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VADIM BRJALIN

Chronic hepatitis C: predictors of treatment response in Estonian patients

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Department of Internal Medicine, University of Tartu, Estonia

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Paper II Brjalin V, Salupere R, Tefanova V, Prikk K, Lapidus N, Jõeste E. Sarcoidosis and chronic hepatitis C: a case-report. World J Gastroenterol 2012;18(40):5816–20.

Paper III Brjalin V, Salupere R, Tallo T, Kuznetsova T, Priimägi L, Tefanova V. Predictors of treatment response in patients with hepatitis C 1b genotype. Cent Eur J Med 2013;8(6):822–9.

Paper IV Kuznetsova T, Tallo T, Brjalin V, Reshetnjak I, Salupere R, Priimägi L, Katargina O, Smirnova M, Jansons J, Tefanova V. Amino acid polymorphisms within the entire HCV NS5A region in Estonian chronic HCV 1b patients with different treatment response. Hepat Mon 2013;13(12):e14481.

Author's personal contribution:

Papers I, II, III: participated in conceiving and designing the study and patients' recruitment, collected demographic and clinical data, performed ultrasound observation with transcutaneous liver biopsies of all studied patients, conducted their treatment and clinical follow-up, performed statistical data analysis and wrote the articles.

Paper IV: participated in the study design, collected demographic and clinical data, performed ultrasound observation with transcutaneous liver biopsies of all studied patients, conducted their treatment and clinical follow-up, collected serum samples for genetic analysis, participated in writing the article.

2. ABBREVIATIONS

aa	amino acid
AE	adverse events
ALT	alanine aminotransferase
AMAM2	antimitochondrial antibodies, M2 fraction
ANA	antinuclear antibody
Anti-HCV	antibody to the hepatitis C virus
AST	aspartate aminotransferase
BMI	body mass index
BOC	boceprevir
CDC	Center for Disease Control and Prevention
CHC	chronic hepatitis C
CPMP	Committee for Proprietary Medicinal Products
CRS	Cytoplasmatic retention signal
CT	computer tomography
DAA	direct acting antivirals
EASL	European Association for the Study of the Liver
ECDC	European Centre for Disease Prevention and Control
EMA	European Medicines Agency
EOT	end of treatment
EVR	early virological response
cEVR	complete early virological response
pEVR	partial early virological response
GGT	gamma glutamyl transferase
ER	endoplasmatic reticulum
FDA	Food and Drug Administration
HBsAg	hepatitis B virus surface antigen
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
Hgb	hemoglobin
HIV	human immunodeficiency virus
IDU	injecting drug use
IFN	interferon
IL28B	interleukin 28B
IRES	internal ribosome entry site
IRRDR	IFN/RBV resistance-determining region
ISGs	interferon stimulating genes
ISDR	interferon sensitivity determining region
LKMA	liver kidney mitochondrial antibodies
LVL	low viral load
NANBH	non A non B hepatitis
NLS	nuclear localization signal
NR	non-response
NS	nonstructural

NS5A	nonstructural protein 5A
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PegIFN	peginterferon
PKR-bd	protein kinase-binding domain
RBV	ribavirin
RL	relapse
RNA	ribonucleic acid
RVR	rapid virological response
SMA	smooth muscle antibodies
SOC	standard of care
ST	patient who stopped treatment
SVR	sustained virological response
Th1	T-helper 1 lymphocyte
Th2	T-helper 2 lymphocyte
TSH	thyroid stimulating hormone
TVR	telaprevir
UTR	untranslated region

3. INTRODUCTION

Hepatitis C virus (HCV) is one of the leading causes of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). With an estimated HCV prevalence of about 2.35% of the world population and 3 to 4 million persons newly infected each year, HCV infection is a serious public health care issue worldwide. Hepatitis C virus is a blood-borne virus with primarily parenteral mode of transmission. The most common transmission route in Europe, accounting for around 78% of cases, is injection drug use (IDU), which has also prevailed in Estonia from the mid-1990s, followed by sexual contacts. The number of patients with chronic hepatitis C (CHC) is increasing and is expected to be an important cause of premature mortality and may exert an extra burden on the health care system in the nearest future.

In Europe the prevalence of HCV ranges between 0.1 and 3.2% with low prevalence in Belgium and higher in Romania. In Estonia up to 1% or even more out of 1.34 million inhabitants are infected with HCV. The HCV has been classified into 6 major genotypes and a number of subtypes with different geographic distributions. In Estonia, like in other Eastern European countries, the most predominant HCV subtypes are 1b and 3a.

Since 2002 therapy with peginterferon alfa-2a/2b (PegIFN) plus ribavirin (RBV) has been recognized as the current standard of care (SOC) for chronic hepatitis C. The aim of antiviral therapy is the eradication of HCV infection in order to prevent the complications of HCV-related liver diseases. The endpoint of therapy is a sustained virological response (SVR). The response to antiviral therapy strongly depends on HCV genotype. In general, HCV genotypes 2 and 3 respond better to combination therapy with SVR over 80%, but results still remain unsatisfactory for genotype 1 with SVR rate ranging from 40 to 52 %.

Thus, predicting non-response (NR) is of major interest for both patient wellbeing and health care expense. Different pre- and on-treatment host and viral factors, as well as treatment-related factors influence treatment outcome in HCV-infected patients. Some of them, such as host (age, stage of fibrosis, genetics) and viral factors (genotype, viral load, viral kinetics during treatment), and adherence to treatment are known with less controversial issues needed for further research. Others, like polymorphisms due to amino acid (aa) substitutions in different regions of the HCV genome, including the non-structural 5A (NS5A) protein, suggested also as an additional potential predictor of SVR, are less studied. The results of different studies on HCV genetics are conflicting and not sufficiently clear, especially regarding European patients.

The SOC for treatment of CHC patients changed in mid 2011. The appearance of new standard “triple therapy” with direct acting antivirals (DAA) has led to significant improvements in response rates even for patients infected with HCV genotype 1 and has reduced the duration of treatment. However, to date a very high cost of these medications might be a barrier for their wide use in low- and middle-income countries.

The PegIFN plus RBV therapy has been available for treatment of CHC in Estonia from 2005 with up to 400 patients treated annually. Regardless the fact that from 2014 two DAA, HCV NS3 protease inhibitors (PIs), telaprevir (TVR) and boceprevir (BOC), were approved for use in the treatment of genotype 1-infected patients, dual antiviral therapy has remained the backbone of treatment and is less expensive for CHC.

There are several studies and doctoral theses dealing with the molecular epidemiology of HCV infection and the relationship between host genetic factors and susceptibility to and clearance of HCV in Estonia (Zusinaite et al 2000, 2005, Tallo et al 2000, 2007, Huik et al 2013). At the same time, studies assessing the efficacy of PegIFN plus RBV therapy and determining the predictors of treatment response in Estonian patients with CHC are so far lacking.

For the first time, the current thesis focuses in a detailed manner on the analysis of different pre- and on-treatment host and viral factors, including polymorphism of NS5A regions, which may be associated with treatment outcome in Estonian treatment-naive HCV infected patients and which could be used as a predictors of treatment response to PegIFN/RBV therapy.

4. REVIEW OF THE LITERATURE

4.1. Hepatitis C virus

4.1.1. Historical overview

The steps towards discovering the hepatitis C virus (HCV) began in the mid-1970s.

Known before as non-A, non-B hepatitis (NANBH), it was first mentioned in 1975 (Alter et al 1975). Novel technologies with the use of recombinant DNA led to the isolation of a single small cDNA clone 5-1-1 from a patient with NANBH, which was distantly related to flaviviridae, and encoded for a protein that binded antibodies only to NANBH virus, which was renamed as hepatitis C virus (Choo et al 1989). Further, the structure of the virus genome encoding for certain enzyme activities like serine protease, helicase and polymerase, was identified (Kato et al 1990).

Hepatitis C virus belongs to the *Flaviviridae* family of viruses, which comprises at least three distinct genera, i.e. Pestiviruses, Flaviviruses, and Hepaciviruses, which currently includes hepatitis C virus and GB virus (Thiel et al 2005). HCV particles are spherical in shape with a diameter of 50 to 70 nm, consist of nucleocapsids (30–35 nm) with multiple copies of core protein containing single – strand RNA, i.e. the HCV genome, and is surrounded by a lipid envelope with two anchored viral glycoproteins, E1 and E2 (Figure 1). HCV virions are stable at lower temperatures, but become sensitive when exposed to high temperatures and different alcohols and antiseptics (Ciesek et al 2010).

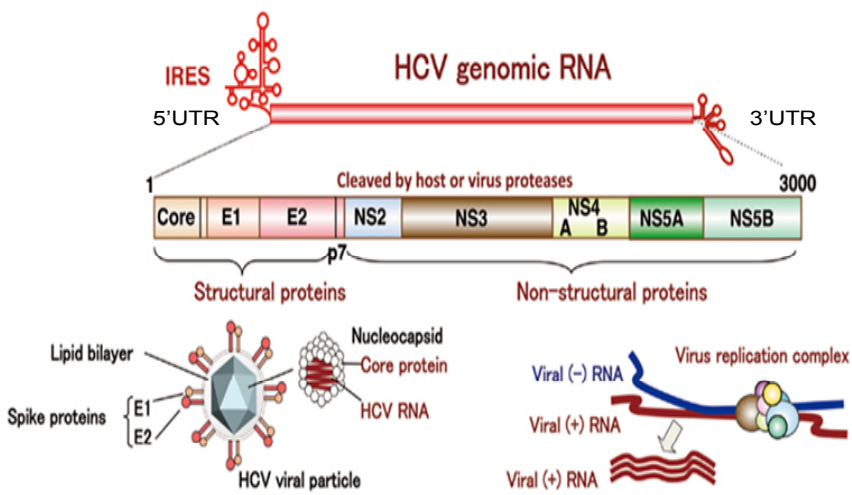

Figure 1. Structure of the HCV genome and particle (Copyright permission from Frontiers Media SA, Moriishi et al 2012)

4.1.2. Genomic organization and function of HCV proteins

The HCV genome consists of one 9.6 kb single-stranded positive RNA molecule with a single open reading frame (ORF) flanked by structured 5' and 3' nontranslated regions (UTR), which are 341 and approximately 230 nucleotides (nt) in length, respectively (Choo et al 1991). The 5'UTR contains an internal ribosome entry site (IRES), which is essential for cap-independent translation of the viral RNA (Rijnbrand et al 2000). The 3'UTR consists of a variable region, a poly U tract and a highly conserved 98-nucleotide RNA element essential for viral replication (Friebe et al 2002).

The linear molecule contains approximately 3000 amino acids, which are processed by host and viral proteases into 10 individual proteins, including structural proteins (core, envelope E1, and E2), hydrophobic short peptide p7 and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B), (Figure 1).

Structural proteins

Besides its role in capsid formation, the core protein can regulate particle assembly, binding and translation of viral RNA (Ait-Goughoulte et al 2006), it may be also involved in other processes such as cell signalling, apoptosis, fibrosis, lipid metabolism and suppression of host immune responses (Tellinghuisen et al 2002, Rehermann 2009).

The two envelope glycoproteins, **E1** and **E2**, are thought to play a pivotal role at different steps in the HCV replicative cycle. There is now strong evidence that they are essential for host-cell entry by binding to receptor(s) and inducing fusion with a host-cell membrane (Bartosch et al 2003). Two hypervariable regions, HVR1 and HVR2, have been identified in E2 glycoproteins. The HVR1 is known as a neutralizing epitope of HCV and together with HVR2 it modulates E2 binding (Zibert et al 1997).

The short peptide **p7** may act as an ion channel and participate in virus assembly and/or release of viral particles (Griffin et al 2003).

Nonstructural proteins

Among the nonstructural proteins, **NS2** is a transmembrane protein with protease activity, which cleaves NS2-NS3 junction of the polyprotein and can inhibit apoptosis (Erdtmann et al 2003).

The **NS3** protein has serine, nucleotide triphosphatase and RNA helicase activities. Together with NS4A it constitutes a protease that is important for polyprotein cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions (Failla et al 1994). The NS3 influences interferon-dependent innate cellular host defence by inhibiting certain pathways (Gale 2003, Rehermann 2009).

The **NS4A** is a cofactor of the NS3 serine protease. It directs NS3 to the endoplasmatic reticulum (ER) and increases its stability (Wölk et al 2000).

The **NS4B** is a small membrane-associated protein localized in ER and is a component of the replication complex (Penin et al 2004).

NS5A is a phosphoprotein with multiple and diverse properties. NS5A participates in viral RNA replication, modulation of cell signalling pathways, pathogenesis and apoptosis regulation, it can interact with other HCV and host cell proteins (Rehermann 2009, Yamasaki et al 2012). Interest in this protein arose when it was shown that NS5A can bind and inhibit IFNα-inducible double-stranded RNA-activated protein kinase, providing molecular explanation of HCV resistance to IFN therapy (Gale et al 1997). The NS5A protein is variable in length and sequence according to the different genotypes (Le Guillou-Guillemette et al 2007).

The **NS5B** is a viral RNA dependent RNA polymerase (RdRp) – a key enzyme in the viral replication process, responsible first for the synthesis of minus-strand RNA from plus-strand RNA template and, further, negative-strand RNA serves as a template for the synthesis of plus-strand RNA molecules. NS5B is a membrane protein with the "fingers", "palm" and "thumb" subdomains (Penin et al 2004). As a crucial enzyme for viral replication, it represents an attractive target for development of new antiviral drugs.

4.1.3. Genetic variability of HCV

The HCV has high genetic diversity, due to the lack of the proofreading activity of its RNA dependent RNA polymerase, and high level of viral replication. The mean frequency of nucleotide mutations varies from 1.4×10^3 to 1.9×10^3 substitutions per nucleotide and per year (Ogata et al 1991, Steinhauer et al 1992). Some of the mutations accumulating during replication are silent or synonymous and have no impact on the amino-acid sequence of the viral protein. Other, so called non-synonymous mutations lead to changes in the protein sequence and to the emergence of variants (Le Guillou-Guillemette et al 2007). HCV genetic variability is characterized by the appearance of heterogeneous but closely related virus particles known as quasispecies (Bartenschlager et al 2000). The appearance of quasispecies allows HCV to escape host immune system's pressure and can contribute to resistance to IFN and ribavirin therapy, which leads to insufficient SVR rate (Wohnsland et al 2007).

The 5'UTR and the core are highly conservative regions, the non-structural regions 2, 3, 5B and the 3'UTR are relatively variable whereas the envelope regions E1 and E2 and the NS4 and the NS5A genes exhibit the highest sequence diversity (Penin et al 2001, Le Guillou-Guillemette et al 2007).

Based on sequence variability within the 5'UTR, core, E1 and NS5B regions or the complete genome, HCV has been classified into six major genotypes designated as 1 to 6, which differ by about 30 percent of their nucleotide sequences (Simmonds et al 2005, Chayama et al 2011). Recently a seventh genotype has been reported among immigrants from Central Africa (Smith et al 2014), (Figure 2). Each genotype is actually a mixture of quasispecies with 70% homology.

Further these genotypes have been classified into at least 67 subtypes, marked as *a, b, c, d, etc*, which have 20–25% sequence difference (Simmonds et al 2005, Smith et al 2014).

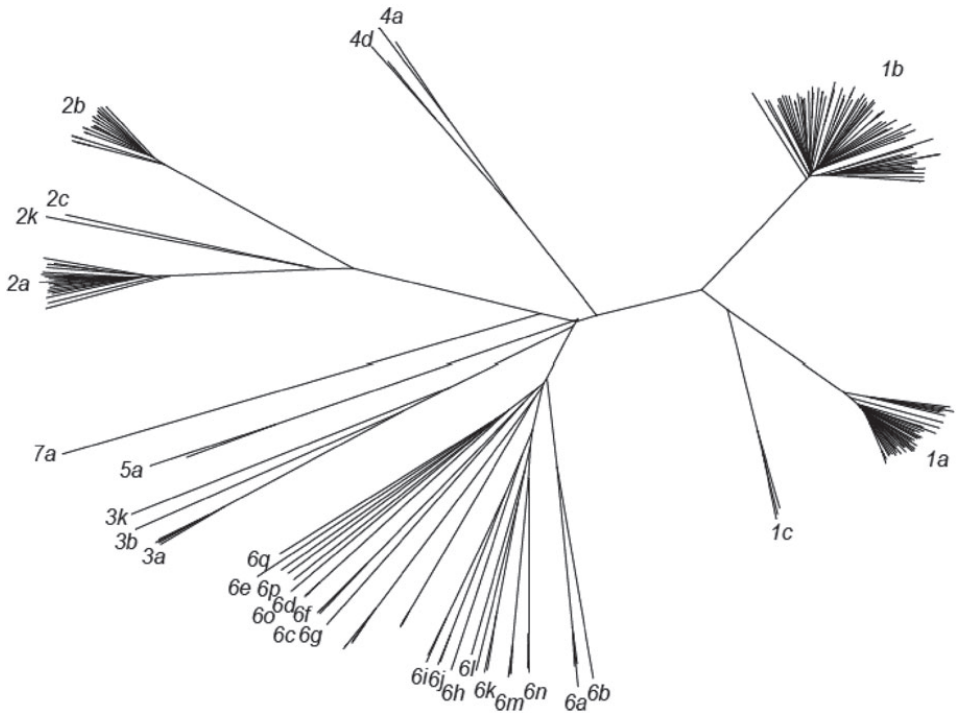

Figure 2. Phylogenetic tree of hepatitis C virus. A phylogenetic tree was constructed using 184 complete nucleotide sequences obtained from the DDBJ/EMBL/GenBank database (Copyright permission from John Wiley & Sons, Chayama et al 2011)

Genotypes differ in distribution both by the geographic region and the mode of transmission. Genotypes 1, 2 and 3 have worldwide epidemic distribution with a high prevalence of genotype 1 followed by 3 and 2 in Europe, Northern America and Japan, while genotypes 4–6 are endemic and prevalent in certain geographical areas (Esteban et al 2008, Averhoff et al 2012, Sievert et al 2011, Chao et al 2011).

Due to immigration from HCV genotype-endemic areas and movement of injection drug users across European and North American borders, genotypes 4–6 have been registered in these countries as well (Esteban et al 2008, Cornberg et al 2011, Averhoff et al 2012, Al Naamani et al 2013).

Like in most countries of Central and Eastern Europe (Jansons et al 2004, Liakina et al 2009, Cornberg et al 2011), mostly genotype 1b prevails in Estonia, followed by 3a and 2a (Tallo et al 2000, 2007, Zusinaite et al 2000).

The significance of the HCV genotypes is mostly associated with specific transmission routes, so genotypes 1a and 3a prevail among IDUs, while 1b and 2 are associated with blood transfusions and unsafe medical procedures in the past (Pawlotsky et al 1995, Esteban et al 2008). Genotypes 4–6 are associated with intravenous drug use as well as with transfusions of unscreened blood products and unsafe medical manipulations in countries with scarce health resources (Khattab et al 2011, Chao et al 2011, Al Naamani et al 2013).

The significance of genotypes in pathogenesis and disease progression remains controversial. The HCV genotype alone cannot be an independent factor of disease progression. However, genotypes are mostly known as a strong predictors of treatment response and duration of antiviral therapy (Manns et al 2001, Fried et al 2002, Hadziyannis et al 2004, Pearlman et al 2007, Shiffman et al 2007).

4.1.4. Epidemiology and routes of transmission

The HCV has worldwide distribution, it can occur among persons of all ages, genders and races. Nowadays it has been estimated that about 2.35% of the global population is infected with HCV, which amounts to 170 million infected people (Lavanchy 2011). Central and East Asia and North Africa/Middle East are estimated to have high prevalence (>3.5%); South and Southeast Asia, sub-Saharan Africa, Central, and Southern Latin America, Australasia, and Europe have moderate prevalence (1.5% – 3.5%); whereas Asia Pacific, Tropical Latin America, and North America have low prevalence (<1.5%) of HCV infection (Hanafiah et al 2013).

In Europe it was estimated that about 9 million people are infected with HCV. A low prevalence of 0.1% has been reported from Belgium (Quolin et al 2007) and a higher prevalence of 3.2% from Romania (Gheorge et al 2010). Each year 86,000 people die because of HCV infection and it accounts for about 60 -70% of hepatocellular carcinoma (HCC) cases and virus-related liver transplantation in Europe (Hatzakis et al 2011, Blachier et al 2013).

In Estonia, notification of hepatitis C started in 1993. Hospitals and family doctors report laboratory-confirmed cases of hepatitis C to the national surveillance system for communicable diseases (NAKIS) at the Estonian Health Board.

In Estonia there are no studies reporting the prevalence of HCV infection in general population, besides studies in specific populations, like health care workers, blood donors, hemodialysis and oncohematological patients and inmates (Priimägi et al 2005, Tefanova et al 2005). In general, it was suggested that up to 1% or even more out of 1.34 mln of Estonian inhabitants may be infected with HCV (Margus et al 2007).

The transmission routes of HCV infection are: intravenous drug use, which has undoubted the highest risk of HCV acquisition (Zaltron et al 2012); non-intravenous recreational exposure to drugs, i.e. cocaine, by sharing inhalatory instrumentation; accidental needle stick exposure among healthcare workers;

healthcare procedures even in developed countries (Gutelius et al 2010, Fabrizi 2013); mother to child vertical transmission (Murakami et al 2012); sexual exposure, which is considered to be very low (Tohme et al 2010) and is a matter of concern in males who have sex with men (Marongiu et al 2012).

Nowadays the most common transmission route in European countries, including Estonia, is injection drug use accounting for around 78% of all reported cases, followed by sexual contacts (ECDC 2013). Due to the asymptomatic nature of HCV infection in up to 30% of cases (Zaltron et al 2012) the route of transmission and source of infection remain unknown.

4.1.5. Control and prevention

Mandatory control of blood donors and blood products, established by the Committee for Proprietary Medicinal Products (CPMP) in the 1990-s, by using anti-HCV tests and later nucleic acid amplification techniques has significantly reduced transmission of HCV infection with the residual risk for acquiring HCV via blood products ranging from 1 to 40 per 10 million transfusions in European countries (ECDC 2010).

There is no vaccine or post-exposure prophylaxis for HCV. Prevention of acute hepatitis C and its sequelae (chronic hepatitis, cirrhosis, HCC) consists of primary and secondary activities.

Primary prevention is aimed to reduce transmission of HCV infection by avoiding unnecessary and unsafe injections, unsafe blood products, use of illicit drugs and sharing of injection equipment, unprotected sex with HCV infected people, tattoos, piercings and acupuncture performed with contaminated equipment (WHO 2013).

Secondary prevention is aimed at people who are infected with HCV. It consists of education and counseling and provision of antiviral treatment to these patients, it also includes vaccination against hepatitis A and B preventing thus coinfection and protecting the liver (CDC 2010).

In Estonia, there exist special national policies of HCV control in blood banks and preventing of HCV in health-care settings, but there is no national or regional standard or national policy on HCV prevention and no general anti-HCV screening programme. Only two specific subgroups, blood donors and prisoners are screened on a regular basis.

4.2. Natural course of HCV infection

4.2.1. Acute hepatitis C

Acute hepatitis C virus infection is a short-term illness that occurs within the first 6 months after exposure to HCV. The incubation period is variable, around 7 weeks (range 4–20 weeks) with detectable HCV RNA and anti-HCV within

1–2 weeks and 8 weeks, respectively, followed by elevation of serum alanine aminotransferase (ALT) 10–30 times of the upper limit at weeks 6–12 before onset of clinical symptoms (Hoofnagle 1997). Spontaneous clearance of infection typically occurs within 3 months from infection in 15% of patients (Santantonio et al 2008). Such factors, as young age, ethnicity, female gender, immune response and host genetics (IL28B CC genotype, CCR5 and CCL5 haplotypes) can contribute to it (Post et al 2009, Huik et al 2013, Grebely et al 2014).

4.2.2. Chronic hepatitis C (CHC)

After acute hepatitis C the disease becomes chronic in up to 85% of all cases. Chronic hepatitis C is a continuous inflammation of the liver caused by HCV infection persisting over time period from four to six months with failed spontaneous viral clearance and with an existence of compatible clinical signs (Mauss et al 2014).

In case of persistent HCV viremia infected liver cells secrete cytokines and chemokines that cause migration of nonspecific mononuclears into liver cells (Larrubia et al 2008) with development of liver damage. Persistent inflammation activates hepatic stellate cells, myofibroblasts and fibroblasts, which cause development of liver fibrosis (Heydtmann et al 2009, Fallahi et al 2012).

Most patients with chronic infection are asymptomatic or have only mild nonspecific symptoms before presenting liver cirrhosis, which may be unrecognized for a long time and is presented in the form of overt hepatic decompensation (Seef 2002).

Aminotransferase level can vary usually from twice up to 5 times above the upper limit of normal, with about one third of patients having normal serum ALT. Patients with persistently normal ALT can have advanced liver disease (Shiffman et al 2006).

Approximately in 20–30% of patients the disease can progress over 10–30 years to liver cirrhosis (Seeff 2002). Such factors as age of acquisition of infection, gender, race, alcohol and/or illicit drugs abuse, co-infection with HIV or/and HBV, comorbidities can contribute to disease progression (Chen et al 2006). Of patients with HCV-related cirrhosis, 1–4% can develop hepatocellular carcinoma (HCC) yearly (Seef 2002).

In Estonia, the surveillance system covers newly diagnosed CHC cases from 2004 and HCV has been found as the main etiological agent of chronic viral hepatitis (Estonian Health Board, annual report).

The HCV is a hepatotropic virus, but it can also exist in extrahepatic tissues, such as bone marrow, central nervous system, endocrine glands, lymph nodes, macrophages and skin cells, that might act as a reservoir and play a role in both HCV persistence and reactivation (Ferri et al 1993). More than 30 extrahepatic manifestations, i.e. concomitant diseases, have been reported during the natural course of chronic hepatitis C (Agnello et al 2004). Their appearance is associated with B-cell hyperactivation and chronic antigenemia (Klenerman et al

2012), and as a result of complex dysregulation of the cytokine/chemokine network (Fallahi et al 2012). Approximately one third of HCV infected patients have at least one extrahepatic manifestation and it may be the first symptom of the disease (Cacoub et al 2000). The best documented manifestations are essential mixed cryoglobulinemia, porphyria cutanea tarda and some others (Zignego et al 2007, Monaco et al 2012).

A relationship between HCV and sarcoidosis has been described (Ramos-Casals et al 2005, Faurie et al 2010). The prevalence of sarcoidosis in HCV-infected patients is reported to be 0.1% – 0.2% (Faurie et al 2010). A relationship between sarcoidosis and HCV infection may emerge in 75% of cases due to antiviral therapy with IFN-α and ribavirin, or owing to unknown factor(s) (Ramos-Casals et al 2005). Sarcoidosis may appear during the treatment or after completion of antiviral therapy. In case of sarcoidosis unrelated to HCV treatment, together with the fact that liver granulomas may be presented in the histological spectrum of chronic hepatitis C, makes several authors speculate that chronic persistent HCV infection *per se* may trigger systemic sarcoidosis (Bonnet et al 2002, Gaya et al 2003).

4.2.2.1. Diagnostics of CHC

The diagnosis of CHC is confirmed by the presence of both HCV antibodies and HCV RNA with compatible clinical signs and liver histology (Mauss et al 2014). Tests for detection of anti-HCV include the enzyme immunoassay, which contains HCV antigens from the core and nonstructural proteins, and the recombinant immunoblot assay. Target amplification techniques with either polymerase chain reaction (PCR) or transcription-mediated amplification are used for HCV RNA qualification, whereas both target amplification and signal amplification techniques are implemented for measurement of HCV RNA levels. The above mentioned assays are necessary for the diagnosis of HCV infection (Ghany et al 2009, EASL 2014).

Despite the use of some non-invasive methods, like Fibroscan and biochemical markers of fibrosis (Degos et al 2010), transcutaneous liver biopsy remains the gold standard for assessment of HCV-related liver disease severity with a very low risk of severe complications (WHO 2014). The widely used METAVIR score system for assessing histological changes in the liver is very advantageous and easy to grade liver changes (Bedossa et al 1996).

4.3. Treatment of CHC

4.3.1. Goals of antiviral therapy and types of virological response

Management of chronic CHC is aimed to eradicate HCV infection preventing thus the complications of HCV–related morbidity and mortality (Backus et al 2011, Veldt et al 2007). Therapy with peginterferon alfa (PegIFN) and ribavirin

(RBV) was approved as the standard of care (SOC). The endpoint of therapy known as a sustained virological response (SVR) (Lindsay 2002, Ghany et al 2009) is the main criterion of treatment efficacy. The SVR is durable with a relapse rate less than 1% (Swain et al 2010).

During PegIFN/RBV therapy there occur several on-treatment and off-treatment types of virological response (Figure 3):

a) rapid virological response (RVR) defined as undetectable HCV RNA at week 4 of antiviral therapy;
b) early virological response (EVR) with HCV RNA detectable at week 4, undetectable at week 12 (complete EVR);
c) nullresponse characterized by a decrease of HCV RNA less than 2 $\log_{10}$ at 12 week of therapy;
d) end-of-treatment virological response (EOT) with undetectable HCV RNA level at the end of therapy;
e) sustained virological response (SVR) defined as HCV RNA negative at 6 months after completion of treatment.
f) relapse (RL) is detectable HCV RNA after completion of therapy in patients with EOT virological response (Ghany et al. 2009, EASL 2014).

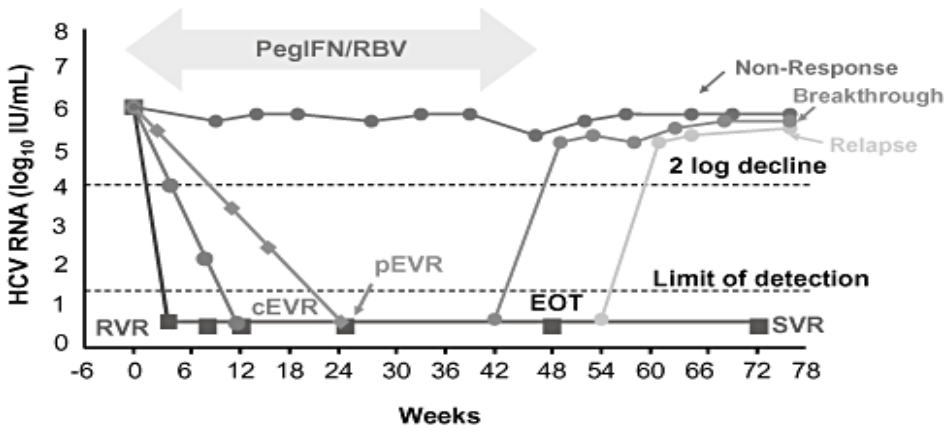

Figure 3. Patterns of on-treatment and off-treatment virological responses (Copyright permission from John Wiley & Sons, Yu 2009)

4.3.2. Overview of antiviral treatment

Interferon alpha (IFN-α) is a naturally occurring cytokine having antiviral, immune modulatory and anti-inflammatory properties (Feld et al 2005), it was first used in a pilot study in 1986 for the treatment of NANBH (Hoofnagle et al 1986). It was suggested that interferon stimulates host's immune system and activates interferon stimulating genes (ISGs) (Zeuzem et al 1996).

Monotherapy with IFN-α dosed at 3 MU 3 times a week with a 24-week duration of therapy showed 6% of SVR (Di Bisceglie et al 1989, Davis et al 1989). A further step was the adding of ribavirin (RBV), a guanosine analogue with a broad spectrum of antiviral activity (Potter et al 1976), to IFN. It was proposed that RBV can modulate host immune responses by shifting a Th2 response in favor of a Th1 phenotype, directly inhibit viral RNA polymerase and cause lethal mutagenesis of HCV RNA genomes (Te et al 2007).

Clinical trials have demonstrated a SVR rate of 13 – 19% with prolongation of monotherapy up to 48 weeks; IFN-α combined with RBV for 24 weeks allowed to attain SVR in 31% – 35% of patients, and when the therapy was prolonged up to 48 weeks, in 38% – 43% (McHutchison et al 1998, Poynard et al 1998).

A significant breakthrough in therapy has been achieved by using of PegIFN. Pegylation is a process that allows, by attaching polyethylene glycol strands to the interferon molecule, to prolong its half-life. Clinical trials demonstrated SVR rates of monotherapy with PegIFNα for 48 weeks to be approximately 39% (Zeuzem et al 2000, Lindsay et al 2001). Based on three randomised trials, rules were established for using combination with PegIFNα plus RBV in treatment for CHC (Manns et al 2001, Fried et al 2002, Hadziyannis et al 2004).

There are two PegIFNs marketed today, PegIFNα-2a (PEGASYS®, Roche) and PegIFNα -2b (PEG-Intron®, Merck). Analogous two forms of RBV are used: Copegus manufactured by Roche and Rebetol manufactured by Merck Sharp and Dohme.

4.3.3. Standard of care for CHC

Antiviral therapy with PegIFN/RBV was implemented in early 2000s and was determined as a SOC for treatment of chronic hepatitis C. It is recommended to all treatment-naive patients with compensated HCV-related chronic liver disease who are willing to be treated, do not have any serious comorbidities and have no contraindication to either medication. SOC is also recommended to patients who failed to eradicate HCV after previous therapy with IFN-α with or without RBV (Ghany et al 2009, EASL 2014).

An individualized therapy, known as a response guided therapy with PegIFN/RBV based on the genotype, baseline viral load and decrease of HCV RNA during weeks 4 and 12 of treatment, has been proposed. It allows, on one hand, by avoiding a large number of side effects without loss of efficacy, to shorten the duration of treatment, and, on the other hand, by prolongation of treatment, to increase SVR rates in particular patient groups.

According to these algorithms, patients infected with HCV genotypes 1 and 4 with a low baseline viral load and RVR, can be treated for 24 weeks, and patients infected with genotype 2 and 3 with a low baseline viral load and RVR, can be treated for only 16 weeks. Patients with detectable HCV RNA at week 4 (genotypes 2 and 3) and week 12 (genotypes 1 and 4) but negative HCV RNA

at week 24, can benefit from treatment prolongation up to 48 and 72 weeks, respectively (Ghany et al 2009, EASL 2014, Margus et al 2007).

In Estonia, IFN-based monotherapy of chronic hepatitis C was introduced in 1997 and the SOC for treatment of CHC has been available since 2005. The treatment of patients is conducted according to the National Guideline on treatment of CHC, which was approved by the Estonian Society of Gastroenterology and the Estonian Society for Infectious Diseases in 2006 and updated in 2007, 2010. Annually around 400 patients with CHC receive antiviral therapy. It is 100% reimbursed by the Estonian Health Insurance Fund for patients covered by health insurance.

4.3.4. Direct acting antivirals in treatment of CHC

Despite the advances in the management of CHC, the present high cost and serious side effects of SOC with yet unsatisfactory treatment results in patients infected with HCV genotype 1, have encouraged researchers to introduce new, more effective agents with favorable safety profiles targeting different sites of the virus genome.

Direct acting antivirals (DAA) are a potentially new class of antiviral drugs aimed at both the structural and nonstructural proteins of the HCV genome and interfere with viral replication (Poordad et al 2012).

In 2011 the Federal Drug Agency (FDA) and the European Medicine Agency (EMA) approved the first-generation protease inhibitors (PIs) boceprevir (BOC) and telaprevir (TVR), both targeting HCV NS3/4A serine protease in combination with PegIFN/RBV, for treatment of both naïve and experienced chronic HCV genotype 1 patients (Ghany et al 2011, EASL 2014). Triple therapy has led to improved response rates of 63-75% with better tolerability and safety of treatment (Poordad et al 2011, Jacobson et al 2011, Sherman et al 2011). Despite promising results, this therapy has demonstrated certain limitations, as it is used only for patients infected with HCV genotype 1. The antiviral efficacy of PIs is affected by the presence of resistance mutations within the targeted NS3 protein (Bartels et al 2013). It still has disappointing results for previous prior partial and null response to PegIFN/RBV and especially for patients with cirrhosis.

Facing these challenges, new antiviral drugs, like sofosbuvir, simeprevir, daclatasvir, faldaprevir etc, which allow to achieve SVR up to 98% – 100% with both interferon-based and interferon-free regimens, are in progress (Asselah et al 2013, Gane et al 2013, Zeuzem et al 2013). Pursuing of goals by using new classes of antivirals for treatment of CHC, which include improved tolerance, high barrier to resistance, pan-genotypic, oral regimen and favorable pill burden, short duration and high SVR rate, will be realistic in the near future (McGowan et al 2012).

4.4. Factors determining treatment response

4.4.1. Pre-treatment host factors

Among the pre-treatment factors, ethnicity is associated with treatment response. Asian patients respond better than patients of other races, with SVR up to 70% in patients with genotype 1 and 4, about 90% in those with genotype 2 and 3, and about 80% in those with genotype 6 (Yu et al 2009). Latinos and African-Americans have more worser results as compared to Asian and Caucasians patients (Melia et al 2011, Rodriguez-Torres 2008).

Age less than 40 years is associated with favorable treatment response (Hadziyannis et al 2004, Bourliere et al 2012). Older patients have a more advanced liver disease at baseline and higher incidence of adverse events during treatment, which lead to premature discontinuation of treatment and lower SVR rate (Huang et al 2010). Nevertheless, patients above 50 years of age infected with HCV genotype 1, with low baseline HCV RNA viral load and without advanced fibrosis, attained SVR rates comparable with those for younger patients (Reddy et al 2009).

Among Asian patients infected with HCV genotype 1, females younger than 40 years had significantly higher SVR rate than males, treatment results became similar at age from 41 to 50 years, and worser at age over 50 (Yu et al 2011). A recent real-life European gender-oriented analysis also has failed to demonstrate differences in response to PegIFN/RBV therapy between males and females, but it has revealed different predictive factors in both gender groups, which could contribute to treatment outcomes (Di Marco et al 2013).

Data about the influence of body mass index (BMI) on treatment response are controversial. A recent large European PROPHESYS trial demonstrated that lower BMI was significantly associated with higher SVR rate (Marcellin et al 2012). Obesity (BMI $\geq$ 30 kg/m^2) in patients with CHC was associated with a worse response to PegIFN/ RBV therapy and with an increased rate of liver steatosis and fibrosis (Hickman et al 2002).

The presence of advanced liver fibrosis and cirrhosis leads to worser treatment response with SVR rates about 51%–31% and 33%–10%, respectively (Cheng et al 2010, Bruno et al 2010). The underlying mechanisms of this are not well understood (D'Ambrosio et al 2012). Liver steatosis has been reported as an unfavorable predictor of treatment response to PegIFN/RBV (Leandro et al 2006). The frequency of steatosis in chronic HCV infected patients was reported to be around 55%, being higher in patients with genotype 3 infection, in particular (Savvidou et al 2012).

Pre-treatment levels of liver enzymes can correlate with treatment response in HCV-infected patients receiving therapy with PegIFN/RBV. Low pre-treatment gamma glutamyl transferase (GGT) level has been associated with better treatment response (Weich et al 2011). Elevated GGT is supposed to be a surrogate marker of advanced fibrosis and high necroinflammatory activity in the liver (Silva et al 2004). In contrast, high baseline ALT level is an independent predictive factor of better treatment response (McHutchison et al 2009).

4.4.1.1. Host genetics

Several studies have investigated different host genetic factors than can contribute to both the natural history (susceptibility, spontaneous clearance and persistence) and treatment outcomes of HCV infection (Selzner et al 2008, Huik et al 2013, Yuan et al 2013). The best studied and recommended for use in clinical practice are single-nucleotide polymorphisms (rs12979860 and rs8099917) near the IL28B gene on chromosome 19, encoding interferon-lambda-3, which is strongly associated with treatment response to PegIFN/RBV in patients infected with HCV genotype 1b and to a lesser extent in patients infected with HCV genotypes 2 and 3 (Mangia et al 2010, Sarrazin et al 2011). Individuals with the CC genotype achieved higher SVR rate as compared to patients with the CT and TT genotypes (Ge et al 2009, Par et al 2011, 2014).

4.4.2. Pre-treatment viral factors

Of the pre-treatment viral factors, virus genotype and viral load are strong predictors of treatment response and can determine the duration of antiviral therapy (Manns et al 2001, Fried et al 2002, Hadziyannis et al 2004). Of the six HCV genotypes found worldwide, genotype 1 is difficult to cure, with SVR rates up to 52% (Fried et al 2002, Hadziyannis et al 2004) and even less than 50% in real-life settings (Mauss et al 2011, Bourliere et al 2012) but up to 80% or more for genotypes 2 and 3 (Zeuzem et al 2004). The reasons for this are not completely clear.

Currently, low or high baseline viral load is known as an issue to guide treatment decisions (Ghany et al 2009, EASL 2014). Low pre-treatment viral load is associated with better treatment response (Marcellin et al 2012). The definition of low baseline viral load (LVL) is under discussion, ranging from 400 000 up to 800 000 IU/ml (Manns et al 2001, Fried et al 2002, Shiffman et al 2007, Zeuzem et al 2006).

4.4.3. On-treatment viral factors

Undetectable serum HCV RNA at week 4 of therapy, known as RVR, is an independent predictor of SVR (Poordad et al 2008). For patients with all genotypes, who achieve RVR, the probability of attaining SVR is 88–100% (Fried et al 2011). Achievement of RVR together with low baseline viral load is a decision point for shortening treatment duration from 48 weeks to 24 weeks in about 15% of patients infected with HCV genotype 1 (Zeuzem et al 2006) and from 24 to 12–16 weeks in patients infected with HCV genotype 2 and 3 (Shiffman et al 2007).

Another important time and decision point is week 12, known as a stopping rule for patients infected with HCV genotype 1. The EVR with undetectable HCV RNA or a > 2 $\log_{10}$ drop in viral load allows to attain SVR in 68–84% and 17–29% of genotype 1 patients, respectively (Berg et al 2006). Patients infected

with HCV genotype 1 who have failed to achieve EVR should stop PegIFN/RBV therapy, because the probability of attaining SVR is very low (Fried et al 2002). About 20% of patients infected with HCV genotype 1, (Berg et al 2006) determined as "slow" virological responders, i.e. HCV RNA detectable at week 12 with > $2\log_{10}$ decline in viral load and HCV RNA negative at week 24, may benefit from extended treatment duration from 48 to 72 weeks (Pearlman et al 2007). Patients infected with HCV genotype 2 and 3 with EVR and negative cofactors, like advanced fibrosis or cirrhosis and high baseline viral load, can benefit from prolongation of treatment up to 48 and even 72 weeks (Hadziyannis et al 2004).

4.4.4. Treatment related factors

Adherence to PegIFN/RBV therapy is another important issue determining treatment outcome. Following the rule, defined as taking no less than 80% of the total dose of PegIFN and/or no less than 80% of total RBV dose and/or during no less than 80% of the total period of treatment, is crucial for achieving SVR (McHutchinson et al 2002). For patients infected with HCV genotype 1b, who take less than 60% of each medication SVR rate is only 7.7%, versus 31% for those who take more than 60% of each medication (Arase et al 2007).

Patient education and close cooperation with medical staff in centres with higher experience (>15 patients/year treated) and facilities, as well as certain host and viral factors (male gender, age, genotype and IFN dosage) allow to enhance adherence to therapy with PegIFN/RBV (Tanioka et al 2009).

4.4.5. Adverse events of antiviral therapy

During PegIFN/RBV therapy adverse events (AE), similar equal for both PegIFNα-2a and 2b, have been observed. As a result, patients experience impaired quality of life (Björnsson et al 2009), they are obliged to reduce the dose of drugs and about 10–15% of patients in clinical trials and even a higher percentage in real-life settings discontinue antiviral therapy.

Premature discontinuation leads to significantly lower SVR rate for patients compared with those who only reduced medication doses (Arase et al 2007). The most common AE are hematologic (anemia, leuco-, neutro- and thrombocytopenia), dermatologic (alopecia, dermatitis, pruritis, injection site reaction), neuropsychiatric (depression, polyneuropathy, convulsions), endocrinological (hypo- or hyperthyroidism), gastrointestinal (nausea, anorexia, abdominal pain, dyspepsia, constipation), pulmonary (cough and/or dyspnea, interstitial pneumonitis), cardiovascular (myocardial ischemia), and ocular (retinopathy) (Sulkowski et al 2011, Monaco et al 2012).

Appropriate monitoring and medical management of treatment-related AE can enhance SVR rate (Sulkowski et al 2011). Step-down strategy of continuous reduction of both PegIFN and RBV until absolute discontinuation is used in the case of hematological abnormalities (Ghany et al 2009, EASL 2014). Several

adjuvant growth factors, such as epoetin α for treatment of anemia, and filgastrim and eltrombopag for treatment of neutro- and thrombocytopenia, which can enhance the SVR rate, are in use (Sulkowski et al 2011).

4.5. Structural polymorphism within the entire HCV NS5A region and treatment response

The mechanism of resistance to antiviral treatment in patients infected with HCV genotype 1 is not thoroughly understood. In order to explain the influence of the viral factor on the efficacy of IFN-based therapy, polymorphisms due to amino acid substitutions within the non-structural 5A protein (NS5A; aa 1973-2419) (Figure 4) have been the main focus in a number of studies (Bouzgarrou et al 2009, Noguchi et al 2011, Muñoz de Rueda et al 2008, Chayama et al 2011, Jardim et al 2009, 2013). Currently, these polymorphisms have been described in terms of a number of random mutations making the sequence divergent from the prototype (Muñoz de Rueda et al 2008, El-Shamy et al 2008). The prognosis for therapy outcome is suggested to be as good as many differences from the prototype are found in the NS5A protein of the viral isolate.

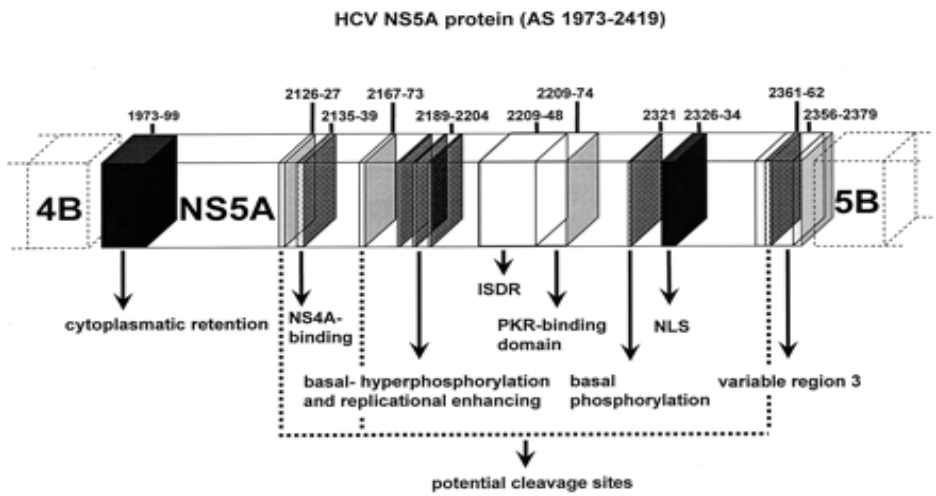

Figure 4. Location of different regions of interest within HCV NS5A protein (Copyright permission from the American Society for Microbiology, Sarrazin et al 2002)

The first positive correlation between number of mutations in the interferon sensitivity determining region (ISDR; aa 2209-2248) within the NS5A protein and SVR rate in Japanese patients infected with genotype 1b was described by Enomoto et al in 1995. Thus, patients infected with the wild type ISDR

sequence (identical to the prototype Japanese HCV strain, HCV-J) did not respond to IFN-based therapy, whereas patients infected with the mutant type, defined as four or more amino acid substitutions in this region, achieved SVR. Following studies from Japan also confirmed those results (Okanoue et al 2009, Watanabe et al 2005, Maekawa et al 2009) while studies from Europe (Veillon et al 2004, Kmieciak et al 2006, Brillet et al 2007) and the United States (Nousbaum et al 2000, Murphy et al 2002) failed to distinguish correlation between ISDR mutations and SVR.

The clinical importance of mutations within the protein kinase-binding domain (PKR-bd; aa 2209-2274), was investigated and PKR-independent effects of NS5A in the IFN response have been also proposed. Concerning the other NS5A regions, significant correlation has been reported between sequence variation in the variable domain (V3; aa 2356-2379) or its upstream region near the carboxy terminus of NS5A, referred to as the IFN/RBV resistance-determining region (IRRDR; aa 2334-2379), and response to INF based therapy (Duverlie et al 1998, Murphy et al 2002, Sarrazin et al 2002, Hofmann et al 2005). The mean number of aa substitutions in the V3 region and in IRRDR was significantly higher for HCV isolates obtained from SVR patients compared with non-SVR patients (El-Shamy et al 2008). The significance of these mutations has been also confirmed by studies carried out in different populations of different countries (Kumthip et al 2011, Yano et al 2012).

Treatment response may be associated also with some specific aa substitutions of NS5A. Thus, it has been demonstrated that patients infected with HCV genotype 1b who had aa mutations in NS5A at positions 2209, 2216, 2217, 2218, 2227, 2360, and 2378 (according to the numeration of the HCV-J prototype strain) achieve SVR more frequently than those without mutations at these positions (Kohashi et al 2006, Muñoz de Rueda et al 2008, El-Shamy et al 2008, Noguchi et al 2011).

But the influence of HCV intragenotypic variability on the combination treatment outcome has not been completely understood so far.

5. AIMS OF THE STUDY

The main objective of the study was to assess the efficacy of PegIFN/RBV therapy in Estonian treatment-naïve patients with CHC in relation to different host and viral factors, including polymorphisms within the entire NS5A region of the HCV genome.

The aims of the study were:

1. To study the efficacy of peginterferon alfa-2α and ribavirin therapy in treatment-naïve patients with chronic hepatitis C genotype 1 and 3 infections (Paper I and II).
2. To determine pre-treatment and on-treatment host and viral factors associated with peginterferon alfa-2α and ribavirin therapy outcome in chronic hepatitis C genotype 1b patients (Paper III).
3. To assess the genetic variability of the non-structural (NS5A) protein of the HCV and its association with peginterferon alfa-2α and ribavirin therapy outcome in chronic hepatitis C genotype 1b patients (Paper IV).

6. MATERIALS AND METHODS

6.1. Study subjects

The study population consisted of the treatment-naïve monoinfected patients with chronic hepatitis C referred to a gastroenterologist of the Internal Medcine Clinic of West-Tallinn Central Hospital (WTCH) between February 2005 and March 2011.

The enrollment of the studied patients is presented in the flow chart (Figure 5).

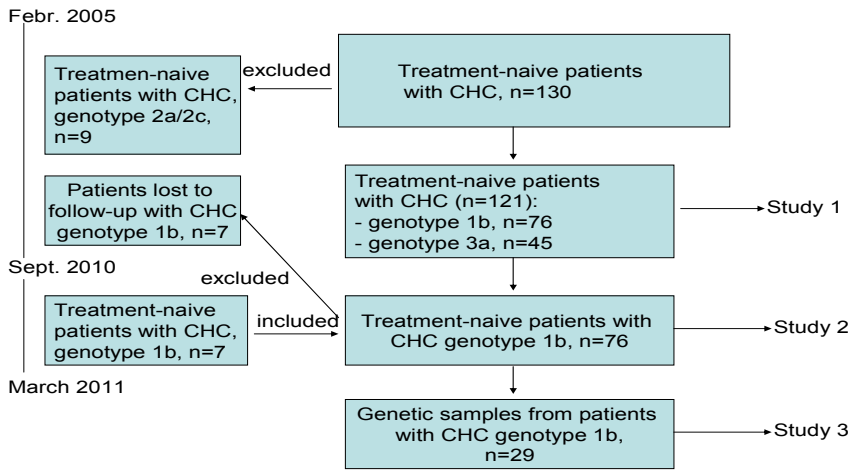

Figure 5. Flow chart of the studied patients.

Additionaly, we present one case report exemplifying relationship between systemic sarcoidosis and chronic hepatitis C in a patient infected with HCV genotype 1b from study 1.

The diagnosis of CHC was based on the presence of anti-HCV antibodies and HCV RNA in the sera, histologically verified fibrosis stage and degree of inflammatory activity and clinical follow-up.

The exclusion criteria for treatment were age <18 years, chronic alcohol intake, decompensated cirrhosis, current injection drug use, depression, co-infections with HIV and HBV, and severe comorbidities (poorly controlled hypertension, heart failure, diabetes and chronic obstructive pulmonary disease).

The treatment decision and further management of patients were conducted according to the Estonian National Guidelines on the treatment of CHC.

Written informed consent for the use of clinical data and serum samples was obtained from all patients prior to the study.

The study was approved by the Tallinn Medical Research Ethics Committee, protocol No.1130.

6.2. Study methods

6.2.1. Laboratory and clinical investigations

Complete blood count, ALT, AST, GGT, ALP, bilirubin, TSH, and autoantibodies (ANA, AMA M2, ASMA, LKMA) were measured before the start of therapy. Complete blood count and liver enzymes were measured in the blood taken from the cubital vein every four weeks during the treatment period and TSH was measured at weeks 12, 24, 36 and 48. Blood tests were performed in a clinical laboratory at WTCH.

Patient's height and weight were measured and body mass index (BMI) before treatment was calculated. Antibodies to HCV in serum prior to treatment were determined by a Abbott HCV EIA 2.0 (Abbott Laboratories, Abbott Park, IL) enzyme immunoassay before 2010 and thereafter by the electrochemiluminescence immunoassay Anti – HCV II test (Roche Diagnostics GmbH). Serum HCV RNA level before therapy and at weeks 4, 12, 24, and at the end of therapy at 24 or 48 weeks, and 24 weeks after treatment were analysed by a quantitative PCR assay. The COBAS Amplicor HCV Monitor version 2.0 test (lower detection limit of 50 IU/ml) was used in 2005–2009 and the COBAS® AmpliPrep/COBAS® TaqMan HCV test (lower detection limit of 15 IU/mL, the linear range of the assay 43 to 69,000,000 IU/mL) was used from 2010.

HCV genotypes were determined by the hybridization technique using a VERSANT HCV genotype assay (LiPA), Bayer Health-Care LLC, Tarrytown, NY.

Antibodies to HCV, HCV RNA measurements and the genotyping assays were performed in the Laboratory of HIV Diagnostics of WTCH.

During clinical visits physical examination was performed with evaluation of adverse events and use of concomitant medications.

6.2.2. Transcutaneous liver biopsy and histology

Ultrasound-guided transcutaneous liver biopsy was performed to all patients at baseline by the author of the thesis at the Department of Radiology of West-Tallinn Central Hospital.

Liver biopsies were assessed by using Metavir score in Pathology Centre of North Estonia Medical Centre. Stage of fibrosis was scored as follows: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with rare septa; F3, numerous septa without cirrhosis, and F4, cirrhosis. Degree of inflammation was scored from 0 to 3 with A0 representing absent activity, A1 minimal, A2 moderate and A3 severe activity (Bedossa et al 1996).

6.2.3. HCV RNA extraction and cDNA synthesis

HCV RNA was extracted from 200 µl of pre-treatment serum using the TriPure isolation reagent (Roche Diagnostics GmbH, Mannheim, Germany) according

to the manufacturer's recommendations. The extracted RNA was reverse transcribed and amplified for full-length NS5A using SuperScript III reverse transcriptase (Invitrogen, Waltham, USA) and random primers (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions.

6.2.4. Entire NS5A amplification, sequencing and phylogenetic analysis

The entire NS5A gene (1341nt/ 447 amino acids; aa) was amplified with external primers 5'-ATGAACCGGCTGATAGCGTT-3' and 5'-CTCCTTGAG CACGTCCCGGT-3' and internal primers 5'-TCCCCCACGCACTATGTGCC-3'and 5'-CGGTARTGGTCGTCCAGGAC-3'. The PCR products were sequenced directly with the PCR internal primers used for amplification and primer 5'-ATTCCAGGTCGGGCTCAA-3'. The obtained sequences were edited with the BioEdit v7.0.8.0 software and aligned with the NS5A region of HCV-J prototype for genotype 1b (GenBank accession number D90208) with the ClustalW multiple alignment utility. Phylogenetic analysis was carried out using the MEGA software (http://www.megasoftware.net) using the Kimura-2 parameter model. The phylogenetic tree was constructed by the maximum likelihood (ML) method using the MEGA 5.2 program. Bootstrap analysis was performed on 1000 replicates.

All sequences reported herein were deposited in the National Center for Biotechnology Information (NCBI) GenBank nucleotide sequence databases with accession numbers JX022751-JX022779.

6.3. Peginterferon alfa-2a and ribavirin treatment

Treatment of the study patients was conducted by the author of the thesis in accordance to National Guideline on the treatment of CHC. All patients, depending on the genotype, were administered 24 or 48 weeks of SOC.

PegIFN alfa-2a/PegIFN (Pegasys, F. Hoffmann La Roche Ltd, Basel, Switzerland) was administered at a dosage of 180 μg/week. The RBV (Copegus, F. Hoffmann La Roche Ltd, Basel, Switzerland) was given per os at a dosage of 1200 mg/day or 1000 mg/day, depending on body weight (above or below 75 kg), to patients infected with genotype 1b; and at a fixed dosage of 800 mg/day, regardless of body weight, to patients infected with genotype 3a. Response-guided treatment algorithms for patients infected with HCV genotypes 1b and 3a are presented in Figures 6 and 7.

Before starting antiviral therapy a nurse demonstrated to all patients how to inject subcutaneously PegIFN.

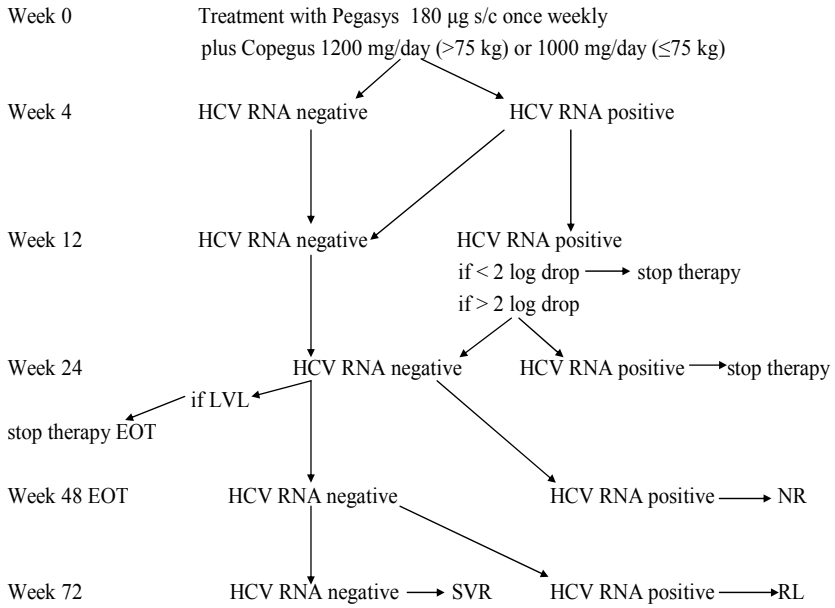

Figure 6. Treatment algorithm for patients infected with HCV genotype 1b. Adopted from the Estonian National Guidelines on treatment of CHC, 2007, 2010.

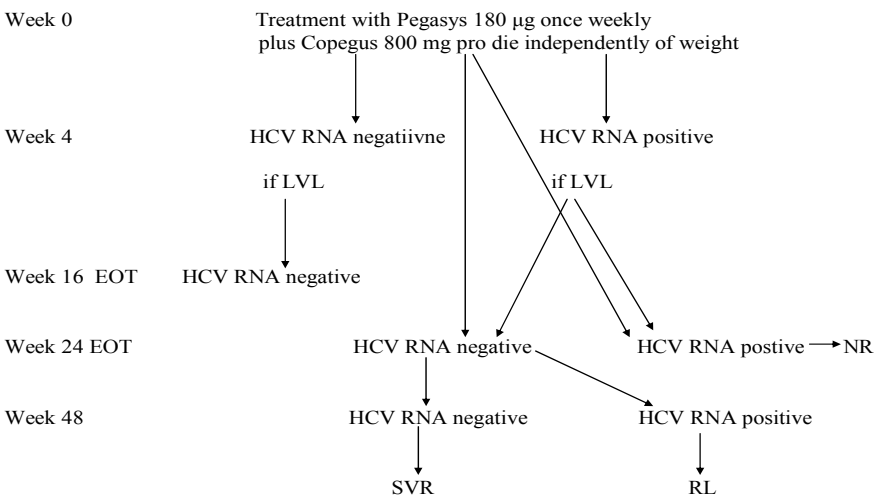

Figure 7. Treatment algorithm for patients infected with HCV genotype 3a. Adopted from the Estonian National Guidelines on treatment of CHC, 2007, 2010.

Modifications and changes in PegIFN alfa-2a doses were made according to recommendations of the product manufacturer in the face of appearance of blood abnormalities and adverse events during antiviral therapy. If the value of leucocytes appeared to be below 1.5×10^9/l, the value of neutrophils below 0.75×10^9/l and the value of platelets below $50x10^9$/l the dose of PegIFN alfa-2a was reduced to 90 μg/week. When blood abnormalities progressed with leuco- neutro- and thrombocytopenia below 1.0×10^9/l, 0.5×10^9/l and 25×10^9/l, respectively, treatment was stopped.

The dose of RBV was modified according to recommendations of the product manufacturer depending on the hemoglobin (Hgb) profile: if Hgb level fell below 100 g/l the RBV daily dose was reduced to 600 mg/day and stopped if the Hgb level was below 85 g/l. Also the doses of both medications were modified and even stopped if the studied patients experienced adverse effects or intolerance of antiviral therapy. Treatment outcomes were assessed as SVR, non-response and relapse.

6.4. Statistical analysis

For statistical analysis, the χ^2 test, Fisher's exact test and Student's t-test were used. A two-tailed *P* value of less than 0.05 was considered statistically significant.

Quantitative variables are expressed as mean ± SD.

Both univariate (Paper III) and multivariate (Paper I) logistic-regression analyses with calculations of odds ratios (OR) and confidence intervals (CI) were performed to explore the baseline factors that could predict the treatment outcomes.

Confidence intervals (CI) are 95% (Paper I, III, IV).

Comparisons between the groups were made by the Student's *t*-test or the Mann-Whitney test for quantitative variables and the chi-square or Fisher's exact test for categorial variables.

Correlation between the variables was estimated by Pearson's or Spearman's coefficients of correlation (Paper IV).

7. RESULTS

7.1. Efficacy of PegIFN alfa-2a and RBV treatment in patients with genotypes 1b and 3a (Papers I and II)

To estimate the efficacy of PegIFN alfa-2a and RBV therapy in Estonian treatment-naïve patients with CHC, a total 121 outpatients aged 19–63 years (76 infected with HCV genotype 1b and 45 with genotype 3a, mean age 42.7 vs 35.1 years, respectively, p<0.01) were enrolled in the study (see the flow chart in Figure 5).

At baseline, the viral load in 75.2% (91/121) of the patients was higher than 600 000 IU/ml. Histologically, 88.4% (107/121) of the patients had fibrosis score F0 – 2, 11.6% (14/121) had advanced fibrosis, F3-4 (Paper I, Table 1).

The overall SVR rate in this setting was 60.3%, being statistically lower in patients with HCV genotype 1b, as compared with genotype 3a patients, 46.1% (35/76) vs. 84.4% (38/45), respectively, (p=0.0004).

The overall non-response and relapse rates were 13.2% (16/121) and 12.4% (15/121), respectively, being significantly higher for patients infected with genotype 1b compared with patients infected with genotype 3a 19.7% (15/76) vs. 2.2% (1/45), p=0.01 and 17.1% (13/76) vs. 4.4% (2/45), p=0.04, respectively (Table 2).

Table 2. Responses to combination therapy with peginterferon and ribavirin in the study patients.

Treatment outcomes	Genotype 1b, n = 76	Genotype 3a, n = 45	All patients, n = 121	*p*-value*
SVR, n (%)	35 (46.1)	38 (84.4)	73 (60.3)	0.0004
Non-response, n (%)	15 (19.7)	1 (2.2)	16 (13.2)	0.01
Relapse, n (%)	13 (17.1)	2 (4.4)	15 (12.4)	0.04
Discontinued treatment, n (%)	13 (17.1)	4 (9.0)	17 (14.1)	0.282

In the present study patients with the viral load below 600 000 IU/ml had higher SVR as compared to patients with the viral load above 600 000 IU/ml, 73.3% (22/30) vs. 57.4% (35/61), respectively, but the difference did not reach significance (p=0.25). Nor was there found significant correlation of SVR with baseline levels of HCV RNA depending on the genotype (p=0.33).

The SVR rate was higher in patients younger than 40 years compared with older patients (> 40 years), 76.4% (42/55) vs. 47.0% (31/66), regardless of the genotype ($p<0.01$).

Allthough females had higher SVR rate than males, 68.7% (33/48) vs. 54.8% (40/73), respectively, the difference between the genders was not statistically significant ($p=0.13$).

The SVR rate for the studied patients with the fibrosis score 0-1 and for the patients with the fibrosis score 2-3 did not differ significantly, being 64.8% (59/91) and 66.7% (14/21), respectively ($p=0.09$). None of the studied patients with cirrhosis (F4), eight patients infected with genotype 1b and one patient with genotype 3a, achieved SVR.

To determine the independent predictors of achieving SVR, such factors, like genotype, age, viral load, stage of fibrosis, and gender, were analysed by multivariate logistic regression analysis. It revealed only two factors that increased the odds of achieving SVR independently and significantly: HCV genotype 3 (OR 6.359; CI 2.525−16.017, $p<0.0001$) and patient's age 40 years or less (OR 0.274; CI 0.125−0.603, $p=0.0014$). Such variables as pretreatment viral load, fibrosis score or male gender did not correlate significantly with SVR rate, according to this analysis.

In our study 96.7% (117/121) of the patients experienced several adverse events shown in Table 3.

Table 3. Adverse events in the studied patients during PegIFN/RBV therapy

	Genotype 1, n = 76	Genotype 3. n = 45	All patients, n = 121	*p*-value
Fatigue, n (%)	70 (92.1)	39 (86.7)	109 (90.1)	0.359
Leucopenia, n (%)	61 (80.2)	35 (77.8)	96 (79.3)	0.878
Neutropenia, n (%)	64 (84.2)	32 (71.1)	96 (79.3)	<0.05
Anaemia, n (%)	31 (40.8)	12 (26.7)	43 (35.5)	<0.05
Depression, n (%)	19 (25.0)	11 (24.4)	30 (24.8)	0.563

Patients infected with genotype 1b experienced more often adverse events than patients infected with genotype 3a, but only neutropenia and anaemia differed significantly, 84.2% (64/76) vs. 71.1% (32/45), $p<0.05$ and 40.8% (31/76) vs. 26.7% (12/45), $p< 0.05$, respectively. However, as adverse events were generally mild and the rate of treatment discontinuation was therefore low, they did not influence significantly SVR rate.

Seventeen patients out of 121 (14.0%) discontinued antiviral treatment. Eight patients, of these 7 with genotype 1b and one with 3a genotype, stopped treatment because of adverse events. Nine patients (7 with genotype 1b and 2 with genotype 3a) were lost to follow-up for various reasons at different weeks (4 weeks to 48 weeks) of antiviral therapy.

Adherence to PegIFN/RBV therapy, as assessed on the basis of self-reports during visits, was high, only thirteen of the 76 patients (17.1%) infected with HCV genotype 1b required dose reduction in PegIFN alfa-2a and/or RBV because of adverse events.

Nevertheless, nine of them (69%) followed the rule of 80% (McHutchinson et al 2002) and achieved SVR (Figure 8).

Neutropenia below 0.75×10^9/l and haemoglobin level <100 g/l were the main reasons for dose reduction in both medications.

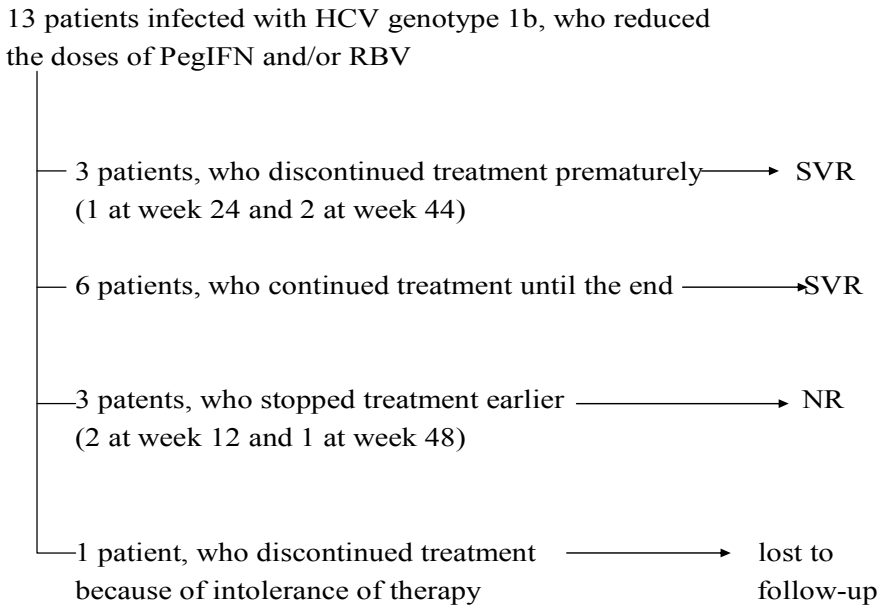

Figure 8. Flow chart of the patients infected with HCV genotype 1b, who reduced the doses of PegIFN and/or RBV.

Out of the 121 study patients, extrahepatic manifestations were diagnosed only in two of them: one had mixed cryoglobulinemia and the other had systemic sarcoidosis. A 25-year-old man, infected with HCV genotype 1b, and with moderately elevated liver enzymes was referred to the gastroenterologist at the WTCH in March 2009. Antiviral treatment was offered, but the patient decided to start it later. Four months later, there developed pulmonary symptoms and erythema nodosum of the left leg. Computer tomography (CT) scans of the chest showed mediastinal and hilar adenopathy and focal lesions in the right upper lung lobe. Transbronchial biopsy of pulmonary lymph nodes revealed epithelioid cell granulomas. Systemic sarcoidosis was diagnosed and corti-

costeroid treatment with prednisolone 20 mg orally was started. After one month of corticosteroid therapy the skin lesions disappeared and the patient discontinued treatment by himself. Three months later transcutaneous liver biopsy was done which revealed hepatic granulomas. Chronic hepatitis C with coexistence of pulmonary and hepatic sarcoidosis was diagnosed. The patient started and completed a 48-week course of PegIFN/RBV therapy. The patient achieved SVR with no reactivation of systemic sarcoidosis. Transcutaneous liver biopsy performed 5 months after SVR showed no remnant granulomas specific for sarcoidosis.

7.2. Predictors of treatment response in patients infected with HCV genotype 1b (Paper III)

7.2.1. Baseline host and viral factors and treatment response

Pre-treatment and on-treatment host and viral factors that are associated with PegIFN/RBV therapy outcome in 76 treatment-naïve patients, 44 males and 32 females, mean age of 43.0 years, with chronic hepatitis C genotype 1b, were described in Paper III. The baseline characteristics of the studied patients are shown in Table 1 (Paper III).

According to treatment response, 38 (50%) out of the 76 studied patients achieved SVR, whereas the remaining 38 (50%) patients did not and were referred to as non-SVR. Of the non-SVR patients 18 (23.7%) were non-responders (NR), i.e. patients in whom serum HCV RNA level remained stable during treatment, and 15 (19.7%) were relapsers (RL), i.e. patients who sero-reverted to HCV RNA during 6 months of follow-up. All patients with SVR and 8 of the 15 (53.3%) RL patients achieved complete EVR, while only 6 of the 18 (30%) NR and 7 (46.7%) of the RL achieved partial EVR. Five (6.6%) of the 76 studied patients stopped treatment due to adverse events.

Five baseline parameters that were associated significantly with high SVR rate were identified by univariate logistic regression analysis: age below 40 years (OR 0.9521; CI 0.9108 – 0.9953, p = 0.0302), normal platelet count (OR 1.0115; CI 1.0029 – 1.0201, p = 0.0084), normal GGT level (OR 0.9901; CI 0.9804 – 0.9999, p = 0.0469), absence of or mild and moderate stages of liver fibrosis (OR 0.6233, CI 0.4250 – 0.9143, p = 0.0156**),** and absence of or mild inflammatory activity (OR 0.5404, CI 0.3272 – 0.8925, p = 0.0162).

However, the other factors, like patient gender, BMI, viral load and ALT levels were not associated with treatment outcomes in our study (Table 4).

Table 4. Comparison of the baseline features in the patients with sustained virological response (SVR) and non-SVR.

	SVR (n = 38)	Non-SVR (n = 38)	P
Gender, n (%) Males Females	 22 (57.9) 16 (42.1)	 22 (57.9) 16 (42.1)	 1
RVR vs nonRVR	6/17	2/17	0.258
Age, n (%) Mean ± SD < 40 yrs > 40 yrs	 39,7±12,0 17 (44.7) 21 (55.3)	 46.1±9.6 7 (18.4) 31 (81.6)	 0.012 0.025
BMI, n (%) Mean ± SD ≤ 25 kg/m^2 > 25 kg/m^2	 25.8±4.3 18 (47.4) 20 (52.6)	 27.5±5.3 12 (31.6) 26 (68.4)	 0.121 0.240
Viral load, n (%) Mean±SD-$\log_{10}$ IU/ml ≤ 0.6 mln. IU/ml > 0.6 mln. IU/ml	 5.98±0.49 10 (26.3) 28 (73.7)	 6.15±0.45 6 (15.8) 32 (84.2)	 0.399 0.136
Fibrosis, n (%) F 0 – 2 F 3 – 4	 36 (94.7) 2 (5.3)	 27 (71.1) 11 (28.9)	 0.012
Inflammation activity, n (%) 0 –1 2 3	 9 (23.6) 21 (55.3) 8 (21.1)	 8 (21.1) 8 (21.1) 22 (57.8)	 0.002
PLT, × 10^9 U/L Range Mean±SD	 128-400 245.1±61.4	 83-361 205.3±58.5	 0.005
ALT, n (%) Mean ± SD, U/l ≤ 42 U/l > 42 U/l	 103.8±100.7 5 (13.2) 33 (86.8)	 91.8±54.9 8 (21.1) 30 (78.9)	 0.250 0.544
GGT, n (%) Mean ± SD, U/l ≤ 61 U/l > 61 U/l	 50.4±45.0 25 (80.6) 6 (19.4)	 91.7±91.6 16 (51.6) 15 (48.4)	 0.028 0.031

The studied variables were then analysed by multivariate logistic regression analysis, which revealed less significant power of association between them and SVR.

In order to find out the predictors of treatment response, the baseline host and viral characteristics for patients with different treatment responses were analysed.

It was found that the NR patients were significantly older (48.3±7.8 years vs. 39.7±12.0 years, p=0.008), more overweight (28.3±7.8 kg/m^2 vs. 25.8± 4.3 kg/m^2, p<0.05), had a more severe stage of fibrosis (F3–4) (38.9% vs. 5.3%, p=0.031), and a higher degree of inflammation (77.8% vs. 21.1%, p=0.0002), lower platelet count (205.3±66.0×10^9 U/L vs. 245.1±61.4×10^9 U/L, p=0.031), and higher GGT levels (76.9% vs. 19.4%, p<0.001) compared with the SVR patients.

High grade of inflammatory activity 77.8% vs. 33.4% (p=0.038) and higher GGT levels, 76.9% vs. 30.8% (p=0.047) prevailed significantly in the NR in comparison with the RL.

There were no significant differences in the characteristics between the SVR and RL patients, while the NR patients differed significantly from the SVR patients practically in terms of all studied parameters.

7.2.2. On-treatment host and viral factors and treatment response

Changes of the on-treatment host factors and changes of the viral load between the groups of patients (SVR vs. NR, SVR vs. RL, NR vs. RL) at weeks 4 and 12 were analysed (Paper III, table 2).

The SVR patients had a more pronounced decrease of the viral load at weeks 4 and 12 as compared to both the non-responders and the relapsers (–3.59 and –5.98 $\log_{10}$ IU/ml, respectively, p<0.01). Similarly, the RL had a more significant decrease of the viral load compared to the NR (–4.89 $\log_{10}$ IU/ml and –1.62 $\log_{10}$ IU/ml, respectively, p<0.01) (Figure 9).

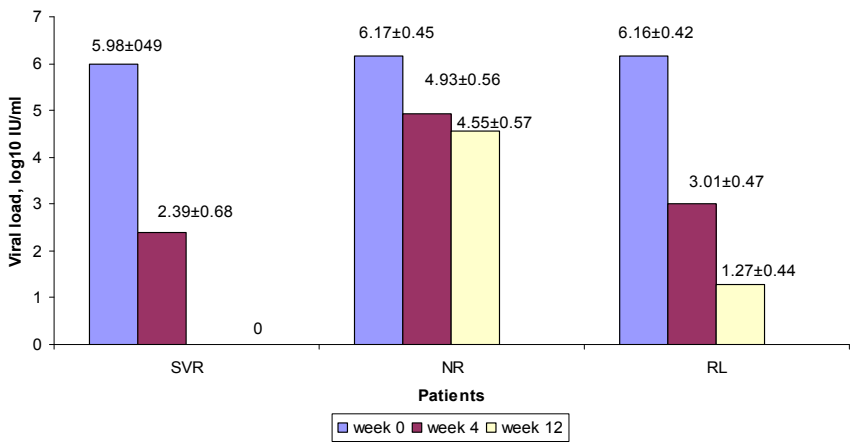

Figure 9. Viral kinetics of the study patients at baseline, weeks 4 and 12.

The other parameters such as ALT (p=0.002) and Hgb (p=0.05) levels, leucocyte (p=0.02), and neutrophil (p=0.018) counts were significantly higher for the NR patients compared to the SVR and RL patients at week 12. There were no differences in these parameters between the SVR, NR and RL patients at week 4.

7.3. Amino acid polymorphisms within the entire HCV NS5A region and treatment response (Paper IV)

7.3.1. Baseline characteristcs of patients and treatment response

Twenty-nine pre-treatment serum samples out of 76, obtained from patients infected with HCV genotype 1b (Paper III) were analysed for nucleotide and amino acid sequences.

Among the 29 patients enrolled in the study, 18 were male and 11 female aged 19 to 60 years, mean age 41.5 years. At baseline, the viral load of 89.7% (26/29) of the patients was higher than 600 000 IU/mL. Seventy nine percent (23/29) of the patients had fibrosis (F) score 0–2.

Out of all patients 12 (41.4%) achieved SVR, 15 (55.6%) failed to achieve SVR and were referred to as non-SVR. Of the non-SVR patients eight (53.3%) were referred to as NR and seven (46.7%) patients were denoted as RL. During follow-up two patients stopped treatment because of side effects. The baseline characteristics of the patients and the response to therapy are summarized in (Paper IV, Table 1).

The baseline characteristics of the SVR and non-SVR patients were similar and did not differ significantly. However, patients with SVR in comparison with non-SVR patients were younger (39.4±12.7 years vs. 45.6±9.5 years), had higher ALT (123.8±151.7 U/l vs. 73.9±U/l) and lower GGT (58.9±55.2 U/l vs. 105±120.2 U/l) levels, respectively.

7.3.2. NS5A polymorphisms and response to combination therapy

Overall, 29 complete NS5A sequences were proof-read twice by both strands, aligned, translated into proteins according to the universal code and compared to the reference strain HCV-J genotype 1b, accession number D90208.

According to the classification by Enomoto et al. (1995), 17.2% (5/29) of the studied patients were infected with wild-type ISDR IFN resistant strains (0 mutations), and 82.8% (24/29) of the patients were infected with intermediate type ISDR strains (1-3 aa mutations). None of the patients was infected with a mutant type strain (≥ 4 mutations).

Examination of baseline aa sequences did not reveal statistically significant associations between the mean number of amino acid substitutions and treatment response either in the PKR-bd including ISDR, V3 or IRRDR regions, or in the entire NS5A for the SVR and non-SVR patients including those with EVR and non-EVR.

Specific amino acid substitutions were found at some positions depending on the treatment response (Paper IV, Figure 3).

Sequence analysis of the cytoplasmic retention signal (CRS; aa 1973–1999) at aa position 1979 revealed D (Aspartic acid) in 91.7% (11/12) of the SVR patients, instead of E (Glutamic acid) in 62.5% (5/8) of the NR patients (p=0.018). Threonine (T) at aa position 1989 was found in 66.7% (8/12) of the patients with SVR, instead of Serine (S) in 75% (6/8) of the NR patients (p=0.028). Twenty five percent (3/12) of the patients with SVR, 14.3% (1/7) of the RL patients and 50% (1/2) of the ST patients carried the aa substitution V2333I in the nuclear localization signal (NLS; aa 2326-2334), but no correlation of this mutation with therapy outcome was statistically significant reliable.

Additionally, aa substitutions at the other three positions throughout the NS5A protein were identified for 62.5% (5/8) of the NR patients, among them T2107A (p=0.004), V2382A (p=0.004), and less statistically significant L2171V (p=0.06).

In our study Arginine (R) at position 2283 was present in 9/12 (75%) of the patients with SVR, and was significantly correlated with viral loads below 1,000,000 IU/mL (CI 35.4–84.8%; $p < 0.05$), instead of Proline (P) in 12/15 (80%) of the non-SVR patients, which correlated significantly with viral loads higher than 1,000,000 IU/ mL, (CI 44.4–85.7%; $p < 0.05$). T at aa position 2319 was found in 100% of the SVR patients (12/12), instead of Alanine (A) in 57.1% (4/7) of the relapse patients (p=0.009). T was also found in 42.8% of the remaining relapsers. Amino acid substitutions throughout the NS5A protein in two ST patients were similar to those in SVR patients.

7.3.3. Phylogenetic analysis

Twenty-nine full-length NS5A nt sequences were used for phylogenetic analysis by ML method (Paper IV, Figure 4).

The analysed sequences exhibited 92–94% maximal identity with HCV 1b strains from Japan, USA, Taiwan, China, France, Australia, Denmark, Switzerland, and Germany (Figure 10).

According to the results of phylogenetic analysis, 24 group-specific nt positions were identified (Paper IV, Table 2). All of the identified nt polymorphisms were translated into aa. Within each subgroup there were found essential aa substitutions common for patients with SVR, NR and RL. Thirteen nt mutations were silent, but eleven nt mutations caused group-specific aa polymorphisms (Paper IV, Table 3). Three of these were found in the V3 region, one in the NLS, one in the CRS region, and six occured in the non-classified region.

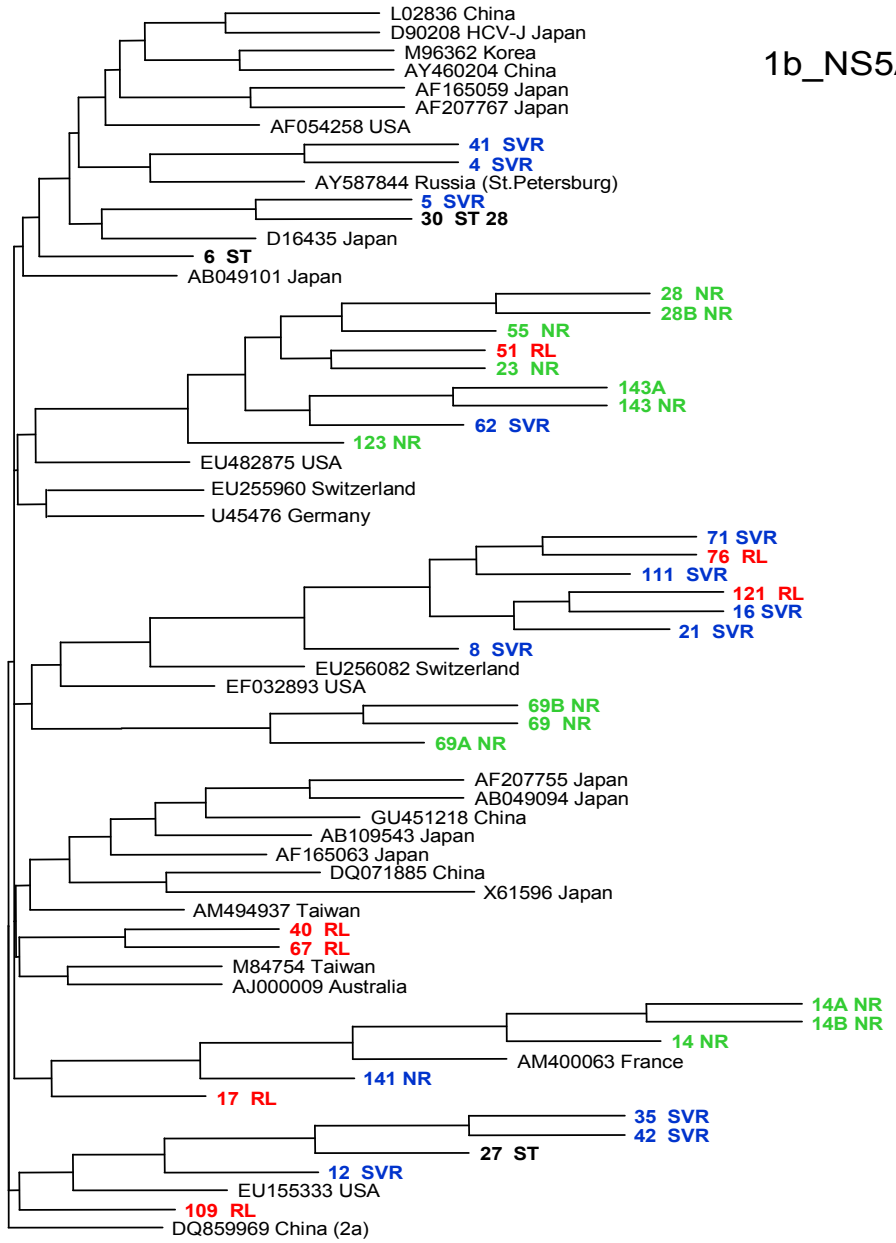

Figure 10. Phylogenetic tree, constructed by using the neighbor joining method, based on the analysis of 36 complete NS5A regions for HCV subtype 1b strains and 28 sequences from GeneBank. The sequences of isolates from GeneBank are indicated according to accession numbers. Bootstrap values were obtained from 1000 replicates. SVR (blue) – patients, who achieved SVR, NR (green) – nonresponders, RL (red) – relapsers.

8. DISCUSSION

8.1. Efficacy of antiviral treatment in patients with genotypes 1b and 3a (Papers I and II)

The results of our study with higher (84%) SVR rate in genotype 3 and lower (46%) in genotype 1 patients are consistent with the results of international clinical and real-life settings trials, which demonstrated SVR rates of 40–52% for patients infected with HCV genotype 1 and over 80% for patients infected with HCV genotype 3 (Manns et al 2001, Fried et al 2002, Hadziyannis et al 2004, Mauss et al 2011, Marcellin et al 2012). The reasons for this discrepancy are not completely clear. It may be explained by differences in viral kinetics: viral clearance in patients with genotype 1, who are IFN responders, is slower than in those infected with the other genotypes (Neumann et al 2000, Zeuzem et al 2001). On the other hand, differences in response to treatment are suggested to be explained by genetic variability between genotypes with the appearance of drug resistant HCV strains (Cannon et al 2008). Despite the fact that SVR(12) rates determined at week 12 after the end of treatment are comparable to standard SVR rate determined at week 24 after the end of treatment, the latter remains the gold standard for assessment of treatment efficacy (Martinot-Peignoux et al 2010). In our study we used SVR determined at week 24 after the end of treatment.

Several studies demonstrated that patients with a low viral load regardless of the genotype can achieve higher SVR compared to those who had a higher viral load (Navaneethan et al 2009). Although our study patients with the viral load below 600 000 IU/ml had higher SVR compared with patients with the viral load above 600 000 IU/ml, 73% and 57%, respectively, the difference did not reach significance (p=0.25). Nor was there found significant correlation between SVR and baseline levels of HCV RNA, depending on the genotype, either. These discrepancies may be explained in part by the fact that the cut-off level of low viral load is not well defined, it is variable, from 400 000 up to 800 000 IU/ml (Manns et al 2001, Fried et al 2002, Shiffman et al 2007, Zeuzem et al 2006). The cut-off level set by us failed to demonstrate significant difference in treatment response. Nor did further statistical analysis reveal significant correlation between viral loads of 400 000 IU/ml and 800 000 IU/ml and SVR rates (p=0.1). We suggest that other factors, like genetic variability of the HCV genome, may be of importance.

Our results confirm that younger age of patients is a strong predictor of treatment response (Saludes et al 2010, Marcellin et al 2012). Thus, SVR rate in the studied patients aged below 40 years was significantly higher as compared with that in the older patients, 76% vs. 47% (p=0.0014), respectively.

In spite of the fact that in our study female patients showed higher SVR rate than male patients (69% vs. 55%, respectively), the difference was not significant. Our finding is in line with the results of other similar studies, which also

failed to demonstrate significant difference between SVR rate and gender (Yu et al 2011, Di Marco et al 2013).

Advanced fibrosis and cirrhosis, due to morphological changes in the liver that prevent optimal interactions between PegIFN/RBV treatment components and hepatocytes, predispose to worse treatment response (Cheng et al 2010, Bruno et al 2010, D'Ambrosio et al 2012). Our study failed to demonstrate association between stage of fibrosis and treatment outcomes in patients with fibrosis F0-1 and F2-3. However, none of the studied patients with compensated cirrhosis (F4), eight patients infected with genotype 1b and one patient with genotype 3a, achieved SVR. At the same time, the small number (9/121) of such patients did not allow us to make any relevant conclusion.

It was shown that during antiviral therapy about 75% of patients may experience one or more systemic side effects and 10 to 16% of them are obliged to discontinue treatment due to adverse events (Arase et al 2006, McHutchison et al 2009). In our study 97% of the patients experienced several side effects with fatigue, different hematologic abnormalities and depression as the most prevalent ones. However, adverse events were generally mild, therefore the rate of treatment discontinuation was low (14%) and did not influence significantly SVR rate.

In spite of the fact that 13 out of the 76 (17%) studied patients infected with HCV genotype 1b required dose reduction of PegIFN alfa-2a and/or RBV because of adverse events, adherence to treatment was rather high and did not affect treatment response. Moreover, nine of these patients who were obliged to reduce the doses of PegIFN and/or RBV achieved SVR.

We suppose that the strict inclusion criteria of the present study (Study Methods) allowed the patients to be in compliance with PegIFN/RBV treatment.

One third of patients can have extrahepatic manifestations (EHM), including sarcoidosis as the first symptom of CHC (Cacoub et al 2000). In the present study HCV 1b genotype-infected patient with systemic sarcoidosis with lung, liver and skin involvement was diagnosed and successfully treated with PegIFN/RBV at standard doses for 48 weeks. The patient achieved SVR with no reactivation of systemic sarcoidosis. In about 75% of cases systemic sarcoidosis is described as interferon-induced during the treatment of chronic hepatitis C (Ramos-Casals et al 2005). Presenting here our case-report of CHC and systemic sarcoidosis unrelated to antiviral therapy, we, like Bonnet and co-authors (2002), speculate that HCV *per se* could be an antigenic trigger for development of sarcoidosis. We suppose that due to persistent antigenic stimulation caused by chronic HCV infection, systemic sarcoidosis may present as an EHM of chronic hepatis C. Achievement of SVR, disappearance of liver granulomas at the second liver biopsy and no reactivation of systemic sarcoidosis confirmed our suggestion. A few cases of EHM in the study can be explained by the relatively young age of our population, with shorter duration of CHC, which predisposed to less manifestations.

8.2. Predictors of treatment response in patients with genotype 1b (Paper III)

We conducted the present study aiming to evaluate the host and viral factors that are associated with treatment response in chronic hepatitis C genotype 1b patients. Genotype 1 was chosen as the most prevalent genotype in Estonia with lower response rate compared with genotypes 2 and 3.

It was shown that age below 40 years, normal platelet count and GGT level, absence of or mild and moderate stages of liver fibrosis as well as absence of or mild inflammatory activity influenced significantly SVR and can be used as predictors of treatment response in patients with CHC genotype 1b. Our results are in line with the findings of similar studies (Mann et al 2001, Fried et al 2002, Hadziyanis et al 2004, Marcellin et al 2012, Weich et al 2011).

Several large prospective studies also failed to demonstrate such association of gender, BMI and ALT with treatment response (Manns et al 2001, Fried et al 2002, Jacobson et al 2007, Mauss et al 2011). In a recent real-life European gender-oriented analysis there were no differences in response to PegIFN/RBV therapy between males and females. However, it was shown that different predictive factors in both gender groups could contribute to treatment outcomes (Di Marco et al 2013). The above analysis revealed absence of correlation between viral load and SVR, which was also discussed in the previous chapter 8.1.

We demonstrated that patients infected with HCV genotype 1b had a significantly higher rate of nonresponders and relapses, which is consistent with the results of analogous studies (Hadziyannis et al 2004, Marcellin et al 2012, Weich et al 2011).

Further we analysed the baseline host and viral characteristics for the studied patients with different treatment outcomes (SVR vs. NR, SVR vs. RL and NR vs. RL).

It was shown that NR patients were significantly older, more overweight, had a more severe stage of fibrosis (F3-4) and a higher degree of inflammation activity, lower platelet count, and higher GGT levels in comparison with the SVR patients. High grade of inflammatory activity and higher GGT levels prevailed significantly in the NR patients as compared with the relapsers. Similar data have been presented by other researchers (Mann et al 2001, Hadziyanis et al 2004, Marcellin et al 2012, Weich et al 2011).

There were no significant differences in the baseline characteristics between the SVR and the RL patients, which makes them difficult to distinguish and cure. Nevertheless, retrospectively it may be speculated that RL patients could benefit from the prolongation of antiviral treatment up to 72 weeks, as was shown previously (Pearlman et al 2007); however, the patients of the present study were treated for 48 weeks. Nowadays some of such patients, i.e. those with advanced fibrosis and cirrhosis (F3-4) can benefit from triple therapy with DAA.

In our study the SVR patients showed a more pronounced decrease of the viral load at weeks 4 and 12 as compared with both the NR and the RL patients. The results of our study are in line with the findings of other studies according to which viral kinetics at weeks 4 and 12, with the achievement of RVR and EVR, are reliable factors that can predict the outcome and duration of antiviral treatment (Pearlman et al 2007, Fried et al 2011, Marcelin et al 2012).

Absence of hematological abnormalities (anaemia, leuco- and neutropenia), which were observed in the studied patients during antiviral therapy at week 12, is typical of non-responders and may be used as a prognostic factor of worse response to antiviral therapy (Navaneethan et al 2009). Escalation of the doses of PegIFN/RBV therapy drugs in order to overcome HCV resistance and to achieve high SVR rate is not recommended by the European, American and Estonian National Guidelines on treatment of CHC, as it does not increase SVR rate.

8.3. Polymorphisms within the entire HCV NS5A region and treatment response (Paper IV)

In our study the genetic variability of the entire NS5A coding sequence, including the PKR-bd domain, ISDR and V3 regions, together with the less studied CRS, NLS and IRRDR regions, in relation to PegIFN/RBV treatment response in Estonian treatment-naïve patients infected with HCV genotype 1b, was investigated for the first time.

8.3.1. Relationship between baseline NS5A amino acid sequences and treatment response

Currently, several aa substitutions within the different regions of NS5A protein, specific for HCV-1b strains, are suggested as potential predictors of PegIFNα/RBV treatment outcome. Thus, patients infected with the wild type ISDR sequence identical to the Japanese HCV prototype did not respond to IFN-based therapy whereas patients infected with the mutant type, defined by four or more amino acid substitutions in this region, achieved SVR. Additionally, correlation was reported between treatment outcome and number of aa substitutions in the other regions within the NS5A protein of the viral isolate (Murphy et al 2002, Hofmann et al 2005, Muñoz de Rueda et al 2008, El-Shamy et al 2007, 2008). However, attempts to extend this approach to European patients were not successful (McKechnie et al 2000, Veillon et al 2004, Kmieciak et al 2006).

In the present study we did not find any mutant viruses carrying four or more amino acid substitutions in the ISDR region. Still, our results about the distribution of the wild and intermediate type ISDR strains (17.2% and 82.8%, respectively) were fairly similar to those reported for European (24.8% and 63.4%, respectively) (Pascu et al 2004) as well as for Tunisian HCV infected patients (26.7% and 66.7%, respectively) (Bouzgarrou et al 2009).

In the present study comparative analysis did not reveal a significant difference between the SVR and non-SVR patients in terms of rate of random mutations within the ISDR, PKR-bd, V3 or IRRDR regions, or in terms of total number of mutations in entire NS5A. Our data does not coincide with the results of several studies performed in different populations of different countries, which revealed significant correlations between number of aa substitutions in the abovementioned NS5A regions and treatment outcome (El-Shamy et al 2007, 2008, Kumthip et al 2011, Yano et al 2012). One explanation for this may be the existence of a methodological bias concerning either selection of patients, or therapeutic regimens (Tan et al 2001, Squadrito et al 2002). The genetic difference between the virus strains circulating in different regions may be another reason for these discrepancies (Schinkel et al 2004, Zhou et al 2011). However, there is still limited information available regarding the correlation of NS5A full-length gene polymorphisms with response to IFN based therapy.

It seems that the polymorphism of the HCV-1b strains isolated from European patients, including Estonian patients, cannot be described in terms of the number of random mutations in comparison with the prototype strain, as has been suggested previously (Aus dem Siepen et al 2005, Brillet et al 2007). On the other hand, reliable analysis of ISDR mutations should consider a representative number of samples of the wild, intermediate and mutant type strains in order to draw any relevant conclusions.

A number of studies have focused on possible correlation between EVR and sequence variation within different parts of NS5A, especially the V3 region and its surrounding regions (Murphy et al 2002, Puig-Basagoiti et al 2005, El-Shamy et al 2007). However, we found no difference in the number of mutations in the analysed regions between the EVR and the non-EVR patients.

Several studies showed a higher pre-treatment viral load in patients with the wild type ISDR sequence compared with this load in patients with the mutant type sequence (Enomoto et al 1996, Toyoda et al 2010). Our findings are consistent with the above data. It should be mentioned that the total rate of SVR in the present study was lower in comparison with that established in our previous report (Paper I). This could be explained partly by the fact that about 90% of the patients included in our study had a higher pre-treatment viral load and no one of the HCV 1b infected patients had the ISDR mutant type strain. Moreover, we found that presence of Arginine at position 2283 correlated significantly with lower viral load, while Proline at the same position correlated with higher viral load. Further studies on a larger patient cohort will are needed to validate the significance of these results.

Previous studies have reported that some specific amino acid substitutions are associated with treatment response. Thus, it has been proposed that patients infected with HCV genotype 1b who had aa mutations in NS5A at positions 2209, 2216, 2217, 2218, 2227, 2360, and 2378, but also some others (according to the enumeration of the HCV-J prototype strain) achieve SVR more frequently than those without mutations at the abovementioned positions (Kohashi et al 2006, El-Shamy et al 2008, Noguchi et al 2011).

In the present study, the association of aa substitution at specific positions in the NS5A protein with response to PegIFN-α plus RBV treatment was investigated as well. We did not find any difference between the sequences obtained from the SVR and non-SVR patients at the above described amino acid positions. However, we found that specific amino acid substitutions at positions 1989 and 2283 correlated significantly with SVR, while mutations at positions 1979, 2107, 2171, and 2382 were associated with non-response to treatment. The single amino acid substitution at position 2319 correlated with treatment relapse.

Recently, three aa substitutions at positions 2268, 2260 and 2278 were associated with unfavorable treatment outcome in HCV-1b patients in Hong Kong (Zhou et al 2011).

In contrast, our data showed that these positions were not informative for prognosis of treatment outcome in Estonian patients.

All these facts can be explained, on the one hand, by genetic differencies between the HCV genotype 1b strains circulating in Estonia and other countries. On the other hand, it deserves to note some limitations of our study like the small number of patients in comparison with previous studies and the selection bias in the enrollment of patients arising from the National Guidelines for treatment of chronic hepatitis C.

8.3.2. Phylogenetic analysis

Previously, some researchers failed to show any clustering association with a specific pattern of response while using phylogenetic analyses of the complete NS5A sequence to predict treatment outcome (Jardim et al 2009).

In the present study, in phylogenetic analysis 1b strains were divided into 4 groups (Paper IV, Figure 4), considering our previous suggestions about several separate introductions of the 1b strain in Estonia (Tallo et al 2007). For the first time we segregated the isolated strains into six subgroups mainly according to treatment outcome (Paper IV, Figure 4). Further, the obtained 1b sequences were translated into proteins and aligned according to the mentioned groups.

Phylogenetically, 24 group-specific nucleotide positions were identified (Paper IV, Table 2), of these thirteen nt mutations were silent and eleven nt mutations caused group-specific aa polymorphisms (Paper IV, Table 3).

The results of our study after validation on studies based on large data sets can be recommended to use as predictors of treatment response in Estonian treatment-naïve patients monoinfected with HCV genotype 1b.

We believe that the results of our study of genetic variability within the NS5A region of HCV genome could shed light on unsatisfactory cure rates, especially in treatment-naïve patients monoinfected with HCV genotype 1b in the face of viral resistance and could help to improve treatment outcome in such patients in the future.

8.4. Considerations and limitations related to the study design

Our study, conducted in one of the five centers in Estonia providing treatment of CHC, is the first one reflecting the experience of PegIFN/RBV therapy for treatment–naïve patients monoinfected with CHC genotypes 1b and 3a in routine clinical practice. Co-existence of HCV/HIV infections is a challenge for the health care system. HIV/HCV coinfected patients, which is an issue of serious concern in Estonia (Uuskula et al 2007), were not included in the study as they are usually referred to infectious disease clinics located in several Estonian counties. A specific population of IDUs having high rate of HIV, HCV and HBV coinfections was excluded from the study, too.

Owing to the use of strict selection criteria, we were able to form a unique study population which differed from analog patient cohorts of other studies (Manns et al 2001, Fried et al 2002, Hadziyannis et al 2004, Mauss et al 2011, Marcellin et al 2012). Our patients were characterized as follows: they were relatively young, with a mean age of 43 years for genotype 1b infected patients and 35 years for genotype 3a infected patients.

Baseline ultrasound-guided transcutaneous liver biopsy was performed to all study patients (100%), which is a higher percentage that those reported in other real-life studies (Mauss et al 2011, Bourliere et al 2012).

The proportion of patients (75%) without or with a mild stage of fibrosis (F0-1) was quite large. Taking into consideration the above circumstances and owing to the natural course of HCV infection, it was unlikely to expect a high number of patients with advanced fibrosis and cirrhosis (F3-4), which definitely diminished the power of statistical analysis.

One of the limitations of this study, which is an issue of discussion, was the relatively small number of patients in comparison with other randomized and real-life setting clinical trials. This could in some cases have diminished the power of statistical analysis in detecting significant associations between different baseline variables and SVR. Yet further delay in the analysis of our data for statistics related reasons would have rendered our study of less importance, especially in the era of DAA.

Another limitation may be that we did not use several host genetic factors, like polymorphisms of the human IL28B gene, which is known as a very important predictor of treatment response to PegIFN/RBV therapy in patients infected with HCV genotype 1 (Ge et al 2009, Par et al 2011, 2014). This can be explained by the fact that at the time of conducting the study the relevant test was not available in routine clinical practice in Estonia.

8.5. Future research

Despite the fact that the incidence of new HCV infections has decreased worldwide, the burden of HCV-related diseases is expected to increase. Triple therapy with PIs (BOC and TVR) has been reported to increase SVR rates up to 63–75% as compared to the standard dual therapy in patients with CHC. In Estonia, the SOC for treatment of HCV genotype 1 was changed from the beginning of 2014 when both of these medications were approved for use in combination with PegIFN/RBV for treatment of CHC genotype 1-infected treatment-naïve and experienced patients with advanced liver fibrosis or compensated cirrhosis.

However, the using of DAAs can lead to the emerging problem of drug resistance due to so-called resistance-associated amino acid variants of the virus. The prevalence of these resistance mutations in patients treatment-naive for PIs has not been fully characterized.

Moreover, the frequency of resistance can vary between the HCV subtypes, being twice as high in patients with subtype 1a compared with subtype 1b. Although subtype 1b has remained predominant, the introduction of subtype 1a into the populations of IDUs and blood donors has been observed in Estonia at least since 2007 (Tallo et al 2011). Thus, we could expect an increase in the number of patients infected with this subtype. Taking into consideration the above circumstances, we are going to assess the efficacy of triple therapy with PIs in patients infected with both HCV subtypes, 1a and 1b, and to compare it with existing dual therapy. Other noteworthy issues of our future plans are study of the presence of primary PIs resistance mutations and follow up of the emergence of PIs resistant variants during triple therapy in order to evaluate their impact on treatment response.

In Estonia there is no state or regional standard or national policy on HCV prevention or a general anti-HCV screening programme. Only two specific subgroups, the blood donors and the prisoners, are screened on a regular basis. The seroprevalence of HCV infection has been well studied only in specific study populations, like IDUs, inmates, hemodialysis and oncohematologic patients (Tefanova et al 2005, 2006, Uuskula et al 2007). As new treatment options are becoming available, there will arise the need to quantify the burden of chronic HCV infection at the national level. The data to be obtained can optimize the cost-effectiveness of CHC treatment in Estonia and hence reduce the burden of HCV-related diseases.

9. CONCLUSIONS

To our knowledge, this is the first systematic study aimed to assess the efficacy of PegIFN/RBV treatment and to evaluate the host and virus factors predicting the outcome of antiviral therapy in treatment-naive patients with CHC in clinical practice in Estonia.

1. The efficacy of PegIFN/RBV therapy was strongly predetermined by the HCV genotype and younger age of the studied patients. The SVR rate for patients infected with HCV genotype 1b was significantly lower compared with SVR rate for patients infected with HCV genotype 3a.

2. Age below 40 years, absence of or mild and moderate liver fibrosis, absence of severe inflammatory activity, normal platelet count and normal GGT level with pronounced changes in viral kinetics at weeks 4 and 12 were valuable predictors of treatment response in treatment-naïve patients with the chronic hepatitis C 1b genotype. The non-responders differed significantly from the patients with SVR in terms of almost all pre- and on-treatment studied parameters. Regarding their characteristics the relapsers were very similar to the patients with SVR.

3. Study of amino acid polymorphisms failed to demonstrate association between number of mutations within the either entire NS5A protein of HCV or its different known functional regions, and different types of response (SVR, non-response or relapse) to PegIFN/RBV therapy in treatment-naïve patients with the chronic hepatitis C 1b genotype.

4. Correlation between treatment response (SVR, non-response or relapse) and specific amino acid substitutions at different previously not described positions within the NS5A protein was identified. Phylogenetic analysis revealed 11 novel aa substitutions in the full-length NS5A protein, which can be used as tags to predict treatment response in Estonian treatment-naïve patients with the chronic hepatitis C 1b genotype.

10. SUMMARY IN ESTONIAN

Krooniline C-hepatiit: ravitulemusi prognoosivad tegurid Eesti patsientidel

C-hepatiidi viirusest (HCV) põhjustatud krooniline hepatiit on suur tervishoiuprobleem, sest haigestunud võib olla ligikaudu 2,3% maailma rahvastikust. Aastas sureb C-hepatiidist tekkinud maksatsirroosi ja maksarakk-kasvaja tõttu umbes 350 000 inimest. Euroopas erineb C-hepatiidi levimus piirkonniti. Eestis on C-hepatiidi viirusega nakatunud hinnanguliselt vähemalt 1% rahvastikust.

C-hepatiidi viirus kuulub *Flaviviridae* sugukonda ja *Hepacivirus*'e perekonda. Sellel viirusel on seitse genotüüpi ja üle 60 alltüübi. Eestis on enam levinud 1b genotüüp, millele järgneb 3a genotüüp. HCV genotüübi teadmisel on kliiniline tähendus, st genotüübist sõltub kroonilise maksahaiguse kulg, viirusvastase ravi tulemused ja ka kestus.

Viirusvastane vaktsiin ja nakatumisjärgne profülaktika puuduvad. Ägeda C-hepatiidiga haigetest tervistub 15%, ülejäänutel tekib krooniline C-hepatiit. Krooniline C-hepatiit võib kulgeda vaevusteta kuni maksatsirroosi tekkeni. Maksatsirroosi tekkeks kulub 10−30 aastat nakatumisajast. Maksatsirroosist omakorda võib 4% haigetel aastas tekkida maksarakk-kasvaja.

Alfa-peginterferoon (alfa-2a-peginterferoon ja alfa-2b-peginterferoon) ja ribaviriin on aastaid olnud kroonilise C-hepatiidi standardravi. Viirusvastase ravi eesmärk on C-hepatiidi viiruse kadumine verest ehk C-hepatiidi viiruse RNA (HCV RNA) puudumine veres. Viirusvastase ravi tõhususe kriteerium on püsiv viirusvastus – HCV RNA on negatiivne 24 nädalat pärast viirusvastast ravi.

Viirusvastase ravi tõhusus tuleneb genotüübist. Teise ja kolmanda genotüübiga patsientidest saavutab püsiva viirusvastuse üle 80%, esimese genotüübiga patsientidest aga vaid ligikaudu 50%.

Viirusvastase ravi tõhusust mõjutavad mitu patsiendist olenevat ja ka mitu C-hepatiidi viirusest tulenevat tegurit. Need on patsiendi vanus, sugu, aminotransferaaside sisaldus veres, maksafibroosi raskusaste ja põletiku aktiivsus maksakoes. Viiruse genotüübist ja viirusekogusest sõltub viirusvastasele ravile allumise tõenäosus ning ravi kestus. HCV genoomi muutlikkus võib samuti mõjutada alfa-peginterferooni ja ribaviriiniga ravimise tõhusust. Näiteks viiruse mittestruktuurvalk NS5A on vajalik viiruse replikatsiooniks ja tema erinevate regioonide polümorfismi seostatakse viirusvastase ravi tõhususega.

Töö eesmärgid

Käesoleva töö eesmärk oli uurida varem viirusvastast ravi mittesaanud (nn ravinaiivsetel) kroonilise C-hepatiidiga patsientidel peremeesorganismist ja C-hepatiidi viirusest tulenevaid tegureid, et prognoosida alfa-peginterferooni ning ribaviriiniga ravimise tõhusust.

Uurimistöö eesmärgid olid järgmised:

1. hinnata alfa-peginterferooni ja ribaviriiniga ravimise tõhusust Eestis varem viirusvastast ravi mittesaanud kroonilise C-hepatiidi esimese ja kolmanda genotüübiga patsientidel (I ja II artikkel);
2. määrata peremeesorganismist ja C-viirusest tulenevad tegurid, mis mõjutavad viirusvastase ravi tõhusust kroonilise C-hepatiidi 1b genotüübiga patsientidel nii enne viirusvastast ravi kui ka ravi ajal (III artikkel);
3. analüüsida kroonilise C-hepatiidi 1b genotüübiga patsientidel HCV mittestruktuurvalgu NS5A muutlikkuse ja ravitulemuse vahelisi seoseid (IV artikkel).

Uuritav materjal ja meetodid

Alates 2005. aasta veebruarist kuni 2011. aasta märtsini uuriti kokku 121 (I ja II artikkel), 76 (III artikkel) ja 29 (IV artikkel) varem viirusvastast ravi mittesaanud kroonilise C-hepatiidiga patsienti. Uuring tehti Lääne-Tallinna Keskhaiglas.

Kroonilise C-hepatiidi diagnoosimine, viirusvastane ravi ja patsiendi jälgimine viirusvastase ravi ajal toimusid kroonilise C-hepatiidi Eesti ravijuhendite järgi. Enne uuringu alustamist allkirjastasid kõik patsiendid nõusolekuvormi ja uuringu kiitis heaks Tallinna Meditsiiniuuringute Eetikakomitee.

Kliinilise vere, biokeemiliste ja autoantikehade näitajad määrati Lääne-Tallinna Keskhaigla diagnostikakliiniku laboris. HCV antikehad, HCV RNA genotüüp ja HCV RNA viirusekogus määrati Lääne-Tallinna Keskhaigla HIV nakkuse referentslaboris.

HCV RNA viirusekogus määrati enne ravi alustamist, 4., 12., 24. ja 48. ravinädalal ning 24. nädalal pärast viirusvastast ravi. Kõikidelt patsientidelt võeti enne viirusvastase ravi alustamist Lääne-Tallinna Keskhaigla radioloogiaosakonnas ultrahelikontrolli all transkutaanne maksabiopsia. Maksakoes hinnati fibroosi staadiumi ja põletiku raskusastet Metaviiri skoori järgi, histoloogiline uuring tehti Põhja-Eesti Regionaalhaigla patoloogiaosakonnas.

NS5A polümorfismi uurimiseks ekstraheeriti HCV RNA, sünteesiti cDNA, sekveneeriti RT-PCR ja HCV genoom terves NS5A piirkonnas (1341 nukleotiidi) ning tehti sekventside fülogeneetiline analüüs. NS5A järjestused tõlgendati valkudeks universaalse koodi järgi ja võrreldi 1b genotüübi referentstüvega. Kõik järjestused anti hoiule NCBI GenBanki nukleotiidide järjestuste andmebaasi registreerimisnumbritega JX022751-JX022779.

Sõltumata viiruse genotüübist manustati kõigile patsientidele viirusvastase ravi käigus alfa-2a-peginterferooni 180 µg kord nädalas ning ribaviriini kehakaalu ja genotüübi alusel. Esimese genotüübi puhul manustati ribaviriini alla 75 kg kehakaaluga patsientidele 1000 mg ja kehakaaluga üle 75 kg 1200 mg ööpäevas. Teise ja kolmanda genotüübi puhul oli ribaviriini annus 800 mg ööpäevas.

Viirusvastase ravi kestus 1b genotüübiga patsientidel oli 48 nädalat, teise ja kolmanda genotüübi puhul 24 nädalat. Patsiendi ravi oli tulemuslik juhul, kui

saavutati püsiv viirusvastus ehk kui HCV RNA oli negatiivne 24 nädalat pärast ravi lõppu.

Statistiliseks analüüsiks kasutati $\chi 2$-testi, Fisheri täpset testi ja Studenti testi. Kvantitatiivseid näitajaid väljendati nagu keskväärtus ±SD. Näitajate korrelatsiooni hinnati Pearsoni või Spearmani korrelatsioonikoefitsiendi abil. Statistiliselt oluliseks peeti kahepoolset P väärtust, mis oli väiksem kui 0.05. Usaldusvahemik (CI) oli 95%.

Peremeesorganismist ja viirusest tulenevate ravi tõhusust mõjutavate tegurite analüüsiks kasutati ühe- ning mitmemõõtmelist regressioonanalüüsi.

Uurimistöö tulemused

1. Viirusvastase ravi tõhusus esimese ja kolmanda genotüübiga patsientidel (I ja II artikkel)

60.3% patsientidest saavutas püsiva viirusvastuse. Esimese genotüübiga patsientidel oli püsiva viirusvastuse saavutamine statistiliselt väiksem kui kolmanda genotüübiga patsientidel (vastavalt 46.1% *vs*. 84.4%).

Ravile mitteallunute arv, sh mittevastajad (*non-responder*, NR) ja tagasilangejad (*relapser*, RL), oli esimese genotüübi rühmas suurem kui kolmanda genotüübi rühmas (vastavalt 19.7% *vs*. 2.2% ja 17.1% *vs*. 4.4%).

Mitmemõõtmeline logistiline regressioonanalüüs näitas, et ainult kolmas genotüüp ja patsiendi vanus (40 aastat või alla selle) suurendasid sõltumatult ning oluliselt püsiva viirusvastuse saavutamist. Sellised tegurid nagu viirusekogus, maksafibroosi raskusaste ja meessugu ei korreleerunud mitmemõõtmelises logistilises regressioonanalüüsis oluliselt püsiva viirusvastusega.

Peaaegu kõigil patsientidel esines viirusvastase ravi kõrvaltoimeid, millest sagedasemad olid väsimus (90%) ja laborianalüüsides neutropeenia (79%). Esimese genotüübiga patsientidest vajas 17% kõrvaltoimete tõttu alfa-2a-peginterferooni või ribaviriini annuste vähendamist. Nendest üheksa patsienti saavutas püsiva viirusvastuse, kolm patsienti osutusid mittevastajateks ja üks patsient katkestas ravi.

Kõigist uuritud patsientidest 14% katkestas ravi eelkõige kõrvaltoimete tõttu.

Uuringus osalenud 25-aastane meespatsient, kellel diagnoositi krooniline 1b genotüübiga C-hepatiit koos süsteemse sarkoidoosiga, saavutas püsiva ravivastuse. Viis kuud hiljem tehtud korduval maksapunktsioonil maksagranuloomid puudusid.

2. Ravivastust prognoosivad tegurid 1b genotüübiga patsientidel (III artikkel)

Esimese genotüübiga patsientidest saavutas püsiva viirusvastuse 50% (76). Ravile mitteallujaist (38/76) oli 24% mittevastajaid (NR) ja 20% oli tagasilangejaid (RL); viis patsienti katkestas ravi kõrvaltoimete tõttu.

Ühemõõtmelise regressioonanalüüsiga avastati viis põhiparameetrit, mis mõjutasid oluliselt püsivat viirusvastust: vanus alla 40 aasta, referentsväärtuses

trombotsüütide arv ja gammaglutamüüli transferaas, maksafibroosi puudumine või kerge kuni mõõdukas fibroosi aste, aktiivse maksapõletiku puudumine või kerge aktiivsus. Korrelatsioone püsiva viirusvastuse, kehamassiindeksi, ravieelse viirusekoguse ega alaniini aminotransferaasi väärtuse vahel ei esinenud. Mitmemõõtmelise logistilise regressioonanalüüsiga ei avastanud neid parameetreid sõltumatute prognoosivate teguritena.

Enne ravi alustamist analüüsiti peremeesorganismist ja viirusest tulenevaid tegureid erinevates rühmades: SVR *vs*. NR, SVR *vs*. RL ja NR *vs*. RL. Mittevastajad (NR) olid statistiliselt vanemad ja ülekaalulisemad ning nende seas oli rohkem raskema maksafibroosi ja suurema aktiivsuse, väiksema trombotsüütide arvu ning suurema gammaglutamüüli transferaasi väärtustega patsiente kui püsiva viirusvastusega patsientide seas. Tagasilangejad (RL) sarnanesid püsiva viirusvastuse saavutanutega.

Püsiva viirusvastusega patsientidel oli aga viirusekoguse vähenemine 4. ja 12. ravinädalal statistiliselt olulisem võrreldes mittevastajate ja tagasilangejatega.

3. Aminohapete polümorfism NS5A regioonis ja ravivastus (IV artikkel)

17% patsientidest esinesid ISDR-i piirkonnas metsiku tüübiga (0 mutatsiooni) ja 83% patsientidest vahetüübiga (1–3 aa mutatsiooni) tüved. Ükski patsient ei olnud nakatunud muteeruva tüübiga (≥ 4 mutatsiooni).

Geneetiline analüüs ei näidanud ei püsiva viirusvastusega patsientidel ja ravile mittevastanud patsientidel statistiliselt olulisi seoseid aminohapete asenduste numbrite ning ravivastuse vahel ISDR-i, PKR-bd, V3 ja IRRDR-i piirkonnas ega NS5A valgus. Aminohapete asendused T1989 ja R2283 korreleerusid oluliselt püsiva viirusvastusega, kuid E1979, A2107, V2171 ja A2382 esinesid mittevastajail. Tagasilangejail avastati T2319A asendus.

Fülogeneetilise analüüsi abil isoleeritud tüved rühmitati ravitulemuse alusel nelja rühma. Avastati 24 spetsiifilist nukleotiidide positsiooni. 13 mutatsiooni olid vaikivad, kuid erineva vastusega rühmades leiti 11 nukleotiidide mutatsiooni. Kolm mutatsiooni avastati V3 piirkonnas, ühekaupa NLS-i ja CRS-i piirkondades ning kuus mutatsiooni leiti klassifitseerimata piirkonnas.

Järeldused

1. Viiruse genotüüp ja vanus alla 40 aasta olid viirusvastase alfa-peginterferooni ning ribaviriiniga ravimise tõhususe olulised prognoosivad tegurid. Kroonilise C-hepatiidi 1b genotüübiga patsiendid allusid ravile halvemini kui 3a genotüübiga patsiendid.
2. Vanus alla 40 aasta, maksafibroosi puudumine või kerge kuni mõõdukas fibroos, aktiivse maksapõletiku puudumine või kerge aktiivsus, referentsväärtuses trombotsüütide arv ja gammaglutamüüli transferaasi väärtus ning viirusekoguse vähenemine 4. ja 12. ravinädalal olid kroonilise C-hepatiidi 1b genotüübiga ravinaiivsetel patsientidel ravivastuse kindlad kriteeriumid.

Ravile mittevastajad (*non-responder*) erinesid viirusvastase ravi tõhusust mõjutavate tegurite poolest oluliselt püsiva viirusvastusega patsientidest nii enne viirusvastast ravi kui ka ravi ajal. Tagasilangejad (*relapser*) sarnanesid püsiva viirusvastuse saavutanud patsientidega.

3. Aminohapete polümorfismi uuringus ravinaiivsetel kroonilise C-hepatiidi 1b genotüübiga patsientidel puudus seos nii HCV terve NS5A valgu kui ka NS5A valgu eri piirkondade mutatsioonide arvu ning alfa-peginterferooni ja ribaviriini ravivastuse (püsiv viirusvastus, *non-response* või *relapse*) vahel.
4. Leiti korrelatsioon ravivastuse (püsiv viirusvastus, *non-response* või *relapse*) ja NS5A valgu varem mittekirjeldatud spetsiifiliste aminohapete asenduste vahel. Fülogeneetilisel analüüsil selgus 11 uut aminohapete asendust terves NS5A valgus, millele tuginedes võib prognoosida ravi tõhusust Eesti kroonilise C-hepatiidi 1b genotüübiga ravinaiivsetel patsientidel.

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