 87% (26/30) CTP C 86% (19/22)
			SOF/LDV+RBV	24	CTP B 89% (24/27) CTP C 87% (20/23)
SOLAR-1†[115] (Cohort B)	1, 4	Compensated/decompensated post-transplant	SOF/LDV+RBV	12	CTP A 96% (25/26) CTP B 85% (22/26) CTP C 60% (3/5)
			SOF/LDV+RBV	24	CTP A 96% (24/25) CTP B 88% (23/26) CTP C 75% (3/4)
SOLAR-2‡[116]	1,4	Decompensated pre- and post-transplant	SOF/LDV+RBV	12	CTP B/C 85% (61/72)
			SOF/LDV+RBV	24	CTP B/C 88% (60/68)
Foster *et al.*[117]	1,3	Decompensated pre-transplant	SOF/LDV	12	71% (20/28)
			SOF/LDV+RBV	12	80% (202/252)
			SOF+DCV	12	73% (11/15)
			SOF+DCV+RBV	12	74% (127/172)
Welzel *et al.*[121]	1,2,3,4,5	Compensated/decompensated pre- and post-transplant	SOF+DCV	24	CTP A 97% (39/40) CTP B 100% (24/24) CTP C 100% (1/1)
			SOF+DCV+RBV	24	CTP A 95% (19/20) CTP B 95% (21/22) CTP C 100% (1/1)
ASTRAL-4[118]	1,2,3,4,6	Decompensated pre-transplant, treatment experienced and naïve	SOF/VPV	12	83% (75/90)
			SOF/VPV+RBV	12	94% (82/87)
			SOF/VPV	24	86% (77/90)

SVR12, sustained virological response at 12 weeks post end-of-treatment; CTP, Child–Turcotte–Pugh class; SOF, sofosbuvir; LDV, lepidasvir; DCV, declatasvir; RBV, ribavirin; VPV, velpatasvir.

* Noncirrhotic post-transplant cohort data not presented.

† Noncirrhotic post-transplant cohort data not presented.

‡ Child–Turcotte-Pugh A/F0-F3 cohort data not presented.

Summary of the significant trials of IFNα-free DAA in decompensated-cirrhotic populations [84].

Appendix 3

Table 5. Treatment recommendations for HCV-monoinfected or HCV/HIV coinfected patients with chronic hepatitis C without cirrhosis, including treatment-naïve patients and patients who failed on a treatment based on PegIFN-α and ribavirin (RBV).

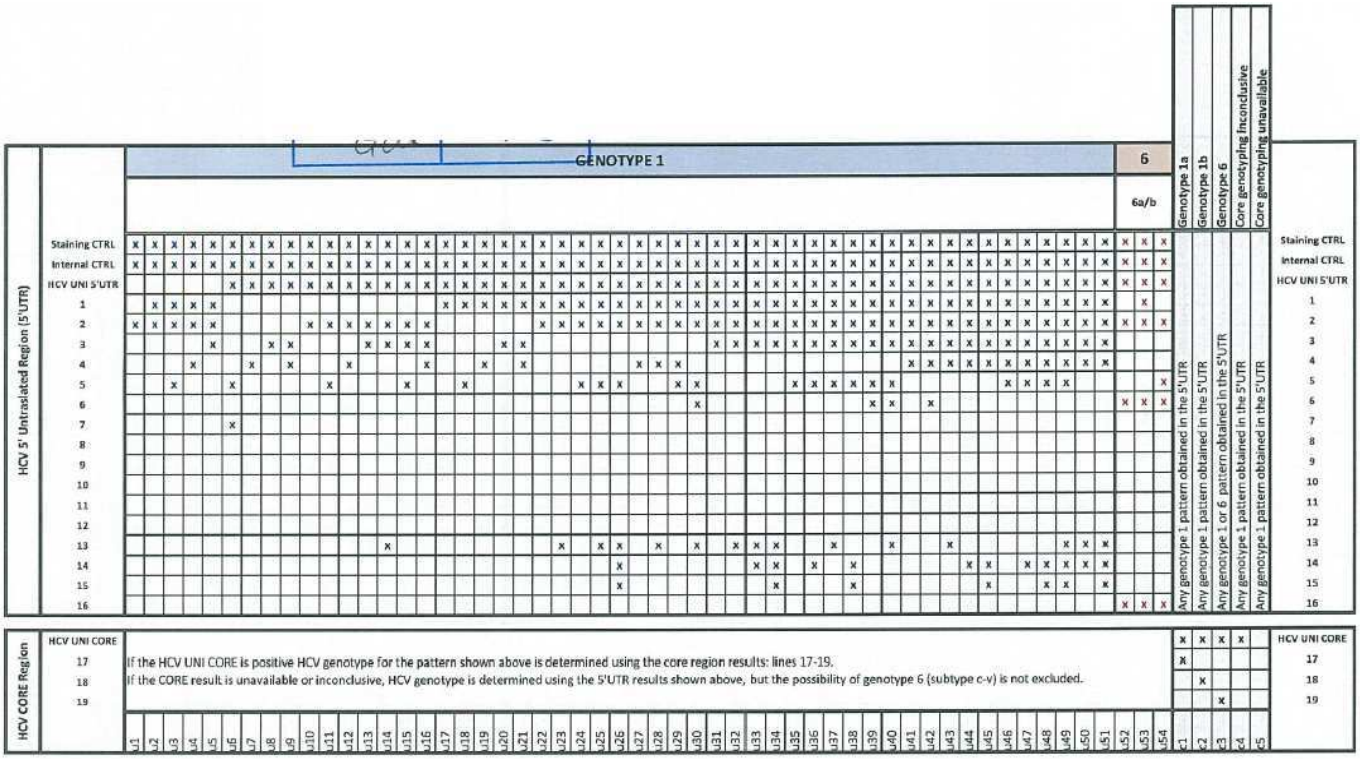
Patients	PegIFN-α, RBV and sofosbuvir	PegIFN-α, RBV and simeprevir	Sofosbuvir and RBV	Sofosbuvir and ledipasvir	Ritonavir-boosted paritaprevir, ombitasvir and dasabuvir	Ritonavir-boosted paritaprevir, and ombitasvir	Sofosbuvir and simeprevir	Sofosbuvir and daclatasvir
Genotype 1a	12 wk	12 wk, then PegIFN-α and RBV 12 wk (treatment-naïve or relapsers) or 36 wk (partial or null responders)	No	8-12 wk, without RBV	12 wk with RBV	No	12 wk without RBV	12 wk without RBV
Genotype 1b					12 wk without RBV			
Genotype 2	12 wk	No	12 wk	No	No	No	No	12 wk without RBV
Genotype 3	12 wk	No	24 wk	No	No	No	No	12 wk without RBV
Genotype 4	12 wk	12 wk, then PegIFN-α and RBV 12 wk (treatment-naïve or relapsers) or 36 wk (partial or null responders)	No	12 wk without RBV	No	12 wk with RBV	12 wk without RBV	12 wk without RBV
Genotype 5 or 6	12 wk	No	No	12 wk without RBV	No	No	No	12 weeks without RBV

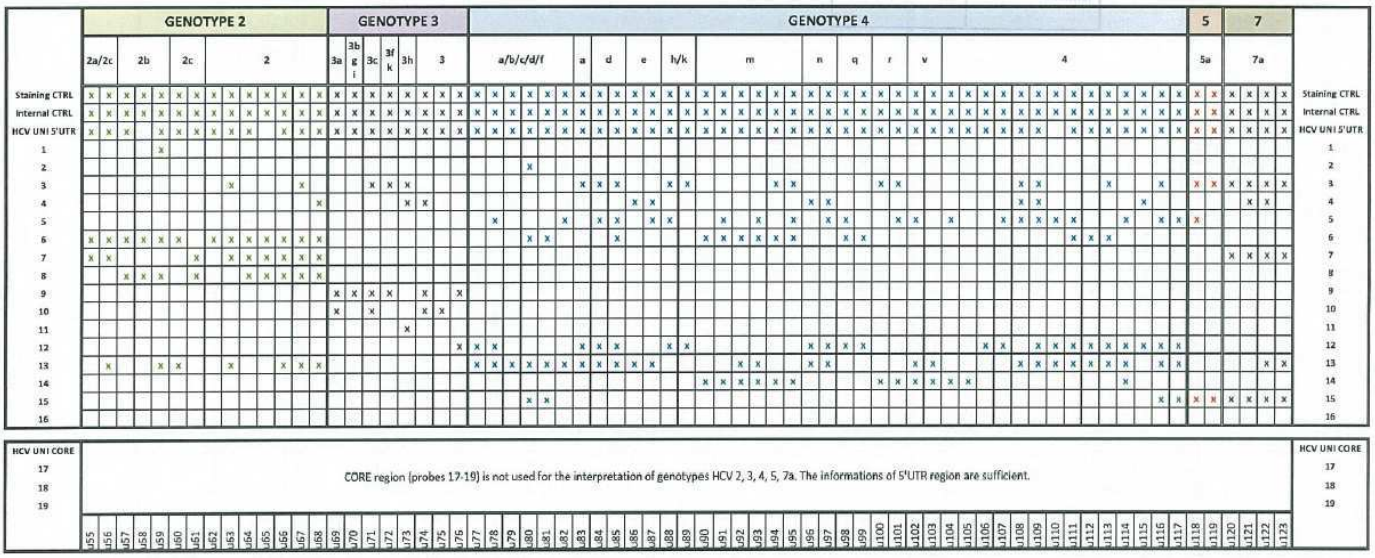
Table 6. Treatment recommendations for HCV-monoinfected or HCV/HIV coinfected patients with chronic hepatitis C with compensated (Child-Pugh A) cirrhosis, including treatment-naïve patients and patients who failed on a treatment based on PegIFN-α and ribavirin (RBV).

Patients	PegIFN-α, RBV and sofosbuvir	PegIFN-α, RBV and simeprevir	Sofosbuvir and RBV	Sofosbuvir and ledipasvir	Ritonavir-boosted paritaprevir, ombitasvir and dasabuvir	Ritonavir-boosted paritaprevir, and ombitasvir	Sofosbuvir and simeprevir	Sofosbuvir and daclatasvir
Genotype 1a	12 wk	12 wk (treatment-naïve or relapsers) or 24 wk (partial or null responders)	No	12 wk with RBV, or 24 wk without RBV, or 24 wk with RBV if negative predictors of response	24 wk with RBV	No	12 wk with RBV, or 24 wk without RBV	12 wk with RBV, or 24 wk without RBV
Genotype 1b					12 wk with RBV			
Genotype 2	12 wk	No	16-20 wk	No	No	No	No	12 wk without RBV
Genotype 3	12 wk	No	No	No	No	No	No	24 wk with RBV
Genotype 4	12 wk	12 wk (treatment-naïve or relapsers) or 24 wk (partial or null responders)	No	12 wk with RBV, or 24 wk without RBV, or 24 wk with RBV if negative predictors of response	No	24 wk with RBV	12 wk with RBV, or 24 wk without RBV	12 wk with RBV, or 24 wk without RBV
Genotype 5 or 6	12 wk	No	No	12 wk with RBV, or 24 wk without RBV, or 24 wk with RBV if negative predictors of	No	No	No	12 wk with RBV, or 24 wk without RBV

Treatment recommendations from EASL [93].

Appendix 4





Interpretative Tables for AMPLIQUALITY HCV TYPE PLUS kit, on the top the Part A of the tables is shown, at the bottom the Part B.

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