**Diplomarbeit**

# Effect of Silibinin on Humoral Immunology in Patients with Chronic Hepatitis C


eingereicht von

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*Eidesstattliche Erklärung*

*Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.*

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# Vorwort:

Etwa drei Prozent der Weltbevölkerung sind chronisch mit HCV infiziert. Schätzungen für Europa gehen von drei bis zu fünf Millionen HCV-positiven Personen aus.

Bei 60 - 80% der Infizierten gelingt es dem Immunsystem nicht, das Virus erfolgreich zu eliminieren, bei ihnen verläuft die Krankheit chronisch.

Bei bis zu 20 Prozent dieser Patienten kann sich eine Leberzirrhose entwickeln. Die Zeitdauer von der Infektion bis zum Vollbild der Zirrhose wird mit 20 bis 30 Jahren angegeben.

Patienten mit einer durch HCV verursachten Zirrhose haben zusätzlich ein hohes Risiko, Leberzellkrebs zu entwickeln.

Etliche Patienten reagieren, bedingt durch Genotyp und anderen Faktoren, nicht auf die herkömmliche Therapie aus Ribavirin und Peginterferon alpha, und neue pharmakologische Ansätze bieten Gelegenheit, auch diesen Patienten Heilung zukommen zu lassen.

Die weltweite Verbreitung und die Anpassungsfähigkeit des Virus erfordern fortwährend die Erforschung neuer Medikamente.

Silibinin ist ein vielversprechender Vetreter dieser neuen Pharmaka zur Behandlung von Hepatitis C bei dem es nun gilt seine Wirkweise zu erforschen und auch die optimale Dosierung zu finden.

**Danksagung:**

**Ich danke all jenen, die mich während meiner Studienzeit und bei der Erstellung dieser Diplomarbeit unterstützt haben, von Herzen.**

# Abstract:

**Background:** Chronic hepatitis C afflicts approximately 3 million individuals and is the leading cause of end-stage liver disease requiring transplantation. The current treatment recommendation for chronic Hepatitis C virus infection is the combination of Peginterferon and Ribavirin for 24 or 48 weeks, depending on the viral genotype. Only approximately half of the patients with chronic hepatitis C achieve a sustained viral response with Peginterferon and Ribavirin. Silymarin, an extract from the seeds of the milk thistle plant *Silybum marianum*, has been used for centuries for the treatment of chronic liver diseases. The mechanism of the antiviral effect of Silibinin is currently unknown. The hepatitis C virus is generally believed to be a noncytopathic virus, and hepatic fibrosis in hepatitis C is thought to be the end result of long-standing inflammation, cell proliferation, cytokines secretion, and the lysis of infected cells. The aim of the present study is to show the effects of Silibinin application on viral load and to assess humoral immunology, of non-responders to standard Ribavirin/Peginterferon therapy, during intravenous Silibinin treatment.

**Methods:** 20mg/kg bodyweight Silibinin (Legalon) was applied daily within two hours for 21 days. After one week of Silibinin application, Peginterferon (Pegasys) and Ribavirin where added for the following two weeks. Peginterferon was applied once a week 180µg parenteral. Serious side effects did not occur in which case the dose would have been reduced to 135µg once a week. Ribavirin was administered oral, 2 times a day 3 pills with 200mg each. HCV RNA levels were determined by COBAS HCV TaqMan/COBAS® AmpliPrep assay (Roche Diagnostics). Cytokines were determined by Bio–Plex 200 multiplex suspension array system with Luminex xMAP–technology. Serum lipids were routinely determined in the clinical laboratory.

**Results:** The statistical analyse shows that there is a significant decrease in viral load concentration at four points of measurement ($F$=5.504, $p$=.029). The pairwise comparison also shows a strong downward trend of viral load between the first and second test point (mean difference 1/2=-4224589, Std. Error1/2=1279242; $p$<.1) where only Silibinin was applied. Cholesterol and Triglycerides showed no significant changes. The measured cytokines show no significant changes. The pairwise comparison of Interleukin 8 shows that there is a significant difference between first and third test point (mean difference t0/t2=37.803, Std. Errort0/t2=9.646; $p$<.05).

**Conclusion:** We did not find any significant changes of the cytokine levels. The drop of the viral load was significant. These results are in accord with in vitro trials and earlier studies that showed reduction in viral load.

## Zusammenfassung:

**Hintergrund:** Die chronische Form der Hepatitis C betrifft weltweit rund 3 Millionen Individuen und das Hepatitis C Virus gilt als Hauptverursacher der transplantationspflichtigen Lebererkrankungen. Die aktuelle Behandlungsempfehlung für chronische Hepatitis C besteht aus einer Kombinationstherapie mit Ribavirin und Peginterferon alpha für 24 beziehungsweise für 48 Wochen, abhängig vom Genotyp. Nur etwa die Hälfte der Patienten erreicht mit dieser Therapie einen Sustained virologic Response. Silymarin ein Extrakt aus den Samen der Mariendistel, *Silybum marianum,* wird schon seit Jahrhunderten zur Behandlung von chronischen Lebererkrankungen eingesetzt. Die genaue Wirkweise der antiviralen Eigenschaften ist noch unbekannt. Nach allgemeiner Auffassung ist das Hepatitis C Virus kein direkt zytopathisches Virus, und die Leberfibrose entsteht durch einen langandauernden Entzündungsprozess, die Ausschüttung von Zytokinen, Zellproliferation und Zelllyse. Das Ziel dieser Studie besteht einerseits darin, die Wirkung von Silibinin auf die Virenlast zu zeigen, und andererseits Auswirkungen von Silibinin auf das humorale Immunsystem festzustellen.

**Methoden:** Es wurden täglich 20mg/kg Körpergewicht Silibinin (Legalon) innerhalb von 2 Stunden für die Dauer von 21 Tagen verabreicht. Nach der ersten Woche, in welcher allein Silibinin verabreicht wurde, kamen für die restlichen 2 Wochen Ribavirin und Peginterferon alpha hinzu. Die Standarddosis von 180µg Peginterferon wurde 1 Mal wöchentlich verabreicht. Nebenwirkungen, die zu einer Dosisreduktion auf 135µg/Woche geführt hätten, traten nicht auf. 3 Tabletten mit je 200mg Ribavirin wurden oral 2 Mal täglich verabreicht. Die HCV RNA Levels wurden mittels COBAS HCV TaqMan/COBAS® AmpliPrep Assay (Roche Diagnostics) bestimmt und die Zytokine Levels mit Hilfe des Bio–Plex 200 multiplex Suspension Array System mit Luminex xMAP–Technologie. Lipidbestimmungen liefen über die Routinemessungen des Kliniklabors.

**Ergebnisse:** In der statistischen Auswertung zeigt sich ein signifikanter Abfall der Virenlast an den 4 Messpunkten ($F$=5.504, $p$=.029). Die paarweisen Vergleiche zeigen bereits in der ersten Woche, in der nur Silibinin verabreicht wurde, eine starke Abwärtstendenz. (mean difference 1/2=-4224589, Std. Error1/2=1279242; $p$<.1). Cholesterin-, Triglyzerid- und Zytokinlevels zeigten keine signifikanten Änderungen. Im paarweisen Vergleich zeigte sich bei Interleukin 8 ein Trend zwischen 1. Und 3. Messzeitpunkt (mean difference t0/t2=37.803, Std. Errort0/t2=9.646; $p$<.05).

**Fazit:** In den Ergebnissen der statistischen Auswertung fanden sich keine signifikanten Änderungen der Zytokine-, Cholesterin oder Triglyceridspiegel. Der Abfall der Virenlast war signifikant, und dieses Ergebnis steht auch Einklang mit früheren Studien die zu diesem Thema durchgeführt wurden.

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*Fig. 21: Boxplots of TNF – alpha at four measurement points. t0=Baseline, t1=one week after t0, t2=two weeks after t0, t3=three weeks after t0.*

# 1. The Hepatitis C Virus

## 1.1 Structure

The hepatitis C virus (HCV) is a positive-sense single-strand RNA virus 60 nm in size with an approximately 3 kb long genome which is similar in organization to that of flaviviruses and pestiviruses, forming the genus Hepacivirus in the Flaviviridae family. The HCV genome contains a single large open reading frame (ORF) that codes for a virus polyprotein, which is cleaved after translation to yield 10 viral proteins. The 5‘ untranslated region and core (C) gene are highly conserved among genotypes, but the envelope (E1 and E2) proteins are coded for by the hypervariable region, which varies up to 35% from isolate to isolate and may allow the virus to evade host immunologic containment directed at accessible virus-envelope proteins and unfortunately often the chemotherapy. The p7 region codes for a small hydrophobic membrane protein. The 3‘end of the genome also includes an untranslated region and contains the genes for six non-structural (NS) proteins NS2, which codes for a cysteine protease that cleaves between NS2 and NS3; NS3, which codes for a serine protease that catalyses processing at all other sites within the NS-region; and an RNA helicase, NS4A, NS4B, NS5A, and NS5B that codes for a RNA-dependent RNA polymerase. (1)

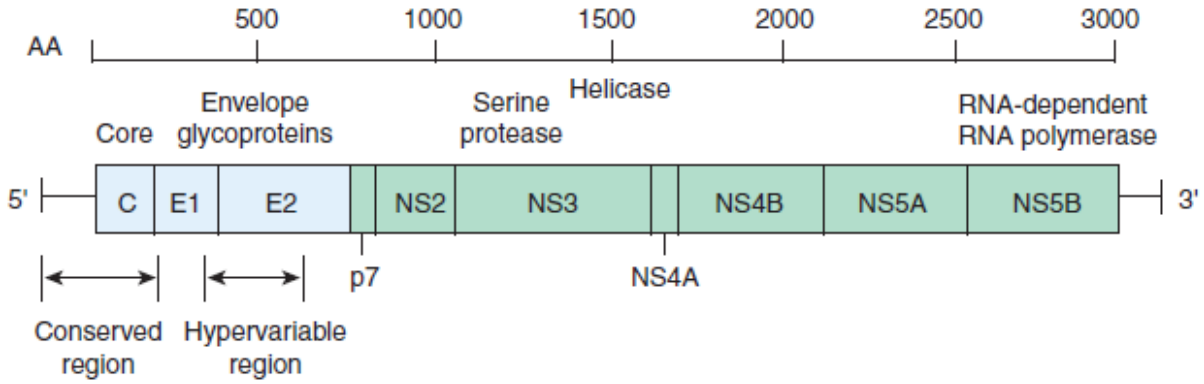



*Fig. 4 Virus structure* (1)

The liver-specific micro-RNA (miRNA) miR-122 binds to the HCV 5‘NCR and enhances viral RNA translation and/or replication. Two seed sequences reside in-between domains I and II, and intensive mutational analyses revealed that both miRNA binding sites are necessary for HCV replication (2)

## 1.2 HCV cell entry mechanism

The invasion of the hepatitis C comprises a large range of interactions which take place simultaneously and/or successively. Interactions between HCV E1–E2 envelope glycoproteins, glycosaminoglycans (GAGs) and the LDL receptor (LDLr), as a proposed capture molecule, contribute to primary binding of the virus particles to host cells. Also the scavenger receptor BI (SR-BI), the CD81 tetraspanin, together with the small tight junction proteins Claudin-1 (CLDN-1) and Occludin (OCLN) synergistically contribute as a „late step“ factors to HCV uptake. HCV entry is strongly reduced in the presence of anti-CD81 and anti SR-BI antibodies. Oxidized LDL and serum amyloid A have been shown to inhibit HCV entry through SR-BI, while HDL enhances HCV entry.

The binding of enveloped viruses to cell surface molecules is followed by fusion of the lipid envelope with a cellular membrane. The hepatitis C virus, enters target cells by clathrin-mediated endocytosis and pH-dependent fusion, which been proposed to occur in the early endosomes and releases the virus particles into the cytosol. (1) (2)

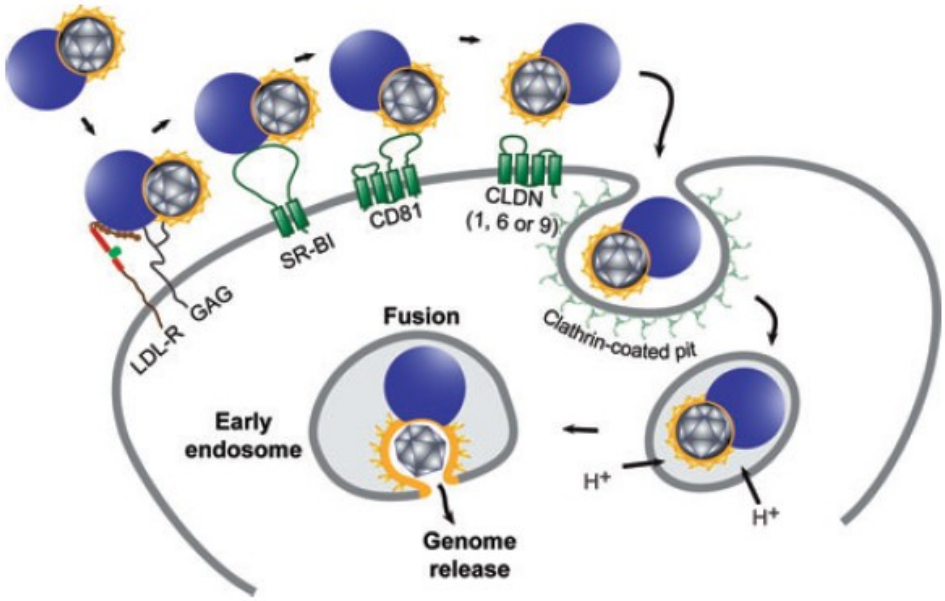



*Fig. 5 HCV entrance mechanism* (2)

### 1.3 Translation and replication:

Viral RNA released into the cytoplasm takes over parts of the cellular components for its replication and is translated via an internal ribosome entry site (IRES) located in the 5‘non-translated region of the genome.
Primarily by the action of NS4B, in conjunction with NS5A, membranous replication vesicles (RVs) are induced and accumulate in the infected cell as distinct structures designated as the membranous web where viral RNA is amplified by the NS5B RNA polymerase and nearly all other NS proteins and host cell factors.
Newly synthesized RNA genomes are used for translation (production of new viral proteins), RNA replication, or formation of new infectious virions (assembly), which appears to happen at the lipid droplets.
RNA could be delivered to the core protein thus triggering nucleocapsid formation, which in turn could explain the (V) LDL-like composition of HCV particles and the incorporation of apoE due to its tight linkage to VLDL synthesis and apoE. (2)

## 1.4 History of Hepatitis C

The ancient history of Hepatitis C remains yet a mystery, due to the circumstance that there are no stored blood-samples of Hepatitis C – positive blood which are older than 50 years. But if we compare it with the evolutionary history of other viruses and consider the fact that it also exists in remote places in the world although it is mainly transmitted through blood to blood contact it seems obvious that the HC-Virus is an old companion to mankind. So on firmer ground than other thesis stands the prediction that the different subtypes of HCV originated approximately 200 years ago and that the six main genotypes of HCV most likely had a common ancestor approximately 400 years ago. (3)

In the mid-1970s, Harvey J. Alter, Chief of the Infectious Disease Section in the Department of Transfusion Medicine at the National Institutes of Health, and his research team demonstrated that most post transfusion hepatitis cases were not caused by hepatitis A or B viruses. (4)

Despite this discovery, international research efforts to identify the virus, initially called *non-non-B hepatitis* (NANBH), failed for the next decade. In 1987, Michael Houghton, Qui-Lim Choo, and George Kuo at Chiron Corporation, collaborating with Dr. D.W. Bradley from CDC, used a novel molecular cloning approach to identify the unknown organism and developed a diagnostic test. (4)

In 1988, the virus was confirmed by Alter by verifying its presence in a panel of NANBH specimens. In April 1989, the discovery of the virus, renamed hepatitis C virus (HCV), was published in two articles in the *Science* journal. (5)

In the 1980's, investigators from the Centre for Disease Control (headed up by Daniel W. Bradley) and Chiron (Michael Houghton) identified the virus in 1989. In 1990, blood banks began screening blood donors for hepatitis C, but it wasn't until 1992 that a blood test was perfected that effectively eliminated HCV from the blood transfusion supply. Now, there is less than one per two million transfused units of blood estimated to be tainted with hepatitis C. Prior to the screening of the blood supply for hepatitis C, approximately 300,000 Americans contracted hepatitis C through blood transfusions or blood products. (6)

## 1.5 Epidemiology

The WHO estimates that about 200 million people, 3% of the world's population, are infected with hepatitis C virus (HCV) and 3 to 4 million persons are newly infected each year with a global number or 170 million chronic carriers at risk of developing liver cirrhosis and/or liver cancer (at least 85% of infected persons become chronically infected and about 70% develop chronic hepatitis) (7)

Although Hepatitis appears everywhere around the world, its grade of distribution depends a lot on the geographic location. Countries with the highest reported prevalence rates are located in Africa (e.g. Egypt with 22%) and Asia (for example Pakistan where most reported rates range between 2·4% and 6·5 %.); areas with lower prevalence include the industrialised nations in North America, northern and western Europe, and Australia. (8)

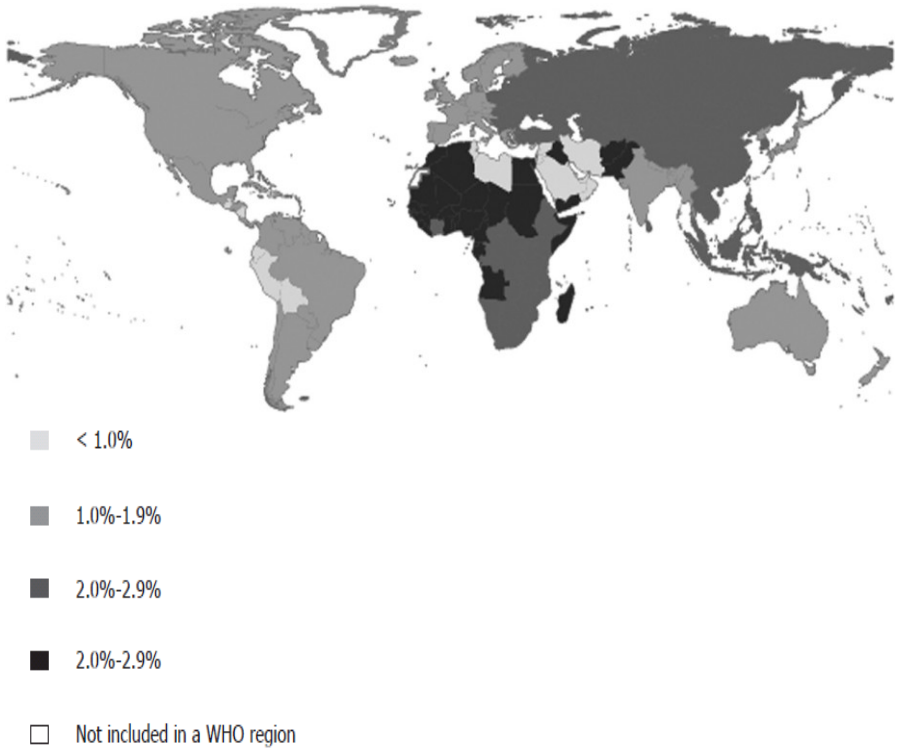



*Fig. 1 Estimated HCV prevalence by region* (9)

The age distribution in the United States and Western Europe shows that most HCV infections occur in adults 30–49 year old, and HCV seems rare in children while In Egypt, high rates of infection are observed in all age groups including young individuals. This indicates that there is an on-going high risk for acquiring HCV infection. (10)

Because chronic liver disease may develop many years after infection, the past incidence is a major determinant of the future burden of HCV-associated complications (10)

## 1.6 Ways of Transmission

**Blood Transfusion**

In developed countries transfusion-associated cases occurred prior to routine donor screening in blood banks with second and third generation enzyme immunoassays, a procedure that resulted in a sharp decline in transfusion-associated HCV transmission, while developing countries were not able to eliminate the risk due to the lack of resources to implement a sufficient donor screening. (10)

Also the largest reduction in the incidence of transfusion - transmitted HCV infection has coincided with adoption of an all - volunteer donor system, but because most blood donations in the developing world still do not come from voluntary, non-remunerated donors, transfusion is probably a major source of HCV transmission throughout the developing world, much as it was in the developed world decades ago. (8)

**Injecting Drug User (IDU)**

The incidence and prevalence of hepatitis C among injection drug users has recently declined in the United States and Europe due to the widespread implementation of harm reduction policies, syringe exchange programs, counselling of IDUs regarding protection from infection, and changing injecting behaviour. (10) But it is still high among old and very young IDUs. Studies among young IDUs with 5 years or fewer of injecting have reported HCV seroprevalence rates of 20–46% and in Norway still 67% of prevalent cases of HCV infection reported a history of injection drug use. (8)

**Sexual Transmission**

Sexual transmission of HCV has been controversial, but there are studies that showed that the chance to acquire HCV increases with the number of lifetime sexual partners, high-risk sexual exposure, and unprotected sex. It is still a problem that studies like these are limited by the potential of confounding habits of IDUs such as sharing items like razors or needles with their sexual partners (10).

Also it should be noted that an infection with HCV is most likely by sexual intercourse when the partner is in the early phase of acute infection; when viral load is high and there is no antibody to complex with the antigen. (8)

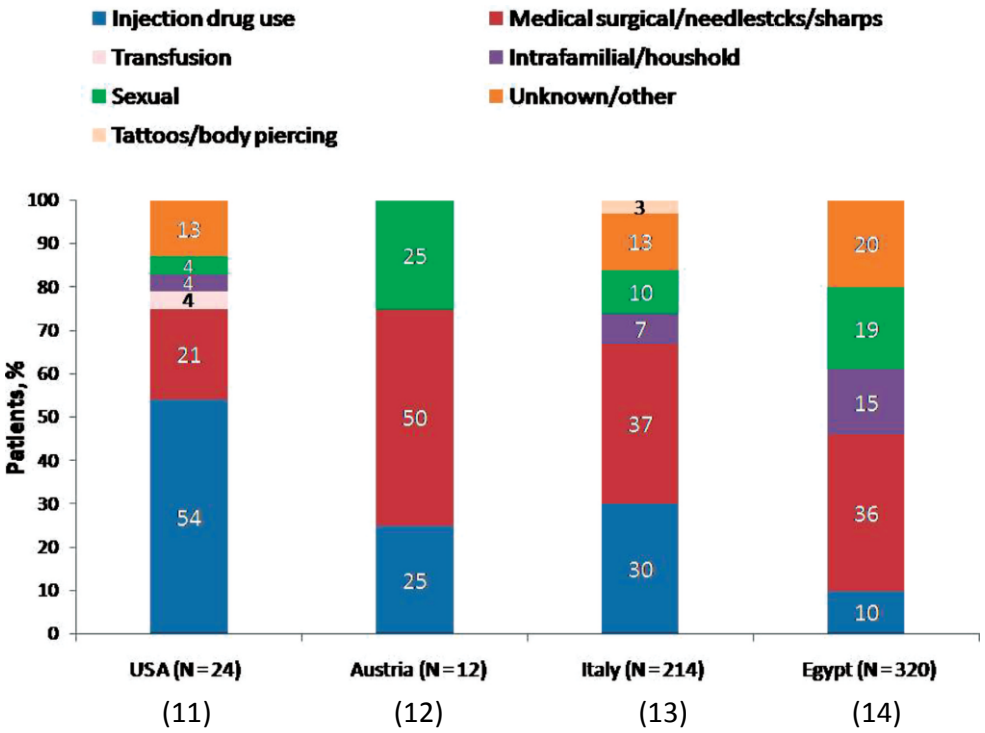
**Nosocomial and Iatrogenic Infection**

There is a high incidence and prevalence of HCV among dialysis patients which can be attributed to several risk factors, including the number of blood transfusions, lack of adherence to infection control practices in dialysis units, transmission through dialysis machines, ultra filtrates, frequent hospitalizations and surgery, which increase their opportunities for exposure to nosocomial infections.

The prevalence of anti-HCV in chronic haemodialysis patients ranges between 10 and 20% in the west and 40 and 85% in some developing countries (10)

Of even greater importance in the spread of HCV, are unsafe therapeutic injections, dentistry, wound treatment, circumcision, excision, and scarification performed by both professionals and non-professionals. It has been estimated that approximately 2 million HCV infections are acquired annually from contaminated health care injections, and may account for up to 40% of all HCV infections worldwide. In India, seroprevalence of HCV infection among patients receiving multiple injections to treat kala-azar was 31, 1% and the reuse of glass syringes during the early campaign to treat schistosomiasis in Egypt appeared to be responsible for the largest outbreak of iatrogenic transmission of a blood borne pathogen ever recorded. (8)

Other ways of transmission: In contrast, the contributions of occupational, which concerns mainly health care workers, perinatal have been relatively constant over time and with substantially less geographic variation. (8)




(11) (12) (13) (14)

*Fig. 2 Ways of HCV transmission*

The disease transmission patterns vary according to the geography, development of the countries and the socioeconomic differences in its population. Some modes are well documented and accepted while others still require further research. But to summarize it is possible to say that the most efficient transmission of HCV is through large or repeated direct percutaneous exposures to viraemic blood.

## 1.7 Diagnosis

**Testing and counseling**

The optimal approach to detect HCV infection is to screen persons for a history of risk of exposure to the virus, and to test selected individuals who have an identifiable risk factor and then to counsel them. (15)

Persons with conditions, behaviour or circumstances associated with a high prevalence of HCV infection including:

_ Persons with HIV infection
_ Persons with haemophilia who received clotting factor concentrates prior to 1987
_ Persons who have ever been on haemodialysis
_ Persons with unexplained abnormal aminotransferase levels
_ Children of HCV positive mothers
_ Sexual partners of HCV-infected persons especially in their early infection phase
_ Prior recipients of transfusions or organ transplants prior to July 1992
_ Occupational endangered health care, emergency medical and public safety workers after a needle stick injury or mucosal exposure to HCV-positive blood
_ Persons who have used illicit intravenous- or nasal applied drugs in the recent and remote past, including those who injected only once and do not consider themselves to be drug users. : (16) (17) (18) (19) (20)

**Laboratory**

In the diagnosis of HCV infection two classes of assays are commonly used: serologic assays that detect specific antibody to hepatitis C virus (anti-HCV) and molecular assays that detect viral nucleic acid, but none of them are a grading of disease severity or prognosis. (21)

**Serologic**

Anti-HCV, which is used for screening and diagnosis, can be detected in the serum or plasma using a number of immunoassays: (21)

Examples for enzyme immunoassays (EIAs) are the Abbott HCV EIA 2.0 and the ORTHO HCV Version 3.0 ELISA which have in third generation a specificity for anti-HCV greater than 99 % (22), one enhanced chemiluminescence immunoassay (CIA) is the VITROS Anti-HCV assay and an example for recombinant immunoblot assay which were originally designed as a more specific, supplemental assay to confirm the results of EIA testing, is the Chiron RIBA HCV 3.0 SIA. (23)

False positive results of the EIAs are more likely to occur when testing is performed among populations where the prevalence of hepatitis C is low, while false negative results may occur in the setting of severe immunosuppression such as infection with HIV, solid organ transplant recipients, hypo- or aggammaglobulinemia or in patients on haemodialysis (24) (25)

**Molecular Assay**

A minority of chronically infected patients will have persistently normal alanine amino - transferase (ALT) levels, and as a result, ALT levels and a positive HCV serology result are not adequate for the diagnosis of chronic HCV; instead, detection of HCV RNA is required to establish the diagnosis. (26)

Nucleic acid tests (NATs) directly detect the presence of HCV RNA using a combination of amplification and detection techniques. Usually NATs have supplanted the recombinant immunoblot assay as the preferred test to confirm HCV infection. (27)

Nucleic acid tests are classified into qualitative tests (qualitative polymerase chain reaction [PCR] mostly with a detection limit of 50 IU/ml, transcription-mediated amplification [TMA]) which is more sensitive, has a detectable limit of 5 IU/ml and is superior to PCR in predicting virological response); and quantitative tests (branched-chain DNA, real time PCR, quantitative reverse transcription PCR) (27)

Qualitative NATs are used to screen low level viremia and to screen blood donations while quantitative techniques appear to predict the likelihood of sustained virological response, spontaneous remission, sexual transmission and determining severity of liver disease (27) (28)

## 1.8 Genotypes

The genotypic heterogeneity in HCV is high as 30– 35% over the complete genome of HCV. This is a consequence of the highly error prone RNA-dependent polymerase of the RNA viruses, which results in heterogeneous mixture of closely related viruses, termed quasispecies, that exist within any individual infected with HCV. (29)

Hepatitis C can be subdivided in 6 major genotypes. Due to the fact that these genotypes respond different to therapy and vary in the ideal duration of it, it is legitimate to genotype all patients who will receive a HCV therapy.
For example, patients who had genotype 2 or 3 were 3 to 6 times more likely to achieve sustained virological response in the 2 large registration trials of Peginterferon of Mans et al. and Fried et al. (30) (31)

The HCV genotype distribution in Austria is: 80.4% type 1, 4.5% type 2, 12.3% type 3, 2.7% type 4, and 0.1% type 6 and co-infections in 0.2%. The major subtypes were 1b (51.7%), 1a (20.4%) and 3a (8.4%). Co-Infection with two genotypes is rare. The distribution is slightly fluctuating due to immigration from countries with different genotype majorities. (32)

Several commercial assays are available to determine HCV genotypes using direct sequence analysis of the 5` non-coding region, that include Trugene 5` NC HCV Genotyping kit, reverse hybridization analysis using genotype specific oligonucleotide probes located in the 5` non-coding region, INNO-LiPa HCV II, and Versant HCV Genotyping Assay 2.0 (21)

## 2. The disease and the treatment

### 2.1 Acute and chronic phase

HCV infection is infrequently diagnosed during the acute phase of infection because of the rareness of the typical symptoms such as jaundice, malaise, and nausea. Clinical manifestations can occur, usually within 7 to 8 weeks (range, 2 to 26) after exposure to HCV, but the majority of persons have either no symptoms or only mild symptoms. An estimated 74 to 86 per cent of persons will have persistent viremia. Most chronic infections will lead to hepatitis and to some degree of fibrosis, which may be accompanied by relatively nonspecific symptoms such as fatigue, and in severe or advanced cases the infection leads to severe complications and even death. (33)

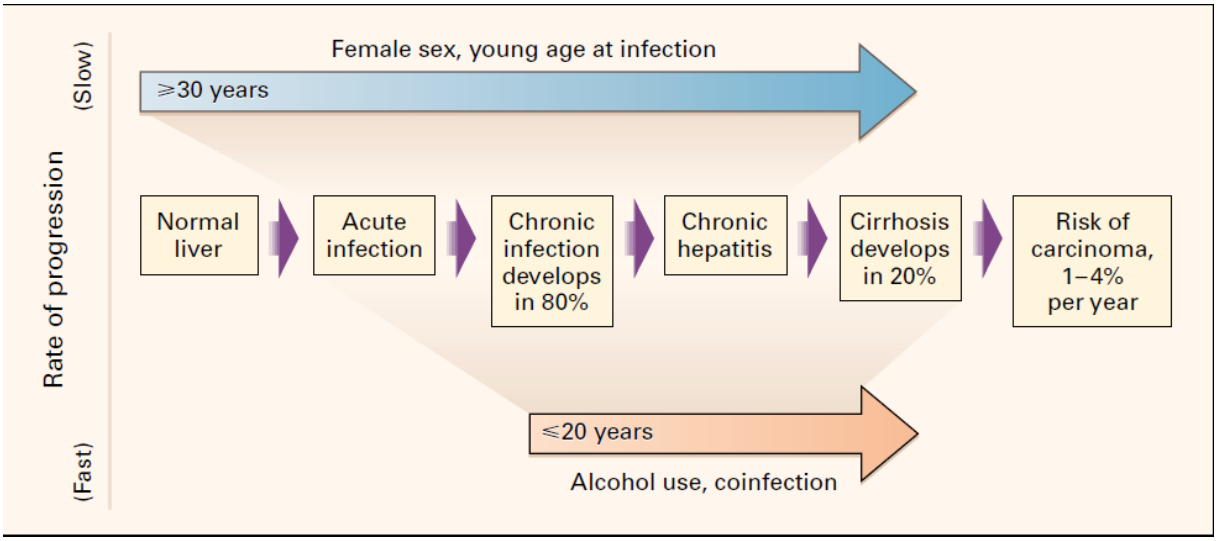



*Fig. 6 Progress of HCV* (2)

The course of infection varies widely among persons. Factors that decrease the risk of progression include female sex and a younger age at infection; factors that increase the risk include alcohol intake, an older age at infection, male sex, and coinfection with other viruses. (33)

The typical morphologic lesions of hepatitis c are similar to those of other viral hepatitis and consist of panlobular infiltration with mononuclear cells, hepatic cell necrosis, hyperplasia of Kupffer cells, and variable degrees of cholestasis. Cell degeneration, necrosis and ballooning of cells happen simultaneously to mitotic cell regeneration. (1)

## 2.2 Liver fibrosis

**Liver biopsy**

The liver biopsy has been the "gold standard" for determining the liver disease status concerning the grading and staging of the liver fibrosis or cirrhosis which will need histological surveillance for hepatocellular carcinoma. Also due to its accuracy it was and often still is a helpful assistance in the decision whether to start therapy and which one. (21)

The 4 most common scoring systems are
The METAVIR Score starting from 0 (correlates with no fibrosis) to 4 (correlates with cirrhosis), the Batts-Ludwig (also ranges from 0 to 4), the International Association for the Study of the Liver (IASL ranges from 0 to 4) and the Ishak score which describes the liver injury staging with 6 gradations. (34)

The high level of accuracy is important because a distinction between F1 and F2 Metavir stage is, inter alia, used to make the decision to treat a patient with antiviral therapy or not, and distinguishing F3 from F4 is important for detecting and preventing complications such as portal hypertension and hepatocellular carcinoma (35)

Disadvantages that make liver biopsy not a satisfying quality criteria are its complications, like transient and moderate pain, along with anxiety and discomfort (5–20%), infrequent vaso vagal episodes, severe complications such as a haemoperitoneum, biliary peritonitis and pneumothorax although they are rare (0.3–0.5), sampling errors, intra- and interobserver variability, expenses on specialists, and the aversion of the most patients to undergo serial monitoring (36) (35)

For the detection of fibrosis and cirrhosis, for which theoretically micro fragments may be sufficient, a 15mm piece of tissue has proved to work well. In addition to the length, the width of the core is of importance, and it has been clearly shown that for accurate staging and grading in chronic liver diseases, that a biopsy obtained with a 16–18G needle is ideal for this purpose. (37)

**Noninvasive tests of fibrosis**

Due to the negative aspects mentioned above, two non-invasive alternatives to liver biopsy have been developed in the past years, which are based on two very different concepts: blood marker panels and liver stiffness measured through ultrasound or more recently through magnetic resonance scanning. (38)

The Fibroscan reaches very fast a high value for the definition of the extension of the cirrhosis. Further advantages are that transient elastography (TE) provides the group of

patients, with cirrhotic livers that have one major limitation of the biopsy, with prognostic approach, it is easy to handle and has a high reproducibility. (39)

Disadvantages of Fibroscan are the higher failure rate in individuals with narrow intercostal spaces and morbid obesity, that it is yet not possible to distinguish an increased liver stiffness as a surrogate of fibrosis or, for example because of necroinflammatory activities during acute hepatitis, and it is also less adequate when assessing the transition from one stage to a higher one. (40) (41)

A relatively new measurement method is the acoustic radiation force impulse (ARFI) imaging technology, which has been introduced as a new technique to diagnose and evaluate liver fibrosis. ARFI imaging involves the mechanical excitation of tissue with use of short-duration acoustic pulses to generate localized displacements in tissue. The displacements resulted in shear wave propagation, which is tracked and recorded in meters per second. Friedrich-Rust et al. show in their study that ARFI has an excellent diagnostic accuracy comparable to that of TE. (42)

It is possible to classify the serum markers into two groups: The so-called „Class II biomarkers“, which are indirect serum markers and are based on the evaluation of common functional alterations in the liver, that do not necessarily reflect extracellular matrix turnover and/or fibrogenic cell changes and the „Class I biomarkers“, that are intended to detect extracellular matrix turnover and/or fibrogenic cell changes. (41)

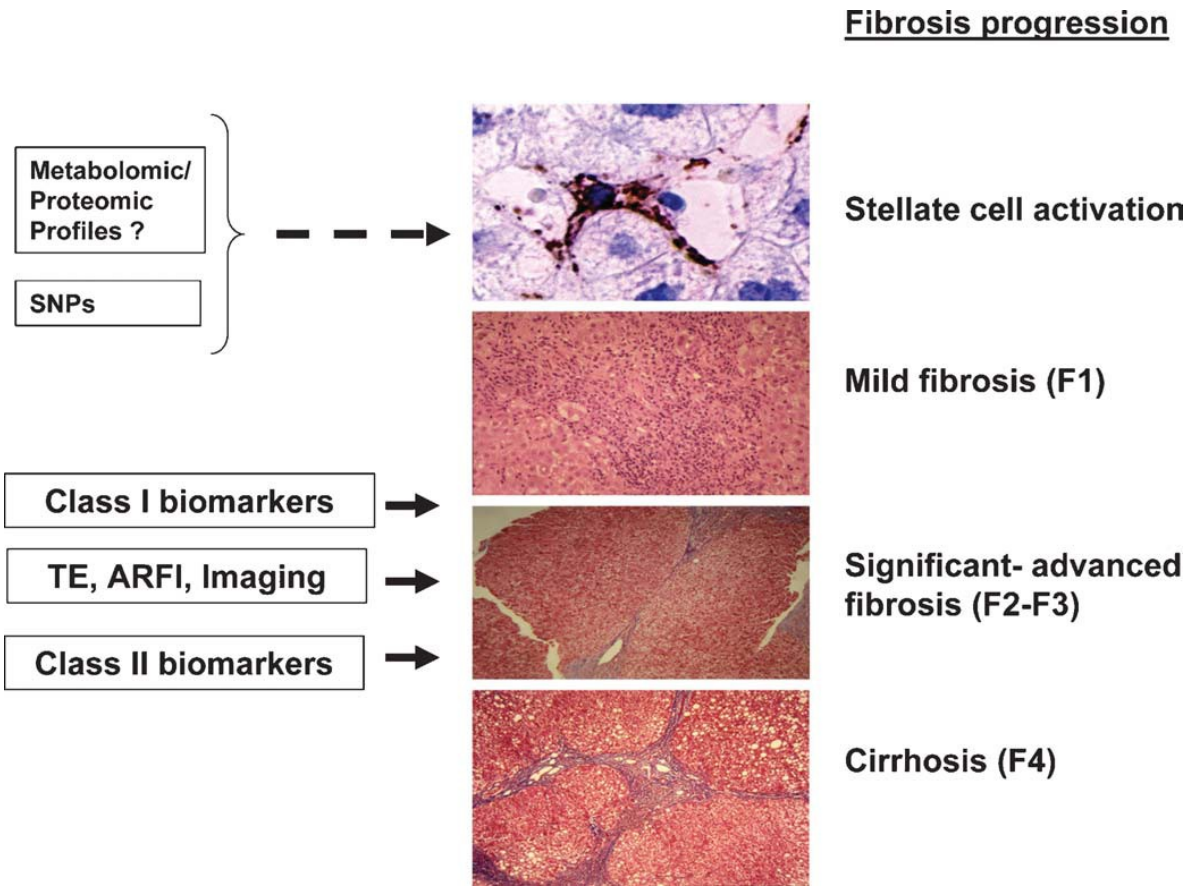



*Fig. 3 Fibrosis progression and classification* (41)

Serum markers are useful for establishing, with acceptable accuracy, the diagnosis of significant or non-significant fibrosis, but are less helpful in assessing the mid-ranges of fibrosis due to the dichotomized approach. (43)

## 2.3 Treatment

### 2.3.1 Treatment History

Interferon alpha was used in a low-dose, long term dosage form since 1986, years before the existence of hepatitis c was validated through cloning techniques, as a treatment of chronic non-A and non-B hepatitis. These promising preliminary data were confirmed by large placebo controlled trials of IFN alpha, given as a single agent (monotherapy) at a dose of 3 million units (MU) three times a week subcutaneously for 24 weeks. But of the 50 % patient that responded with an ALT-level normalisation within the 24 weeks, only 16 to 23 % of the patients with genotypes 2 and 3 and 2% to 9 % with the predominant genotype 1 had

sustained virological responses. The next improvement was made by prolonging the treatment duration to 48 weeks. (44)

Ribavirin, a nucleoside analogue in use for the treatment of respiratory syncytial virus, that was the most promising and efficiency increasing combination drug, lowers ALT levels in many patients with chronic hepatitis C. When used in combination with IFN-alpha, it increases the end of treatment response and reduces post-treatment relapse, which was confirmed by randomised placebo-controlled trials in the USA and other countries. The 12 month combined therapy increases the sustained virological response among patients with genotype 1 up to 28 to 31% and of patients with non-genotype 1 up to 65% even after only 6 month. These findings led to the recommendation that patients infected with genotype 1 require a course of 48 weeks of combination therapy whereas a 24-week combination therapy was sufficient for patients with genotypes 2 and 3. (44)

### 2.3.2 Current treatment options

**Peginterferon und Ribavirin** (standard of care)

Natural history studies indicate that 55% to 85% of individuals who develop acute hepatitis C will remain HCV-infected. The risk of developing cirrhosis ranges from 5% to 25% over periods of 25 to 30 years and hepatocellular carcinoma might be a consequence. The goal of therapy is to prevent these complications and death that may result from HCV infection. (21)

Because of the slow evolution of chronic HCV infection over several decades, it has been difficult to demonstrate the effectiveness of drugs. Short-term outcomes can be measured biochemically (normalization of serum ALT levels), virologically (absence of HCV RNA from serum by a sensitive PCR-based assay), and histologically (improvement in necroinflammatory score with no worsening in fibrosis score). (21)

**Current treatment dosage**

The current standard of care (SOC) is a combination of Ribavirin with one of the two pegylated Interferon alpha 2, alpha-2a (40 KD) or -2b (12 KD). Pegylation is the process of covalent attachment of polyethylene glycol polymer chains to the interferon molecules to hide it from the immune system and as a consequence to minimize the immune response and to prolong its circulatory time. The SOC Ribavirin dosage is 1,000-1,200 mg and 800 mg for patients infected with HCV genotype 1 and 2/3, respectively. The optimal dose of Peginterferon alfa-2b is 1.5 µg/kg/week dosed according to body weight whilst Peginterferon alfa-2a is administered at a fixed dose of 180 µg/week. (21)

A large prospective study showed that in late virological responders with genotype 1, the sustained virological responder rate was higher with extended 72 weeks (57% sustained virological response) of treatment compared to 48 weeks (44% sustained virological response) of treatment. Although discontinuation rates were not reported in all the studies, overall extended therapy had higher discontinuation rates compared to standard treatment (20% vs. 11%), so it should be considered if a therapy extension is useful to the patient. (45)

Trials with high dose Peginterferon alpha regimen (3 µg/kg weekly for 1 week, 1.5 µg/kg/weekly for 3 weeks and 1 µg/kg weekly for 44 weeks) were associated with a faster rate of viral clearance compared with the standard regimen, but the level of undetectable HCV RNA at the end of therapy was similar to the results of standard dosage. An increase in Ribavirin dose (1,600 to 3,600 mg per day) had in a small trial with genotype 1 patient's great success (90% SVR) but is hardly an option due to its sometimes serious adverse effects such as anaemia. (46) (47)

For patients with HCV genotype 4 infections, combination treatment with pegylated Interferon plus weight based Ribavirin administered for 48 weeks appears in trials to be the optimal therapy, just like with genotype 5 and 6, but here are hardly any sufficient trial data are on-hand due to its low world-wide frequency. (48)

Due to drug toxicity, discontinuation and therapy costs, it is important to find an individually tailored regimen for groups with different response to Ribavirin and Peginterferon alpha.

### 2.3.3 Results of therapy

SVR: Sustained virological response. HCV RNA is negative 24 weeks after completion of treatment.

EVR (partial or complete): early virological response. 2 log reduction in HCV RNA level compared to the baseline HCV RNA level (partial EVR) or HCV RNA negative at treatment week 12 (complete EVR).
The absence of an EVR is the most robust means of identifying non-responders. Ninety-seven to 100% of treatment-naive patients with HCV genotype 1 infection who did not reach an EVR failed to achieve an SVR. On the other hand only 65% to 72% of subjects who achieved an EVR ultimately attained an SVR. (21) (49)

RVR: rapid virological response. No detectable HCV RNA through PCR after 4 weeks of treatment.
Achieving an RVR is highly predictive of obtaining an SVR independent of genotype and regardless of the treatment regimen. RVR affects 66% of genotype 2 and 3, but only 15-20% of genotype 1, nevertheless is an absence of RVR not a basis for discontinuing treatment. (50)

ETR: end of treatment response. No detectable HCV RNA through PCR after 24 or 48 weeks of treatment.

Relapse: recurring HCV after a successful therapy end.

No responder: Failure to clear HCV RNA from serum after 24 weeks of therapy.

Null responder: Failure to decrease HCV RNA by 2 logs after 24 week of therapy.

### 2.3.4 Adverse events

Adverse events are a major reason for patients to discontinue therapy or a dose adaption has to be made. In the trial of Peginterferon alfa-2a and 2b plus ribavirin, 14% of patients had to discontinue therapy. The most common adverse events were influenza - like side effects such as fatigue, headache, fever and rigors, which occurred in more than half of the patients, and psychiatric side effects (depression, irritability, and insomnia), which occurred in up to 31% of patients. Laboratory abnormalities are the most common reasons for a dose reduction. Among these are neutropenia, which occurs in up to 20% of the patients, and anaemia observed in approximately one-third of patients, reaching a nadir within 6 to 8 weeks and requiring a dose reduction in 15% of the patients. (30)

Interferon - induced depression appears to be composed of two overlapping syndromes - a depression - specific syndrome characterized by mood, anxiety and cognitive complaints that respond well to serotonergic antidepressants, and neurovegetative symptoms, characterized by fatigue anorexia, pain and psychomotor slowing that may be treated with catecholamin modulators. (21)

### 2.3.5 Further Treatment Option

**New interferons**

Albinterferon is a genetic fusion polypeptide of Albumin and Interferon alfa-2b with a longer half-life than pegylated Interferons and similar sustained virologic response rates as Peginterferon but with a better tolerability. Peginterferon-λ is a pegylated type III Interferon that binds to a unique receptor with more limited distribution than the type I Interferon receptor. IFNl3 may yet be developed as an alternative HCV treatment, particularly in those

with the IL28B risk polymorphism, and genotyping for IFNl3- related single nucleotide polymorphisms may become part of a treatment decision algorithm. (51) (52)

**Protease Inhibitors**

The NS3/4A protease has key functions in the hepatitis C virus replication cycle as it generates the N-termini of the NS4A, NS4B, NS5A, and NS5B proteins and also works as an RNA helicase and a nucleotide triphosphatase. Monotherapy with protease inhibitors ciluprevir, telaprevir and boceprevir was shown to be effective in lowering the viral load but both protease inhibitors showed a rapid occurrence of drug resistant HCV strains within 2 weeks of therapy, indicating that protease monotherapy is not sufficient for treatment of patients with chronic hepatitis C, but Peginterferon alfa-2a and Ribavirin were effective in preventing the rapid occurrence of resistance. From large studies, it has become clear that response - guided therapy with Telaprevir is no inferior to 48 weeks of treatment. In therapy-naive patients with eRVR and 12 weeks of telaprevir-based triple therapy is required for the optimal virologic response. In patients with genotype 1 it appears that telaprevir undertakes the role of ribavirin as an additional drug to Peginterferon. (53) (52)

**Polymerase Inhibitors**

Two classes of NS5B polymerase inhibitors have been developed, nucleoside, which mimic the natural substrates of the RNA-dependent RNA polymerase and are incorporated into the elongated RNA, where they act as chain terminators, and non-nucleoside polymerase inhibitors, representing a heterogeneous group of compounds that bind to different allosteric enzyme sites, resulting in a conformational protein change before the elongation complex is formed. Both are targeting the HCV replication. New studies reported that 75% of patients who received twice daily 400 mg of ANA598, a non-nucleoside palm I site inhibitor, achieved undetectable HCV RNA levels at treatment week 12 (complete early virological response (cEVR)). Subject to further trials is BMS-790052, a NS5A inhibitor which shows rapid virological response rates (83% and 92%) in patients who received 10 and 60 mg once daily. In addition, undetectable HCV RNA levels at week 12 (cEVR) were observed in 83% of patients. In general it appears that polymerase inhibitors will find their place in a tailored triple therapy with Peginterferon and ribavirin. (54)

**Emerge Inhibitors**

These new drugs include inhibitors of HCV entry, internal ribosome entry site inhibitors, inhibitors of HCV assembly and release, and a-glucosidase inhibitors, and base on the fact that chronic hepatitis C is characterized by a high turnover of infected cells and continuous de novo infection of target cells. Cyclophilin inhibitor Debio 025 (Alisporivir) and SCY-635, which address a protein that participates in HCV replication, showed antiviral activity in patients

infected with different HCV genotypes (1–4) during monotherapy. Both showed selection of HCV resistant variants with mutations clustering in the NS5A gene.
Nitazoxanide (Alinia) is a thiazolide that appears to inhibit viral glycoproteins at the post-translational level has been shown in vitro to have activity against both hepatitis B virus and HCV and further studies are necessary. (54)

**Vaccine**

Immunoglobulin is ineffective in preventing hepatitis C and is no longer recommended for post exposure prophylaxis in cases of perinatal, needle stick, or sexual exposure. Although prototypes like epitope vaccines, vector vaccines, DNA vaccines and recombinant protein vaccines, that induce antibodies to HCV envelope proteins have been developed, currently, hepatitis C vaccination is not feasible practically. Genotype and quasispecies viral heterogeneity, as well as rapid evasion of neutralizing antibodies by this rapidly mutating virus, conspire to render HCV a difficult target for immunoprophylaxis with a vaccine. (1) (55)

## 2.4 Silibinin

### 2.4.1 Profile

Silybum marianum is an annual to biannual plant of the family Asteraceae growing up to 1.5 m. The milk thistle is native to southern Europe, southern Russia, Asia Minor and northern Africa and is naturalized in North and South America as well in South Australia. The commercial drug originates principally from the cultivated sources, partly from Germany, but primarily from China, Argentina, and Romania. The freshly milled fruits have a cocoa-like odour and an oily and bitter taste. (56)

Milk thistle has been used since the time of ancient physicians and herbalists to treat a range of liver and gallbladder disorders, including hepatitis, cirrhosis and jaundice, and to protect the liver against poisoning from chemical and environmental toxins, including snakebites, insect stings, mushroom poisoning and alcohol. (56)

The primary drug contains proteins, lipids in the form of triglycerides, and sugars, like arabinose, rhamnose, xylose, glucose, but the ingredients mainly responsible for the activities are the flavonolignans, which are a mixture of flavonoids also known as Vitamin P, a plant secondary metabolites and lignans, a phytoestrogen. (57)

This mixture, known as Silymarin, represents 1.5–3% of the dry drug weight and consists of Silibinin (approximately 50% to 60%), Isosilibyn (about 5%), Silychristin (about 20%) and Silydianin (about 10%), as well as Silimonin, Isosilychristin, Isosilibinin. (58)

### 2.4.2 Pharmacokinetic

The bioavailability of Silibinin, considered being the main active flavonolignan of silymarin, is low and depends on several factors such as the content of accompanying substances with a solubilizing character, as other flavonoids, phenol derivates, amino acids, proteins, tocopherol, fat, cholesterol and other substances found in the preparation, and the concentration of the preparation itself. (59)

The systemic bioavailability can be improved by adding solubilizing substances to the extract, by complexing it with phosphatidylcholine or β-cyclodextrin and possibly by the choice of the capsule material. This explains why the variations in the content, dissolution and (oral) bioavailability of Silibinin between different commercially available silymarin products – despite the same declaration of content – are significant. (60) (61)

Silibinin inhibits 'in vitro' denitronifedipine oxidation (CYP3A4; IC50 = 29 μM) and S (-)-warfarin 7-hydroxylation (CYP2C9; IC50 = 43μM) and also the dextromethorphan metabolism at the low affinity site.
It had no apparent effect on indinavir plasma concentrations, which is a powerful protease inhibitor used to treat HIV Infections, did also not affect the function of CYP3A4 and UGT1A1 in cancer patients treated with irinotecan or the kinetics of alcohol after a single dose of Silibinin. (62) (63) (64)

After the single oral administration of a standardised dose of 100–360 mg Silibinin in men, peak plasma levels were reached after approximately 2 h and ranged between 200 and 1,400 ng/ml Silibinin, of which approximately 75% was presented in the conjugated form. (65)

Only a small part is excreted through the urine and the major part is excreted via faeces (unchanged, not absorbed). Silibinin levels in bile reach approximately 100 × higher concentrations than in serum (10–5 to 10–4 mol/l of Silibinin in bile), with peak concentrations within 2 – 9 h. No accumulation is observed after multiple dosing. (60)

### 2.4.3 Modes of action

**Antiinflammatory and immuno-modulation activity**

The tumour necrosis factor-α receptor family, located in the cell membranes, contains several members with homologous cytoplasmic domains known as death domains (DD), important in initiating apoptosis and other signalling pathways following ligand binding by the receptors.
A pre-clinical study showed that Silibinin prevented the effect of TNF-α induced with α-amanitin in hepatocytes possibly by reactive oxygen species (ROS)-dependent mechanisms
Kang et al. demonstrate in their study that Silymarin directly inhibits the expression of cell adhesion which mediates the recruitment of monocytes the sites of inflammation. (66)

Silymarin inhibits dose dependent the TNF-alpha induction of NF-kB transcription and the CXCL-8 transcription a NF-kB target gene. The reasons a currently unknown, but because HCV infection induces oxidative stress and inflammation NF-kB is the best-characterized redox responsive transcription factor, it is therefore possible that Silymarin elicits anti-inflammatory actions by inhibiting NF-kB via antioxidant actions. It is also possible that Silymarin modulates cellular membranes and/or membrane receptor functions. (67)

Silymarin also inhibits the TNF-α- induced activation of mitogen-activated protein kinase and c-Jun *N*-terminal kinase and abrogated TNF-α-induced cytotoxicity and caspase activation (68)

Silibinin proved to be an immune-response modifier in vivo, inhibiting intrahepatic expression of tumor necrosis factor, Interferon gamma, Interleukin (IL)-4, IL-2 and iNOS, and augmenting synthesis of IL-10. (58)

**Enhanced protein synthesis**

Rats with administration of Silymarin, showed increased synthesis of DNA, RNA, protein and cholesterol suggesting the regeneration of liver, which is probably caused by a specifically stimulation of RNA polymerase. These observations were made only in those after a partial hepatectomy, where 70 per cent of the tissue was removed, and not in healthy controls, in hepatoma cells or other neoplastic cells. (69)

**Antifibrotic activity**

The excessive accumulation of extracellular matrix proteins including collagen, the remodelling of liver architecture and its results, liver failure, portal hypertension, hepatic encephalopathy and often required liver transplantation, are most likely influenced by the treatment with Silymarin. Silymarin inhibits NF-kB, protein kinases and other kinases and also retards hepatic stellate cells activation. (58) (70)

In animal studies Silymarin suppressed the expression of profibrogenic Pro-collagen-α1 (I) and TIMP 1, a natural inhibitor of the matrix metalloproteinase, most likely via down-regulation of TGF-β1 mRN. Silymarin, administered for 3 years, retarded the development of alcohol-induced hepatic fibrosis in baboons. (71) (72)

**Antioxidant effects**

Reactive oxygen species (ROS) are free radicals, like the superoxide radical, hydroxyl radical, hydrogen peroxide, and lipid peroxide radicals, are produced as a normal consequence of biochemical processes in the body and have been implicated in liver diseases. (73)
The mechanism of free radical damage includes ROS-induced peroxidation of the polyunsaturated fatty acid in the bilayer cell membrane, which causes the chain reaction of lipid peroxidation, thus damaging the cellular membrane and causing further oxidation of membrane, lipids and proteins. (74)

The cytoprotective effects of Silymarin are mainly attributable to its antioxidant and free radical scavenging properties. It reacts rapidly with HO- radicals in free solution at approximately diffusion controlled rate and it was shown that *in vitro* incubation with Silymarin in a therapeutic dosage concentration markedly increased the expression of superoxide dismutase in lymphocytes in patients with alcoholic cirrhosis. (73) (58)

Silymarin administration lowers the malondialdehyd levels in lung and brain tissues, and reverses the decrease in glutathione levels. In animal trials carbon tetrachloride ($CCl_4$) treatment caused significant decrease in Glutathione in liver homogenate tissue, while Silymarin increased significantly the level. Also found was an increasing in lipid peroxidation level in $CCl_4$ treated rats and significant decreasing for plant extract and silymarin. (75)

**Toxin blockade**

In many trials Silymarin has turned out to be a good candidate to treat iatrogenic and toxic liver diseases. It has a regulatory action on cellular and mitochondrial membrane permeability in association with an increase in membrane stability against xenobiotic injury. In case of intoxication with amanita and galerina mushrooms that contain, inter alia, the potent cytotoxin amanitin, it can prevent the absorption of toxins into the hepatocytes by occupying the binding sites, as well as inhibiting many transport proteins at the membranes. The phalloidin - transporting system, which is most probably also transport system for amanitin, belonging to the hepatocyte-specific organic anion uptake transporters OATP2 is inhibited in a competitive way by silymarin with no influence on membrane fluidity. (58)

An analysis based on 154 cases of intoxication with *Amanita phalloides* reported in Germany from 1983–1992, showed a mortality of 15.2% in 38 not Silibinin-treated cases vs. 8.3% in the remaining, Silibinin-treated patients. (68)

**Metabolism**

Silymarin and the polyphenolic fraction (PF) of Silymarin significantly reduce cholesterol absorption in rats fed on high cholesterol diet and caused significant decreases in plasma and VLDL cholesterol and content of cholesterol and triacylglycerol in the liver. The level of HDL cholesterol was significantly increased after silymarin, but not after administration of PF. It is assumed that the inhibition of cholesterol absorption caused by Silymarin and its polyphenolic fraction could be a mechanism contributing to the positive changes in plasma cholesterol lipoprotein profile and in lipid content in liver. (76)

Huseini et al. showed in their studies that Silymarin treatment significantly lowered the HbA1c and fasting blood glucose levels in diabetic patients. An explanation might be that the elevation of glucose and free fatty acid (FFA) levels in patients with DM II leads to the generation of reactive oxygen species and oxidative stress and as a result to insulin resistance, β-cell dysfunction and impaired insulin secretion. (77)

**Milk thistle and endocrine system**

Di Pierro et al. point out, that women orally treated for 63 days with Silymarin showed a clear galactagogue role for the product with an increase of 85.94% of the daily milk production (placebo:+32.09%) without affecting the main milk biochemical characteristics. (78)

Administration of silymarin to rats significantly increases their uterine weight and endometrial height, as well as hypertrophy of luminal epithelium, which have been established as reliable indices of estrogenic effects. Silymarin also prevents the bone loss in rats, induced by ovariectomy (OVX), significantly, maybe due to additive beneficial effect of Silymarin on bone either due to direct interaction with oestrogen receptor B or increasing bone formation parameters including ALP, osteocalcin and PTH. (79)

### 2.4.4 Combination therapy

**Cancer therapy**

The main role of Silymarin in cancer therapy originates from its antiradical potential, and consequently from its cytoprotective activity, which reduces the cytotoxicity of both, cancer treatment and cancer-induced substances: Silymarin significantly decreases the incidence of urinary bladder neoplasms and preneoplastic lesion caused by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine, azoxymethane-induced colon carcinogenesis in rats and inhibits skin carcinogenesis induced by benzoyl peroxide or 12-*O*-tetradecanoylphorbol-13- acetate. It also

protects the liver in a case of promyelocytic leukaemia receiving 6- mercaptopurine and methotrexate. (73)

Silibinin does show additive and synergic anti - cancer activity in prostate cancer models when combined with cisplatin, carboplatin, doxorubicin and/or mitoxantrone. (80)

Another very important fact is that silymarin also has a variety of features that directly interfere with the growth and dissemination of cancer. It has been reported to suppress the proliferation of tumor cells in various cancers including prostate, ovarian, breast, lung, skin, and bladder, through induction of G1 arrest and/or G2-M, activation of apoptosis-pathways procaspase-8, caspase 9, caspase 3, caspase 2 and poly(ADPribose) polymerase (PARP) cleavage. (81)

Gagan Deep et al. show that Silymarin has a remarkable antimetastatic effect against many cancers in various preclinical models. The antimetastatic efficacy of Silibinin has been reported through pleiotropic mechanisms including the inhibition of epithelial-to-mesenchymal transition (EMT) in cancer cells. (82)

The anti-angiogenic potential of silymarin has been demonstrated in various cancers. It inhibits the growth and survival of human umbilical vein endothelial cells (HUVECs) by inhibiting capillary tube formation, and induction of cell cycle arrest and apoptosis together with a reduction in invasion and migration, decreases the secreted vascular endothelial growth factor (VEGF) levels in prostate and breast cancer cells and regulates MMP2 and CD34 in human hepatoma cell lines down. (81)

**Silymarin in the therapy of hepatitis c**

Ferenci et al. showed in their study that intravenous applied MK-001 (a rigorously standardized Silymarin preparation) is a potent antiviral agent in patients with chronic hepatitis C not responding to standard antiviral combination therapy. After using a 15- and 20-mg/kg/day Silibinin infusion dose for the period of 14 days, HCV RNA was <15 IU/mL in 6 of 14 patients without any serious adverse effects, except a transitional sensation of heat. In other studies it appears that the antiviral effect of the standardized Silymarin may be due in part to stimulation of the Jak–Stat pathway and induction of an IFN antiviral response. (67) (83)

Other in vitro-trials showed that a standardized preparation of Silymarin, MK001, has the ability to dose dependently inhibit T-cell proliferation and proinflammatory cytokine secretion in vitro. Furthermore, MK001 was able to inhibit NF-kB-dependent transcription in Jurkat T cells stimulated through T-cell receptor engagement. Combined with the results mentioned above it proposes a model for potential silymarin efficacy in HCV infection that includes both antiviral and anti-inflammatory effects. (84)

# 3. The study

## 3.1 Aim of Study

The mechanism for the antiviral effect of silibinin is currently unknown. Possible mechanisms may include direct inhibition of viral replication and/or stimulation of cellular and humoral immune responses against HCV. The aim of the present prospective study is to show the effects of silibinin application on viral load and to assess humoral immunology during intravenous silibinin treatment of HCV nonresponders.

To assess a possible effect of silibinin on humoral immune function, plasma levels of various cytokines have been determined using the Luminex assay which enables analysis of multiple cytokines in a single sample. The following cytokines have been determined: IFN-gamma, TNF-α, IL1ß, IL4, IL6, IL7, IL8, IL10, IL15

**Duration and Dose of Therapy**

20mg/kg body weight Silibinin (Legalon) was applicated daily within two hours for 21 days. After one week of Silibinin application Peginterferon (Pegasys) and Ribavirin where added for the following two weeks. Peginterferon once a week 180µg parenteral. Serious side effects did not occour in which case the dose would have been reduced to 135µg once a week. Ribavirin was applicated oral 2 times a day 3 pills with 200mg each.

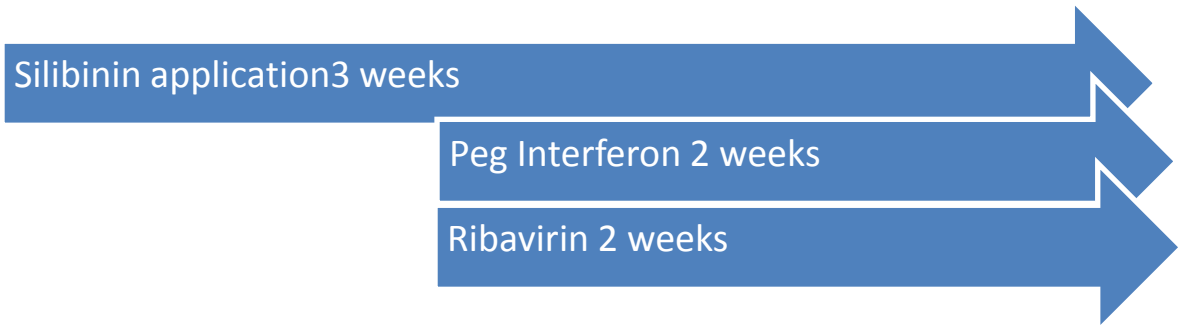



*Fig. 7 Duration of and starting points of drug administration.*

**Literature investigation**

Literature research was done by searching though common scientific publication-finding engines on the internet like Pubmed, Medline, Cochrane library and OvidSP embase. Further information where found in specific library literature and other gastroenterological and hepatological journals.

## 3.2 Target population

Male and female patients who did not respond to a prior IFN or IFN/Ribavirin combination therapy (standard or pegylated) for at least 12 weeks with serologically proven chronic Hepatitis C in the care of outpatient clinics specialized in hepatology are eligible for screening.

**Inclusion criteria**

- Male and female patients with chronic hepatitis C, not responding to a prior or on-going treatment with Peginterferon/ribavirin (standard of care). Patient must have received at least 12 weeks of full dose Peginterferon/ribavirin
- Age between 18 and 65 years
- Serologic evidence of chronic hepatitis C infection by an anti-HCV antibody test
- Present with at least one elevated serum alanine-aminotransferase (ALT) level higher than normal in the last 6 months before therapy start
- Positive HCV-RNA level in serum (determined with quantitative PCR (COBAS AMPLICOR® MONITOR HCV test v 2.0)
- Laboratory parameters (within 35 days prior to study start):
  - Hemoglobin values > 12 g/dl in women or > 13 g/dl in men
  - Leukocyte count (WBC) > 3 000 /µl
  - Platelets count > 100 000/µl
  -Creatinine not 1.5 times higher than normal
  - Normal TSH
  - Normal uric acid with a maximum tolerance of 15 % in patients without history of gout (or uric acid within this range achieved by treatment before study start
- Laboratory parameters (within 3 months prior to study start):
  - Hepatitis A anti – IgM negativity
  - HIV-Ab negativity
  - HbsAg negativity

- Negative urine or blood pregnancy test (for women of childbearing potential) documented within the 24-hour period prior to the first dose of study drug
- All fertile males and females receiving ribavirin must be using two forms of effective contraception during treatment and during the 6 months after treatment end
- Written informed consent obtained
- A liver biopsy within 6 months prior to study start (exception: in genotype 2 or 3 patients abdominal sonography within 3 months prior to therapy)

**Exclusion criteria**

- Class B or C cirrhosis as coded by Child Pugh classification
- Women with ongoing pregnancy or breast feeding
- Therapy with any systemic anti-neoplastic or immunomodulatory treatment (including supraphysiologic doses of steroids and radiation) 6 months prior to the first dose of study drug
- Any investigational drug 6 weeks prior to the first dose of study drug
- Drug addiction (including alcohol dependence) within 1 year prior to study start.
- Diabetes mellitus in patients receiving an insulin therapy
- History or other evidence of a medical condition associated with chronic liver disease other than HCV (e.g., hemochromatosis, autoimmune hepatitis, metabolic liver disease, alcoholic liver disease, toxin exposures)
- History or other evidence of bleeding from esophageal varices or other conditions consistent with decompensated liver disease
- History of severe psychiatric disease, especially depression. Severe psychiatric disease is defined as treatment with an antidepressant medication or a major tranquilizer at therapeutic doses for major depression or psychosis, respectively, for at least 3 months at any previous time or any history of the following: a suicidal attempt, hospitalization (for other reason than drug abuse) for psychiatric disease, or a period of disability due to a psychiatric disease. Exception: if there is a current psychiatric report which certifies there is no contraindication to interferon therapy, patient may be included
- History of immunologically mediated disease (e.g., inflammatory bowel disease, idiopathic Thrombocytopenic purpura, lupus erythematosus, autoimmune haemolytic anaemia, scleroderma, severe psoriasis, rheumatoid arthritis etc.)
- History or other evidence of chronic pulmonary disease associated with functional limitation
- History of a severe seizure disorder or current anticonvulsant use
- History of severe cardiac disease and severe coronary heart disease within the last 6 months (angina pectoris, congestive heart failure, recent myocardial infarction, severe hypertension or significant arrhythmia). If there is clinical suspicion of coronary heart disease cardiologic workup of the patient prior to study entry is recommended.

- History of thyroid disease poorly controlled on prescribed medications, elevated thyroid stimulating hormone (TSH) concentrations with elevation of antibodies to thyroid peroxidase and any clinical manifestations of thyroid disease
- History or other evidence of severe illness, malignancy or any other conditions which would make the patient, in the opinion of the investigator, unsuitable for the study
- History of major organ transplantation with an existing functional graft
- Evidence of severe retinopathy (e.g. CMV retinitis, macula degeneration)
- Inability or unwillingness to provide informed consent or abide by the requirements of the study Additional exclusion criteria concerning ribavirin:
- *Male partners of women who are pregnant*
- Any patient with an increased baseline risk for anaemia (e.g. thalassemia, spherocytosis, history of GI bleeding, etc.) or for whom anaemia would be medically problematic
- Patients with documented or presumed coronary artery disease or cerebrovascular disease should not be enrolled if, in the judgment of the investigator, an acute decrease in haemoglobin by up to 4 g/dL would not be well-tolerated

## 3.3 Observed Cytokines

Cytokines are proteins secreted by the cells of innate and adaptive immunity, which mediate many of the functions of these cells and are produced in response to microbes and other antigens. Different cytokines stimulate diverse responses of cells involved in immunity and inflammation and are therefore important to be considered to understand the mechanisms of pathogenesis and the effects of drugs. In the activation phase of immune responses, cytokines stimulate the growth and differentiation of lymphocytes, and in the effector phases of innate and adaptive immunity they activate different effector cells to eliminate microbes and other antigens. (85)

**Interleukin 7** is a four alpha helical cytokine, secreted by bone marrow stromal cells, that stimulates survival and expansion of immature precursors committed to the B and T lymphocyte lineages. Knockout mice lacking IL – 7 or its receptor are lymphopenic, with a decreased number of T and B cells. In vitro, IL – 7 is also mimicking the action of IL – 2 in terms of its growth factor property for mature T cells. (85)
Studies show that in HCV-infected rhesus macaques, the administration of recombinant simian IL - 7 during IFN-alpha therapy eliminates the lymphopenic effect of IFN-alpha, keeping the important CD 4+ and CD 8+ population at the same level. (86)

**Interleukin 4** is the major stimulus for the production of IgE antibodies and for the development of TH2 cells from naive CD4+ helper T cells. The principal cellular sources of IL – 4 are CD4+ T lymphocytes, activated mast cells and basophils. IL – 4 is the only cytokine that activates the STAT6 protein which is responsible for most of the actions of IL – 4. It is the principal cytokine that stimulates B cell Ig switching to the IgE isotype. Knockout mice lacking IL – 4 have less than 10 % of IgE levels. IL – 4 stimulates the development of TH2 cells from naive CD4+ cells and functions as an autocrine growth factor for differentiated TH2 cells. (85)
In the inflammatory state, IL-4 can inhibit proinflammatory cytokines and chemokine production in monocytes, and it enhances the expression of VCAM-1 on endothelial cells. (87)

**Interleukin 10** is an inhibitor of activated macrophages and is thus involved in the homeostatic control of innate immune reactions and cell mediated immunity responses. IL – 10 is mainly produced by activated macrophages and is therefore an excellent example of a negative feedback regulator. Its biological effects result from the inhibition of activated macrophages: IL – 10 stops the production of IL – 12 and TNF and as a consequence the production of IFN gamma. IL – 10 inhibits the expression of co-stimulators and class II MHC molecules on macrophages which as a consequence prevents the T cell activation and cell mediated immune reactions. The physiological role of IL - 10 during hepatitis C is likely to reduce tissue damage resulting from the unfavourable and excessive effects of inflammation. However, an inappropriate production of IL-10 may compromise the effectiveness of the immune system, allowing fulminant or persistent infection. (85)

**Interleukin 15** is a cytokine produced by mononuclear phagocytes and other cell types in response to viral infection and other triggers to the innate immunity. Structurally IL – 15 and its receptor are homologous to Interleukin 2. The best documented function of IL – 15 is to promote the proliferation of NK cells within the first few days after infection which is caused by its early synthesis. IL – 15 may also act as a T cell growth and survival factor, especially for long lived CD8+ memory cells. Knockout mice have a greatly reduced number of these cells. (85)

**Interleukin 1** works, similar to TNF alpha, as a mediator of the host innate inflammatory response to infections. IL – 1 major source, activated mononuclear phagocytes, also produce a natural inhibitor of IL – 1. The production is induced by bacterial products and other cytokines such as TNF. There are two forms of IL – 1, alpha and beta, binding to the same cell surface receptors and mediating the same biological activities, but the main IL – 1 population is beta. The biologic effects depend on the quantity of cytokines produced: In low concentrations IL – 1 works as a mediator of local inflammation and increases the expression of surface molecules that mediate leukocyte adhesion such as ligands for integrins. When secreted in larger quantities, IL – 1 enters the blood stream and exerts endocrine effects.

Systemic IL – 1 shares with TNF the ability to cause fever, to induce synthesis of acute phase plasma proteins by the liver, and to initiate metabolic wasting. (85)

**Interleukin 6** is a cytokine that functions in both innate and adaptive immunity and is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts and other cells in response to microbes and the cytokine IL – 1 and TNF. It activates the JAK/STAT pathway. In innate immunity IL – 6 stimulates the synthesis of acute phase proteins by hepatocytes and thus contributes to the systemic effects of inflammation the so called acute phase response. In adaptive immunity IL – 6 stimulates the growth of B lymphocytes that have differentiated into antibody producers. It also acts as a growth factor for neoplastic plasma cells and myelomas autonomously secrete IL – 6 as an autocrine growth factor. HCV induces production of IL – 6 and IL – 8 through a mediation via Toll-like receptor 2 (TLR2) and lead to increased B-cell proliferation *in vitro. It* may play a role in the pathogenesis of hepatitis C–associated MC and B-NHL. (85)

**Interleukin 8** is produced by monocytes and macrophages through the stimulation of LPS, live bacteria, and other, early proinflammatory cytokines such as TNF and IL – 1. IL-8 is relatively unique since it may be produced early in the inflammatory response but will persist for a prolonged period of time, even days and weeks. IL-8 is exquisitely sensitive to oxidants, a characteristic that plays a role in ischemia-reperfusion states and the induction of reactive oxygen intermediates but it is resistant to temperature and proteolysis and relatively resistant to acidic environments. IL-8 bears principal responsibility for recruitment and activation of neutrophils, the signature cell of the acute inflammatory response. (85) (88)

**Tumor Necrosis Factor** is the principal mediator of the acute inflammatory response and is responsible for many of the systemic complications of severe infections. The major cellular source is activated mononuclear phagocytes, although NK cells, stimulated T cells and mast - cells can also secrete this protein. TNF has two distinctive receptors, TNF – RI and TNF – RII, and knockout mice show that most biologic effects are mediated through TNF – RII. The principal physiologic function of TNF is to stimulate through several actions on vascular endothelial cells and leukocytes, the recruitment of neutrophils and monocytes to sites of infection and to activate these cells to eradicate microbes. TNF cause vascular endothelial cells to express integrins and endothelial selectins, which make the surface adhesive for neutrophils monocytes and lymphocytes. TNF induces the production of chemokines that induces chemotaxis and recruitment of IL – 1 producing cells, a cytokine which functions almost like TNF itself. In severe infections, TNF is produced in large amounts causes systemic clinical and pathologic abnormalities. TNF acts via hypothalamus as an endogenous pyrogen and increases the synthesis of prostaglandins. TNF acts on hepatocytes to increase synthesis on serum proteins like amyloid A and fibrinogen. The combination of hepatocyte derived plasma proteins induced by TNF, IL – 1 and IL – 6 constitutes the acute phase response to

inflammatory stimuli. Prolonged production of TNF causes the metabolic alterations of cachexia through appetite suppression and inhibition of lipoprotein lipase. The nature of TNF to cause intravascular thrombosis, due to its stimulation of tissue factors secretion of and inhibition of thrombomodulin is eponymous.
Increased titres of TNF-a have been associated with high levels of transaminases in patients with chronic HCV infection. It seems that the inhibitory effect of TNF-alpha on T-cell proliferation and activation is the key factor for resistance to IFN-a therapy. (85) (89)

**Interferon gamma** is the principal macrophage activating cytokine and serves critical functions in innate immunity and in specific cell mediated immunity. It has slight antiviral properties but mainly it functions as a signature cytokine of the TH1 subset of T helper cells. IF – γ is produced by NK cells, CD4+ TH1 cells and CD8+ T cells and functions as a mediator of innate and adaptive immunity.
IFN – γ is the macrophage activating cytokine that provides the means by which T lymphocytes and NK cells activate macrophages to kill phagocytized microbes by stimulating the synthesis of reactive oxygen intermediates and nitric oxide. IFN – γ stimulates expression of class I and class II MHC molecules and co-stimulators on APCs. IFN – γ promotes the differentiation of naive CD4+ T cells to the TH1 subset and inhibits the proliferation of TH2 cells. IFN – γ acts on B cells to promote switching to certain IgG subclasses, notably IgG2a, and to inhibit switching to IL – 4 dependent isotypes such as IgE and IgG1. (85)

## 3.3 Methods of measuring

### 3.4.1 Cytokine measurement

We used the Bio–Plex 200 multiplex suspension array system, which is a complete biomarker assay system employing Luminex xMAP–technology for the simultaneous detection and quantitation of multiple bioanalytes (proteins, peptides, DNA, RNA) in a single microplate well requiring only very small sample volumes. It is a fully integrated and validated system combining hardware, Bio–Plex Manager software, and system validation and calibration tools. The system uses a liquid suspension array of microscopic beads that are conjugated with different capture molecules. The beads are colour–coded with different ratios of two spectrally distinct fluorophores.

The array reader is equipped with two lasers: a red classification laser (635 nm) for bead discrimination and a green reporter laser (532 nm) for detection and quantitation of captured

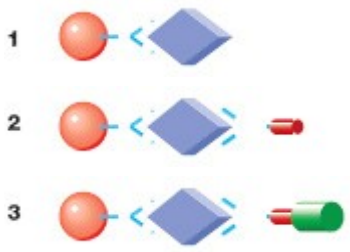
analytes. Potential applications include immunoassays (cytokines, phosphoproteins), enzyme assays, receptor–ligand assays and DNA⁄RNA hybridization assays.

**Multiplexing Bead Technology**

Currently, the most common application of this technology is the quantitation of cytokines in body fluids. Microscopic beads – each bead is conjugated to antibodies against a different cytokine – are incubated with plasma, serum, or cell lysate under scrutiny. Biotin– labeled detection antibodies specific for each cytokine together with fluorescently labeled streptavidin are used for detection and quantitation of the captured cytokines. The level of each cytokine bound to beads is indicated by the intensity of the reporter signal as measured with the green reporter laser. In essence, the Bio–Plex system allows for the analysis of multiple cytokines in one run which would normally require doing the equivalent number of single ELISAs, thus saving precious sample as well as time and costs.

**xMAP Technology**

The Bio–Plex Technology is based on a sandwich immunoassay technique:




A capture molecule conjugated to a colour–coded bead binds to a target analyte

Binding with a specific biotinylated detection antibody

Binding with streptavidin–PE

*Fig. 8 Sandwich immunoassay technique*

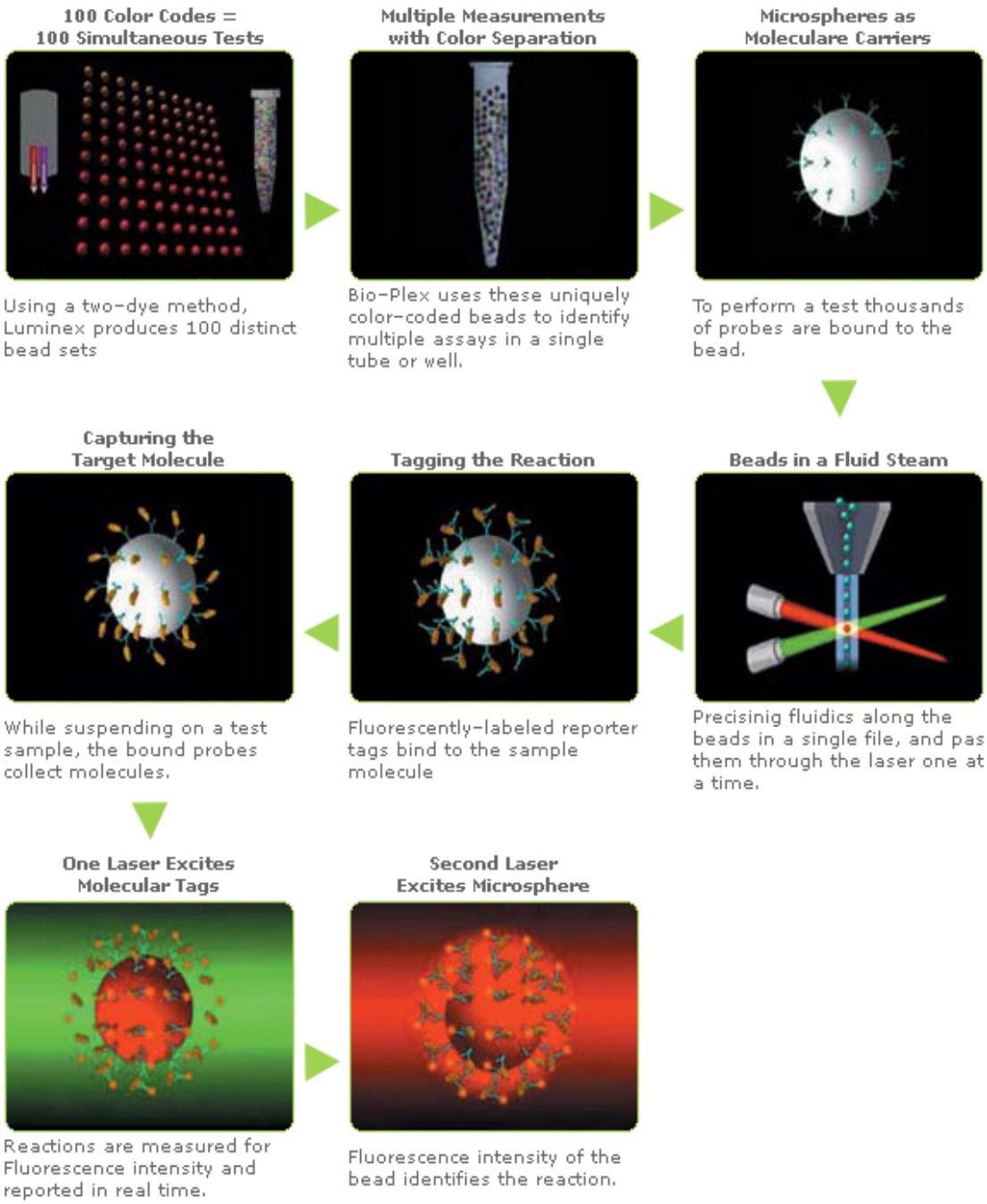



*Fig. 9 XMAP steps of procedure* (90)

To gain cytokine information all steps were taken according to the instructions of the manufacturer.

- Prewet wells and prepare serum
- Incubate antibody-coupled beads with sample for around 30 minutes
- Wash, then incubate with biotinylated detection antibody for another 30 minutes

- Wash, then incubate with streptavidin-PE for around 10 minutes
- Read on the Bio-Plex suspension array system for around 30 minutes (90)

### 3.4.2 Viral load

PCR: COBAS® AmpliPrep/COBAS® TaqMan® System:

Virus levels in peripheral blood can be quantitated by direct measurement of HCV RNA in plasma using nucleic acid amplification technologies such as polymerase chain reaction (PCR) and real-time PCR combined with reverse transcription (RT) of the viral RNA or signal amplification methods such as branch DNA and transcription mediated amplification (TMA) assays. The COBAS® AmpliPrep Instrument isolates RNA or DNA targets for real-time PCR using generic capture with silica-coated glass magnetic beads. This instrument works with both the COBAS® TaqMan® Analyser and this HCV test is based on three major processes: 1. Specimen preparation to isolate HCV RNA 2. Reverse transcription on the target to generate complementary DNA using the thermostable enzyme Thermus specie DNA polymerase, and 3. Simultaneous PCR amplification of target cDNA and detection of cleaved dual-labeled oligonucleotide detection probe specific to the target.

## 3.5 Statistical evaluation

The data out of the PCR and Cytokine counter were entered into a computerized database (Excel 2010, Microsoft Corporation, Redmond, Washington) in order to do further statistical evaluations and for illustration and better understanding Excel and SPSS were used

The data was processed with SPSS 17.0 (SPSS, Chicago, Illinois). Repeated measures ANOVAs were performed to analyse mean comparisons in examined parameters in dependency of time. As an illustration boxplots of all examined parameters were included.

# 4. Results

## 4.1 Viral load

The statistical analyse (GLM repeated measures) shows that there is a significant decrease in viral load concentration ($M_1$=4771884, $SD_1$=3656080; $M_2$=547295, $SD_2$ =1258874; $M_3$=57469, $SD_3$ =180087; $M_4$=35365, $SD_4$ =111605) at four points of measurement ($F$=5.504, $p$=.029). Additionally, the analyses of the within subject effects demonstrate a significant difference in serum concentration of viral load (F=13.749, p<.05). The pairwise comparison (adjusted with

Bonferroni correction) shows that there is a significant difference between first and third test point (mean difference $_{1/3}$=-4.714, Std. Error$_{1/3=}$1147613; $p$<.05). The pairwise comparison also shows the strong downward trend of viral load between the first and second test point (mean difference $_{1/2}$=-4224589, Std. Error$_{1/2}$=1279242; $p$<.1) where only Silibinin was applied. The mean values are shown in Figure 10.

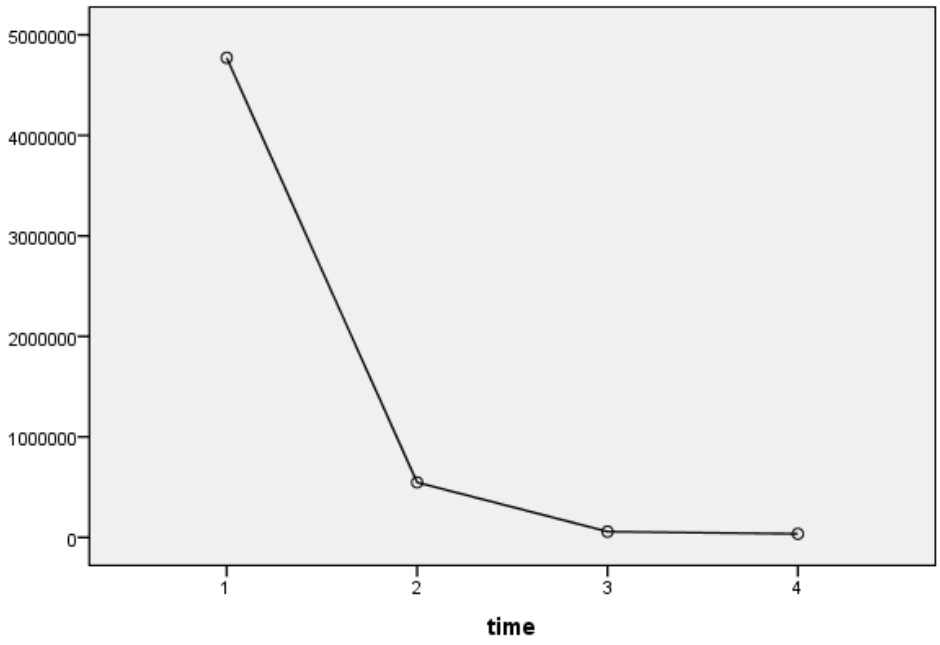



*Fig. 10: Mean values of viral load at four measurement points. 1=Baseline, 2=one week after 1, 3=two weeks after 1, 4=three weeks after 1.*

## 4.2 Cholesterol

The statistical analyse (GLM repeated measures) shows that there are no significant changes in the concentration of Cholesterol ($M_1$=191.90, $SD_1$ =33.38; $M_2$=206.60, $SD_2$ =31.76; $M_3$=199.40, $SD_3$ =38.92; $M_4$=196.90, $SD_4$ =31.54 at four points of measurement (1 – 4; $F$= .922, ns.). The mean values are shown in Figure **11.**

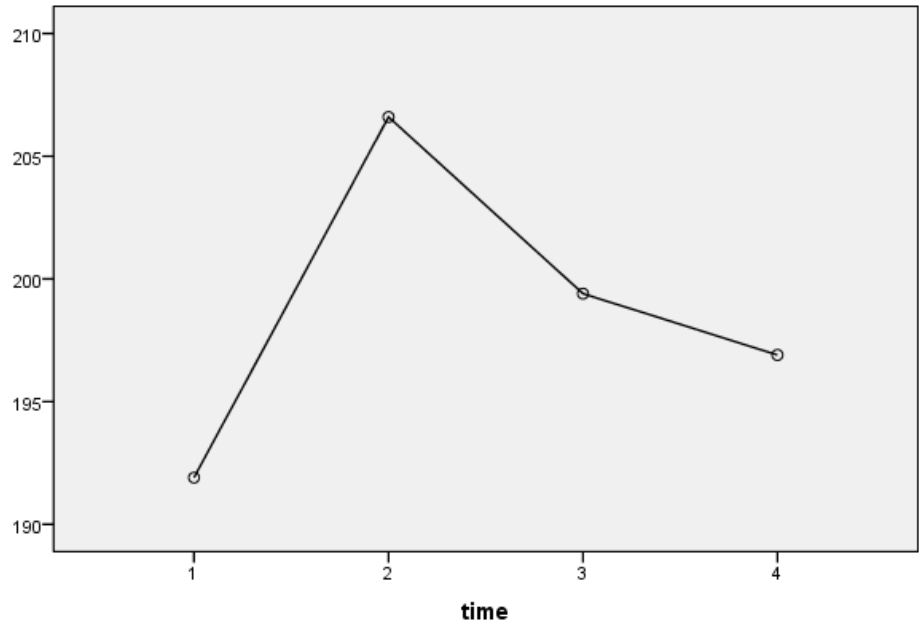



*Fig. 11: Mean values cholesterol at four measurement points. 1=Baseline, 2=one week after 1, 3=two weeks after 1, 4=three weeks after 1.*

## 4.3 Triglycerides

The statistical analyse (GLM repeated measures) shows that there are no significant changes in the concentration of Triglycerides ($M_1$=141.50, $SD_1$ =80.94; $M_2$=171.60, $SD_2$ =131.23; $M_3$=164.60, $SD_3$ =101.17; $M_4$=191.60, $SD_4$ =137.62 at four points of measurement (1 – 4; $F$= .922, ns.). The boxplots are shown in Figure **12.**

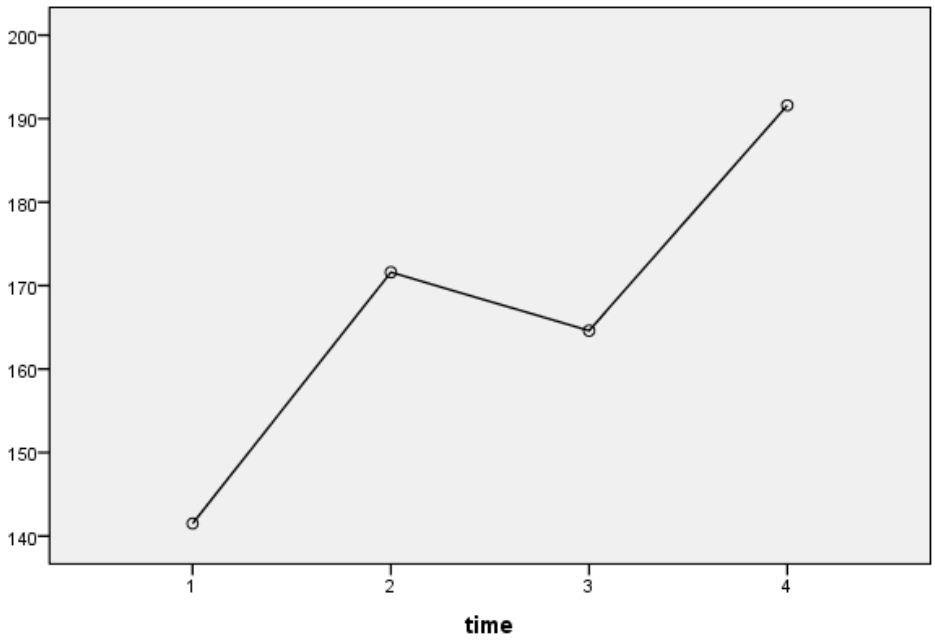



*Fig. 12: Mean values of Triglycerides at four measurement points. 1=Baseline, 2=one week after 1, 3=two weeks after 1, 4=three weeks after 1.*

## 4.4 Cytokines

### IFN gamma

The statistical analyse (General Linear Model (GLM) repeated measures) shows that there are no significant changes in the concentration of Interferon gamma ($M_{t0}$=6.58, $SD_{t0}$ =6.15; $M_{t1}$=2.18, $SD_{t1}$ =1.94; $M_{t2}$=2.24, $SD_{t2}$ =2.02; $M_{t3}$=16.82, $SD_{t3}$ =36.09 at four points of measurement (t0 – t3; *F*= .922, ns.). The boxplots are shown in Figure 13.

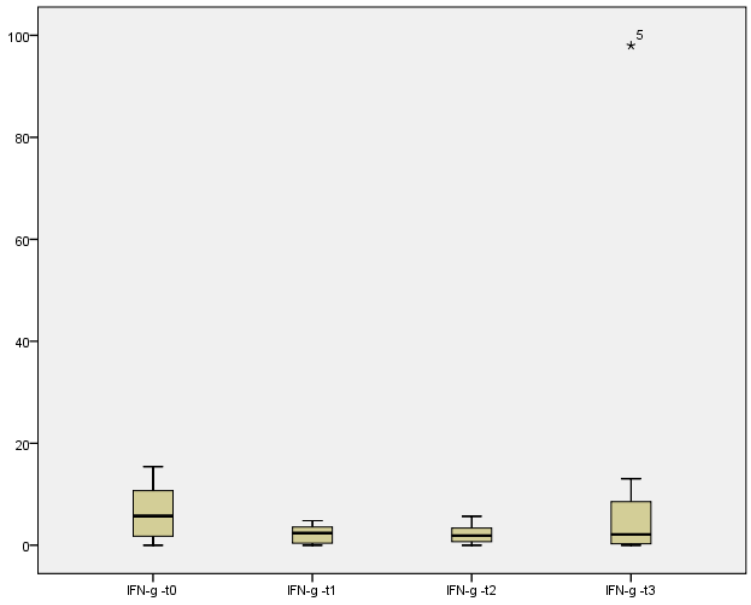



*Fig. 13: Boxplots of Interferon gamma at four measurement points. t0=Baseline, t1=one week after t0, t2=two weeks after t0, t3=three weeks after t0.*

**IL – 1b**

The statistical analyse (GLM repeated measures) shows that there are no significant changes in the concentration of Interleukin 1b ($M_{t0}$=1.95, $SD_{t0}$ =4.77; $M_{t1}$=.00, $SD_{t1}$ =.00; $M_{t2}$=2.86, $SD_{t2}$ =6.25; $M_{t3}$=.32, $SD_{t3}$ =.77 at four points of measurement (t0 – t3; $F$= 1.00, ns.). The boxplots are shown in Figure 14**.**

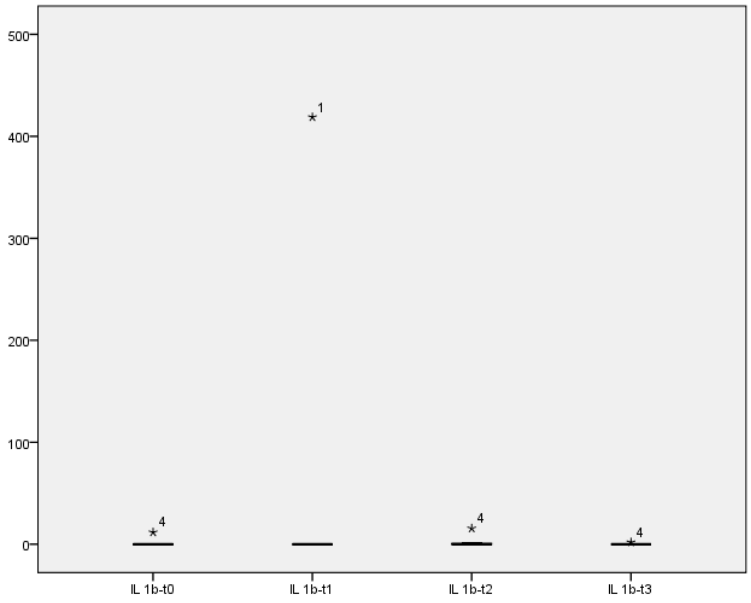



*Fig. 14: Boxplots of Interleukin 1b at four measurement points. t0=Baseline, t1=one week after t0, t2=two weeks after t0, t3=three weeks after t0.*

**IL – 4**

The statistical analyse (GLM repeated measures) shows that there are no significant changes in the concentration of Interleukin 4 ($M_{t0}$=129.02, $SD_{t0}$ =67.87; $M_{t1}$=147.78, $SD_{t1}$ =135.13; $M_{t2}$=228.20, $SD_{t2}$ =211.47; $M_{t3}$=192.13, $SD_{t3}$ =217.76 at four points of measurement (t0 – t3; $F$= 2.619, ns.). The boxplots are shown in Figure **15.**

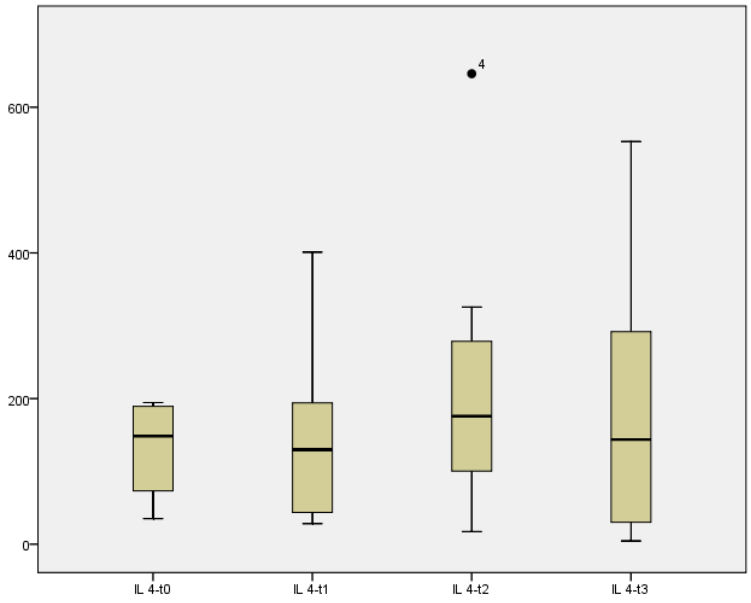



*Fig. 15: Boxplots of Interleukin 4 at four measurement points. t0=Baseline, t1=one week after t0, t2=two weeks after t0, t3=three weeks after t0.*

**Interleukin 6**

The statistical analyse (General Linear Model (GLM) repeated measures) shows that there are no significant changes in the concentration of Interleukin 6 ($M_{t0}$=.57, $SD_{t0}$ =.76; $M_{t1}$=.25, $SD_{t1}$ =.62; $M_{t2}$=.46, $SD_{t2}$ =1.12; $M_{t3}$=.58, $SD_{t3}$ =1.10 at four points of measurement (t0 – t3; $F$= 2.265, ns.). The boxplots are shown in Figure 16.

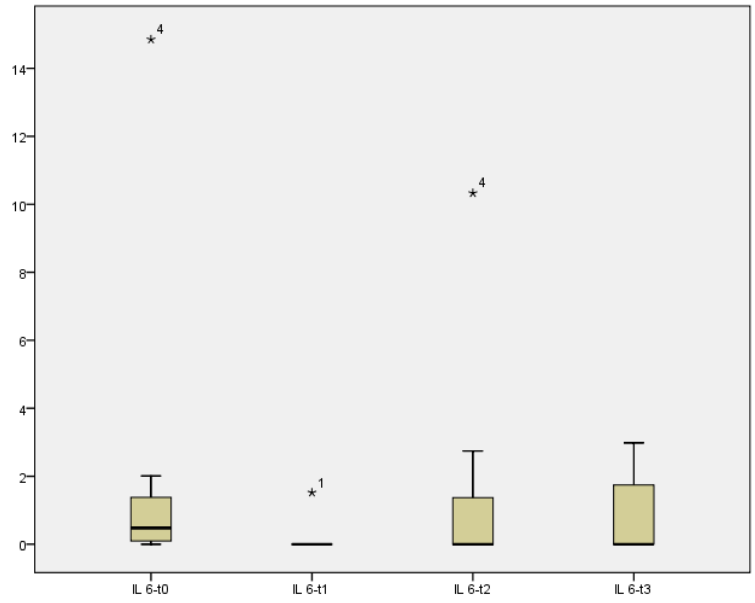



*Fig. 16: Boxplots of Interleukin 6 at four measurement points. t0=Baseline, t1=one week after t0, t2=two weeks after t0, t3=three weeks after t0.*

**Interleukin 7**

The statistical analyse (General Linear Model (GLM) repeated measures) shows that there are no significant changes in the concentration of Interleukin 7 ($M_{t0}$=2.87, $SD_{t0}$ =4.43; $M_{t1}$=0.47, $SD_{t1}$ =0.81; $M_{t2}$=4.19, $SD_{t2}$ =5.71; $M_{t3}$=0.23, $SD_{t3}$ =0.62 at four points of measurement (t0 – t3; $F$= 1.578, ns.). The boxplots are shown in Figure **17.**

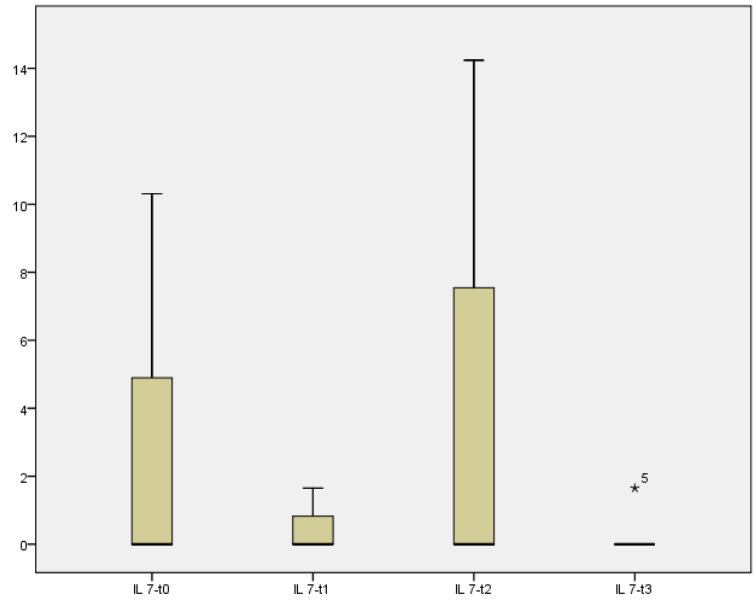



*Fig. 17: Boxplots of Interleukin 7 at four measurement points. t0=Baseline, t1=one week after t0, t2=two weeks after t0, t3=three weeks after t0.*

**IL – 8**

The statistical analyse (GLM repeated measures) shows that there is a clear shift in Interleukin 8 concentration ($M_{t0}$=73.37, $SD_{t0}$=29.76; $M_{t1}$=40.72, $SD_{t1}$ =30.82; $M_{t2}$=35.56, $SD_{t2}$ =25.14; $M_{t3}$=28.58, $SD_{t3}$ =22.64) at four points of measurement ($F$=5.081, $p$=.075). Additionally, the analyses of the within subject effects demonstrate a significant difference in serum concentration of Interleukin 8 (F=3.619, p<.05). The pairwise comparison (adjusted with Bonferroni correction) shows that there is a significant difference between first and third test point (mean difference $_{t0/t2}$=37.803, Std. Error$_{t0/t2=}$9.646; $p$<.05). The boxplots are shown in Figure 18.

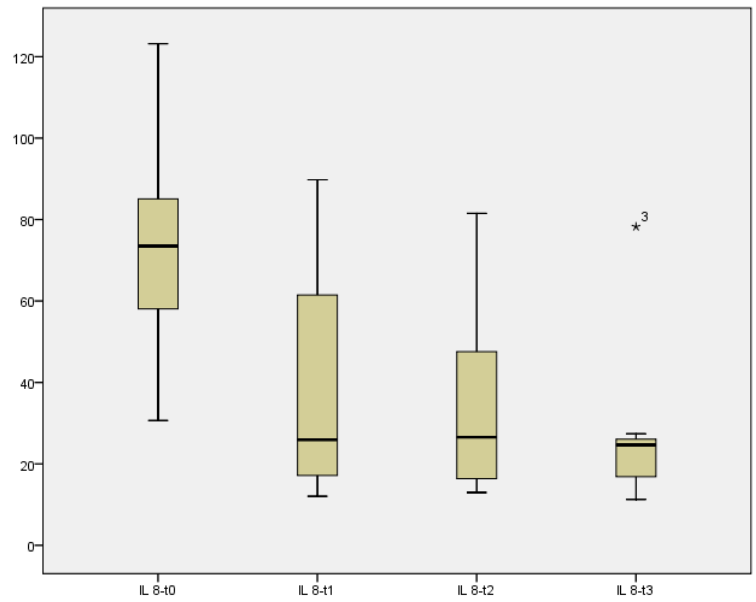



*Fig. 18: Boxplots of Interleukin 8 at four measurement points. t0=Baseline, t1=one week after t0, t2=two weeks after t0, t3=three weeks after t0.*

**IL - 10**

The statistical analyse (GLM repeated measures) shows that there are no significant changes in the concentration of Interleukin 10 ($M_{t0}$=2.65, $SD_{t0}$ =1.70; $M_{t1}$=1.69, $SD_{t1}$ =1.58; $M_{t2}$=2.56, $SD_{t2}$ =3.51; $M_{t3}$=4.35, $SD_{t3}$ =3.84 at four points of measurement (t0 – t3; $F$= 1.963, ns.). The boxplots are shown in Figure **19.**

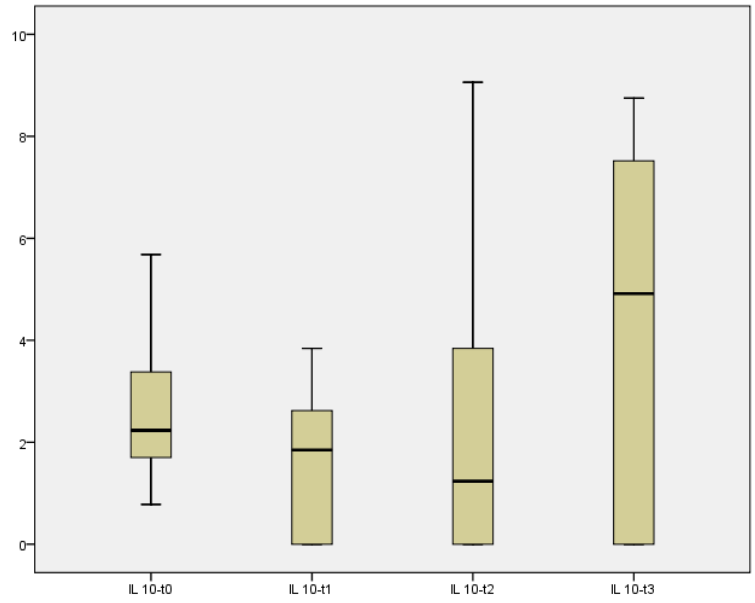



*Fig. 19: Boxplots of Interleukin 10 at four measurement points. t0=Baseline, t1=one week after t0, t2=two weeks after t0, t3=three weeks after t0.*

**IL – 15**

The statistical analyse (GLM repeated measures) shows that there are no significant changes in the concentration of Interleukin 15 ($M_{t0}$=2.91, $SD_{t0}$ =4.30; $M_{t1}$=1.16, $SD_{t1}$ =1.54; $M_{t2}$=3.64, $SD_{t2}$ =5.77; $M_{t3}$=2.32, $SD_{t3}$ =2.84 at four points of measurement (t0 – t3; $F$= .924, ns.). The boxplots are shown in Figure 20.

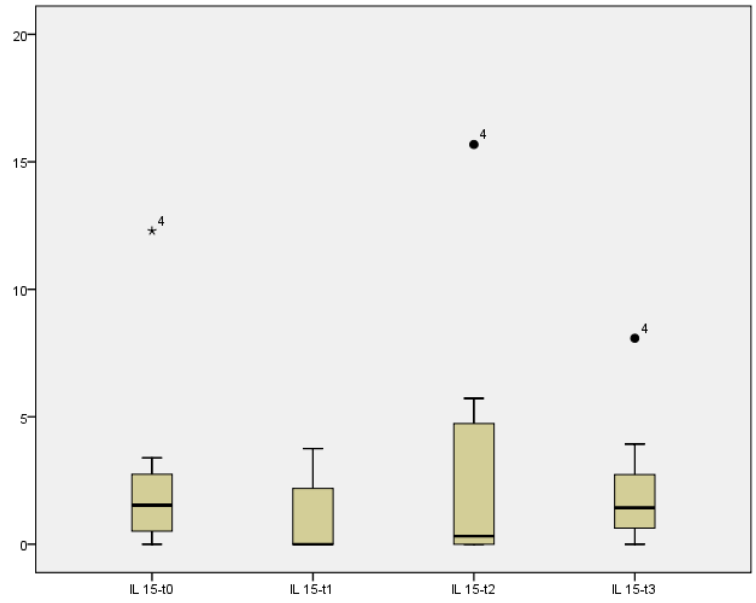



*Fig. 20: Boxplots of Interleukin 15 at four measurement points. t0=Baseline, t1=one week after t0, t2=two weeks after t0, t3=three weeks after t0.*

**TNF alpha**

The statistical analyse (GLM repeated measures) shows that there are no significant changes in the concentration of Tumor Necrosis Factor ($M_{t0}$=11.31, $SD_{t0}$ =3.18; $M_{t1}$=10.62, $SD_{t1}$ =3.08; $M_{t2}$=11.71, $SD_{t2}$ =4.52; $M_{t3}$=14.10, $SD_{t3}$ =6.34 at four points of measurement (t0 – t3; $F$= 1.873, ns.). The boxplots are shown in Figure **21.**

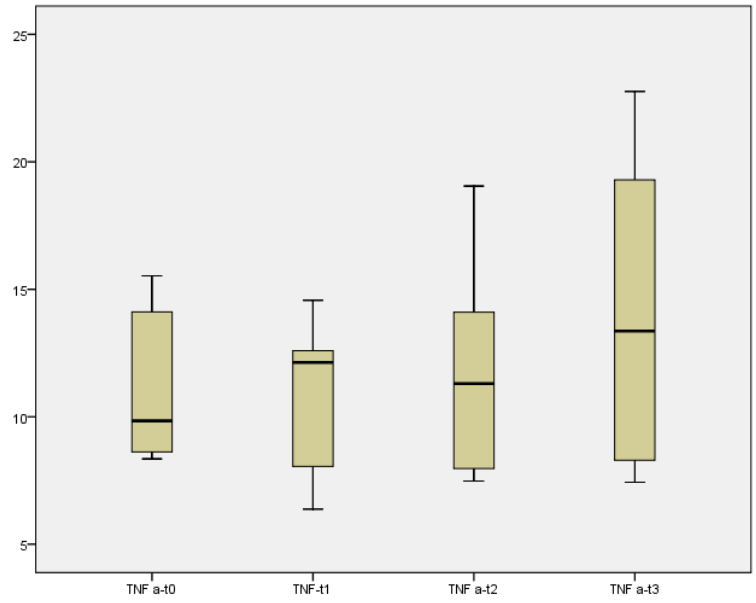



*Fig. 21: Boxplots of TNF – alpha at four measurement points. t0=Baseline, t1=one week after t0, t2=two weeks after t0, t3=three weeks after t0.*

# 5. Discussion and Conclusion

## 5.1 Discussion

**PCR**

The results of the weekly PCR show that the application of 20mg/kg body weight Silibinin for 3 weeks significantly lowers the viral load in patients with chronic hepatitis C which were not responding to prior standard antiviral combination therapy. These results are in accord with in vitro trials and earlier studies that showed reduction in viral load.

Ferenci et al. administered 15- and 20-mg/kg/day dose, and as a result HCV RNA dropped below 15 IU/mL in 6 of 14 patients after the Silibinin infusion period of 14 day. (83)
The antiviral properties of Silibinin can be considered as established even though earlier studies were not able to find significant changes in HCV RNA titers. This can be explained by the complexity of Silibinins pharmacokinetic, the various bio-availability of intravenous versus oral application and the dose- and treatment-duration–dependent effectiveness.

Silibinin was safe and, apart from rare reports of transient sensation of heat, well tolerated in our study. No significant differences were observed in the nature or frequency of adverse events seen during Silibinin and placebo treatment with the overall incidence being 23% and 19%, respectively, and gastrointestinal adverse effects predominating. (91)

For adult patients 20mg/kg for 21 days appears as an optimal treatment duration and dose, which is also recommended by the manufacturer (MADAUS GmbH, 51101 Köln, Germany, drug registration number 4178.00.00). Because Peg-interferon alpha 2a and ribavirin have been reported to synergistically impair the mitochondrial function triggering formation of reactive oxygen species, free radicals, and the initiation of lipid peroxidation, it might be helpful to administer the antioxidant Silibinin before RBV/IFN, to reduce tissue damage and fibrogenesis (92)

**Lipoproteins**

Triglycerides and cholesterol showed no significant changes but triglycerides level had tendencies to rise. Studies show that chronic HCV infection is associated with hypocholesterolemia and hypotriglyceridemia, which can be reversed by successful eradication of HCV. The clinical significance of hypolipidemia is reversal among SVR patients, such as the risk of coronary artery or cerebral vascular disease. (93)

Harrison et al. discovered in a large retrospective analysis derived from a multicentre trial that patients with baseline elevated LDL levels or low HDL levels have improved SVR rate. These findings therefore lead to a re-evaluation of statins in the treatment of Hepatitis C. (94)

Lipid abnormalities have also been associated with IFN therapy, with the most notable changes being an increase in total and very low-density lipoprotein triglycerides and a decline in high-density lipoprotein cholesterol. The underlying mechanism(s) for such IFN-induced disturbances in lipoprotein metabolism is unclear but some studies suggest that they may be associated with inhibition of lipoprotein lipase activity.

**Cytokines**

Although we did not find any significant changes in the cytokine levels there are studies where a dose – independent inhibition of TNF alpha production of monocytes was observed in vitro. (95)
Polyak et al. have also shown that Silibinin inhibits basal and TNF-alpha induced activation of NF-kappaB, and a direct antiviral effect, which may be due in part to stimulation of the Jak–Stat pathway and induction of an IFN antiviral response. The possible reason for the discrepancies between the results of our study and the in vitro experiences with Silymarin, might be located in the different study type and in relatively high doses of in vitro studies (100 – 300 µmol/L or ca. 50 –150 µg/mL). (67)
The backgrounds for the measured change of Interleukin 8 concentration need to be evaluated, due to the fact that to our knowledge there a no studies concerning the effect of Silibinin on Interleukin 8.

## 5.2 Conclusion

Due to the fact that we did not find any significant changes in cytokine levels we suggest that the strong antiviral properties of Silibinin are induced by other ways of interaction, such as direct virostatic effects or as entrance inhibitor in target cells. The impressive drop in viral load already during week one corroborates the assumption that Silibinin is not only active as part of a combination therapy. Mehrab-Mohseni et al. showed that Silibinin starts to downregulate HCV RNA in a dose-dependent manner after 2 h, after 6 h of treatment they already observed significant effects. (96)

In recent studies of Ahmed-Belkacem et al. it was shown that Silibinin inhibits HCV RNA-dependent RNA polymerase (RdRP) function and also HCV genotype 1b sub-genomic replicon replication and HCV genotype 2a strain JFH1 replication in in-vitro cell cultures, which might be because Silibinin components could induce cellular antiviral effectors and/or Silibinin components could directly inhibit vital HCV functions. (97)

Mehrab-Mohseni et al. also found out that Silibinin treatment is also associated with downregulation of HMOX-1 mRNA and upregulation of Nrf2 protein. Nrf2 gene expression leads to a better protection against oxidative damage in HCV – replicating cells. (96)

The results of our study corroborate the role of Silibinin as a trend-setting therapy for patients which not responding to standard therapy. Further studies are needed to gain complete insight into the background of Silibinins effectiveness.

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