Chronic hepatitis C: new diagnostic tools and therapeutic strategies
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Citation for published version (APA):
Chapter 1

GENERAL INTRODUCTION

The hepatitis C virus (HCV) is a member of the Flaviviridae family, which includes viruses such as yellow fever, dengue, and West Nile. HCV is a positive-stranded RNA virus that infects the liver and causes hepatitis C. The virus is characterized by a single-stranded RNA genome of approximately 9500 nucleotides, which is encoded by a single open reading frame (ORF) that produces a polyprotein. This polyprotein is then processed by proteases to yield multiple structural and non-structural proteins.

1. HCV STRUCTURE AND MORPHOLOGY

1.1. Genome and polyprotein products

The hepatitis C virus is a single-stranded positive-sense RNA virus and belongs to the family of Flaviviridae. Within the flaviviridae family, which includes the yellow fever and dengue viruses, HCV was classified as a separate genus. The HCV genome consists of a single-stranded RNA molecule (ssRNA) and contains one large open reading frame (ORF) which encodes a polyprotein of about 3000 amino acids (aa). This polyprotein is subsequently cleaved into multiple smaller structural and non-structural proteins by host and viral encoded proteases.

Figure 1:

Schematic representation of the HCV genome, the polyprotein and the subsequently cleaved structural and non-structural proteins.

Source: Reed KE, Rice CM. Molecular characterization of hepatitis C virus.

Chapter 1

GENERAL INTRODUCTION
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After the introduction of hepatitis B surface antigen (HbsAg) screening in the 1970s, it became apparent that another 'serum' hepatitis (non-A, non-B hepatitis) was responsible for a considerable proportion of post-transfusion hepatitis cases. Support for this concept came from studies in drug abusers and hemophilia patients, which described multiple attacks of hepatitis in the same individual.

For cloning of the hepatitis C virus (HCV) sequence, high-titered pooled plasma from chimpanzees with non-A, non-B hepatitis was used. Nucleic acid was extracted from this plasma and a denaturation step was included to allow both DNA and RNA to be used as templates for reverse transcription with random primers into complementary cDNA. Millions of resultant cDNAs were inserted into a cloning vector. The expressed proteins were screened using serum from patients with non-A, non-B hepatitis. This led in 1989 to the discovery of the 5-1-1 clone. Viral RNA could be characterized further by determining overlapping cDNA clones and by comparison with distantly related viruses. Because the majority of non-A, non-B hepatitis cases proved to be caused by the newly characterized virus, it was subsequently called hepatitis C virus.

1. HCV STRUCTURE AND MORPHOLOGY

1.1. Genome and protein products

The hepatitis C virus is a single-, positive-stranded RNA virus and belongs to the family of flaviviridae. Within the flaviviridae family, which includes the pestivirus and flavivirus genera, HCV was classified as a separate genus. The HCV genome consists of about 9500 nucleotides (nt) and contains one large open reading frame (ORF) which encodes a polyprotein of about 3000 amino acids (aa). This polyprotein is subsequently cleaved into multiple smaller structural and non-structural proteins by host and viral encoded proteases.

Figure 1:
Schematic representation of the HCV genome, the polyprotein and the subsequently processed structural and non-structural proteins.

The organization of the HCV genome, the encoded proteins, and the processing into various viral proteins are schematically shown in figure 1. The 5' untranslated region (5'UTR) of about 341 nt precedes the coding sequence and is highly conserved among the various HCV isolates. This region shows extensive secondary structures with several stems and loops, which have been predicted to play a role in the translation of the viral genome. It was shown that the 5'UTR and part of the region encoding for the core protein (nt 40-370) contains an internal ribosomal entry site (IRES), which specifically directs ribosomes to a start codon for translation initiation.

In the processing of the HCV polyprotein, three categories of cleavage can be distinguished (figure 1). The structural proteins are cleaved from the polyprotein by the cellular enzyme signalase. NS2/NS3 cleavage is dependent on zinc and polypeptide sequences on both sides of the cleavage site. The third type of cleavage is mediated by a viral proteinase which is located in the NS3 protein. The NS4A protein acts as a cofactor for the NS3 proteinase. This proteinase complex directs intramolecular cleavage (NS3/NS4A) as well as intermolecular cleavages (NS4A/NS4B, NS4B/NS5A, NS5A/NS5B). The crystal structure of the NS3 proteinase domain, which has recently been determined, can be an important tool for the development of specific proteinase inhibitors for the treatment of hepatitis C.

Table 1: Summary of the known functions of the HCV proteins.

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core</td>
<td>nucleocapsid</td>
</tr>
<tr>
<td>E1/E2</td>
<td>envelope</td>
</tr>
<tr>
<td>P7</td>
<td>unknown, associated with E2</td>
</tr>
<tr>
<td>NS2</td>
<td>transmembrane protein in endoplasmatic reticulum (ER)</td>
</tr>
<tr>
<td>NS3</td>
<td>protein with a complex crystal structure, containing domains with three different enzymatic activities: proteinase, ATP-dependent nucleotide triphosphatase and helicase</td>
</tr>
<tr>
<td>NS4A</td>
<td>cofactor for NS3 proteinase, anchorage for replication complexes</td>
</tr>
<tr>
<td>NS4B</td>
<td>unclear, probably functional in HCV replication complexes</td>
</tr>
<tr>
<td>NS5A</td>
<td>unclear; it may play a role in reduced susceptibility to the host immune response, by interaction with the IFN-induced protein kinase (PKR), which is a mediator of IFN-induced antiviral resistance. This would be an explanation of the sensitivity of HCV to IFN after a mutation in a discrete region of NS5A</td>
</tr>
<tr>
<td>NS5B</td>
<td>RNA dependent RNA polymerase</td>
</tr>
</tbody>
</table>

Sequence analysis of the 3'UTR region revealed a tripartite structure, including a conventional 3'end, a poly(U) region which is extremely heterogenous between different isolates, and a highly conserved region. Computer modelling has predicted that this...
conserved region can fold in a stem-loop structure, which might play an important function in viral replication. Besides NS3 proteinase, other enzymes and structures which are specific for HCV replication, such as the NS3 helicase, the NS5B RNA dependent RNA polymerase and the 5'UTR IRES, might be important targets for HCV specific anti-viral treatment.

1.2. Sequence heterogeneity
In an HCV infected patient, the turnover of HCV virions has been found to be approximately $3.7 \times 10^9$ virions per day. The half life of the virus in humans is around 7 hours (personal communication, Gretch 1996). During viral replication errors may occur and consequently mutations may develop. Hence, HCV infected patients typically carry a population of HCV quasispecies. Besides this error-prone RNA replication, the evolution of a quasispecies population within a person may be influenced by the duration of infection, anti-viral treatment and immune selection. When HCV isolates from various geographical areas are compared, a considerable sequence heterogeneity is observed. The extent of sequence heterogeneity is not the same in different parts of the HCV genome. The most variable sequences are situated in the envelope region and part of the 3'UTR region (55% and 26% homology respectively). The most conserved sequences are situated in the 5'UTR of the HCV genome (92% homology), which makes this region suitable for HCV-RNA detection by amplification techniques. Calculated substitution rates in the HCV genome vary from nearly zero in highly conserved regions (e.g. 5'UTR) to $2.97 \times 10^{-3}$ per site per year in highly variable regions (e.g. 3'UTR). Calculations of the time of divergence between HCV isolates may be important in finding routes of transmission.

Figure 2:
Phylogenetic tree of the NS5B sequences in HCV isolates from several countries. The length of the lines between the different isolates represents the phylogenetic distance. Source: Simmonds P. Variability of the hepatitis C virus genome. In: Reesink HW (Ed), Hepatitis C virus, Current Studies in hematology and blood transfusion 1998, Basel, Karger; no 62: 38-63.
Simmonds et al. have classified different HCV isolates into six major genotypes (1-6) and a number of subtypes. The phylogenetic analysis for this classification system was initially performed on sequences of the NS5 region (figure 2), but this analysis was also applicable to other regions of the genome, including the 5'UTR. A number of HCV genotyping assays based on HCV-RNA amplification techniques are published.

Worldwide, the different HCV genotypes are not equally distributed, as is shown in the geographical survey of HCV genotypes (Table 2). Applying the known substitution rates of the different HCV regions to HCV genotypes, it can be calculated that the divergence of virus types started to occur 300-500 years ago. This viral history might explain the current geographical distribution pattern of HCV. A long period of endemic infection is likely if a single HCV genotype with numerous subtypes is prevalent in a specific area (e.g. Northern India, type 3; Egypt, type 4; Southern Africa, type 5; South-East Asia, type 6). If, on the other hand, more genotypes with a few subtypes are prevalent, recent introduction of virus from endemic areas is likely (e.g. Western Europe and USA, types 1,2,3 and Japan, types 1,2). Also, the origin of newly emerging viral strains can be determined. Genotype 3 is usually found in young individuals with a history of intravenous drug abuse. The widespread prevalence of genotypes 1 and 2 might be due to large scale parenteral medical interventions, such as the administration of yellow fever and vaccinia virus vaccines.

<table>
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<tr>
<th>Region</th>
<th>no of samples</th>
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<th>3</th>
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<td>14</td>
<td>28</td>
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<td></td>
<td></td>
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<tr>
<td>Netherlands, Hungary)</td>
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</tr>
<tr>
<td>Taiwan, Japan</td>
<td>133</td>
<td>63</td>
<td>37</td>
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<tr>
<td>Hong Kong, Macau</td>
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<td>8</td>
<td>7</td>
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<td>29</td>
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<td>50</td>
<td>1</td>
<td>49</td>
<td></td>
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<tr>
<td>Malaysia, Thailand)</td>
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<td>8</td>
<td>4</td>
<td>56</td>
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</tr>
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<td>USA (Pittsburg)</td>
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<td>76</td>
<td>17</td>
<td>5</td>
<td>2</td>
<td></td>
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<tr>
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<td>19</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2

HCV genotype distribution in 15 countries as identified by restriction fragment length polymorphism of the 5'UTR.

1.3. **Morphology**

Identification of HCV structure is delayed by the lack of an efficient in-vitro replication system. Recently, some progress has been made with an in-vitro infection system using Daudi cells (a human B-cell line). In this Daudi cell system, virion particles of...
approximately 50 nm are visualized in cytoplasmatic vesicles by electron microscopy. Apart from studies on HCV morphology, an in-vitro HCV replication system could provide infectious recombinant HCV virions for studies of the full-length genome.

From several studies of the density of viral particles, HCV infected serum was shown to contain a low density, infectious fraction and a high density, non-infectious fraction, both of which associated with HCV. The density of HCV in the infectious fraction of serum suggests an association with low density lipoprotein (LDL) and very low density lipoprotein (VLDL). Therefore, the entry of HCV into hepatocytes may occur via the LDL receptor. The density of HCV in the non-infectious fraction of serum suggests an association with anti-viral antibodies. Recently, it was found that HCV particles, via the envelope-2 (E2) protein, can bind to CD81, a receptor on different human cell types. The identification of the CD81 and HCV interaction may have important implications for the pathogenesis of HCV-mediated disease and future treatment of HCV infection.

2. METHODS FOR DETECTION OF HCV INFECTION

2.1. Viral and immunological parameters following HCV infection

The first assays for the detection of HCV infection were antibody assays that recognized C100 antigen. HCV antibody assays were further developed and refined. Currently, third generation screenings- and confirmation assays are generally applied in Western Europe. The IgM antibody response was shown to be absent, late or persistent after primary infection, and therefore is not a reliable tool for the diagnosis of early HCV infection. HCV IgG antibodies become detectable after a window period of about 82 days (range 54 to 192 days) (figure 3). This window period can be shortened to about 23 days when HCV-RNA detection methods are applied. The window period has important implications for blood transfusion safety. Furthermore, antibody assays can't distinguish between ongoing and resolved infection; this distinction is important for patient counseling. Therefore, HCV-RNA amplification assays are generally applied to confirm HCV infection.

Figure 3:
Schematic representation of the chronology of appearance of HCV-RNA, HCV antibodies and elevated ALT levels in serum after infection with HCV.
2.2. First- and second-generation antibody assays

In figure 4 the nomenclature and localisation of proteins used as antigens in various HCV antibody assays are shown in figure 4.
First generation anti-HCV assays applied recombinant C100 antigen and were characterized by low specificity and limited (60-80%) sensitivity, because anti-C100 develops relatively late during the course of HCV infection\(^5\). \(^\text{51-58}\)

Since 1991, the most commonly used second-generation anti-HCV screening assay was an ELISA (ELISA-2), which utilized recombinant antigens from the core (C22) and NS3/NS4 region (C100/C200). With the application of this assay, the sensitivity of detection of HCV infection has increased. The additional antigens in this assay accounted for earlier detection of seroconversion (at about 6 weeks) in the course of the HCV infection\(^6\).\(^\text{59-60}\). Because many false-positive reactions were observed using the ELISA-2, second generation HCV confirmatory assays were developed. The most commonly used assay, RIBA-2, applied recombinant antigens from core (C22), NS3 (C33), and NS4 (C100 and 5-1-1). This assay proved to be much more specific than the ELISA-2\(^6\).\(^\text{61-62}\). However, a number of indeterminate reactivities (only one antigen reactive) was produced by the RIBA-2\(^6\).\(^\text{63-64}\).

2.3. Third-generation antibody assays

Since 1993 third-generation anti-HCV screening assays have been available. Besides antigens from the second-generation ELISAs, an antigen from the NS5 region was included. In seroconversion panels, the third-generation ELISAs have shown an improved sensitivity, largely due to improvement of the antigens which were incorporated in the second generation assays\(^6\).\(^\text{65-67}\). However, the addition of the NS5 antigen resulted in a new group of possible false-reactive anti-HCV test results\(^6\).\(^\text{65-67}\). The third-generation confirmatory assays were introduced shortly after the screening assays in 1993. A frequently used assay (HCV-RIBA-3; Chiron Corporation, Emmeryville, CA, USA) utilizes antigens from the core (C22), NS3 (C33), NS4 (C100) and the NS5 (NS5) region. Synthetic peptide mixes have been used as substitutes for the recombinant antigens C22 and C100. RIBA-3 resolved the majority of indeterminate results obtained with RIBA-
2, due to improved sensitivity of the C33 and the C100 antigens (chapter 3). The introduction of the NS5 antigen, which has a lower sensitivity and specificity than the other antigens, was associated with an additional group of aspecific reactive samples (chapter 3). In another third-generation anti-HCV confirmation assay, designated Liatek-III (Innogenetics, Belgium), a synthetic E2/NS1 antigen is applied in addition to the antigens mentioned above. This E2/NS1 antigen did not contribute to the improved sensitivity of the assay. Experimental E2 antigens were shown to be highly immunoreactive, but did not shorten the serological window period after HCV infection.

One of the problems concerning anti-HCV testing is the antigenic variation among HCV genotypes. As currently used in antibody assays, antigens are almost all based upon HCV genotype 1. Thus, the third-generation assays may be suboptimal for screening populations in which other genotypes are prevalent. Therefore, it has been proposed that type-specific components be included in the next generation anti-HCV assays.

2.4. Nucleic acid amplification techniques
Detection of the viral genome using nucleic acid amplification techniques (NAT) has become an important tool in HCV testing. For clinicians HCV-NAT testing is important for distinguishing ongoing and resolved infection and for monitoring treatment. For epidemiologists NAT testing can be helpful in studying possible sources of transmission. HCV-NAT tests will be introduced in blood safety testing in the near future to minimize HCV transmission during the serological window phase. An introduction to HCV-RNA amplification techniques and their application in various settings for HCV testing is presented in chapter 2 of this thesis.

3. NATURAL HISTORY OF THE HCV INFECTION
3.1. Epidemiology and transmission of HCV infection
At present, HCV accounts for 20% of cases of acute hepatitis, 70% of cases of chronic hepatitis and 30% of cases of end-stage liver disease in the USA. As the hepatitis C virus is a blood born virus, transfusion of blood and blood products were important routes of transmission before the introduction of anti-HCV screening in 1989, in Western countries. At present, circumstances associated with acute hepatitis C (in the USA) are intravenous drug use (IVDU) (43%), low socio-economic level and promiscue life style (30%), sexual/household relationship with HCV positive patients (18%), occupational exposure (4%), blood transfusions (4%), and unknown (1%). In 49% of cases no clear parenteral risk factor is found. In a study among volunteer blood donors, conducted between 1991-1994 in the USA, anti-HCV positivity was associated with a history of blood transfusion, intranasal cocaine use, IVDU, sexual promiscuity and ear piercing in men. In this study, a parenteral risk factor was found in 75% of anti-HCV positive individuals. Whether or not HCV is transmittable sexually is still controversial. Support for a role of sexual transmission comes from studies reporting HCV sequence homology between isolates from sexual partners and studies reporting acute hepatitis C after previous sexual exposure. However, other shared risk factors can't be excluded in these studies. In this context, the possible facilitating role of HSV-2 infection in the sexual transmission of HCV, as described by Shev et al, is of interest. On the other hand, in sex-partner studies which carefully excluded other risk factors for HCV infection, no evidence of sexual transmission of HCV was found. Furthermore, in groups at high risk for sexually-
transmitted diseases, the prevalence of HCV is much lower than that of other sexually-transmitted infections. Only in the presence of a high HCV-RNA load, as in concomitant HIV infection, has sexual transmission of HCV been established. Based on these findings, it is generally recommended that percutaneous exposure to blood or blood-contaminated items (e.g. razor, toothbrush) should be avoided. Barrier precautions for stable, monogamous sexual partners are generally not recommended.

When sustained infections in the neonate are studied, perinatal spread of HCV infection appears to be uncommon. As the vertical transmission rate correlates with the HCV-RNA load, the risk of vertical HCV transmission is much higher when there is concomitant HIV infection (18%) than in HCV monoinfection (4.5%). A more recently recognized transmission route is the iatrogenic transmission of HCV during invasive medical procedures (other than blood transfusions) and nosocomial transmission to immune compromised patients with skin- and mucosal lesions. Other potentially important iatrogenic transmission routes are intravenous or intramuscular injections and vaccination programmes in which no disposable needles and syringes are used.

Nevertheless, a large number of HCV cases are of unknown origin or ‘community acquired’, especially in developing countries. Other possible transmission routes have been postulated, such as shaving by a barber or hairdresser, traditional or cosmetic tattooing, and traditional acupuncture or other skin perforating folk remedies. Furthermore, HCV transmission through a biologic vector (e.g. an insect) has been postulated. Some evidence for this was found in a study among the inhabitants of an isolated Italian village: a correlation between improved sanitary conditions in the population and a decreased incidence of HCV infection was observed.

3.2. Natural course of HCV infection

While acute HCV infection is anicteric and subclinical in 90% of cases, about 10% of HCV-infected patients develop jaundice and/or other clinical symptoms. HCV infection is persistent in 80-90% of patients. Development of fulminant hepatitis C is very rare. Recently, it was postulated that not all individuals exposed to HCV, develop acute HCV infection and anti-HCV. In anti-HCV negative, non-viremic persons with occupational exposure to HCV, and in anti-HCV negative, non-viremic spouses of HCV-infected patients, HCV-specific lymphocyte reactivity was observed. Whether these reactivities represent protective immunity is currently unclear.

Clearance of acute HCV infection or the development of chronic HCV infection is dependent on viral and host factors. Viral factors include susceptibility to the humoral and cellular immune response of the host. Host factors include the cellular and humoral immune response to the virus. At present, the exact mechanisms responsible for development of the chronic HCV carrier state are still unclear.

The relevance of various viral and host factors to the clinical course of chronic HCV infection has been studied. The mode of HCV transmission may be important since transfusion-acquired HCV appears to be associated with more aggressive inflammatory activity and a higher viral load than HCV infection transmitted by IVDU. The relevance of the different HCV genotypes is still controversial: in various studies genotype 1 has been associated with severe progressive liver disease but in other studies the HCV genotype did not appear to influence the course of the disease. In several studies the viral load and diversity of HCV quasispecies increased with progression of chronic liver
The significance of ALT elevation in chronic hepatitis C is controversial. Studies in anti-HCV positive individuals have shown that HCV viremia correlates with the histological severity of chronic liver disease, regardless of ALT levels. On the other hand, viremic patients with normal liver histology and persistently normal ALT levels have been observed, suggesting a benign HCV carrier state in some individuals.

In a recent study among 2235 HCV positive patients, it was found that age at the onset of infection may be an important factor in relation to the course of infection: among patients infected below the age of forty, the rate of progression to fibrosis and cirrhosis was significantly lower than that among patients infected above the age of forty. Furthermore, the rate of progression to fibrosis was extremely low among patients infected in their first decade, whereas this rate was extremely high for patients infected above the age of fifty. Probably, some immune tolerance develops when HCV infection occurs at a very young age.

In additional studies, chronic HCV infection was found to be strongly associated with development of cirrhosis and hepatocellular carcinoma (HCC). In Figure 5 the mean interval from blood transfusion to the diagnosis of HCV-associated liver disease is presented; the data are from a cross-sectional study of 131 post-transfusion HCV positive patients with a mean age of 57 years (range 21-81) at the time of transfusion. Cirrhosis developed in 45.8% of the patients after a mean period of 20.6 years and HCC in 10.7% after a mean period of 28.3 years.

**Figure 5:**
Cross-sectional study showing the mean (±SD) interval from HCV transmission by blood transfusion and the diagnosis of HCV related diseases in 131 patients.
Various extra-hepatic manifestations of HCV infection have been described. These include mixed-cryoglobulinemia type II and various auto-immune phenomena (e.g. membranoproliferative glomerulonephritis, vasculitis, Sjögren’s syndrome and auto-immune thrombocytopenia). The exact pathogenic mechanisms responsible for these extrahepatic manifestations are still unclear; possibilities include cross-reactivity between viral and auto-antigens and formation of virus-antibody complexes. Furthermore, in several studies HCV infection has been shown to be associated with the development of certain subtypes of B-cell non-Hodgkin lymphoma (NHL), particularly in patients with mixed-cryoglobulinemia type II. HCV infection has been shown to be more prevalent among patients with B-cell NHL than among patients with other hematologic malignancies, and HCV-RNA and HCV-related proteins have been found among epithelial cells of a parotid NHL, and also in bone marrow and lymph node specimens from NHL patients. However, the possible mechanisms of this association with B-cell NHL remain unclear.

3.3. Treatment
Since the characterization of HCV about 10 years ago, progress with treatment of HCV-infected patients has been made. At present, chronic HCV infection can be treated with IFN-alpha with a successful outcome in 20-40% of cases (chapter 9+10). Recent studies of combination treatment with IFN-alpha and Ribavirin have shown promising results. Furthermore, specialized techniques, e.g. measurement of viral load and determination of genotype, have become available to enhance routine patient monitoring. It is anticipated that additional techniques that are relevant for monitoring these patients, will become available in the near future (e.g. determination of drug resistance). A review of treatment of HCV-infected patients is presented in chapter 9 of this thesis.

REFERENCES


