gastroenterology insights

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Review

Hepatitis C Virus: History and Current Knowledge

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Abstract: According to the World Health Organization (WHO), the incidence of HCV remains high (around 1.5 million new patients every year), and 80% of patients with acute infection will progress to chronic hepatitis and develop cirrhosis and even liver cancer. Furthermore, some extrahepatic pathologies may be correlated with HCV (such as mixed cryoglobulinemia, porphyria cutanea tarda, lichen planus, glomerulonephritis, Sjogren's syndrome, Hodgkin and non-Hodgkin cell lymphoma, and others). In view of these secondary complications, together with the substantial risk of liver damage, the objective of this review was to research and suggest, based on the scientific evidence, the appropriate clinical use of drugs with direct antiviral action (AAD) according to the criteria of international medical organizations. This is to maximize the clinical benefits for patients and to facilitate access to DAA therapy for all patients with chronic hepatitis C. According to the WHO, no vaccine is currently available, and therapies using new antivirals and their combinations are now an effective and safer solution for patients than they have been in the past with the use of interferons. This study aims to analyse the history and knowledge of the pathogenic biomolecular mechanisms and current therapies for HCV.

Keywords: HCV; hepatology; hepatitis; extrahepatic conditions; antivirals

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1. Introduction

Hepatitis C virus (HCV) infection represents a public health problem of primary importance worldwide. The World Health Organization (WHO) estimated that, globally, 58 million people have chronic hepatitis C virus infection, with about 1.5 million new infections occurring each year. In 2019, approximately 290,000 people died from hepatitis C, mostly from cirrhosis and the primary liver cancer known as hepatocellular carcinoma (HCC) [1]. Antiviral medicines can cure more than 95% of people with hepatitis C infection, but access to diagnosis and treatment is poor, and currently, there is no effective vaccine against hepatitis C. Data that have recently become available showed that, in the WHO European Region, an estimated 15 million people have and live with hepatitis C (2.0% of adults). Two-thirds of people infected with hepatitis B and C are in eastern Europe and Central Asia. Hepatitis C causes about 86,000 deaths per year in the WHO European Member States [1]. The European Centre for Disease Prevention and Control (ECDC), for the year 2019, recorded 37,733 cases of hepatitis C in 29 EU/EEA Member States; excluding the cases reported as acute, 37,660 remain, which corresponds to an estimated incidence rate of 8.9 cases per 100,000 inhabitants. The most-reported infection modality was intravenous drug use, which accounted for 45% of the cases with complete information on transmission status [2]. Finally, in its latest 2019 report for HCV, the Centre for Disease Control and Prevention (CDC) reported 4136 cases with a rate of 1.3 acute cases per

100,000 people with acute infections, with an estimated 57,500 cases (bootstrap confidence interval: 45,500–196,000) [3]. HCV is currently classified into 8 genotypes and 93 confirmed subtypes based on geographical distribution: (a) Western Europe, Japan, and the USA; (b) Japan and China; (c) Northern Europe, India, and South America; (d) Eastern Africa; (e) Central Africa; (f) South Africa and Holland; and (g) Southeast Asia. The numbers of people chronically infected with HCV in each WHO region are as follows: Eastern Mediterranean Region and European Region—12 million; Southeast Asia Region and Western Pacific Region—10 million; African Region—9 million; and America—5 million [3,4].

Based on these epidemiologic data, in 2016, the WHO started a project to eradicate HCV infection around the world by 2030. To date, important results have been achieved, and several documents have been produced to guide health professionals and the general population to reach the target on time [5]. On the other hand, several obstacles remain (i.e., no adherence to protocols; unavailability of testing and/or effective therapies, especially in low-income countries; and no globally available vaccine).

HCV is responsible for both hepatic and extrahepatic damage (extrahepatic manifestations of HCV) [6]. The variety of extrahepatic diseases potentially associated with HCV has led to the term "HCV disease", meaning that this infection should be considered a systemic disease, with broad internal impacts, rather than being considered as a strictly hepatological disease [7–9]. For some extrahepatic manifestations of HCV, the association is clear; for others, it is highly suspicious, even if it has not been definitively confirmed, or an association is only suspected [6].

HCV is transmitted through exposure to infected blood. In the past, the main apparent parenteral transmission modality was through blood transfusions and blood products from infected donors. Today, thanks to the serological and molecular screening of HCV markers of blood donor, this risk has been significantly reduced. Currently, the main apparent mode of transmission is intravenous drug use with the exchange of syringes [10,11]. Injections play a key role in HCV transmission, for example, through medical procedures (e.g., dental procedures, contaminated medical equipment, invasive diagnostic procedures), acupuncture, accidental injury with contaminated needles or sharp instruments, and cosmetic treatments. The risk of transmission by sexual contact is low. This risk is increased for men if they have sex with men and for people who have multiple sexual partners. Perinatal transmission and sexual transmission are possible but infrequent [4].

2. The Historical Route of Hepatitis

In the ancient era, hepatitis was mentioned in medical texts and literary references. We can identify it as a manifestation of jaundice that can be common; however, jaundice also occurs in other diseases (such as tuberculosis, malaria, syphilis, yellow fever, spirochaetosis, scrub typhus, haemolytic anaemia, liver cancer, exposure to toxic substances, etc.) [12–15]. Jaundice means "the appearance of yellow colour of the skin and eye sclera sometimes in full well-being, sometimes preceded by a period of generalized malaise with loss of appetite, nausea, vomiting, low-grade fever, generalized osteo-muscular pains" [16]. The first evidence relating to jaundice manifestations in humans and their connection with the liver can already found in some manuscripts from the ancient civilizations of China (6th millennium BC), Mesopotamia (3rd millennium BC), and the Egyptian, Jewish, Greek, and Roman eras [17,18]. In fact, descriptions of diseases affecting the liver are present in the Babylonian Talmud (5th century BC) that refer to "inflammation" of the liver and jaundice was attributed to the malignant creature Ahhazut that attacks the liver (in the chapter "On Purities" or "Taharot Order") appears in ancient Chinese medical texts such as the *Nei Jing*; later, in an Egyptian manual from the second half of the 1st century AD, there are invocations and spells against the evil one as well as to cure the black jaundice (Figure 1) [18–21].

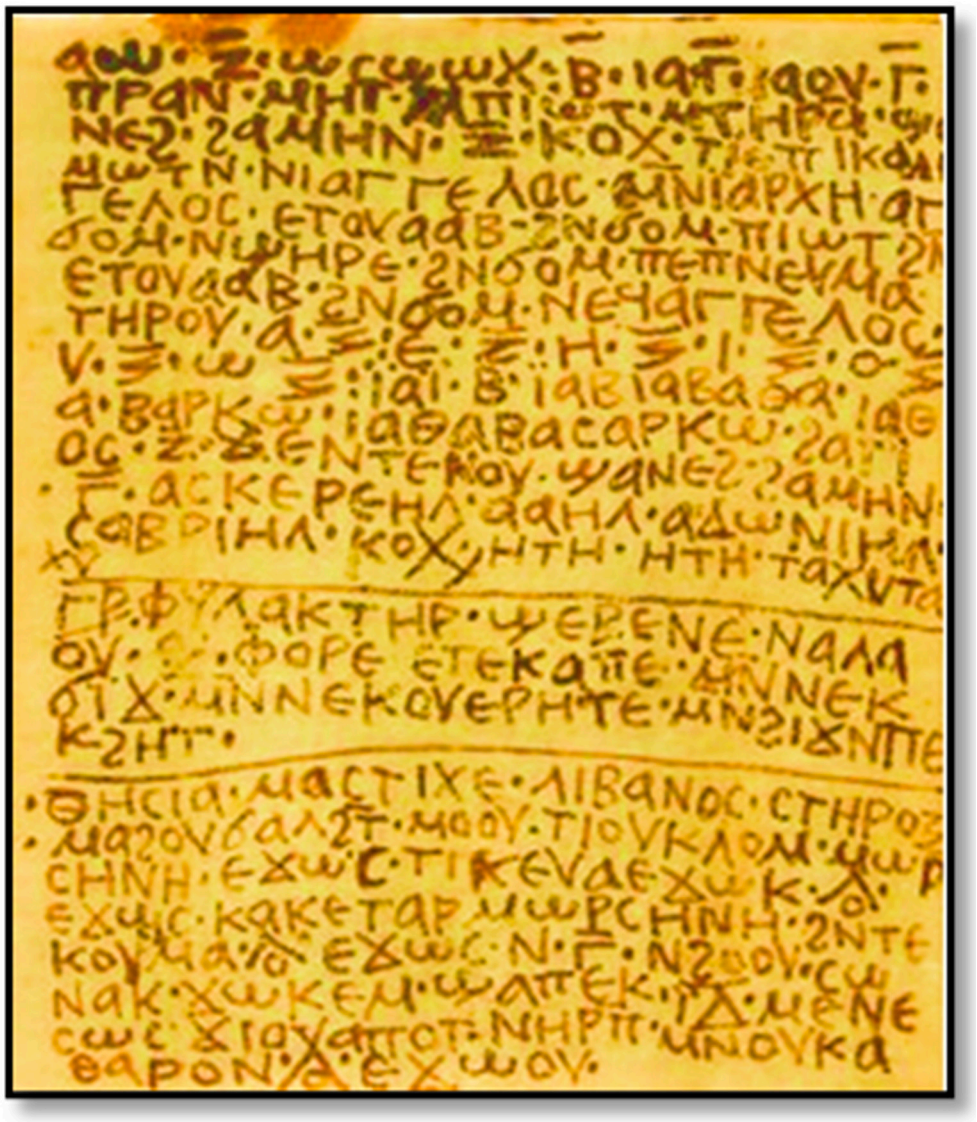

Figure 1. A 20-page bound parchment of the ritual power codex manuscript found in Egypt, which dates to 1300 years ago and was written in Coptic (Greek alphabet with the addition of seven signs of Egyptian demotic scripture). Source: https://it.pinterest.com/pin/789748484632236613/ (accessed on 31 January 2024).

In European paleo-medicine, the first written evidence of jaundice appears in the texts of *Ιπποκρατικό σώμα* (Corpus Hippocraticum), such as in the first and second part of *Περί Επιδημιών* (*De morbis popularibus*, or *Epidemics*) by Hippocrates. He describes several variants of jaundice in relationship with the seasons, but he excludes the divine origin of the symptom, using the word ἴκτερος (ikteros = a displacement of yellow bile under the skin) to define the clinical condition of the patient. Hippocrates would therefore have identified a distinction between infectious jaundice and obstructive jaundice and was the first to guess the origin of jaundice as "predominantly infectious" and that its origin could be found in the liver [22]. Indeed, in the text *Αφορισμοί* (*Aphorisms*, chapter 4, part 62), he states: "...*πυρέσσοντι ἰδρὼς ἐπιγενόμενος, μὴ ἐκλείποντος τοῦ πυρετοῦ, κακόν: μηκύνει γὰρ ἡ νοῦσος, καὶ ὑγρασίην πλείω σημαίνει*..." (...When jaundice supervenes in fevers before the seventh day, it a bad symptom, unless there be watery discharges from the bowels...) [23]. Later, Rufus of Ephesus (Ῥοῦφος ὁ Ἐφέσιος, 70–110 AD), in his text *Περί Ικτέρου* (*De Ictero*), describes these clinical signs and points out that not all jaundice is febrile or originating from a liver disease (hepatitis). He refers to three types: (a) that which occurs on critical days in fiery fevers (if it appears before the 7th day of fever, jaundice is

fatal; meanwhile, after seven days, it brings down the fever); (b) that which occurs due to bad heat of the liver, with coloured (biliary) urine; and (c) that which occurs due to an obstruction of the liver that prevents bile from flowing out, with discoloured urine and stools. He also includes a toxicological reference to types that can occur after a poisonous bite (such as that from a viper) caused precisely by the haemolysis that we are aware of today [18,24]. Another eminent physician was Aretaeus of Cappadocia (Ἀρεταῖος, 2nd century AD), who, in the works *Περί αιτίων και σημείων οξέων παθών* (*De causis et signis acutorum morborum*) and "περὶ Ἰκτέρου," Κεφ. ιε (*On Jaundice or icterus vol. 2*, chapter XV), described the clinical signs and points out that an inflammation of the liver, also infectious, can cause the manifestation of jaundice: "ἢν φλεγμαίνῃ μὲν ἢ σκίρρον ἴσχῃ τὸ ἧπαρ, ἄτρεπτον δὲ τὸ ἐς ἐργασίην ἔῃ, τίκτει μὲν ἐν τῷ ἥπατι χολὴν, καὶ διακρίνει τήνδε ἡ ἐν ἥπατι οὖσα κύστις…" ("…if the liver become inflamed or contract scirrhus, but remain unchanged with regard to its functional duty, it produces bile, indeed, in the liver, and the bladder, which is in the liver, secretes it…"). Later, other doctors such as Galen (Γαληνός, 129-216 AD) also maintained the same assumptions [25,26].

Later, during the Middle Ages, the doctors of the Roman Christian Empire (also called Byzantium today) in the capital New Rome were the first to write based on the medicine of the classical era about liver pathologies and both their symptoms and treatment. One example is the physician Chrysobalantes Theophanes, or Nonnos (Χρυσοβαλάντης Θεοφάνης, about 10th century AD), who writes the *Σύνοψις εν Επιτομή της ιατρικής απάσης τέχνης* (*Synopsis in an Epitome of All Medical Art*), a work of 297 chapters. He refers to the previous Greek doctors of the Empire such as Oribasius of Pergamum (4th century AD), Aetius of Amida (second half of the 5th century), and Alexander of Tralles (6th century AD) regarding various diseases (such as the liver's) and their drug therapies, and was the first to use the term "παρακέντησης" (paracentesis) for the practice of the penetration of the abdomen of patients with ascites. Subsequently, the knowledge passes to the medical schools of Salerno (Southern Italy) and Montpellier (France) [26,27]. Between the 17th and 19th centuries, scholars such as Willis (1678), Herman Boerhaave (1668–1738), Jean-Baptiste Morgagni (1682–1771), Rudolf Virchow (1821–1902), and others have described the various causes of jaundice without offering a classification of them (such as the infectious one) [28]. Morgagni pointed out that gallstones are a cause, and Boerhaave tends to attribute hepatitis with jaundice based on epidemic observations such as those in the 17th and 18th centuries in Germany, especially in 1629, during the Thirty Years War and the Seven Years' War and subsequently in the Napoleonic wars. In the early 20th century, Rudolf Virchow argues that hepatitis is related to an obstruction of the bile ducts. In fact, the pathologist demonstrated through evidence from autopsies that the inflammation of the common bile duct is found in catarrhal jaundice [28].

In the second half of the 20th century, when new serological approaches and developments took place, the first suspicions about the existence of the HCV virus were recorded in 1975, when patients who developed hepatitis after blood transfusions were found to be seropositive to the virus hepatitis A and B [29–31]. In the 1980s, the viral cause was already investigated in 1984, when it was reported that a proportion of chronic viral hepatitis cases did not have antibodies to hepatitis A (HAV) or hepatitis B (HBV). In fact, for the first time, HCV was discovered in 1989 by a group of American scholars with the California biotechnology company Chiron, in collaboration with the CDC. Through applied studies on the chimpanzee, the presence of a viral agent that can be transmitted through blood products and in the serum of blood donors was discovered. Subsequently, thanks to the development of a new molecular cloning approach to identifying the unknown virus, it will be used as a diagnostic test [29,30]. The Nobel Prize in Physiology or Medicine was subsequently awarded to three scientists for their discovery of the hepatitis C virus: the Americans Harvey J. Alter, Michael Houghton, and Charles M. Rice. Their scientific work helped this clinical form of viral hepatitis to become a distinct disease caused by an RNA virus, now known as Hepatitis C (HCV). Rice helped identify the viral cause of HCV-related chronic hepatitis, while Alter devoted his scientific work to blood transfusion

studies, showing that this Hepatitis C virus caused chronic hepatitis in blood transfusion patients. Houghton isolated the virus, and Rice showed in his research that this virus itself causes hepatitis [5,31].

3. Characteristics of the Virus

HCV is a spherical, enveloped virus belonging to the Flaviviridae family, which is subdivided into three genera: Flavivirus, Pestivirus, and Hepacivirus. The Hepacivirus genus includes the hepatitis C virus (HCV), GB-virus b, and the recently identified NPHV (non-primate hepacivirus), as well as the RHV (rodent hepacivirus) and BHV (bat hepacivirus) viruses [32,33]. The genetic material of HCV consists of single-stranded RNA, inhomogeneous, and mutates to eradicate the virus from the immune system. Thus, viruses have in common a small spherical particle, provided with an external envelope with surface projections and a nucleocapsid. In the case of HCV, the particle size is approximately 50 nm. The HCV consists of three structural proteins: two glycoproteins E1 and E2, which are assumed to together form a complex at the level of the envelope, the core protein of the nucleocapsid. In addition, a small p7 non-structural polypeptide belonging to viroporins, capable of forming ion channels thanks to two transmembrane domains, is expressed on a viral surface and seems to play a pivotal role in hepatocyte entry and viral assembly. However, its function is not completely understood (Figure 2) [34–36].

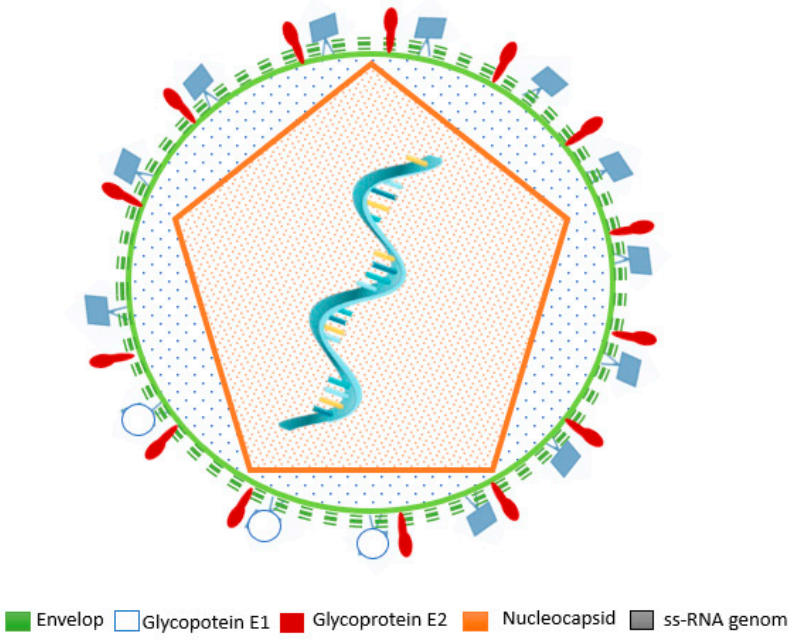

Figure 2. Viral structure of the HCV. The viral particle is composed of a lipoprotein envelope, and the envelope, which surrounds a capsid, is made up of the core protein; the viral genome is enclosed within the capsid. Credits: Original figure by I.A. Charitos.

HCV-RNA has a significant genetic variability, and it is estimated that it is subject to mutations whose frequency is approximately 10^{-4} replacements per site per year. The ability to constantly change seems to be one of the strategies adopted by the virus to escape the control of the host's immune system and allow the persistence of the infection in the long term; additionally, this hypermutability would also support the resistance of HCV to treatment with IFN-α (inflammatory cytokine produced by cells in response to a viral infection) [37]. In fact, even its association with Ribavirin (RBV), IFN-α does not allow for a complete recovery in all patients treated, and about 30–40% of patients show a relapse at the end of treatment. Recent studies have confirmed this observation, attributing greater resistance to IFN to certain genotypes (1a and 1b) of HCV [38–40]. The high tendency of mutations means that viral forms are found in the host (which differ from each other for

few mutations), defined as "quasi-species" [41]. Therefore, in most cases, it appears as a population of RNA molecules which differ in serum, hepatocytes, and peripheral blood mononuclear cells (PBMCs). Some regions of the viral genome in which the mutational frequency is particularly high ("hot spots") have been identified in the sequences that encode the E2 glycoprotein of the envelope; at the 5′ portion of this domain, there is a hypervariable sequence (HVR1) characterized by an elevated level of non-synonymous mutations (e.g., mutations that lead to amino acid substitutions) [42]. Some sequences that encode non-structural proteins (in particular, NS5A), as well as the one that encodes the capsid, show a lower, albeit still significant, level of variability. On the contrary, the untranslated 5′ region is highly conserved among different isolates, although mutations may be identified (Figure 3) [42]. It often turns out that a patient is infected with more than one of the eight HCV genotypes [43].

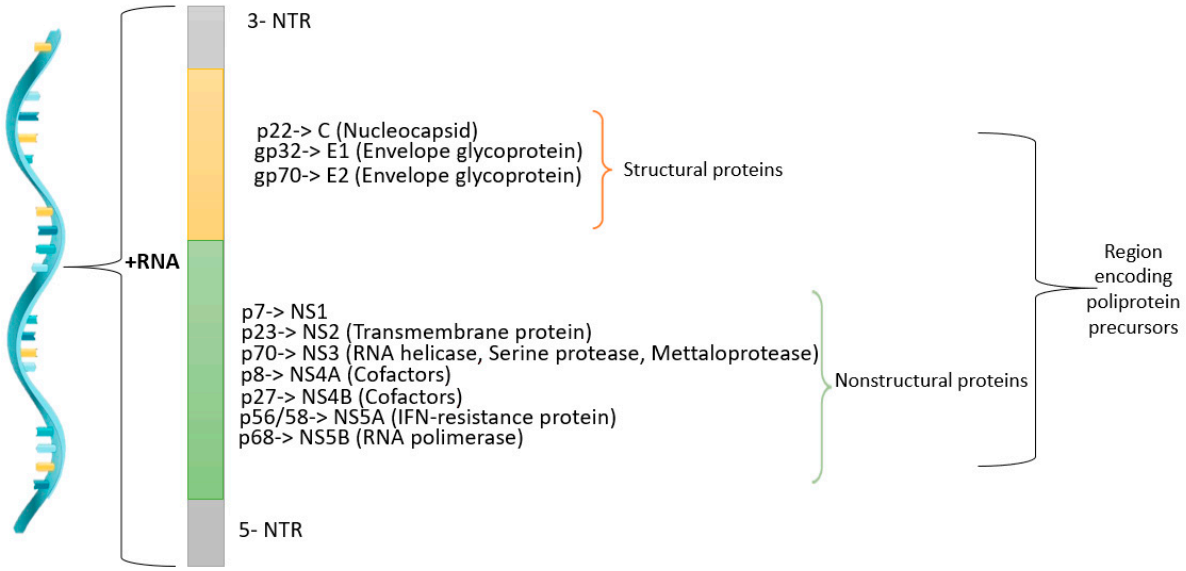

Figure 3. The genome consists of a single-stranded RNA molecule of positive polarity (therefore, of the messenger type), at the ends of which are two non-coding regions with a regulatory function: the 5′-UTR and 3′-UTR, respectively. These flank a large central region encoding a polyprotein of 3010 amino acids. This polyprotein is processed at the co- and post-translational level by cellular and viral proteases to produce structural proteins (core C and E1, E2 glycoproteins of the viral envelope) and non-structural proteins (NS1, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) which intervene in the cleavage of the polyprotein and in viral replication [42–44]. Credits: Original figure by I.A. Charitos.

Overall, the viral genome consists of an untranslated 5′ region (5′NTR), a long ORF encoding a polycistronic protein which is subsequently processed in a series of distinct products, and a 3′ untranslated region (3′ NTR) [42,43]. The beginning of the HCV ORF transcription does not require a cap structure at the end 5′ but is driven by the 341 nucleotide RNA segment found in the 5′ NTR. This region functions as an internal ribosome entry site (IRES) and allows direct binding of the ribosomes near the start codon of the ORF [44]. The untranslated 3′ region (3′ NTR) has a tripartite structure composed of a variable sequence of about 40 nucleotides downstream of the codon of stop of the ORF; this is an internal poly-U/UC tract of heterogeneous length with a highly conserved sequence of 98 nucleotides essential for replication in vivo, for the initiation of synthesis of the negative filament. The HCV polycistronic protein is co- and post-transcriptionally processed in ten different products [45]. The C-NS2 region is processed by host peptidases, which cleave at the level of the junctions C/E1, E1/E2, E2/p7, and p7/NS2. The production of a processing intermediate such as protein E2-p7-NS2 indicates that not all cleavages within the structural region occur co-transcriptionally. Furthermore, a second post-transcriptional processing closes the carboxy region terminal of the core protein, removing the signal sequence for

E1 via the cellular enzyme signal peptidase [46]. The processing between NS2 and NS3 is a rapid intramolecular reaction performed by the NS2-3 protease. Processing of the NS3-5B region is mediated by the NS3 protease, which, however, does not follow a precise order of cleavage: NS3/4A, NS5A/B, NS4A/B, NS4B/5A. The processing at the NS3/4A site consists of an intramolecular co-transcriptional reaction, while cleavage of the other sites can be intermolecularly mediated [47]. The translation process can begin thanks to the presence of IRES (Internal Ribosome Entry Site) sequences in the 5'-UTR region, which direct the binding of the ribosome to RNA. The core protein (molecular weight 21 kDa) is processed by a cellular endoprotease at the C/E1 site, corresponding to the specific SPP (signal peptide peptidase) sequence. Its localization is cytoplasmic, anchored to the membrane of the endoplasmic reticulum thanks to its C-terminal end. It forms homodimers and aggregates with viral RNA, thus forming the icosahedral nucleocapsid of the virus [44]. It also interacts with the non-coding 5' region and with the E1 glycoprotein for the assembly of the virion; it has been demonstrated that the core protein can modulate the transcriptional activity of genes that regulate cell proliferation, such as *RB*, *c-myc*, and *c-fos*, or apoptosis, such as *p53* and *p21*. The core can interact with different cellular proteins (such as lymphotoxin β or members of the tumour necrosis factor receptor [TNFr] family) and have an anti- or pro-apoptotic effect. Finally, P16 (molecular weight 16 kDa) is a minor form of the core protein founded in the nucleus (Figure 4) [48].

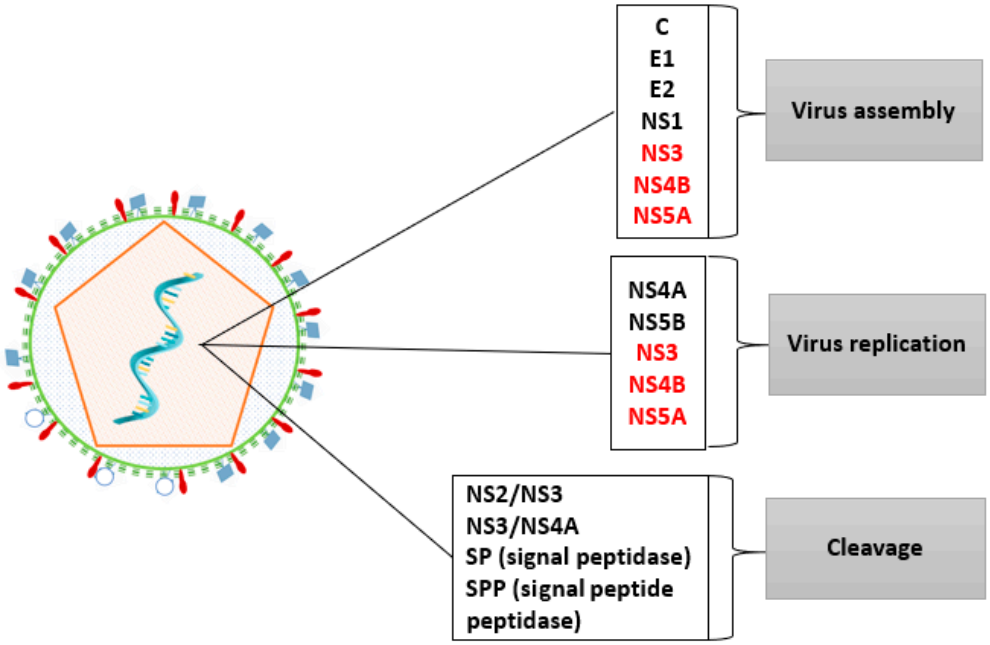

Figure 4. This figure illustrates the viral proteins with their related functions. Red spikes: E1 glycoprotein; black spikes: E2 glycoprotein [44,48]. Credits: original figure by I.A. Charitos.

Studies have shown that the HCV core protein, when expressed in hepatocytes, can in some cases induce hepatic steatosis and its ability to interact with lipids and accumulate in the lipid droplets of the cell, a crucial factor in the onset of steatosis and in liver pathogenesis [49,50]. Therefore, the core protein plays a key role in viral pathogenesis, both in chronic infection and in cellular transformation, modifying the mechanisms of apoptosis and modulating the immune response (Figure 5).

The role of E1 and E2: E1 (30kDa) and E2 (70kDa) are two transmembrane glycoproteins that make up the viral envelope. They are anchored to the endoplasmic reticulum through their C-terminus, and after glycosylation, they are non-covalently associated with each other in the form of a heterodimer [51]. The C-terminal region of E1 contains the signal sequence (found in the C-terminal region) for the cleavage of E2 by cellular proteases. Currently, E1 seems to share its functions with E2, especially in the case of the interaction with cell

receptors. The acquisition of the envelope occurs at the time of budding (common for all Flaviviridae) at the level of the endoplasmic reticulum membrane, where the two proteins, E1 and E2, are located [52]. The E2 protein appears to be involved in the fixation of the virus on the cell surface by interacting with the CD81 receptor. p7 is a hydrophobic peptide that is localized at the level of the membrane of the endoplasmic reticulum and mitochondria, with the N- and C-terminal groups oriented inside the organelle with a hydrophilic part facing the cytosol [35]. When expressed, these form hexamers, which function as ion channels. This protein could therefore belong to the viroporin family (a group of proteins present in several viruses), which participates in a number of viral functions, including the improvement of the trans-membrane ion passage. NS2 (23 kDa) is a non-glycosylated trans-membrane protein processed by a zinc-dependent metallo-protease starting from the viral polyprotein [53,54]. After phosphorylation, it is degraded by the proteasome NS2 that is not necessary for the replication of the viral genome. The only known function is the cleavage in *cis* between NS2 and NS3, which requires a zinc-dependent mechanism (an important element for the integrity of the protein structure of NS3). A zinc binding site has been found in the N-terminal region of NS3 involved in this proteolytic activity, and therefore also for its serine-protease activity [55,56]. The NS3 protein (70 kDa) has a double role: proteolytic activity in the N-terminal domain (responsible for four of the five cleavages of the non-structural region, at the junctions located downstream of NS3) and RNA helicase and NTPase activity (hydrolysis of triphosphate nucleosides) in the two thirds C-terminals, in which they participate in the development of RNA during the replication of the viral genome [57,58]. The NS4B (27 kDa) is a small trans-membrane protein found in the endoplasmic reticulum wall, oriented towards the cytoplasm. Its function is unclear, but it may be part of the replicative complex. The NS5A has two protein forms (due to the diversity in its phosphorylation) with different molecular weights of 56 and 58 kDa, respectively, and are found near the membranes of the endoplasmic reticulum. Inside, the replication complex is linked to other non-structural proteins with a role in the regulation of RNA-dependent RNA polymerase activity. It could also influence the antiviral response induced by interferon through its interaction with the double-stranded RNA-dependent protein kinase (PKR) protein [58,59]. Finally, it can also trigger specific signalling pathways for STAT3 activation. The NS5B constitutes the RNA-dependent RNA polymerase of the virus that interacts with the 3′ end of the viral RNA, which, through polymerization, leads to the synthesis of a copy of the entire genome [60,61]. The interaction between the virus and the cell can involve one or both glycoproteins of the viral envelope. Furthermore, an undefined number of cellular receptors may participate, and four putative receptors have been identified: (a) cluster of differentiation 81(CD81) of tetraspanin proteins family; (b) "human scavenger receptor class B type 1"; (c) prototype C-type lectin (DC-SIGN) family receptors; and (d) the receptor for LDL (low lipoprotein density) [59–61]. CD81 is a molecule that participates in numerous cellular functions, such as cell adhesion, activation, proliferation, and the differentiation of B cells, T cells, and other cell types. The importance of the role of CD81 in the mechanism of virus entry into the host cell has been demonstrated in vitro (Figure 6) [59,62,63]. Finally, DC-SIGN lectin in the liver (expressed on the surface of dendritic and sinusoidal endothelial cells) binds to the viral particles and glycans of the soluble E2 glycoprotein. However, the interaction of DC-SIGN with viral particles does not represent the only mechanism of the tropism of the virus for hepatocytes that do not express this lectin other Flaviviridae, and so the viral particles penetrate the target cells in a process of endocytosis that occurs from the presence of a receptor [64,65]. Subsequently, the fusion of the cell membranes and the HCV will take place within the endosomes, thanks to the lowering of the pH (acidification). Then, the RNA is released, and its translation begins in order to initially produce the various viral proteins used to replicate HCV. Afterwards, through the polymerase present in the virus, the RNA molecule is copied, forming an intermediate molecule of negative polarity, which acts as a matrix for the synthesis of multiple positive RNA strands [65]. These filaments are used for the translation of HCV proteins, for its replication, and for assembly within the completed viral

particles. During replication is the translation of the HCV- RNA, which at first produces a polyprotein composed of 3000 amino acids. The proteolytic process of the single precursor through the proteases present (two viral and two cellular) leads to the generation of ten complete proteins: C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [66]. The HCV replication complex contains the non-structural viral proteins from NS3 to NS5B, along with a yet-unknown number of cellular proteins. The cellular enzymes peptidases of the signal sequence and SPP (signal peptidase peptidase) are together responsible for the maturation of structural proteins, from C to p7 [67,68]. The viral protease NS2/3 is responsible through an autocatalytic event for the cleavage between NS2 and NS3, while the NS3-NS4A complex contains the protease responsible for the generation of the other non-structural proteins. The main enzymatic components responsible for the replication of the viral genome are RNA helicase and RNA-dependent RNA polymerase (RdRp), respectively, expressed by NS3 and NS5B [69,70].

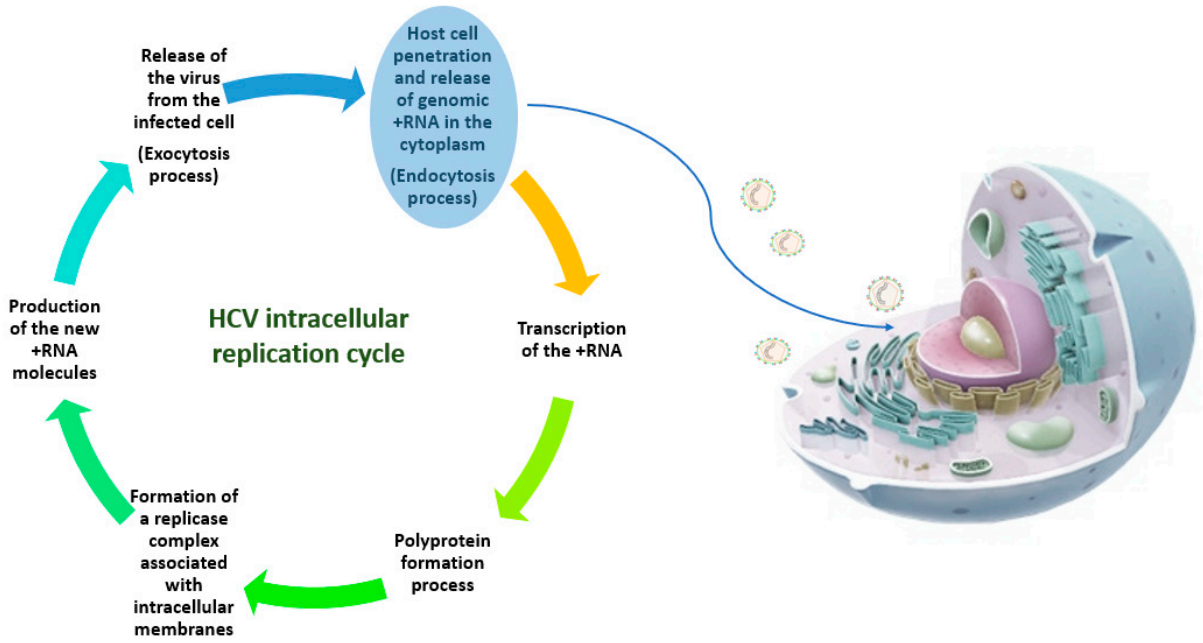

Figure 5. The HCV replication phases occur only in the cytoplasm. The replication of the viral genome occurs within membrane structures that have been observed under an electron microscope in cells containing a self-replicative HCV sub-genomic RNA. These membrane structures could therefore serve as a support for the virus replication complex, formed by the association between RNA dependent RNA polymerase and non-structural proteins ribosome at the level of the IRES region; thus, the 40S subunit of the ribosomes and the translation initiation factor eIF3 bind directly to the RNA without the intervention of the co-factor eIF4G. Finally, at the end of the replication cycle, the virion is assembled in the endoplasmic reticulum, but the details of the mechanisms involved in the assembly, and especially in the maturation and release of the viral particles, remain undefined [50–52]. Credits: original figure by I.A. Charitos.

Some studies report that the LDL receptor could mediate virus entry; this is because, in the serum of infected patients, HCV virions are associated with a low-density lipoprotein receptor (VLDL or LDL). According to one recent hypothesis, HCV may not have a type II fusion protein because it may lack a well-defined lipid bilayer. Since the viral particle is coated with a variable layer of lipids and apolipoproteins, the mechanics of the fusion should be different from that of a bilayer, but this biomolecular mechanism is unknown. Together, it should also be considered whether HCV has a fusion protein. If we think that there is a greater similarity of HCV to lipidic particles than flaviviruses, the mechanisms of lipid homeostasis could be the response to how HCV realizes the infection [66,70,71].

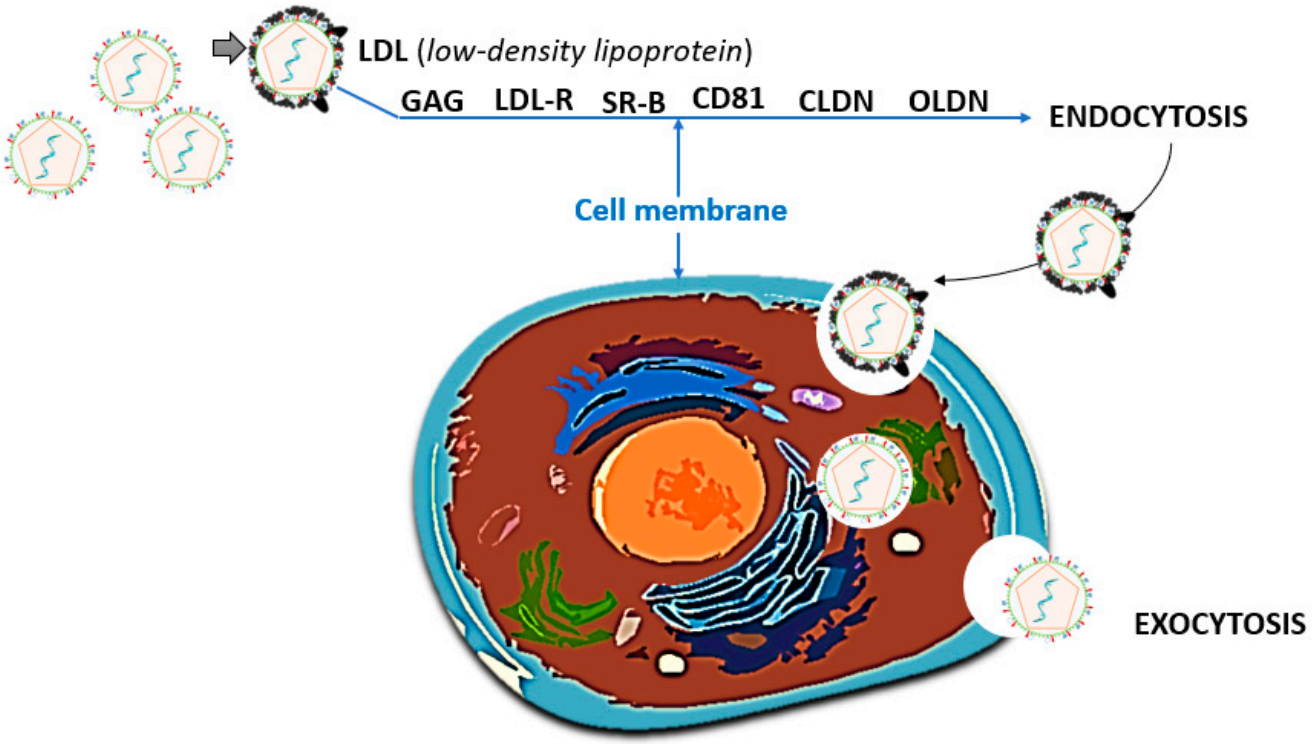

Figure 6. The HCV replication: the viral particle uses the low-density lipoprotein (LDL) to flank the cell, and so thanks to glycosaminoglycan (GAG) protein and/or the low-density lipoprotein receptor (LDLR), it establishes itself on the membrane through a weak bond. Subsequently, the viral particle "builds" a high affinity bond with cluster of differentiation 81 (CD81) protein gene and scavenger receptor class B type 1 (SR-B1) at the surface of the cell membrane. Hence, E2 binds to host cell receptors CD81 and SR-BI, and pH is important, as it is regulated by p7 during the exit. With additional necessary coreceptors, claudin 1 coreceptor association (CLDN) and occludin (OCLN) induces its own endocytosis. The endosome containing the viral particle virus/LDL with the creation environment with a low pH release the nucleocapsid in the cytoplasm of the host cell. Then, with a process of denudation, the exposure of its genome takes place. But how the pH is detected after endocytosis remains unknown. Then, the biomechanism continues with HCV-RNA, which is translated into a polyprotein and processed, thus producing viral proteins now capable of assembling and forming new viral particles. Eventually, these new HCVs build the envelope via the endoplasmic reticulum of the cell and leave the host cell via the process of exocytosis [64,65]. Credits: original figure by I.A. Charitos.

4. Viral Infection and Related Diseases

4.1. Viral Hepatitis Pathogenesis

HCV infection is characterized by a predominance of mostly asymptomatic acute forms and a high frequency of evolution towards the chronic form. Acute hepatitis occurs in the first 45 days of infection, but it is asymptomatic in most cases (80–90%). Up to 50% of acute hepatitis cases (symptomatic or not) from HCV are considered spontaneously self-recovering. Instead, about 50–80% of patients develop a chronic infection, and 20% of these infections develop into liver cirrhosis, which in turn can lead to HCC [72,73]. Symptoms can be arthralgia, headaches, moderate fever, severe asthenia, and, in rare cases, jaundice. In 10–20% of symptomatic cases, the clinical signs are different and non-specific, and thus do not allow the diagnosis of hepatitis of other etiological causes. HCV infection therefore remains very often unknown and is diagnosed only at the chronic hepatitis stage [74,75]. During acute hepatitis, the rise in serum transaminases is moderate (an increase of a factor of ten above normal values) [76,77]. Healing is defined by a lasting normal level of transaminases and the inability to trace the viral RNA in the serum after at least 6 months

from infection, despite the persistence of anti-HCV antibodies [78]. The most relevant feature of hepatitis C is the evolution towards a chronic infection in 50–80% of cases. The patients remaining can be (indeed often are) asymptomatic even in the chronic stage. In this clinical form, however, there can be (infrequently) an increase in transaminases, as well as the presence of the viral genome and anti-HCV antibodies in the serum, which is a clear sign of a chronic infection [79]. It becomes essential to perform a liver biopsy to better define the histological alteration evolution stage of HCV disease, and thus investigate the state of the liver's structure from a histological point of view. The METAVIR classification is used for this purpose based on necrotic–inflammatory histological criteria and the degree of fibrosis. The main feature of chronic virus C hepatitis is steatosis (a phenomenon more frequent than other chronic viral hepatitis) for 50–70% of cases [80–82]. Fibrosis and lymphoid aggregates or nodules present in the portal region can be observed and are sometimes also found in alterations of the interlobular biliary canaliculi. Some patients with chronic virus C hepatitis may have a benign disease course, but others may have a 20% risk of developing cirrhosis due to infection over 20 years, the age at the time of contagion (beyond 40 years), and the association with alcohol abuse [82–84]. Cirrhosis due to infection is one of the main causes of liver transplantation, despite the almost systematic reinfection of the transplanted organ (the 5-year survival of those transplanted for cirrhosis is of the order of 70%) [84]. The evolution of cirrhosis into HCC is performed through hepatocellular necrosis and consequent regeneration, and some viral proteins play a direct role in this process [85,86]. Mortality due to HCC is increasing in Western countries, as is the number of cases of HCV-related HCC. HCV infection is one of the main causes of liver disease that can lead to irreversible organ dysfunction and that indicates the need for transplants. With the transplant, a liver with a large amount of HCV is replaced by a non-infected liver [82,86–89]. However, the virus remaining in the serum infects the transplanted liver immediately and begins to replicate; in patients with HCV-RNA detectable in serum at the time of transplantation, the postoperative relapse of HCV infection is immediate and universal, and all patients undergo reinfection of the gifted organ. Recurrence of hepatitis C after liver transplant constitutes a problem of great importance, since it is the most frequent cause of death and loss of the transplanted organ in these patients, causing about two thirds of transplant failures. The recurrence of hepatitis C after transplant differs radically from hepatitis C in non-transplant patients [84]. This is because some factors only act in the transplanted liver, causing a different clinical course of hepatitis C. First, the immune system is in a chronic state of infection in an HCV-positive liver transplant recipient. Therefore, the relapse rate of hepatitis C after transplant differs from acute or chronic hepatitis in a non-transplant patient, which is about both the consequences at the level liver and the host's immune response. Furthermore, the transplant is followed by the administration of immunosuppressive drugs to prevent organ rejection [90,91]. The use of immunosuppressants influences the evolution of hepatitis after transplant, particularly with regard to the extent of immunosuppression and the composition of the scheme immunosuppressive influence on the progression of fibrosis. HCV-RNA levels are higher after transplant than preceding the procedure, and this is associated with an unfavourable long-term outcome. More specifically, serum HCV-RNA levels rise rapidly starting from the second post-transplant week and reach maximum levels at the fourth post-transplant month. The main predictors of disease severity and survival for transplanted patients are summarized in Figure 7 [92,93].

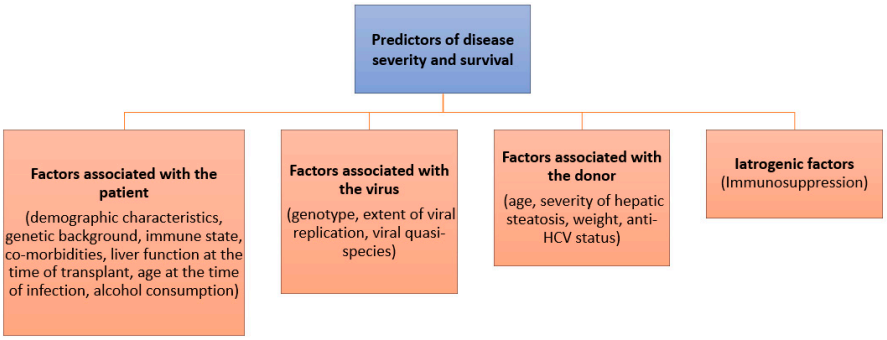

Figure 7. Main predictors of disease severity and survival in patients receiving liver transplantation: the female gender is associated with a worse course, and a consequent lower survival of the patient. Race influences the outcome of patients with HCV relapse, as Caucasian patients have a better prognosis than non-Caucasian patients [94–96]. The age of the donor is associated with the severity of the disease and patient survival. Only 14% of recipients who have an organ from a donor under the age of 30 develops cirrhosis related to recurrence of HCV infection, while recipients of an organ from a donor aged between 30 and 60 and over 60 develop cirrhosis, respectively, in 45% and in 52% of cases [96,97]. The course of HCV infection is accelerated in transplant patients as opposed to immunocompetent patients, with rapid progression fibrosis and a high risk of early clinical decompensation and liver failure after transplant. There is a close correlation between the development of high-grade cirrhosis and immunosuppression, bolus of methyl-prednisone, use of anti-lymphocyte immunoglobulins, and a cumulative dose of steroids. In addition, the degree of necro-inflammatory activity, the stage of fibrosis, and specific histological features such as cholestasis, balloniform degeneration of hepatocytes, and confluent necrosis—if observed in one liver biopsy early after transplantation—are useful factors in predicting the progression to a severe form of hepatitis [97,98].

4.2. Virus and Extrahepatic Diseases Manifestations

There is a whole series of extrahepatic diseases for which an association with HCV infection has been suggested (Figure 8). These can include idiopathic pulmonary fibrosis, lichen planus, Sjogren's syndrome, autoimmune thyroiditis, and porphyria cutanea tarda. In some cases, an association is suggested only by isolated anecdotal observations. The existence of a pathogenetic link between HCV and these disorders has sometimes been suggested by the observation of response to antiviral therapy. However, there are also cases in which the same antiviral treatment with interferon has been deemed to be the real culprit of the detected association [98–101].

Extra-hepatic manifestations of HCV infection

Haematologic	Integumentary system	Endocrine system	Respiratory system	Urinary	Nervous system	Immune system	CV system	Eye	Reproductive
• Mixed cryoglobulinemia (complete or incomplete clinical syndrome) • Non-Hodking B-cell lymphomas • Monoclonal gammapathies	• Porphyria cutanea tarda • Lichen planus • Chronic urticaria • Chronic itching • Vitiligo • Psoriasis • Necrotic migrating erythema • Kaposi's pseudo-sarcoma	• Thyroid cancer • Diabetes Mellitus type II (DM2)	Alveolitis - Pulmonary Fibrosis	• Cryoglobulinemic and non-cryoglobulinemic nephropathies • Type I membranoproliferative glomerulonephritis (MPGN-1) • Mesangial glomerulonephritis, and focal and segmental glomerulonephritis • Renal cell carcinoma	• Peripheral and central non-cryoglobulinemic neuropathies	• Autoimmune thyreopathies • Siogren's syndrome • Chronic polyarthritis • Rheumatoid arthritis • Polyarteritis nodosa • Adamantiades-Behcet syndrome • Poly/dermatomyositis • Fibromyalgia	• Cirrhotic cardiomyopathy (CCM)	• Mooren's corneal ulcers	• Erectile dysfunction

Figure 8. List of extrahepatic manifestations demonstrated or suggested associated with infection from HCV [98,99,102,103].

An aspect that makes the causes of HCV infection interesting is its association with extrahepatic manifestations, which refer to the marked lymph tropism that it demonstrates by infecting the mononuclear cells of the peripheral blood. The mechanisms involved in lymphoproliferative disorders, both virus-dependent and virus-independent, are complex and not yet fully understood. In favour of this phenomenon, different hypotheses have been proposed through different observations [103,104]. Observations support the hypothesis of a "compartmentalization" of HCV genomes. In fact, the existence of lymphotropic strains of the virus have been previously observed. The presence of HCV-RNA of negative polarity was noted in PBMCs samples isolated from infected chimpanzees by observing that the ability of the virus to infect PBMCs and/or liver cells in the same animal (as well as human lymphocyte cell lines in vitro) varied between different HCV strains [105–107].

The negative strand of HCV-RNA (indicating an active viral replication in progress) was found in all cell subpopulations in some studies; meanwhile, in others, it was found in specific cell subpopulations (B lymphocytes and monocytes). The differences in the distribution of quasi-species in these tissues tending to favour the growth and selection of viral variants may originate from cellular factors present both in the liver and in PBMCs [107–109]. Thus, infecting non-hepatic cells leads to the hypothesis that a reserve network of viral particles is produced, and that it could favour the selection of both HCV variants that are effective in escaping from immunity, therefore providing viral persistence. Infection of PBMCs and bone marrow cells could promote specific cellular alterations, which can be interpreted as potential pathogenetic strategies that are adopted by the viral agent to induce lymphoproliferative disorders that frequently occur during chronic HCV infection. The association between HCV virus infection and extrahepatic manifestations has been confirmed by epidemiological studies in some areas of the world (but not all), supporting the role of the virus and/or host-related factors (Figure 9) [99,110–112].

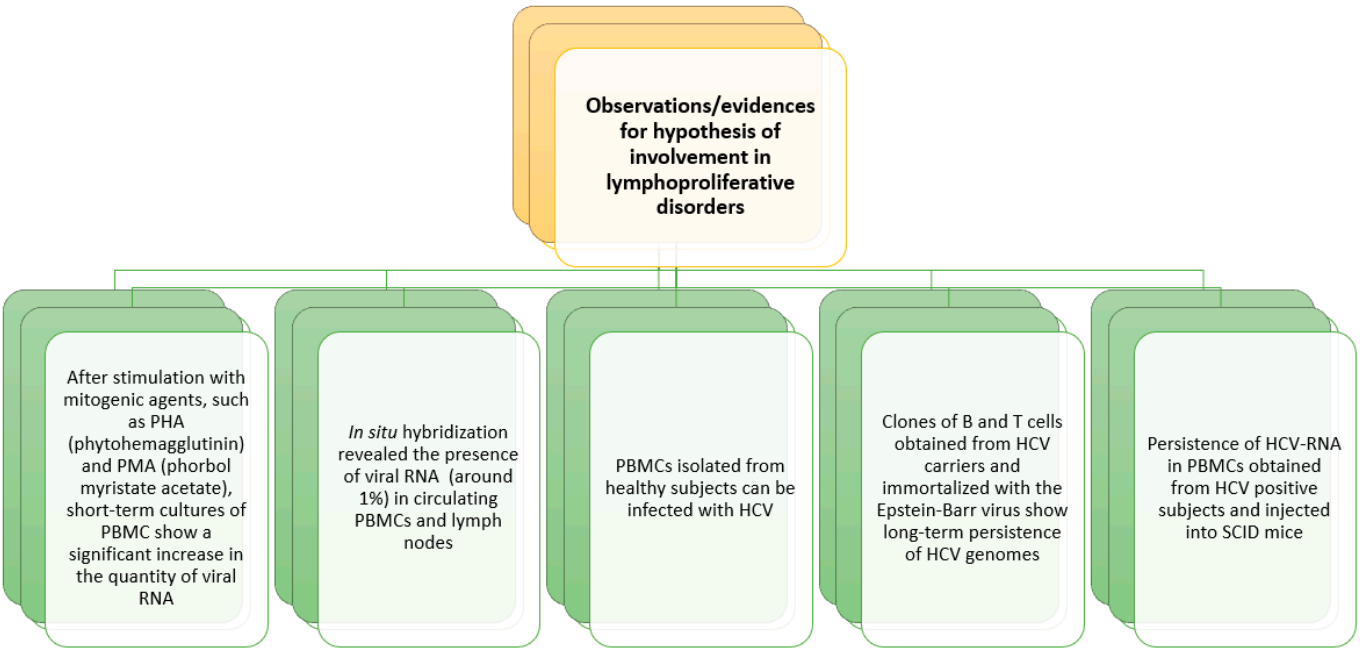

Figure 9. An important aspect of HCV infection is its association with extrahepatic manifestations, related to the marked virus lymph tropism, which infects the mononuclear cells of the peripheral blood. There are several observations reported in the figure that support this hypothesis. Studies both in vitro and in vivo, and based on the analysis of the HVR1 sequence, have shown different quasi-species profiles between serum, liver, and human PBMCs [112–115].

The B cell DLP which they have been most frequently associated with during chronic infection is mixed cryoglobulinemia, B cell non-Hodgkin's lymphoma (NHL), and mono-

clonal gammopathies [116,117]. The mechanisms involved in lymphoproliferative disorders, both virus-dependent and virus-independent, are complex and not yet fully understood. Mixed cryoglobulinemia is the most associated with HCV infection, characterized by circulating immune complexes and produced by a B-cellular lymphoproliferative process [118–120]. The determination of a state of cryoglobulinemia is based on laboratory data. This explains the presence in the serum of one or more immunoglobulins (Ig) characterized by precipitating at temperatures below 37 °C and redissolving upon heating the serum. Therefore, these cryo-precipitation immune complexes represented by the mixed cryoglobulins IgG and IgM constitute the serological marker of mixed cryoglobulinemia. G-type immunoglobulins are autoantigens, while M-type immunoglobulins are endowed with rheumatoid factor activity and constitute autoantibodies [121–123]. The type of mixed cryoglobulinemia is determined by the presence of polyclonal (III type) or monoclonal (II type) IgM, which is a clinically distinct syndrome characterized by purpura, asthenia, arthralgia, membranoproliferative glomerulonephritis, peripheral and central neuropathies, skin ulcers, and diffuse vasculitis [121,124]. This syndrome frequently coexists with aspects of the bone marrow typical of NHL and evolves in up to about 8% of cases into an outright B cell malignancy (mixed cryoglobulinemia is considered a "borderline" disorder, thus being something between "benign" and "malignant"). Clonal proliferation of IgM-producing B cells was found in patients with mixed cryoglobulinemia HCV correlation. Regarding organ involvement and clinical course, mixed cryoglobulinemia type II and type III are similar; mixed cryoglobulinemia, unlike type II, can evolve into lymphoma [125–128].

For the pathogenetic mechanisms involved in the benign lympho-proliferation underlying mixed cryoglobulinemia and in its evolution into lymphoma, a sequential progression process has been suggested, which various host and virus factors contribute to: (a) stimulation antigenic sustained by chronic viral infection; (b) virus lymph tropism; (c) virus proteins, especially E2; (d) rearrangement of the antiapoptotic gene of leukaemia 2/B cell lymphoma (*bcl-2*); (e) reciprocal translocation; and (f) micro-RNAs 30. Many studies have suggested that some HCV antigens may be involved in the development of both mixed cryoglobulinemia and lymphoma [128–130].

The B cell receptor (BCR) of the monoclonal population of B cells overexpressed in patients with mixed cryoglobulinemia and immunocytoma (as well as the RF + IgM component of the cryoprecipitate) show cross-reactivity with the HCV NS3 antigen [131,132].

It has also been reported that the HCV E2 antigen interacts with the CD81 tetraspannin also present on the surface of B cells, and this link is hypothesized to be responsible, along with other factors, for the polyclonal expansion of B cells by lowering the level of basal activation of these cells [133]. Furthermore, E2 appears to mimic human Ig observed from the study of the N-terminal region of the protein, which is antigenically and structurally like the hypervariable domain of human Ig [134]. This chromosomal aberration involves the JH union segments of the Ig heavy chain (IGH) gene. At the junction point of the two genes, the N segments, which are the insertions of variable length due to the random addition of nucleotides, do not present in the original recombinant sequences and determine a specific nucleotide profile for each translocated clone [135,136].

The micro-RNAs (small linear RNA molecules) that function as post-transcriptional regulators of genes play various roles that are not physiological but are also pathological (such as favouring neoplastic progression and the manifestation of tumours) in the cell [137]. Indeed, when patients with HCV-related NHL and mixed cryoglobulinemia HCV-related performed on a pool of selected micro-RNAs, mir-26b was significantly downregulated in these patient populations compared to an HCV positive control population with no lymphoproliferative manifestations. Levels that were restored after anti-HCV eradication therapy were noted, demonstrating a clear role of this miRNA in the manifestation of ongoing HCV lymphoproliferations [138,139]. A high prevalence of thyroid disorders was noted in patients with chronic hepatitis C. All forms of thyroid disorders can be observed, namely hypo- or hyperthyroidism, Hashimoto's thyroiditis, or the presence of autoantibodies in the absence of other manifestations [140–142]. Indeed, the prevalence of various thyroid

dysfunctions and circulating antithyroid antibodies is high in patients with HCV infection when compared with patients with hepatitis B or D, or with healthy control subjects [143]. The frequency of a high titre of circulating anti-thyroid antibodies in HCV-positive patients has been noted. Thus, different methodological approaches, including different patient selection criteria, can account for conflicting observations regarding thyroiditis [143,144].

Sjögren's syndrome is an autoimmune disease that affects the exocrine glands and usually presents with xerostomia and xerophthalmia (due to the involvement of the salivary and lacrimal glands) in the presence of immunological alterations represented by the identification of antinuclear and anti-ENA autoantibodies (SSA/Ro, SSB/La) [145–147]. In the absence of an associated systemic autoimmune disease, patients with similar manifestations can be defined as having primary Sjögren's syndrome. The symptomatology varies from an autoimmune exocrinopathy to a systemic process with different extra-glandular manifestations. A correlation between Sjögren's syndrome and HCV infection was first reported in 1992 [145,146]. Subsequently, epidemiological and clinical studies have shown that a Sjögren syndrome with the same clinical–histological features observed in primary Sjögren may be present in patients with chronic virus infection. The existence of a close correlation between SS and the virus has been reported, and the presence of mixed cryoglobulinemia has been suggested in patients with associated HCV–Sjögren's syndrome. In fact, in an in vivo study with transgenic animals (mice) carrying the genes of the HCV outer coating, it was demonstrated that they can develop an exocrinopathy of the salivary and lacrimal glands, similar to Sjögren's syndrome. In view of the frequent association between Sjögren's syndrome and mixed cryoglobulinemia in HCV-positive patients, as well as the possible evolution of Sjögren's syndrome in a B-cell NHL, the syndrome observed in infected patients can be interpreted as one of the possible clinical manifestations of HCV-related lymphoproliferative disorder [146,147].

The existence of a pathogenetic link between HCV infection and idiopathic pulmonary fibrosis has been suggested based on the observation of increased lymphocyte and neutrophil counts in bronchoalveolar lavage of patients with chronic HCV infection, which suggests that HCV infection may trigger alveolitis [148]. A second factor that makes its pathogenetic link is the higher frequency of HCV markers in patients with idiopathic pulmonary fibrosis than in normal controls, as well as from the observation of cases of HCV-related chronic hepatitis treated with interferon alpha that developed an idiopathic pulmonary fibrosis [149,150].

HCV infection has also been associated with different skin disorders. A strong association between the sporadic variant of porphyria cutanea tarda (a metabolic disorder characterized by a reduced hepatic activity of uroporphyrinogen decarboxylase) and HCV infection was suggested by the observation of a high prevalence [151,152]. Another skin pathology would be Lichen planus, which is a mucocutaneous disease characterized by inflammatory infiltrates with a prevalence of CD4 positive cells, vacuolar degeneration of the deeper basal epithelium, and the presence of acidophilic bodies that could be keratocytes in apoptosis. The aetiology is unknown, but a cell-mediated immune response is present towards antigens expressed on mucosal epithelial cells [153–155]. A higher frequency of the HLA-DR6 allele was observed in subjects with oral lichen planus and HCV infection compared to anti-HCV-negative patients. In favour of the correlation between lichen and HCV infection, the presence of HCV-RNA chains with positive and negative polarity has been demonstrated by means of PCR techniques carried out on biopsy samples of oral mucosa during lichen. Given the high sensitivity of PCR, these results could be consequent to the contamination of biopsies with infected blood [153–155]. The possible association between diabetes mellitus and HCV infection remains much discussed. A high prevalence of type II diabetes mellitus has been reported in patients with chronic viral infection. In patients with HCV infection, the onset of type II diabetes is associated with high insulin resistance, but without the presence of pancreatic islet antibodies. On the other hand, alpha interferon (IFN) therapy is associated with the development of autoimmunity against pancreatic islets and the onset of type I diabetes mellitus, which is sometimes severe [156–159].

Chronic HCV-related polyarthritis can be observed in HCV-positive subjects with different clinical patterns, both within a CM and isolated. Rheumatoid arthritis proper, meeting the classic criteria, seems to be uncommon in HCV-positive subjects, while an intermittent oligoarthritis, non-erosive and involving the large and medium joints, is frequently observed. This association was especially evident in the case of the active replication of HCV [160,161].

The presence of anti-HCV in patients with hypertrophic cardiomyopathy and those with dilated cardiomyopathy with statistically significant differences was highlighted compared to the rate of anti-HCV present in healthy subjects. However, other studies have not confirmed this association. The recent determination of significantly higher prevalence of aortic atherosclerosis in patients with HCV infection is also noteworthy [162,163].

The association between chronic HCV infection and glomerulonephritis has been recognized for some time (Figure 10). Several studies have revealed the presence of deposits of genetic material or viral proteins in the glomeruli (such as C22-3 virus fragments, envelope glycoprotein E2/NS1, C33, NS5). Glomerular changes related to HCV infection can occur both in the native kidney and in the transplanted kidney [164]. At present, three kidney diseases have been recognized in association with HCV: (a) mixed cryoglobulinemia (cryoglobulinaemic nephropathy); (b) membranoproliferative glomerulonephritis (GNMP); and (c) membranous nephropathy (NM). In adults, the most frequent form of renal involvement in HCV infection is cryoglobulinaemic nephropathy [165,166]. Renal disease associated with mixed cryoglobulinemia manifested by haematuria, proteinuria (sometimes in the nephrotic range, e.g., with values greater than 3 g/24 h), declining oedema, and renal insufficiency of varying degrees. The histological manifestations of CM-associated renal disease are similar to those of idiopathic membranoproliferative glomerulonephritis, from which they are distinguished by the presence of capillary thrombi at light microscopy [167]. The histological examination highlights a thickening of the membrane basal cell glomeruli, cell proliferation, and infiltration of circulating macrophages. Characteristic lesions of cryoglobulinaemic nephropathy include (a) intraluminal thrombi formed by precipitated cryoglobulins, (b) diffuse deposits of IgM in the capillary loops, and (c) subendothelial deposits that appear on electron microscopy as complex structures that resemble fingerprints (subepithelial deposits are rare) [167–169]. Some patients with HCV have kidney disease, upon which, histological examination is classified as membranoproliferative glomerulonephritis without the simultaneous presence of cryoglobulins. This form of kidney disease was common until the 1980s, but now it is rare. It has been hypothesized that the apparent disappearance of the disease may be due to a lower frequency of HCV infection [170,171].

The association between HCV and type I membranoproliferative glomerulonephritis varies significantly in case studies. Membranous nephropathy is another glomerular disease that has been reported in association with an HCV infection. Hypocomplementemia and rheumatoid arthritis factor in this glomerular disease. Histologically, membranous nephropathy is characterized by the thickening of the glomerular basement membranes, which contain deposits immune to microscopic examination electronics, but the proliferative aspects are absent [172–175]. Clinically, membranous nephropathy does manifest with nephrotic syndrome. In HCV, the major cause would be the deposition of immune complexes and/or the presence of cryoglobulinemia. Renal damage can manifest in the presence of hepatic type I squamous cell carcinoma with concomitant type II cryoglobulinemia due to impaired immune response, but there is also an association between HCV infection and renal cell carcinoma (RCC) risk. Blood urea nitrogen testing (BUN), electrolytes dosage, plasma electrophoresis, complement proteins, urinary protein (proteinuria) of 24 h, and kidney ultrasound are fundamental in better defining and following up on the clinical state of these patients [170,172,175].

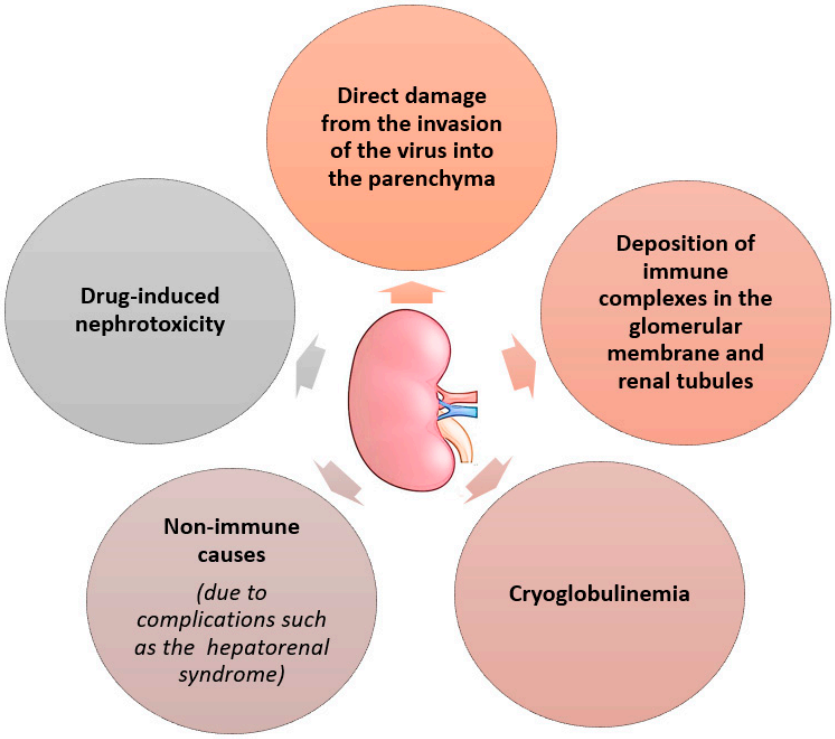

Figure 10. The main causes of the renal injury during HCV infection. Credits: original figure by I.A. Charitos [164,169].

The differential diagnosis between the three forms mentioned previously requires a histological assessment. The indications of whether to perform renal biopsy in a patient with HCV and signs of renal damage are not standardised. The presence of proteinuria greater than 1–2 g/24 h, acute renal failure, "active" urinary sediment characterized by haematuria, or cylindruria in association with another of these factors may indicate the need for a kidney biopsy. However, the presence of a single kidney, small kidneys, changes in haemostasis or thrombocytopenia, or a lack of understanding of informed consent are contraindications to biopsy [164,171,174].

5. Laboratory Diagnosis

Many immunological and molecular tests have been developed for detecting and monitoring HCV infection. We can distinguish between indirect tests, which identify the presence of antibodies against HCV antigens, and direct tests, which determine the presence of HCV-RNA in the host's blood (Figure 11) [4,175,176]. The determination of specific antibodies against HCV antigens is the first step in diagnosing an HCV infection. The enzyme immunoassays (EIAs—enzyme immunoassays) currently in use contain recombinant proteins as antigenic determinants encoded by the NS3, NS4, NS5, and core regions, conserved in the various viral genotypes [177]. These tests have high sensitivity and specificity equal to 99%. The presence of anti-HCV antibodies indicates exposure to the virus, but does not differentiate between acute, chronic, or resolved infection. In addition, anti-HCV antibodies persist for many years or even for life in recovered or SVR patients (sustained virologic response) after therapy. For this reason, patients with positive HCV antibodies must undergo tests capable of revealing the presence of HCV-RNA to determine whether they have cleared the infection or if they have an active infection [178]. A new indirect test, the HCV core antigen test (HCVcAg), was developed in the last two decades to detect the circulating HCV core antigen. It has a high specificity (up to 99.4%) and a lower sensitivity (up to 87.1%) and may be helpful in diagnosing active infection, acute or reactivated, especially in the health settings of low-income countries, because of the limited costs compared to molecular tests [179].

A direct search of the viral genome (HCV-RNA) is based on a polymerase chain reaction preceded by a retro-transcription (RTPCR) [179]. There are two types of direct HCV-RNA detection exams: qualitative and quantitative tests. Qualitative tests report whether HCV-RNA is present in serum, but not the amount of present HCV-RNA. These tests are for screening and are no longer used in clinical practice. Quantitative tests, on the other hand, measure the levels of HCV-RNA present in the serum [180]. For this purpose, it is necessary to evaluate the viral genotype and the extent of viral replication (viral load) by means of quantification of HCV-RNA at the beginning of antiviral treatment, except for pan-genotypic treatment. Next, it is necessary to monitor the virological response during therapy; this is because, for patients undergoing antiviral treatment, the persistent negativity of viral RNA in serum ensures the duration of the drug result. The identification of the genotype can be performed by several means; of these, the most accurate is PCR, followed by the direct sequencing of the NS5B or E1 regions. However, these approaches are not widely used in clinical practice because they are extremely complex [181]. The genotype can also be determined by other methods, such as reverse hybridization with genotype-specific probes, analysis of the length of the restriction, or amplification fragments of the 5′ UTR region of the viral genome by PCR. The Inno-LIPA test is frequently used, which is an aligned probe-based exam on reverse hybridization with the use of genotype-specific probes for UTR region 5′ of the viral genome [4,182,183].

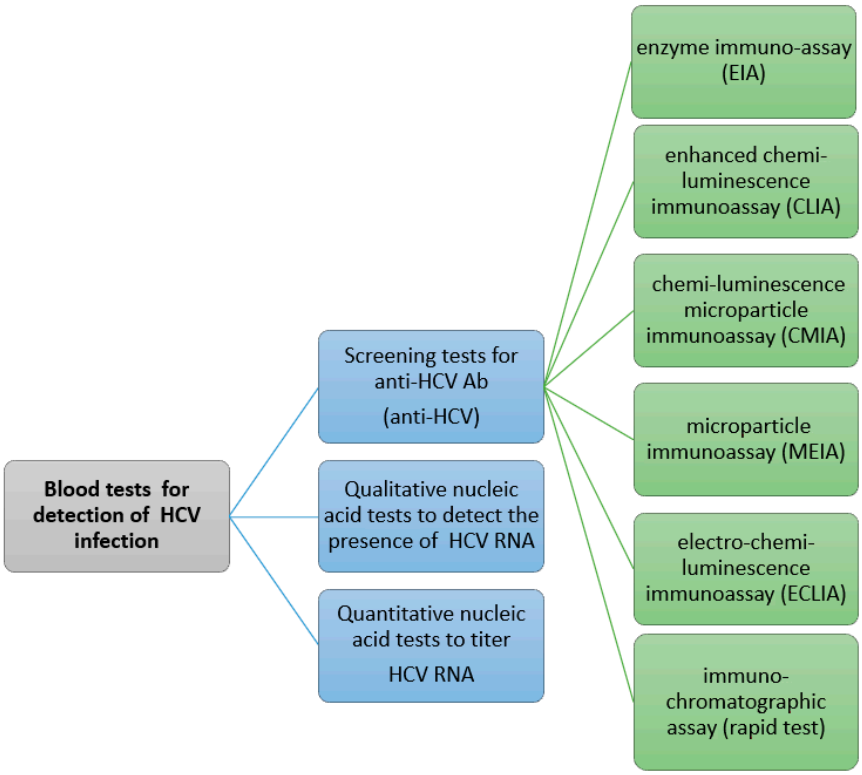

Figure 11. The blood tests that can detect HCV infection. In the WHO principles of good practice for the provision of HCV health services, from the 2018 Guidelines for the care of patients with chronic hepatitis C, 3 new recommendations emerge: (a) the integration of hepatitis testing, care, and treatment with other services to increase the efficiency and reach of hepatitis services; (b) the presence of decentralized services testing and treatment at primary healthcare facilities or harm reduction centres to promote access to care; and (c) task-sharing practices, supported by the training and mentoring of health professionals and colleagues [183]. However, molecular techniques allow for a better understanding of the viral genome organization and function involved in both hepatic and extrahepatic conditions, including liver cancer [184,185].

6. Current Treatment Landscape

In recent years, there has been a radical update of therapeutic prospects for HCV patients. In fact, the previous standard of care was the combination of pegylated interferon and ribavirin with a fair percentage of cure rates, but this caused side effects in a non-negligible number of patients, which led to the suspension of treatment in 10–20% of cases. Cases of induced or exacerbated glomerular damage have been reported with interferon [186,187]. The appearance or increase in proteinuria after the start of interferon therapy was described in a study on average after 12 days caused by mesangial proliferative or membranoproliferative glomerulonephritis or glomerular sclerosis, which disappears after the suspension of anti-viral treatment. The possibility that the drug induces glomerular disease has also been suggested by reports of cases in which interferon was used to treat tumours or other non-liver diseases, and cases of glomerular diseases improved by the administration of the antiviral have been described. Therefore, there is currently insufficient evidence to claim that interferon treatment is contraindicated in the presence of kidney disease. Finally, the clearance of ribavirin is reduced in patients with renal insufficiency, and dialysis does not remove the drug. The use of this antiviral drug is therefore not recommended for subjects with a creatinine clearance rate of less than 50 mL/min [185–188].

Subsequently, the first direct-acting antivirals (DAAs) belonging to the class of protease inhibitors were marketed in 2011: boceprevir and telaprevir. These inhibit the protease that determines the cleavage between the NS3 and NS 4A fragments of the viral polyprotein. Their use involved an association with interferon and ribavirin in patients with genotype 1 hepatitis C (with the exclusion of patients with decompensated cirrhosis) [189]. Their effectiveness, however, was associated with significant side effects that added to those of interferon and ribavirin, and the marketing of new direct-acting antivirals such as boceprevir has limited their clinical use [185,190]. Another possible antiviral is sofosbuvir, a nucleoside inhibitor of NS 5B RNA polymerase, which belongs to the same class as boceprevir and telaprevir, as well as daclatasvir (inhibitor of the NS 5A protein). Another drug in this group was simeprevir, which was discontinued in late 2017 because it was replaced by new drugs with higher cure rates [191,192]. Then, according to pangenotypic therapy protocols, there are pre-established combinations, such as sofosbuvir–ledipasvir (NS 5A protein inhibitor), paritaprevir–ritonavir–ombitasvir (to be combined when needed with dasabuvir), elbasvir (NS5A protein inhibitor)–grazoprevir (protease inhibitor), sofosbuvir–velpatasvir (NS5A protein inhibitor) (the first available pangenotypic drug), glecaprevir–pibrentasvir (protease inhibitor–NS5A protein inhibitor) (a further combination of pangenotypic drugs), and sofosbuvir–velpatasvir–voxilaprevir (inhibitors of the NS5A protein, inhibitors of the NS5B RNA polymerase and of the NS3—NS4A viral protease) [193–200]. Paritaprevir is boosted with ritonavir, while ombitasvir belongs to the same class as daclatasvir and ledipasvir. Dasabuvir is a non-nucleoside inhibitor of NS 5B RNA polymerase. These HCV antivirals allow for healing (defined as sustained viral response, or SVR) in a high percentage of cases over 70% (in certain cases over 90%), which varies according to genotype, degree of fibrosis, and the progression of liver disease (Figure 12) [201,202].

These also are more tolerable and safer than those containing previous-generation protease inhibitors (such as telaprevir and boceprebvir) and allow for shorter treatments in certain patients. Contraindications to direct-acting antiviral (DAA) treatment are rare and are represented by (a) decompensated liver disease (CPT B and CPT C), contraindication to the use of the Glecaprevir/Pibrentasvir, Grazoprevir/Elbasvir, and Sofosbuvir/Velpatasvir/Voxilaprevir regimens; (b) pregnancy and the inability to use effective contraception; (c) Sofosbuvir-based combinations used with caution in renal insufficiency (eGFR <30 mL/min); and (d) the choice of therapeutic regimen based on careful consideration of any concomitant therapies (among these, remember the impossibility of co-administration of DAA and amiodarone due to the risk of severe bradycardia with the possibility of cardiac arrest) [187,203]. Their advantage relies on the possibility of using them in combination with each other without interferon (and in some cases without ribavirin), reducing the toxicity of the therapy and thus increasing the probability of recovery

and compliance for the patient. Therefore, it is possible to effectively treat the most difficult genotypes in those compromised patients and for those who have not had any benefit from interferon (both because they do not tolerate it and/or they cannot take it due to contraindications) (Figure 13) [187,203,204].

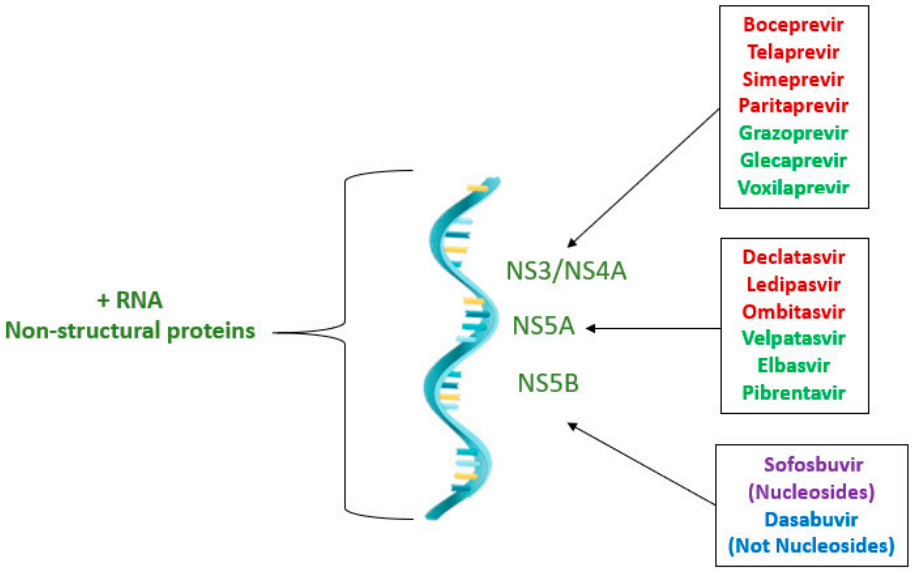

Figure 12. This figure represents the genome of the hepatitis C virus and the targets of the non-structural viral polyprotein with the terminal part of the drug name identifying the mechanism of action, and therefore the class to which it belongs. By convention, the protease inhibitors at the NS cleavage site 3/NS 4A terminate in "-previr", inhibitors of the protein NS 5A terminate in "-asvir", and inhibitors of RNA polymerase NS 5B terminate in "-buvir" (the first-generation antivirals are in red, and the second-generation ones are in green) [201,202]. Credits: original figure by I.A. Charitos.

AASLD treatment recommendations

Naive genotype 1a without cirrhosis	Naive genotype 1b without cirrhosis	Naive genotype 1a with compensated cirrhosis	Naive genotype 1b with compensated cirrhosis
glecaprevir/pibrentasvir 8 weeks ledipasvir/sofosbuvir 12 weeks (8 weeks for patients who are HIV-uninfected and whose HCV RNA level is <6 million IU/m), sofosbuvir/velpatasvir 12 weeks or elbasvir/grazoprevir 12 weeks	elbasvir/grazoprevir 12 weeks glecaprevir/pibrentasvir 8 weeks ledipasvir/sofosbuvir 12 weeks ledipasvir/sofosbuvir (for patients who are HIV-uninfected and whose HCV RNA level is <6 million IU/mL, 8 weeks) sofosbuvir/velpatasvir 12 weeks	ledipasvir/sofosbuvir 12 weeks sofosbuvir/velpatasvir 12 weeks glecaprevir/pibrentasvir 8 weeks	elbasvir/grazoprevir 12 weeks ledipasvir/sofosbuvir 12 weeks sofosbuvir/velpatasvir 12 weeks glecaprevir/pibrentasvir 8 weeks

Figure 13. This figure summarizes the initial treatment of adults with HCV Infection by the American Association of the Study of Liver Diseases (AASLD) [205].

However, during treatment, there is the ability to select resistant viral variants that can be responsible for a therapeutic failure. The various available antivirals differ in the extent of the genetic barrier and the genetic variants of the virus in their ability to adapt and their speed of replication [204,205]. The greater or lesser probability of therapeutic failure derives from the interaction between these factors. When there is therapeutic failure, a new therapeutic strategy must be defined according to the drug history of the individual patient in relation to their clinical condition and age (Figure 14). The isolation of resistant variants and the characterization of mutations can represent a useful cognitive element, even if there is little knowledge to date of the clinical effects of the results obtained in vitro [206–208].

WHO RECOMMENDATIONS		
Points of priority areas: ○ use of DAA treatment in adolescents and children age ≥3 years ○ simplified service delivery (decentralization, integration and task sharing) ○ HCV diagnostics – use of point-of-care (POC) HCV ribonucleic acid (RNA) viral load testing and reflex HCV RNA viral load testing.	Treatment using pangenotypic DAA regimens for children 3 years and above with chronic hepatitis C infection, regardless of stage of disease: ○ Younger children (3–5 years): conditional recommendation; very low certainty of evidence ○ Older children (6–11 years): strong recommendation; moderate/very low certainty of evidence ○ Adolescents (12–17 years): strong recommendation; moderate/low certainty of evidence ○ Adults (≥18 years): strong recommendation; moderate certainty of evidence	The use of the following pangenotypic DAA regimens in younger children (3–5 years), older children (6–11 years) adolescents (12–17 years) and adults (18 years and above): ○ sofosbuvir/daclatasvir* for 12 weeks**: certainty of evidence: high (adults), high (adolescents and older children); very low (younger children) ○ sofosbuvir/velpatasvir for 12 weeks: certainty of evidence: high (adults), low (adolescents and older children); very low (younger children) ○ glecaprevir/pibrentasvir for eight weeks: certainty of evidence: high (adults), moderate (adolescents and older children); very low (young children).

most widely used regimen in adults due to availability of quality-assured, low-cost generics
*** in those without cirrhosis. Treatment for 24 weeks in those who are treatment-experienced or with compensated cirrhosis*

Figure 14. This figure shows the treatment of HCV infection according to the WHO [206,207].

Thus, the antiviral regimens ensure excellent cure rates in patients infected with any viral genotype, regardless of most virus and host characteristics, with failure rates in subjects with decompensated liver disease or who develop drug resistance [208]. However, also due to potential interactions between antivirals and other drugs, when making the decision of whether to subject a patient to therapy with antivirals, one must consider the following measures: careful evaluation of the patient and their home therapy approaches; particular attention should be given to their clinical history (and physical examination) to identify the risk factors, duration of infection, alcohol use, drug use, and use/exposure to hepatotoxic substances (Figure 15). The following comorbidities must also be evaluated: (a) patient age and life expectancy; (b) pathologies that may lead to poor adherence to treatment; (c) extrahepatic manifestations of HCV infection; (d) low creatinine clearance values; (d) biochemical and virological tests (HCV genotype, HCVRNA, HBsAg, HBsAb, HBcAb, and HIV-Ab); (e) instrumental examinations such as upper abdomen ultrasound, Fibroscan, and Esophagogastroduodenoscopy (EGD) (in presence of cirrhosis); and (f) liver biopsy (in selected cases) [209–211].

The search for the presence or absence of cirrhosis is mandatory in the pre-treatment evaluation for direct-acting antivirals (DAAs). Therefore, treatment with glecaprevir/pibrentasvir for 8 weeks may be recommended for treatment-naïve patients with GT-3 CC. It is particularly important to carefully identify the history of decompensation in patients with compensated cirrhosis who should be treated with glecaprevir/pibrentasvir S-Gf-P regimens. The 12-week S-Gf-P regimens of sofosbuvir/velpatasvir are recommended for previous treatment-naïve patients without cirrhosis or with compensated cirrhosis, as mentioned in EASL recommendations. The sofosbuvir/velpatasvir S-Gf-P regimen should be limited to GT-3 naïve patients (considered suitable for S-Gf-P regimens) in treatment without cirrhosis. Assessment of renal function does not appear necessary if the treatments that are applied are anti-HCV S-Gf-P. In this context, the new WHO guidelines recommend the use of DAA according to the conditions of liver function, i.e., the presence of compensated cirrhosis (Figure 16) [207,212].

EASL recommendations on HCV treatment				
Sofosbuvir	**Sofosbuvir/velpatasvir**	***Sofosbuvir/ velpatasvir/ voxilaprevir**	***Glecaprevir/ pibrentasvir**	**Grazoprevir/elbasvir Grazoprevir/elbasvir**
Administered at the dose of 400 mg (1 tablet) once per day.	Available in a two-drug fixed-dose combination containing 400 mg of sofosbuvir and 100 mg of velpatasvir in a single tablet.	Available in a three-drug combination, fixed-dose, containing 400 mg of sofosbuvir, 100 mg of velpatasvir and 100 mg of voxilaprevir in a single tablet. The recommended dose of the combination is 1 tablet taken orally once daily with food.	Available in a two-drug fixed dose combination containing 100 mg of glecaprevir and 40 mg of pibrentasvir. The recommended dose is 3 tablets taken orally once daily with food.	Available in a two-drug fixed-dose combination containing 100 mg of grazoprevir and 50 mg of elbasvir in a single tablet. The recommended dose of the combination is 1 tablet taken orally once daily with or without food.
** Treatment with pangenotypic regimens, including sofosbuvir/velpatasvir or glecaprevir/pibrentasvir, can be initiated without knowledge of the genotype and subtype with a high probability of success.*				

Figure 15. The actual HCV guidelines for DAA treatment by the EASL [101].

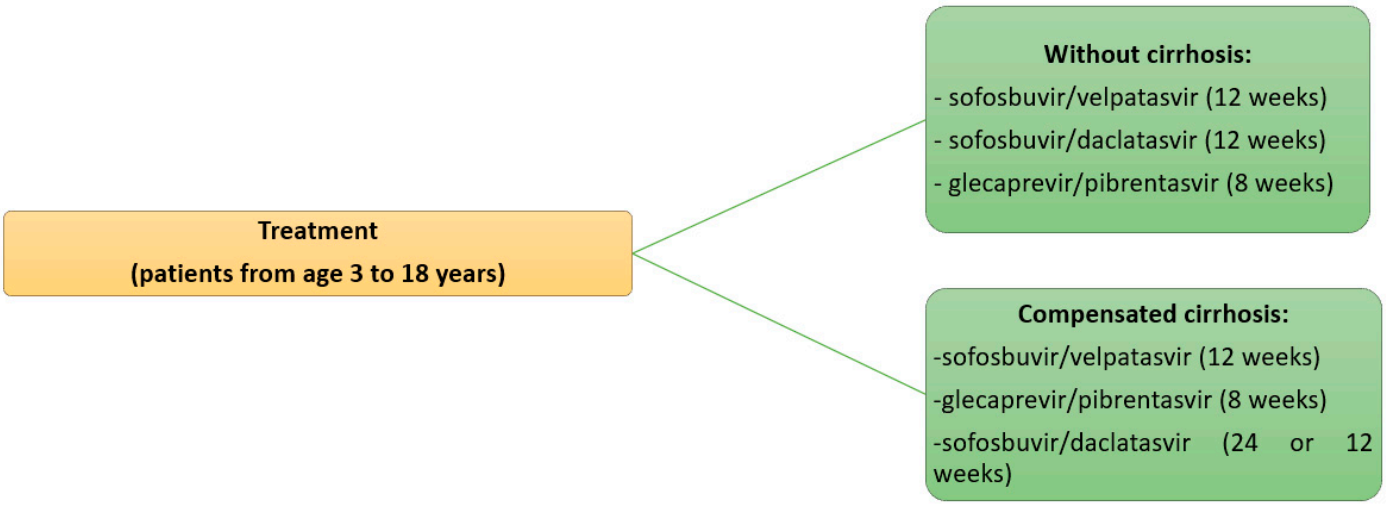

Figure 16. This figure demonstrates the treatment in relationship with the presence of cirrhosis according to WHO guidelines [207].

The availability of therapeutic strategies in transplant patients must be highly effective and with minimal interactions with immunosuppressive drugs, making the use of therapeutic schemes, including inhibitors of the HCV NS3/4A protease, which is not a first choice in this category of patients. It is advisable that an antiviral regimen with the least interaction with immunosuppressive drugs is chosen, especially in the early post-transplant period [208,209]. The sofosbuvir/velpatasvir combination represents the regimen with the best profile of interactions, while the glecaprevir/pibrentasvir combination represents a therapeutic alternative. However, based on the data available in the literature, close clinical monitoring is recommended for the possibility of interactions; additionally, a duration of therapy of no less than 12 weeks is advised [210,212]. For effective prevention of the recurrence of post-transplant HCV, it is necessary to maintain a suppression of viral replication for at least 30 continuous days before transplantation. If the goal of treating the patient with pre-transplant antiviral therapy is to prevent this recurrence, then the estimated waiting time on the list must be carefully evaluated to allow for this goal to be achieved. It also seems important to carefully consider the option of treating patients with decompensated liver disease before transplantation supported by HCV genotype 3, as it represents to date

the genotype with the highest, albeit contained, rate of failure to antiviral therapy, for which no effective retreatment strategies are currently available [213–215]. If the waiting time on the list for liver transplantation exceeds a certain duration (e.g., six months), then patients with decompensated liver disease without HCC (a MELD score > 18–20) should be treated prior to transplantation, depending on their clinical circumstances [216,217].

Organs from positive HCV-RNA donors can be transplanted into either negative or positive HCV-RNA recipients. The treatment of HCV infection in HCV-RNA-negative and HCV-RNA-positive organ recipients is identical to the treatment of chronic hepatitis C in HCV-infected solid organ transplant recipients. Complete and clear informed consent must be obtained prior to transplantation, and post-transplant DAA therapy must be ensured as soon as possible after the stabilization of immunosuppressive therapy. Finally, transplantation of HCV-RNA-positive organs with greater-than-moderate fibrosis is not recommended. Thus, the management of the patient receiving a new transplanted liver includes the prevention of re-infection in the donated organ [218–222]. If HCV is not present in the peripheral blood prior to liver transplantation, then the reinfection of the transplanted organ by HCV can theoretically be prevented, as the infected liver, which is the reservoir of the virus, is removed during surgery [209,210]. Therefore, interferon-free therapy performed before liver transplantation can prevent the reinfection of the transplanted organ after surgery. The main goal of treating HCV infection in transplant patients is to prevent the onset of liver damage related to a recurrence of infection and consequently to increase the survival of the transplanted organ and the patient, thus achieving a sustained virological response (SVR). Early post-transplant treatment can increase the percentage of SVR but prevent the onset of damage to the transplanted organ, improving long-term outcomes. However, there are no published studies regarding the best timing for initiating preventive therapeutic strategy with DAAs after liver transplantation [210,216]. It has been hypothesised that, due to the rapid decline in the number of liver transplants seen after 2014 because of the important effect of DAA, we cannot rule out the epidemiological change of the infection. Most patients treated with DAA were in fact born between 1945 and 1965 (“baby boom”; i.e., in the period of the most births); therefore, their suitability for transplantation is progressively decreasing due to aging. Finally, as reported by the USA CDC, there has not been an effective vaccine development because the virus has multiple genotypes and subtypes, and it mutates rapidly to evade the immune system. However, new vaccines have been developed and explored, which may be candidates based on advanced molecular technology [4,217,218].

It is noteworthy that recent studies have highlighted the role of gut microbiota in facilitating or stopping possible viral hepatitis, but this relationship has not been fully explored or confirmed [223]. The most important data emerging from these studies show that certain microbial compositions of gut microbiota play effective roles in this process, so while lacking a vaccine, its modulation could provide important support for HCV infection prevention and improve time for recovery in patients receiving DAAs [224–226].

7. Conclusions

Hepatitis C virus (HCV) infection remains a major public health problem worldwide; this is due not only to the serious complications of chronic infection (such as cirrhosis, liver failure, and the development of hepatocellular carcinoma) and extrahepatic manifestations, but is also due to a lack of prevention and access to new therapies among certain populations. Chronically infected people have a reduced quality of life compared to the general population. However, thanks to the development of new techniques and the consequences of the biomolecular mechanisms of viral replication and its genotypes, we have been able to develop and establish new drugs and therapeutic schemes for therapy. The goal of treatment is to cure the infection in order to avoid liver-related complications and extrahepatic manifestations. The treatment for chronic HCV infection has proven inadequate for many years. Now, more than 32 years after the discovery of HCV, significant reduction or eradication is still a long way off. Morbidity and mortality due to complications of chronic

hepatitis C could increase in the next few years without adequate prevention and therapy. Treatments today are safer and achieve virus eradication in 90% of patients. In fact, with new antivirals, the end of the Peg-IFN began (since 2014) thanks to their effectiveness; in addition, they can be administered orally and be adapted to each patient, and any HCV genotype countries must coordinate their international shared efforts to reduce the cost of drugs against hepatitis C, which will allow HCV eradication in the future. To achieve the elimination or reduction in hepatitis from HCV, programmed control schemes are required in symptomatic patients, as well as the widespread application of new therapies on a large scale.

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