HEPATITIS C VIRUS INFECTION IN LIVER TRANSPLANT PATIENTS.

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1. Prologue

There are many persons who I would like to thank because of their support and knowledge during the realization of this dissertation.

First of all I would like to thank my thesis supervisor Prof. Dr. Philip Meuleman for his support and guidance during the two-year lasting process of this dissertation.

In addition, I would like to thank both of my parents for giving me the opportunity to realize this education and for their endless support and good care.

I also want to thank my brother Klaas, sister Heleen and all of my other friends for their friendship, cheers and their support at the times most needed. And last but definitely not least Matthias, for his uplifting stories, reviving coffee and helpful advice.
## 2. List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AR3</td>
<td>antigenic region 3</td>
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<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
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<td>ALT</td>
<td>alanine aminotransferase</td>
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<td>CD81</td>
<td>cluster of differentiation 81</td>
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<td>CLDN1</td>
<td>claudin-1</td>
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<td>CNI</td>
<td>calcineurin inhibitors</td>
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<td>CYP3A4</td>
<td>cytochrome P450 3A4</td>
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<td>DC</td>
<td>dendritic cell</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<td>HCV</td>
<td>hepatitis C virus</td>
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<td>HCVpp</td>
<td>hepatitis C virus pseudoparticle</td>
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<td>HVR</td>
<td>hypervariable region</td>
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<td>IFN</td>
<td>interferon</td>
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<td>IFNAR</td>
<td>interferon-α/β receptor</td>
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<td>IL28B</td>
<td>interleukin-28B</td>
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<td>IMPDH</td>
<td>inosine monophosphate dehydrogenase</td>
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<td>IRF</td>
<td>interferon regulatory factors</td>
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<td>ISG</td>
<td>interferon-stimulated gene</td>
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<td>ISGF</td>
<td>interferon-stimulated gene factor</td>
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<tr>
<td>ISRE</td>
<td>interferon-stimulated response element</td>
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<tr>
<td>IU</td>
<td>international unit</td>
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<td>Jak</td>
<td>janus kinase</td>
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LDLr  low-density lipoprotein receptor
LTx  liver transplantation
mAb  monoclonal antibody
nM  nanomolar
NK  natural killer
NS3  nonstructural protein 3
OCLN  occludin
PAMP  pathogen-associated molecular patterns
PCR  polymerase chain reaction
Peg  pegylated
PP2A  protein phosphatase 2A
RBV  ribavirin
RIG-I  retinoic-acid-inducible gene I
RNAi  RNA interference
siRNA  small interfering RNA
SOCS3  suppressor of cytokine signaling 3
SR-B1  scavenger receptor class B type 1
STAT  signal transducer and activator of transcription
SVR  sustained virological response
Th1/2  T helper 1/2
TLR  toll-like receptor
TRIF  toll-interleukin-1 receptor-domain-containing adaptor inducing IFN-β
USP18  ubiquitin-specific peptidase 18
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4. Abstract

Introduction
The problem of hepatitis C virus infection and its development to chronic HCV, liver cirrhosis or hepatocellular carcinoma is an important research topic nowadays. Reinfection of the liver graft after transplantation is almost unavoidable, even if the transplant patients receive antiviral therapy in order to prevent HCV recurrence. By mechanism of mutation, HCV is capable of evading host immune response and becoming resistant to antiviral therapy. Standard therapy nowadays is the combination of pegylated interferon alpha and ribavirin and this therapy is proven to be efficient. However, because of severe side effects and limited sustained virological response, new antiviral agents are being developed, of which monoclonal antibodies, protease inhibitors and RNA-dependent RNA polymerase inhibitors are the most important. These antiviral agents attack specific targets and thereby interact with the HCV life cycle.

Methodology
To gain insight in the processes and targets on which antiviral therapy can act, sources were obtained using the electronic databases PubMed and ISI Web of Knowledge. This thesis is a systematic literature review concerning the development of an effective antiviral therapy against hepatitis C virus. Literature was found mainly by using following terms combined with “Hepatitis C virus” MeSH term. “Standard therapy”, “interferon receptor therapy”, “therapy receptor block”, “Monoclonal antibodies”, “AP33”, “CD81 receptor” and “SR-B1 receptor”.

Results
Antiviral therapy can be initiated before transplantation to reduce viral load to a minimum, shortly after transplantation when viral load is at its lowest and fibrosis is not yet reported or finally, when recurrence has already occurred. This last approach intends to slow down viral progression. The combination therapy of pegylated interferon alpha and ribavirin, two immunomodulators, acts on the mechanisms developed by HCV to escape host immune response. Secondly, monoclonal antibodies against HCV envelope glycoproteins (E1 and E2) and cell membrane receptors, of which CD81 and SR-B1 are the most important, interact with their specific targets and thereby inhibit the viral entry process. MAb AP33 and mAb 3/11 are the most effective monoclonal antibodies so far. Next to these two main components of viral entry, monoclonal antibodies are also developed to act against co-receptors and lectins. The second major step in the viral life cycle after entering the hepatocyte is the viral replication on which protease inhibitors, like boceprevir and telaprevir and RNA polymerase
inhibitors, like silymarin act. By blocking this crucial replication process, HCV is no longer able to reproduce and viral load is reduced.

**Conclusion**

The addition of one of the new antiviral therapies results in an amelioration of the virological response. However, adding an antiviral agent like a protease inhibitor entails several adverse effects. Therefore, the combination of pegylated interferon alpha and ribavirin remains standard antiviral therapy until now, but the newly developing therapies offer promising results to reach a higher virological response.
Nederlandstalige abstract

Inleiding

Hepatitis C virus infectie en de evolutie van acute naar chronische infectie, levercirrose of hepatocellulair carcinoom is een belangrijk actueel onderwerp voor onderzoek. Herinfectie van het donororgaan na levertransplantatie is zo goed als onvermijdelijk, ook al wordt een antivirale therapie opgestart om net dit te voorkomen. Hepatitis C virus is in staat tot mutatie en ontwikkelt op die manier resistentie tegen zowel het immuunantwoord van de gastheer en de antivirale therapie. De standaard behandeling tot op heden bestaat uit de combinatie van gepegyleerd interferon alfa en ribavirine. Deze therapie heeft zijn werkzaamheid bewezen, maar door de aanwezigheid van ernstige nevenwerkingen en een toch slechts beperkte blijvende virologische respons is men gestart met de ontwikkeling van nieuwe antivirale medicamenten waaronder monoclonale antilichamen, protease inhibitoren en RNA polymerase inhibitoren de belangrijkste zijn. Deze antivirale middelen vallen specifieke doelwitten aan en beïnvloeden de virale levenscyclus op deze manier.

Methodologie

Om inzicht te verwerven in de verschillende mechanismen waarop antivirale therapie kan inwerken, werden bronnen gezocht via de elektronische databanken PubMed en ISI Web of Knowledge. Deze literatuurstudie handelt over de ontwikkeling van een effectieve antivirale therapie tegen het hepatitis C virus. Daarbij werd literatuur voornamelijk bekomen met behulp van volgende zoektermen gecombineerd met de MeSH term “Hepatitis C virus”. “Standard therapy”, “interferon receptor therapy”, “therapy receptor block”, “Monoclonal antibodies”, “AP33”, “CD81 receptor” en “SR-B1 receptor”.

Resultaten

Antivirale therapie kan op drie verschillende momenten in het behandelingproces gestart worden. Men kan de therapie starten vóór de transplantatie met als doel het aantal viruspartikels tot een minimum te herleiden. Anderzijds kan men de therapie aanvangen net na de transplantatie, alvorens fibrose gedetecteerd kan worden en de virale lading op zijn laagst is. Tenslotte kan men de antivirale therapie aanwenden als de HCV infectie zich reeds gemanifesteerd heeft na transplantatie. Het voornaamste doel van dit laatste beleid is het vertragen van de HCV progressie.

De therapie bestaat uit twee pijlers. Enerzijds de immunomodulatoren, gepegyleerd IFN-α gecombineerd met ribavirine die inwerken op de mechanismen die HCV heeft verworven om aan de immuunrespons te ontsnappen. Anderzijds de nieuwe antivirale middelen die op het virus zelf inwerken. Monoclonale antilichamen inhiberen het entry mechanisme door in te werken op zowel de
Envelope glycoproteïnen E1 en E2 als de cellmembraan receptoren, waaronder CD81 en SR-B1 de belangrijkste zijn. MAb AP33 en mAb 3/11 zijn de meest doeltreffende monoclonale antilichamen tot nu. Naast de envelope glycoproteïnen en de receptoren zijn onder meer co-receptoren en lectines mogelijke doelwitten van monoclonale antilichamen. De tweede grote stap in de virale levenscyclus een het virus de levercel is binnengedrongen, is de virale replicatie waarop protease inhibitoren, zoals boceprevir en telaprevir, en RNA polymerase inhibitoren, zoals silymarin, aangrijpen. Door dit cruciale proces te blokkeren is HCV niet langer in staat zich te repliceren en op die manier wordt de virale lading gereduceerd.

Conclusie

Het toevoegen van één van de nieuwe antivirale middelen resulteert wel degelijk in een verbetering van de virologische respons. Toch brengen deze medicijnen nog verscheidene, niet te verwaarlozen nevenwerkingen met zich mee. De therapie bestaande uit de combinatie van gependeglyeerd IFN-α en RBV blijft voorlopig nog de gouden standaard als behandeling voor HCV patiënten, maar de nieuwe behandelingen in ontwikkeling tonen veelbelovende resultaten om een hogere blijvende virologische respons te behalen.
5. Introduction

At present, Hepatitis C Virus (HCV) is a worldwide growing health problem. In 2008 3-4 million new infections were reported and WHO estimated in 2011 that 180 million people (3% of the world population) are infected with HCV. Seventy-five to ninety percent of patients infected with hepatitis C virus progress to chronic hepatitis C virus infection. Progression to liver fibrosis, cirrhosis and hepatocellular carcinoma are dangerous complications of HCV that emphasize the importance of the disease and the need for a reliable therapy.

The incidence in developing countries is still growing. Transmission by parenteral routes, such as blood transfusion, intravenous drug use, contaminated medical equipments and tattoos is the most important and frequent route of infection. Sexual or perinatal transmission on the other hand is rather rare. Since the 1990s there has been reported a decrease in incidence of acute hepatitis C in the USA and Western Europe due to the improved blood donor screening, needle exchange and education of injecting drug users (Maheshwari et al., 2008).

Hepatitis C virus is an enveloped positive-stranded RNA virus from the Flaviviridae family. There are 6 different genotypes and the virus is subdivided in over 70 subtypes. This variety of genotypes and subtypes results in many different responses on therapies and different evolution of disease and persistence. HCV has a high replicative rate and the HCV-polymerase lacks a proof-reading function.
by which replication errors can be corrected. These two characteristics of HCV result in a wide range of viral variants that may lead to persistence of the virus, caused by evasion of host immune response (Gale and Foy, 2005).

The virus encodes a single polyprotein of approximately 3000 amino acids, which consists of structural and non-structural proteins. The envelope glycoproteins and core proteins are structural genes and the non-structural genes include proteases, helicases and the RNA-dependent RNA-polymerase, responsible for the assembly of the ribonucleotides (Fig. 1). The E2 envelope glycoprotein contains two regions, designated HVR1 and HVR2 which have the characteristic of being hypervariable and this may explain the variation of the hepatitis C virus.

Clinical diagnosis of HCV infection is difficult because of absence of specific symptoms. A raise in concentrations of liver enzymes could suggest an infection, but is not HCV specific as these enzymes also rise with other liver diseases. It is an indication for further research though. Detection of antibodies against HCV by immunoassay is an unreliable way to identify infection as the appearance of antibodies may be delayed at the onset of symptoms. The most sensitive tests to detect HCV infection is the detection of HCV RNA by polymerase chain reaction (PCR), branched DNA assays (bDNA) and transcription-mediated amplification (TMA). Liver biopsy remains the golden standard in the detection of fibrosis and inflammation.

HCV associated liver disease is the most common cause for liver transplantation and even though transplantation offers an effective treatment, reinfection of the liver graft and accelerated disease progression due to immunosuppression is an almost inevitable complication. This highlights the need of new therapies that prevent reinfection of liver grafts after orthotopic liver transplantation.

At the moment the “standard therapy” of recurrent HCV in LTx patients consists of the combination of ribavirin and pegylated interferon. However, the tolerability of this therapy is rather poor and adverse effects often lead to the need of dose reduction. Furthermore, a sustained virological response (SVR) is only achieved in 25-45% of all cases (Peveling-Oberhag et al., 2010).

Because of this poor tolerability and low effectiveness of standard therapy, a lot of research is being done to develop new therapies that prevent reinfection and hepatitis C virus recurrence. The mechanisms by which reinfection occurs are currently being investigated and once understood, these mechanisms may offer a possibility to develop therapies that influence the reinfection process. Therapies that interfere with the host immune response and with different stages of the viral life cycle offer promising results on this area.
The interferon pathway, in which a lot of components play an important role, and the activity of neutralizing antibodies against HCV infection provide the largest contribution to the host immune response. As for the viral life cycle, more specifically viral entry, several factors are involved and all of these factors could be a possible target for HCV therapy. The major factors operating in this multistep process of viral entry, are the viral envelope glycoproteins E1 and E2 and the different receptors present on the hepatocyte membrane.

In the first part of this literature study, the various mechanisms of immunity and viral entry are being described. This background information provides insight into the possible targets for hepatitis C virus therapy. In the second part, these targets are discussed one by one and the current progress on the development of antiviral therapy is clarified.
6. Methodology

This dissertation is a systematic literature review. First of all, general information about hepatitis C virus was found in the fifth edition of the reference book “Fields Virology” by Knipe D.M and Howley P.M. Once this information was obtained, specific exploration of the initial subject “Reinfection of liver grafts after transplantation in the context of hepatitis C virus and prevention of reinfection” started. Sources were found using different databases such as PubMed, ISI Web of Knowledge, MeSH and clinicaltrials.gov.

Search queries were found using the MeSH database. To obtain more information about epidemiology and HCV itself, “Hepatitis C virus” and “epidemiology” were the terms used. Exact statistics on the incidence were found on the World Health Organization site. More extensive information on the subject of therapy was found using “hepatitis C virus” combined with the following terms. “Standard therapy”, “interferon receptor therapy”, “therapy receptor block”. By using the function of “related articles” other articles on the subject were obtained.

The next step in the process was looking for articles about antibodies as a therapy. Some authors were suggested by my thesis supervisor Prof. Dr. P. Meuleman and these authors were used as search queries together with the subject they wrote about. “Neumann [AU] AND silibinin”, “Pawlotsky [AU] AND silibinin”. More general articles about this subject were found by using “Monoclonal antibodies AND HCV”, “Polyclonal antibodies AND HCV”, “AP33 AND HCV”, “XTL AND HCV” each time a new interesting antibody was mentioned in an earlier found article.

As receptor blockers are possible therapies as well, “CD81 receptor AND HCV” and “SR-B1 receptor AND HCV” were used to learn more about the role that these receptors play in the process of HCV infection and reinfection.

Finally, literature about hepatitis C virus infection kinetics and the role of B-cells in the infection process was found by using “Neumann [AU] AND HCV AND kinetics” and “Stamatakis [AU] AND B cells” as MeSH terms.
7. Results

7.1 Hepatitis C virus infection

Hepatitis C virus infection usually results in a chronic stimulus of the immune system. Similar to the “survival of the fittest” principle, the most infectious viral clones that can easily escape host immunity are being selected. Several mechanisms by which HCV affects host immunity are listed below.

7.1.1 Antiviral response and HCV’s escape from this response

The immune response is activated once the patient is infected with HCV and it is this immune response that causes hepatocyte damage as infected liver cells are being targeted by immune cells such as cytotoxic T cells and natural killer (NK) cells (Munir et al., 2010). HCV possesses several mechanisms by which it can escape from the host immune response. Once escaped, hepatitis C virus has the ability to replicate and infect other cells in a very efficient manner. The philosophy in the development of new therapies is that if we can interfere with the mechanisms by which HCV succeeds to escape host immunity, we can possibly block HCV infection and reinfection after liver transplantation.

7.1.1.1 Interferon in response to hepatitis C virus infection

As soon as HCV enters the body, the host innate immune system together with an adaptive response are strongly activated. Interferon plays an essential role in the innate immune response to viruses. Numerous classes of soluble cytokines are involved.

The interferons of importance during hepatitis C virus infection are IFN-α, IFN-β, IFN-γ and IFN-λ. Each interferon has a different effector pathway, which is being influenced by interferon regulatory factors (IRF), of which IRF-3 is the most important. There two different cell receptors, RIG-I and TLR3, that sense pathogen-associated molecular patterns (PAMPs) present in viral proteins and RNA. Once PAMP is bound to its receptor, the host response is triggered.

PAMP binds to the retinoic-acid-inducible gene I (RIG-I) and consequently activates a signaling cascade that results in phosphorylation and activation of IRF-3 (Gale and Foy, 2005). C-terminal phosphorylation of IRF-3 results in its transport to the nucleus where it forms a transcriptional
complex together with the IFN-β promoter. This transcription finally results in synthesis of IFN-β. Next to RIG-I, Toll-like receptor 3 (TLR3) in stellate cells, Kupffer cells and dendritic cells in the liver, has been described as PAMP receptor. Together with Toll-interleukin-1 receptor-domain-containing adaptor inducing IFN-β (TRIF), they induce phosphorylation and activation of IRF-3 as well (Fig. 2) (Gale and Foy, 2005).

IRF-3 also regulates transcription of IFN-λ and IFN-α is synthesized by IRF7 following the same mechanism (Lemon, 2010).

**Fig. 2. IFN-β production pathway.** Viral PAMP binding to RIG-I or TLR3 results in the phosphorylation and activation of IRF-3. The phosphor-IRF-3 dimer translocates to the cell nucleus and binds together with its transcription partners to the DNA positive regulatory domain (PRD) of the IRF-3 target genes. This binding results in activation of IRF-3 and this in turn results in the production and secretion of IFN-β. NS3/4A cleaves RIG-I and TRIF and disturbs viral PAMP signaling. (Gale and Foy, 2005).

IFN-β then binds its receptor, the type I IFN-α/β receptor (IFNAR). This binding activates the Jak-STAT (Janus Kinase - Signal Transducer and Activator of Transcription) signaling pathway. This
cascade finally results in binding of IFN-stimulated gene factor 3 (ISGF3) to the IFN-stimulated response element (ISRE) within the IFN-stimulated genes (ISGs), that provide direct antiviral activity against HCV by disturbing viral RNA translation.

Hepatitis C virus is known to possess mechanisms to interfere with the IFN-β signaling pathway and thereby inhibit immune response. First of all, the HCV NS3/4A protease complex blocks TIG-I and cleaves TRIF and thereby blocks TLR3 signaling pathway as well. This blockage results in a disruption of the phosphorylation and activation of IRF-3.

**Fig. 3. IFN signaling pathway and mechanisms of suppression.** IFN-α/β and IFN-λ bind to its receptors on the plasma membrane and activate the Jak-STAT signaling pathway, which in turn activates transcription factor ISGF3 which is responsible for the transcription of ISGs. The HCV core protein may disturb this pathway by interacting with STAT1. PP2A induces other proteins and in that way indirectly smothers phosphorylated STAT1. Finally, USP18 and SOCS3 confound immune response by inhibiting the IFN-receptor and Jak1 respectively. See text for details. (Lemon, 2010).
Hepatitis C virus can act on different levels in the signaling pathway (Fig. 3). STAT1 is directly inhibited by overexpression of HCV core proteins, leading to reduced phosphor-STAT1. Phosphor-STAT1 in turn is indirectly inhibited by protein phosphatase 2A (PP2A) which may be produced by endoplasmatic reticulum stress. Besides the inhibition of STAT, SOCS3 (suppressor of cytokine signaling 3) inhibits Jak1 and USP18 (ubiquitin-specific peptidase 18) interacts with the type I IFN-α/β receptor. All these inhibitory factors may explain the suppression of the Jak-STAT signaling pathway in HCV infection (Lemon, 2010).

7.1.1.2 T-cell response in HCV

Innate immunity is activated shortly after HCV infection, but it is the adaptive immunity that is responsible for the resolution of HCV infection. HCV-specific T-cells are activated but fail to eradicate HCV infection in the majority of chronically infected patients. A few hypotheses could explain this observation. The number of epitopes recognized and the number and function of responding T-cells decrease. HCV has the ability to mutate and produce escape variants that are no longer recognized by T-cells. Furthermore, T-cells remain focused on viral sequences encountered early in HCV infection and these initial T-cells are insufficient to act against the escape variants. As a result, T-cell response may be suboptimal in countering the escape variants formed by selective pressure.

In addition, infections with rapidly replicating viruses and a persistent high viral load result in a collapse in HCV CD4+ T-helper cells, required for the CD8+ T cells resulting in a decline of T-cell response (Dustin and Rice, 2007).

7.1.1.3 B-cell proliferation following the detection of HCV antigens

Antigen presenting cells like dendritic cells (DC) recognize HCV antigens and present those antigens to B-cells with the help of T-helper cells. The B-cells start proliferation and production of antibodies that form immune complexes and neutralize the virus (Fig. 4) (Landau et al., 2007).

Chronic HCV continuously stimulates the immune system and this leads to an uncontrolled proliferation and clonal expansion of B-cells. This may be the cause of multiple HCV associated B-cell disorders like mixed cryoglobulinemia vasculitis and B-cell lymphoma. There are several mechanisms that may contribute to the loss of regulatory control. For example, B-cell receptors like Fas and soluble TNF receptors I and II are elevated in HCV patients and genetic events like bcl-2 translocation and overexpression (Landau et al., 2007).
Neutralizing antibodies produced after recognition of HCV antigens, reduce viral load and prevent cell infection. There are three possible mechanisms by which antibodies may decrease viral load but these mechanisms are not yet fully understood (Eren et al., 2006).

1) Antibodies bind to the circulating viruses and thereby remove them from the blood circulation.
2) The antibodies form immune complexes and human phagocytes provide phagocytosis of these complexes.
3) Antibodies induce complement or antibody-mediated cytotoxicity.

A major target of neutralizing antibodies is viral entry, an important step of HCV infection and more specifically viral envelope proteins (E1 and E2). HCV is a virus that has the possibility to mutate and mutations in the region of the envelope glycoproteins may result in the alteration of viral entry and escape from neutralizing antibodies (Fafi-Kremer et al., 2010).

**7.1.1.4 Mode of action of anti-HCV antibodies in the immune response**

**7.1.2 Entry mechanisms**

The major targets for the newer antiviral therapies are the mechanisms by which the virus enters the hepatocytes. Blocking viral entry means blocking viral spread and thus control of HCV infection. First we have to understand the process of entry itself before we can develop therapies that block this
mechanism. The exact mechanism however is not yet completely known, partly because representative in vitro cell culture systems do not completely mimic the natural infection of the liver.

Studies on HCV attachment, entry and antibody-mediated neutralization were first performed using hepatitis C virus pseudoparticles (HCVpp). These pseudoparticles are composed of the viral envelope proteins E1 and E2 and have been showed to be excellent reagents to study the entry step of the HCV life cycle (Fafi-Kremer et al., 2010).

The key role of viral entry is attributed to the HCV envelope proteins E1 and E2. Those envelope proteins are highly glycosylated proteins. These glycosylations are highly conserved and this may point out that those sites are necessary for envelope protein folding structure and function (Falkowska et al., 2007). E1 and E2 bind to the cell surface receptors CD81 and scavenger receptor class B type 1 (SR-B1) located on the hepatocytes (Owsianka et al., 2005). This binding results in fusion of the viral and cell membranes and internalization, most likely by endocytosis, of the virus. Fusion of the membranes is a pH-dependent process and conformational rearrangements caused by acidification provide the energy necessary for fusion. This mechanism results in the hypothesis that modifying pH results in reduction of infectivity (Bartosch et al., 2003). Once the virus has entered the hepatocyte, its RNA genome is released into the cytoplasm where translation and replication of the virus can take place (Vercauteren et al., 2012).

The CD81-binding domain is supposed to be located in the HVR1 region of the E2 glycoprotein or in its close proximity (Fig.5). Deletion in the HVR1 region increased binding to CD81 but decreased binding to SR-B1. Infectivity was reduced and the remaining infectivity may be explained by the residual binding to SR-B1 or the increased binding to CD81 (Bartosch et al., 2003). Furthermore, the removal of some of the N glycosylation sites resulted in a reduced HCV entry mediation by E2 (Falkowska et al., 2007). These results suggest that HVR1 and HVR2 are important determinants for HCV binding and entry.

Fig. 5. The HCV E2 ectodomain. The N (Y) and O (Ψ) glycosylation sites with their percentages of conservation are shown. The CD81 binding site is located in the central region of E2 (HCR1 through HCR2. HCR: hyperconserved region. (Falkowska et al., 2007).
The tight junction proteins occludin (OCLN) and claudin-1 (CLDN1), predominantly expressed in the liver, mediate HCV entry as well. They most likely play a role in the late entry stage, just before viral internalization (Ploss et al., 2009). Indeed, a significant correlation between the levels of those tight junction proteins and the HCV RNA levels after liver transplantation could be demonstrated. HCV binds to the CD81 receptor and this complex is transferred into the tight junctions followed by entry into the hepatocyte. Therefore, OCLN and CLDN1 play an important role in the paracellular permeability, the cell adhesion and the cell-to-cell transmission of HCV and its recurrence (Mensa et al., 2011).

Next to the most important receptors and co-receptors CD81, SR-B1, CLDN-1 and OCLN, the mannose-binding lectins, DC-SIGN and L-SIGN have been proposed as entry receptors for HCV. They bind high-mannose N-glycans of HCV E2 and facilitate entry. L-SIGN is selectively expressed in liver sinusoidal endothelial cells and this may contribute to the presentation of HCV to the liver (Bartosch et al., 2003).

Finally, the low-density lipoprotein receptor (LDLr) has been proposed to play a role in viral entry, however the exact contribution of the LDLr is still under discussion. Viral particles are associated with lipoprotein molecules that are internalized by this cell-surface receptor. As a result, the virus uses the plasma lipoprotein uptake mechanism for its attachment to the hepatocyte surface (Heo et al., 2006). Moreover, HCV uses cholesterol and other lipids for its RNA replication and therefore lipoprotein uptake through the LDLr is necessary. In conclusion, we can say that the LDL receptor contributes to the HCV life cycle, but it does not seem to lead to viral infection by itself (Albecka et al., 2012).

### 7.2 Factors influencing HCV recurrence in liver transplant setting

Liver transplantation is an effective therapy for hepatitis C virus induced liver disease, but outcome mainly depends on the prevention of reinfection of the graft, which is almost unavoidable thus far. Reinfection of the graft severely impairs graft and patient survival. Statistics from 2008 showed that 75-90% of patients developed chronic HCV infection and 5-30% progressed to cirrhosis within 5 years after liver transplantation.

Numerous factors influence the outcome and severity of recurrence (table 1). Factors like female gender, increasing age of the donors (> 40 years old), African American ethnicity and genotype 1 HCV are rather hard to change and do have a negative effect on the outcome (Roche and Samuel, 2008).
Furthermore high pre-transplant and early post-transplant serum HCV RNA levels mean worse outcomes. Coinfection with cytomegalovirus or HIV and the presence of metabolic syndrome as well have a negative effect on the severity of recurrent hepatitis C. This means that those infections need to be treated as good as possible and insulin resistance in metabolic syndrome patients has to be controlled.

Naturally, the quality of the donor organ influences the survival of the graft after transplantation. The older the donor, the worse the survival of the graft and a long period of cold ischemia should be avoided.

<table>
<thead>
<tr>
<th>Factors affecting severity of hepatitis C recurrence</th>
<th>Factors affecting graft survival</th>
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<tbody>
<tr>
<td><strong>- Viral factors:</strong></td>
<td><strong>- Viral factors:</strong></td>
</tr>
<tr>
<td>• High HCV RNA level early post-liver transplantation</td>
<td>• Pre-transplant high HCV RNA level</td>
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<tr>
<td>• Genotype I HCV</td>
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<tr>
<td><strong>- Recipient factors:</strong></td>
<td><strong>- Recipient factors:</strong></td>
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<tr>
<td>• Female gender</td>
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<td>• African American ethnicity</td>
<td>• African American ethnicity</td>
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<tr>
<td>• HIV and Cytomegalovirus coinfection</td>
<td>• HIV and Cytomegalovirus coinfection</td>
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<tr>
<td>• Metabolic syndrome</td>
<td>• Older age (&gt;40 years old)</td>
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<tr>
<td><strong>- Donor-related factors:</strong></td>
<td><strong>- Donor-related factors:</strong></td>
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<tr>
<td>• Older donor age</td>
<td>• Older donor age</td>
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<tr>
<td>• Prolonged cold ischemia time</td>
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<tr>
<td><strong>- Immunosuppression</strong></td>
<td><strong>- Immunosuppression</strong></td>
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Table 1. Factors influencing HCV recurrence after liver transplantation and graft survival. (Aytaman et al., 2010)

Finally, another important affecting factor is the use of an immunosuppression therapy. More specifically, the intensity of treatment and rapid changes in the immunosuppressant level influence
treatment outcome significantly. Transplant patients are under a life-long immunosuppression therapy to avoid graft rejection. In this way, host immunity is suppressed to avoid host-versus-graft reaction. This suppression of host immune mechanisms allows the hepatitis C virus to infect healthy hepatocytes of the donor graft and recurrence of HCV occurs. Nowadays, the two most used immunosuppressive agents are corticosteroids and the calcineurin inhibitors.

a) **Corticosteroids**

The effectiveness of corticosteroids as a treatment of acute rejection has been proven, but several adverse effects are reported when corticosteroids are used for a long period. In addition, it also has a major influence on the development of HCV recurrence. More bacterial infections, metabolic complications and accelerated liver fibrosis are some of these adverse effects. In addition, they increase HCV RNA levels and HCV disease severity (Aytaman et al., 2010).

b) **Calcineurin inhibitors (CNI)**

Cyclosporine and tacrolimus belong to this category of immunosuppressive agents. These medications also come with certain side effects like renal dysfunction, metabolic complications and cardiovascular disease and those adverse effects contribute to posttransplant morbidity. Comparison studies between cyclosporine and tacrolimus pointed out that cyclosporine has a favorable effect during interferon therapy, while tacrolimus is associated with less acute rejection episodes. But those studies are somewhat conflicting concerning the risk of histological HCV recurrence and overall survival. Some point out that there is no difference between cyclosporine and tacrolimus while others claim the benefit of cyclosporine regarding histological recurrence but a disadvantage on survival compared with tacrolimus (Aytaman et al., 2010). But those differences are very little.

SVR after Peg-IFN-α and RBV therapy was achieved in 48% of patients with cyclosporine whilst only 38% with tacrolimus. In general, the advice is to start therapy with tacrolimus to minimize the frequency of rejection episodes and switch to cyclosporine afterwards (Aytaman et al., 2010).

So far, the optimal approach may be the “low and slow” approach in which an adequate immunosuppression with a calcineurin inhibitor is maintained to minimize rejection, combined with a low initial steroid dose and very gradual increasing of this corticosteroid dose over 6-12 months (Tamura and Sugawara, 2008). Nevertheless, no ideal immunosuppression therapy is yet developed and more research has to be done. The perfect balance between prevention of rejection and avoidance of excessive immunosuppression is a challenge on that area.

Directly after transplantation viral load decreases, but the following two weeks HCV RNA levels raise again with a peak level after 3-4 months. Within the first six months, recurrence is already a fact.
The composition of viral population following liver transplantation changes and selected variants with clear advantages regarding viral entry and escape from neutralizing antibodies develop (Lacek et al., 2012).

The benefit of retransplantation is somewhat disappointing and prognosis is poor. Patient survival was 20% to 30% lower than survival in patients who underwent primary liver transplantation. In any case, retransplantation should be considered and performed at an early stage (Tamura and Sugawara, 2008).

### 7.3 Therapy

The ultimate goal of antiviral HCV therapy is to achieve a sustained virological response. One can speak about SVR if HCV RNA in serum is undetectable (<50 IU/mL) 24 weeks after the end of a year treatment (Guedj and Neumann, 2010). An early virological response, low baseline HCV RNA before treatment and a HCV genotype other than genotype 1 was related to a higher SVR-rate. A sustained virological response in turn was related to better outcome parameters like liver enzymes, cirrhosis development and mortality (Berenguer et al., 2008).

During HCV replication, the virus can mutate and these mutations can cause resistance to antiviral therapy and escape from viral eradication. However, those resistant viruses can only become dominant if its relative resistance is high enough to compensate for the lower relative fitness of the resistant clones (Guedj and Neumann, 2010).

### 7.3.1 Standard therapy in chronic HCV patients

The initial choice of therapy was purely coincidental and it was initiated in 1991, even before hepatitis C virus was identified (Tamura and Sugawara, 2008).

Standard therapy for HCV infection consists of the combination of pegylated interferon alpha and ribavirin for up to 48 weeks. In general, a SVR of approximately 45%-50% and 80% was achieved in respectively HCV genotype 1 and genotype 2 or 3 (Reesink et al., 2010). Various factors like the virus genotype and the use of immunosuppression, may influence the therapy outcome. In addition, the optimal timing of onset and duration of antiviral treatment is still unclear.
The major issue on this therapy is the presence of numerous adverse effects and consequently its tolerability. The most important side-effects are severe decompensation, development of cytopenia together with hemolytic anemia, uncontrolled sepsis or flu-like symptoms (e.g., fever, headaches, fatigue, arthralgia, myalgia (Neumann et al., 2006)) and these effects sometimes ask for dose reduction or drug discontinuation (Roche and Samuel, 2008). This dose reduction in turn affects the effectiveness of the therapy.

The outcome of antiviral therapy and transplantation for HCV can be “predicted” by the nucleotide sequence near the interleukin-28B (IL28B) gene, an IFN-λ gene on chromosome 19. Single nucleotide polymorphisms in this gene or in its proximity are associated with the degree of viral clearance and response to antiviral therapy. The C/C genotype is associated with a better response and a faster and higher degree of viral clearance whereas the T/T genotype more rapidly evolves to fibrosis and show a lower effectiveness (Burra and Freeman, 2012). To illustrate this, in European patients, about 80% of C/C genotype patients cleared the virus while only 30% of patients with the T/T genotype did so (Afdhal et al., 2011). The exact mechanisms by which IL28B contributes to HCV RNA clearance is still unknown but it is assumed that IFN-λ, and hence IL28B acts in the same way as IFN-α and β, using the Jak-STAT pathway (see above).

Although IL28B is a proper outcome predictor, it should not be used as the only factor on which the decision of starting antiviral therapy depends. On-treatment viral response at week 4 is also a predictor that should be taken into account (Afdhal et al., 2011). The standard therapy has been pushed forward after years of research and the evolution is outlined below.

### 7.3.1.1 Ribavirin and interferon monotherapy

Ribavirin is a guanosine analogue that has activity against RNA and DNA viruses. The exact mechanisms by which ribavirin exerts its antiviral effect is not fully understood but several hypotheses have been proposed. First of all, ribavirin provides a direct antiviral effect against the HCV RNA-dependent RNA polymerase. Secondly, high concentrations of RBV may cause the misincorporation of nucleotides and thereby leading to lethal mutagenesis of HCV. The third hypothesis suggests an effect on the adaptive immune response by inducing a shift from Th2 to Th1 cells. Finally, RBV is an inhibitor of inosine monophosphate dehydrogenase (IMPDH) leading to depletion of GTP pools. As a result of this decrease, TLR signaling pathway seemed to enhance resulting in better antiviral activities of TLRs (Chung et al., 2008). Furthermore, a study by Cattral et al. reported that ribavirin inhibits a lot of viral-induced parameters of which tumor necrosis factor, interleukin-1 and interleukin-4 are the most important. Thus ribavirin is a modulator of host immune response (Cattral et al., 1999).
Both ribavirin and interferon monotherapy are insufficient to achieve a sustained virological response and patients remained viremic. For example, a negligible SVR of 2.5% was reported with interferon (Curry, 2004). The reasons for the low hepatitis C antiviral effect of interferon are its characteristics. It is a glycoprotein with poor stability, a short half-life and potential immunogenicity, and that’s why its efficiency is limited (Lampertico et al., 2009). However, a biochemical and histological amelioration was reported in patients who received IFN (Schmidt et al., 2010) or a full-dose RBV, 1200 mg per day during 24 weeks to be precise (Tamura and Sugawara, 2008). Indeed, a study in 1998 already observed normal AST values in respectively 85% and 43% of patients after a 24-weeks lasting therapy with ribavirin and interferon alpha (Gane et al., 1998).

### 7.3.1.2 Ribavirin and interferon combination therapy

After somewhat disappointing results, the following step in the development of a successful antiviral therapy was the combination of RBV and IFN-α. Results on viral eradication were still low. But compared to the results of monotherapy, the combination of IFN-α and RBV did have a beneficial effect on the outcome parameters like the normalization of ALT values and virological response. A study in 1998 revealed a SVR of 36% with combination therapy compared to 18% with interferon alpha monotherapy (Schalm, 1998) and an earlier study in 1996 showed normalization of ALT values in 43% compared to 11% of patients 24 weeks after discontinuing respectively an IFN-α plus RBV therapy and an IFN-α monotherapy (Lai et al., 1996). More recent studies revealed a serum clearance in 24% of patients and a varying SVR between 5% and 33% with this combined therapy (Tamura and Sugawara, 2008). On the contrary, adverse effects were more frequently reported with this combination therapy.

### 7.3.1.3 Pegylated interferon alpha and ribavirin combination therapy

Finally, the combination of pegylated interferon alpha and ribavirin was found to be the best antiviral therapy. Pegylated interferon is produced by attaching one or more polyethylene glycol molecules to the interferon. By pegylation, interferon gains a longer half-life. This means that pegylated IFN is longer available in blood and dose intervals can be prolonged. This results in less adverse effects and a better compliance (Lampertico et al., 2009).

SVR obtained after therapy with pegylated interferon alpha and RBV compared to standard interferon and RBV separately was 24% and 20% respectively (Peveling-Oberhag et al., 2010). Tolerability and safety were comparable with standard IFN therapy. Less than 60% were able to complete therapy due
to the adverse effects (Charlton, 2011). A histological benefit (Tamura and Sugawara, 2008) and a benefit on several disease endpoints like liver fibrosis progression, hepatic decompensation and death was obtained. At the same time, therapy with pegylated IFN-α and RBV was a good alternative for patients who failed under standard RBV and IFN therapy.

Viral kinetics during the first 24-48 hours after an IFN-based antiviral therapy is characterized by a rapid dose-dependent decline of viral load. This rapid decrease is attributed to the reduction of the rate of viral replication and release. The second phase is characterized by a slower decline, caused by the progressive loss and elimination of infected cells by interferon therapy (Guedj and Neumann, 2010).

### 7.3.2 Standard Peg-IFN/RBV therapy in liver transplant patients

Hepatitis C virus therapy in LTx setting can be administered at three different stages (Roche and Samuel, 2008; Aytaman et al., 2010; Peveling-Oberhag et al., 2010).

#### a) Pre-transplant antiviral therapy

The main goal of this approach is to achieve sustained virological response and to eradicate the hepatitis C virus before transplantation and thereby reducing the risk of HCV recurrence. Indeed, more graft loss and more severe recurrence is reported when viral loads before transplantation are high.

Because of several adverse effects, the applicability of this approach should be limited and reserved to patients who have favorable disease characteristics (Child-Pugh class A or early B, table 2).

<table>
<thead>
<tr>
<th>Score</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
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<tbody>
<tr>
<td><strong>Ascites</strong></td>
<td>None</td>
<td>Mild</td>
<td>Moderate/severe</td>
</tr>
<tr>
<td><strong>Hepatic encephalopathy</strong></td>
<td>None</td>
<td>Mild</td>
<td>Marked</td>
</tr>
<tr>
<td><strong>Bilirubin (µmol/L)</strong></td>
<td>&lt; 34</td>
<td>34-50</td>
<td>&gt; 50</td>
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<tr>
<td><strong>Albumin (g/L)</strong></td>
<td>&gt; 35</td>
<td>28-35</td>
<td>&lt; 28</td>
</tr>
<tr>
<td><strong>Prothrombin time</strong> (seconds over normal)</td>
<td>&lt; 4</td>
<td>4-6</td>
<td>&gt; 6</td>
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</tbody>
</table>

**Table 2. Child-Pugh classification of liver cirrhosis.** Child-Pugh class A (a score of 5-6), class B (a score of 7-9) or class C (a score of 10 and more). (Kumar & Clark’s Clinical Medicine seventh edition, 2009)
b) Early post-transplant prophylactic or pre-emptive therapy

This therapy is started immediately after transplantation (within the first two weeks) to prevent hepatitis C virus progression. Shortly after transplantation viral load is at its lowest and fibrosis is still absent. Therapy initiated at that moment can delay or even prevent recurrence. The major disadvantage of this therapy is the limited tolerability and effectiveness influenced by immunosuppression therapy given to prevent graft rejection. A study by Chalasani et al. demonstrated that patients treated with pegylated IFN-α as pre-emptive therapy did show lower HCV RNA levels, but a SVR was only achieved in 8% of patients (Roche and Samuel, 2008).

c) Post-infectious antiviral therapy

Once infection has established and there is histological evidence of recurrence, antiviral therapy can be started. The main purpose is to slow disease progression. Not all hepatitis patients are suitable for post-infectious therapy. Short-term prognosis and rapidly evolving fibrosis on biopsies are contraindications (Roche and Samuel, 2008) and therefore standard therapy (see below) should not be used.

A SVR of approximately 30% was reported after an interferon-based therapy in liver transplant patients (Schmidt et al., 2010).

### 7.3.3 Novel therapies for HCV

Several studies are ongoing in order to develop therapies that act against the vital mechanisms of the viral life cycle. The most important findings on this research area are listed below. However, pegylated IFN and RBV still remain vital components of antiviral therapy.

#### 7.3.3.1 Antibodies

Viral entry is the major target of neutralizing antibodies and monoclonal antibodies consequently provide promising results for new effective anti-HCV therapy. As mentioned above, they prevent cell infection by neutralizing the circulating viruses and thereby altering the interaction between HCV glycoproteins and the cell surface receptors. More specifically, mAbs can be directed against either the envelope glycoproteins E1 and E2 or against the cell surface receptors like CD81 and SR-B1. Next to
these two major components of viral entry, mAbs can also be directed against attachment factors like lectins and against co-receptors like OCLN and CLDN1 (Vercauteren et al., 2012).

a) Monoclonal antibodies against the envelope proteins

The virus contains several conserved regions, the antigenic region 3 (AR3) on E2 for example (Law et al., 2008), important for viral infection. Administration of monoclonal antibodies against these conserved regions, forces the virus to mutate and as the mutations occur in regions important for HCV replication, the virus becomes less infectious (Burioni et al., 2008). A diversity of monoclonal antibodies against the E2 envelope glycoprotein is capable of inhibiting entry and thereby inhibiting recurrence of HCV infection. Moreover, they can also neutralize infection by viral variants, resistant to host-neutralizing responses (Fafi-Kremer et al., 2010).

The development of antibodies against HCV is a long-term investigation project and the first antibodies tested, like AbXTL68, were found to be inefficient. This anti-E2 monoclonal antibody only showed a transient HCV RNA decrease and did not succeed to achieve a SVR after LTx (Schiano et al., 2006). As a result, this mAb is not suitable for prevention of recurrence in LTx patients.

**Fig. 6. Viral clearance.** Treatment of HCV-infected mice by HCV-AB 68 and 65. HCV RNA was measured 1 day (day 18 after transplantation) and 5 (day 22 after transplantation) days post-treatment. (Eren et al., 2006)

The human antibodies HCV-AB 68 and HCV-AB 65 have the ability to neutralize HCV, although the HCV RNA level decrease is rather poor and transient. They act on different epitopes in the E2 envelope glycoprotein. The human monoclonal antibodies AB 68 and AB 65 have three major functions. They fix complement, form IgG immune complexes and induce phagocytosis of these
immune complexes. By these mechanisms, they ensure viral clearance (Fig. 6) with a reduction of HCV RNA of 80%, neutralization (Fig. 7) and inhibition of infection in vitro. The effect is dose-dependent and a higher dose leads to a stronger effect. It is estimated that a dose of more than 500 mg of monoclonal antibody is needed to achieve a reduction of HCV RNA in liver transplant patients (Eren et al., 2006).

The mouse monoclonal antibody (mAb) AP33 was shown to inhibit infection by interacting with the E2 glycoprotein. MAb AP33 recognizes a highly conserved region located next to the HVR1 region on E2 (amino acids 412-423). The high preservation of this region indicates that those key residues are critical for protein conformation, function and binding (Tarr et al., 2006). Mutations in the amino acids in this region resulted in a reduction of antibody binding. Rat mAb 3/11 recognizes an overlapping yet different epitope in the E2 area recognized by mAb AP33.

There is a difference in binding affinity to E1E2 between mAb AP33 and mAb 3/11. Experiments showed that the binding affinity of mAb 3/11 was 10-fold lower than the affinity of AP33 (Fig. 8). Furthermore, other studies demonstrated that the neutralization potency of AP33 was also higher than mAb 3/11. The results were obvious. MAb AP33 was able to neutralize the virus in chronic HCV patients by between 80% and 99% while mAb 3/11 by only 10% to 80% (Tarr et al., 2006).
Monoclonal antibodies against the viral envelope glycoproteins did not show very successful results regarding HCV reinfection, probably because of the variety of these envelope proteins. On the other hand, monoclonal Abs can be targeted against the HCV entry receptors including CD81, SR-B1 and CLDN1 and these antibodies did show highly promising results.

Both anti-CD81 and anti-SR-B1 monoclonal antibodies have already been tested in vitro and in vivo and have been proven to be effective as antiviral therapies. Experiments showed that CD81 density is directly proportional to the susceptibility to HCV infection (Koutsoudakis et al., 2007) and SR-B1 expression is correlated with viral decline (Lacek et al., 2012). Therefore, inhibiting CD81 and SR-B1 by antibodies reduces hepatitis C virus reinfection in liver transplant patients and this in a dose-dependent manner. In vitro studies also revealed that anti-CLDN1 mAbs inhibited viral infection, even in patients that developed viral variants, resistant to neutralizing antibodies, after LTx (Fofana et al., 2010).
7.3.3.2 Polymerase inhibitors

7.3.3.2.1 Silymarin, silibinin

Silymarin is extracted from the milk thistle *Silybum marianum* and its main components are silibinin A and B, representing 50%-60% of all components, the diastereoisomers isosilibinin A and B, silicristin and silidianin (Ahmed-Belkacem et al., 2010). Silibinin A is found to have the strongest antiviral effect. Like the monoclonal antibodies, silibinin acts in a dose-dependent manner and together with the route of administration, these factors seem to influence antiviral results. Indeed, first-pass metabolism, influenced by liver disease, and pharmacokinetics depend on the route of administration and these influence drug concentration, which determines the antiviral effect (Ferenci et al., 2008).

Silibinin is an inhibitor of the viral RNA polymerase NS5B, an enzyme complex that participates in HCV replication (Neumann et al., 2010). By blocking this polymerase with a high dose of an intravenous mixture of silibinin A and B (Legalon SIL), hepatitis C viral load can be reduced and replication can be inhibited, even in patients who failed to achieve a virological response with pegylated IFN-α and RBV therapy. In this hypothesis, silibinin attacks vital functions of the hepatitis C virus. Other authors presume that antiviral effect of silibinin is caused by a cellular antiviral effect, namely through both Jak-STAT and IFN-dependent and -independent cellular pathways (Ahmed-Belkacem et al., 2010).

In addition, silibinin is an anti-oxidative and an anti-fibrotic agent. Hepatitis C virus induces oxidative stress and this stress contributes to fibrosis and carcinogenesis, thus silibinin can act against these effects of HCV (Ferenci et al., 2008).

![Fig. 9. HCV RNA levels with intravenous silibinin therapy.](image-url) Silibinin therapy was started 8h after LTx. (Neumann et al., 2010)
The antiviral activity of silibinin therapy is recently reported in two different case studies. Two Child-Pugh class C patients infected with HCV who failed IFN-based therapy were treated with silibinin monotherapy. One started silibinin treatment shortly after the anhepatic phase for 14 days (Neumann et al., 2010) and the other 15 days prior to transplantation (Beinhardt et al., 2011). HCV RNA levels became undetectable in both patients respectively on day 9 (Fig. 9) and on day 22 after LTx. Success or failure of silibinin monotherapy was influenced by the HCV RNA level at the time of LTx (Berg et al., 2011). These results are limited of course and larger studies on these polymerase inhibitors have to be performed.

There is uncertainty about the mechanism by which silibinin operates, but one agrees on the ability of silibinin to significantly reduce viral load and prevent reinfection after LTx. The silibinin therapy is well tolerated and there are no serious adverse effects reported. (Ferenci et al., 2008). The exact mechanism and metabolism of silymarin and silibinin however is not yet known and more research on these drugs needs to be done to gain more information about pharmacokinetics, the ideal dose etc.

### 7.3.3.2.2 Other RNA-dependent RNA polymerase inhibitors

By using nucleoside/nucleotide analogues as antiviral therapy, false substrates are incorporated in the RNA and transcription is thereby inhibited. These nucleoside or nucleotide analogues mimic the natural substrates of RNA that are incorporated by RNA-dependent RNA polymerases, without having the same function. As a result, HCV RNA is reduced. On the other hand, even a single amino acid substitution in the HCV virus can provide resistance against the nucleoside/nucleotide analogues therapy. However, the resistant variants have a low fitness in the presence of the drug, so overall resistance is limited (Sarrazin et al., 2012).

### 7.3.3.3 Protease inhibitors

#### 7.3.3.3.1 Telaprevir and boceprevir

Telaprevir or VX-950 and boceprevir are competitive inhibitors of the nonstructural 3/4A (NS3/4A) serine protease complex, exclusively of hepatitis C virus genotype 1 (Jensen, 2011). NS3/4A is needed for RNA replication and virion assembly (Ghany et al., 2011). In addition, NS3/4A blocks the RIG-I and TLR3 signaling pathway needed for the IFN-mediated immune response. By inhibiting this serine protease complex, viral replication and formation is blocked and host immune response is restored,
resulting in suppression of infection. As mentioned above, telaprevir and boceprevir show no or very limited activity against HCV genotype 3 and 4.

On the field of virological response, boceprevir showed an increase of almost 30% in SVR in comparison to standard therapy in chronic HCV patients. PROVE 1 clinical trial showed a rapid virological response 4 weeks after initiation of telaprevir therapy (Lang, 2007). Relapses were also less frequent (Sarrazin et al., 2012). The achievement of SVR was better in patients who had a prior response to standard therapy with pegylated IFN-α and RBV. Null-responders are defined as patients who achieve a $< 2 \log_{10}$ HCV RNA level decline during the first 12 weeks, whereas partial responders do achieve a $\geq 2 \log_{10}$ HCV RNA level decline during this period, but HCV RNA remains detectable. Relapsers are patients who achieve SVR but relapse after therapy cessation (Sarrazin et al., 2012). Boceprevir and telaprevir treatment is therefore appropriate in partial responders and relapsers but less for null-responders (Ghany et al., 2011).

Next to the inhibition of the NS3/4A serine protease complex, telaprevir and boceprevir act against the enzyme cytochrome P450 3A4, responsible for the metabolism of the calcineurin inhibitors cyclosporine and tacrolimus (Charlton, 2011). As they inhibit the enzyme responsible for the degradation of the calcineurin inhibitors, the blood concentrations of cyclosporine and tacrolimus rise.

Telaprevir and boceprevir are both cleared by a hepatic metabolism and only a minor part by renal metabolism (Charlton, 2011). This results in higher levels of the protease inhibitors in patients with liver disease due to reduced clearance. The recommended dose of boceprevir and telaprevir is respectively 800 mg for 24-44 weeks and 750 mg for 12 weeks three times a day. Boceprevir should be preceded by a four weeks lasting lead-in therapy with pegylated IFN-α and RBV. On the other hand, telaprevir treatment should be followed by a pegylated IFN-α plus RBV treatment of 12-36 weeks (Ghany et al., 2011). The duration of therapy is still controversial. Studies revealed that patients with advanced liver disease benefit from a longer therapy of 48 weeks of treatment with a protease inhibitor. On the contrary, they also claim that patients with an early virological response should receive a short therapy of 24 weeks (Sarrazin et al., 2012).

Resistance of HCV against a protease inhibitor in monotherapy is easily developed. To avoid resistance, the administration of boceprevir should be stopped if HCV RNA is higher than 100 IU/mL at treatment week 12 or if HCV is still detectable at week 24. Telaprevir should be discontinued at HCV RNA levels of 1,000 IU/mL or more at treatment weeks 4 or 12 or if still detectable at week 24 (Ghany et al., 2011). The recommendation is to combine these protease inhibitors with pegylated IFN-α and ribavirin and administer this triple therapy to avoid the problem of resistance.

The most important adverse effects were anemia, rash, dry skin and dysgeusia (Jensen, 2011). Hemolytic anemia can be an adverse effect of standard therapy with interferon and ribavirin. By
adding telaprevir or boceprevir, the anemia can be worsened due to bone-marrow suppression (Sarrazin et al., 2012). Furthermore, as they inhibit CYP3A4, drug-drug interactions frequently occur due to a reduced demolition of medicines that are metabolized by CYP3A4.

### 7.3.3.3.2 Other NS3/4A protease inhibitors

A variety of NS3/4A protease inhibitors are under investigation and they are expected to have better pharmacokinetics and higher tolerability than telaprevir and boceprevir. SVR rates were similar to those of telaprevir and boceprevir. MK-5172 is an example of a second-generation NS3/4A protease inhibitor and it expressed a high antiviral activity against HCV, even against the HCV genotypes with amino acid substitutions which caused resistance to the first-generation protease inhibitors (Sarrazin et al., 2012).

TMC435 is found to be a potent NS3/4A protease inhibitor as well. It has a good tolerability and no severe adverse effects are reported up till now. It provides a rapid decline of viral load and this virological response is maintained, suggesting a continued suppression of HCV replication (Reesink et al., 2010). The best SVR rates in HCV patients were achieved using TMC435 combined with Peg-IFN-α and RBV as it has an additive effect on this standard therapy.

### 7.3.3.4 RNA interference-based antiviral therapy

A gene silencing mechanism has also showed activity against hepatitis C virus. This mechanism, called RNA interference (RNAi), is induced by small interfering RNA (siRNA) and it is to this siRNA that HCV RNA is very vulnerable (Jahan et al., 2011). Producing this RNAi silencing mechanism could provide a new antiviral therapy for HCV.

siRNA can be directed against a lot of components of the HCV entry pathway like the cell surface receptors CD81, SR-B1 and LDLR or the envelope glycoproteins, especially E2. Experiments showed that the receptors were indeed inhibited after adding receptor-specific siRNA, respectively siCD81, siSRB1 and siLDLR. The effect is dose-dependent and maximum inhibition could be obtained by using the optimal dose of 100 nM siRNA (Jahan et al., 2011). Interference with the cell entry receptors results in a reduction of viral load up to 67% with siCD81, 58% with siLDLR and 51% with siSRB1 in chronic HCV patients. Furthermore, combinations of siRNA showed a higher decrease in viral load, especially the combination of siCD81 + siLDLR with a reduction of 83.5% and siSRB1 + siLDLR with a reduction of 73% (Jahan et al., 2011).
By inhibiting both receptors and envelope glycoproteins, siRNA-induced RNA interference is able to inhibit hepatitis C virus entry. For this reason, the production of RNAi molecules is a promising option for antiviral HCV therapy.
8. **Discussion**

Hepatitis C virus infection is an important health problem as its incidence is growing and the majority of patients develop chronic HCV after infection. The greatest impact of hepatitis C virus infection on the liver is the progression to cirrhosis and hepatocellular carcinoma. Transplantation is considered to be the best therapeutic approach, but the problem of liver graft reinfection is the biggest issue at the moment.

Hepatitis C virus has a high replicative rate and its RNA polymerase lacks a proof-reading function. The combination of these two characteristics gives the virus the ability to evade host immunity. By mechanism of mutation, HCV develops a very efficient viral entry mechanism on which immune factors are no longer able to interact. Even more, these viral mutants are able to develop resistance against several antiviral therapies.

Key components of this host immunity response are IFN-α, IFN-β, IFN-γ and IFN-λ. They activate IFN-stimulated genes through the Jak-STAT pathway. Transcription of these genes enables antiviral activity. Hepatitis C virus is able to suppress this interferon pathway by inhibiting crucial steps in the antiviral pathway. This ensures the virus to disturb antiviral response.

The first antiviral therapy with a reasonable virological response developed to treat hepatitis C virus infection was based on this immune mechanism. Administration of pegylated interferon alpha combined with ribavirin has a favorable outcome, although viral clearance achieved with this therapy was not sufficient enough. Moreover, the most important disadvantage is the adverse effects of the therapy of which severe decompensation, sepsis and cytopenia are the most important. Tolerability is poor and therapy is often terminated before reaching a sustained virological response.

Because of these gaps in current therapy with Peg-IFN-α and RBV, researchers started to look for new antiviral therapies. Therefore, the mechanism of viral entry and replication was investigated with the aim to find new targets on which therapeutic agents could act.

The entry pathway is a very important process of the viral life cycle. Viral entry can be divided into two major parts: the viral attachment to the hepatocyte and membrane fusion. The viral envelope glycoproteins E1 and E2 fuse with the cell membrane by interacting with the HCV entry receptors, of which CD81 and SR-B1 are the most important. After fusion of both the viral and host cell membranes, the virus is incorporated into the hepatocyte, where it initiates its viral replication. As a result, both envelope glycoproteins E1 and E2 and cell membrane receptors CD81 and SR-B1 are key proteins in the viral entry mechanism. HCV succeeds to escape from host immune response as a result of mutations in the surrounding area of domains responsible for binding of E1 and E2 to the cell.
membrane receptors. Those domains are called hypervariable regions and alterations in these regions highly affect HCV binding affinity and entry.

Beside the envelope glycoproteins and the cell membrane receptors as mentioned above, there is a very wide range of other factors participating in the entry process. Tight junction proteins like OCLN and CLDN1 form complexes with the cell membrane receptor CD81 and the LDL receptor is used to mediate entry as HCV is bound to lipoproteins. Thereby the virus uses the LDL lipoprotein uptake pathway of the liver to enter the hepatocytes.

Thus, the entry factors are a possible target for antiviral therapy. Blocking a step in the entry process means inhibition of viral entry and thereby inhibition of viral replication and recurrence. Monoclonal antibodies use this inhibitory mechanism to exert an antiviral effect. They are directed against the envelope glycoproteins, the cell membrane receptors and the co-receptors as mentioned above. Moreover, they can also be directed against conserved regions that are important for viral replication. Changes in these regions force the virus to mutate into less viable variants.

The most promising monoclonal antibodies are mAb AP33 and mAb 3/11. They both attack a conserved region in the envelope glycoprotein E2. E2 loses its ability to mediate viral entry by interacting with the cell membrane receptors and as a result, HCV is neutralized. The binding and neutralization capacity of mAb AP33 is greater compared to mAb 3/11.

Antibodies can also be directed against HCV entry receptors and thereby inhibiting viral attachment and subsequently viral entry. Both anti-CD81 and anti-SR-B1 monoclonal antibodies have been proven to be an effective antiviral therapy in vitro and in vivo and anti-CLDN1 mAbs already showed its antiviral activity in vitro.

siRNA against different components of the viral entry mechanism could also be used as an antiviral therapy. Receptor-specific or envelope glycoprotein-specific siRNA inhibit their own targets, resulting in a reduction of viral load.

Beside monoclonal antibodies, RNA-dependent RNA polymerase inhibitors harbor antiviral capacities as well. Silymarin with its components silibinin A and B, is the most important actor of this class of antiviral agents. They inhibit RNA polymerase, such as NS5B, resulting in viral replication failure as the RNA transcription, mediated by RNA polymerase is no longer correct and functional. This therapy is well tolerated and a reduction of viral load is achieved.

Finally, protease inhibitors like telaprevir and boceprevir act against cytochrome P450 3A4 and nonstructural protease complexes like NS3/4A, needed for viral replication and virion assembly. They should be given as a triple therapy, combined with Peg-IFN-α and RBV to avoid resistance of HCV against therapy with telaprevir and boceprevir. The virological response obtained with these antiviral
agents was better than SVR reached with standard therapy and relapses were less frequent. The disadvantage of adding a protease inhibitor was the worsening of the adverse effects caused by ribavirin and Peg-IFN-α. More research is being done to develop other protease inhibitors with a higher tolerability.

HCV therapy in liver transplant patients can be initiated at three different points of time in the pre- and post-transplantation period. Pre-transplant antiviral therapy is used to achieve viral eradication before transplantation. By reducing viral load to a minimum, the risk of reinfection of the healthy liver graft is reduced. There are a lot of adverse effects associated with this therapy, so it should only be given to patients who have favorable disease characteristics. Secondly, antiviral therapy can also be initiated within the first two weeks after transplantation, the so-called pre-emptive therapy. Therapy is started before viral breakthrough is reported and indeed, a beneficial effect is reported. Finally, therapy can be initiated when histological recurrence has already been detected. The main purpose of this approach is to slow down viral progression.

Non disputable statistics on sustained virological responses achieved with the different classes of antiviral therapy are not available as results vary depending on the study design. In general, a sustained virological response of approximately 50% in genotype 1 and 80% in genotype 2 or 3 is achieved with standard ribavirin and pegylated interferon alpha combination therapy. According to recent studies, this virological response can be augmented by adding one of the newer antiviral therapies.

In conclusion, future hepatitis C virus therapy in transplant patients focuses on two major pillars. First, ribavirin and Peg-interferon-α therapy targets the eradication of hepatitis C virus itself. Secondly, the new antiviral agents act against the viral life cycle. Monoclonal antibodies inhibit viral entry, polymerase inhibitors disturb viral replication and protease inhibitors prosecute an antiviral effect by restoring the immune response and disturbing viral replication. Nevertheless, the combination therapy with pegylated interferon alpha and ribavirin remains hitherto the golden standard in hepatitis C virus treatment in both chronic and LTx patients. However, research on new therapies should be continued as studies show promising results regarding virological response and clearance.
9. References


