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HEPATITIS C VIRUS: GENOTYPE IDENTIFICATION AND INTERACTIONS BETWEEN VIRAL PROTEASES

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ABBREVIATIONS

AIH	autoimmune hepatitis
AMA	antimitochondrial antibodies
ANA	antinuclear antibodies
ARA	antireticulin antibodies
BPV-1	Bovine Papillomavirus type 1
DEPC	diethylpyrocarbonate
ER	endoplasmic reticulum
FITC	fluorescein isothiocyonate
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCV RNA	hepatitis C virus ribonucleic acid
IRES	internal ribosome entry site
IVDU	intravenous drug users
LKMA	liver-kidney microsomal antibodies
NOSA	nonorgan-specific autoantibodies
NS	nonstructural protein
PBMC	peripheral blood mononuclear cells
PCA	parietal cell antibodies
RT-PCR	reverse transcription – polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RFLP	restriction fragment length polymorphism
SDS-PAGE	sodium dodecylsulphate polyacrylamide gelelectrophoresis
SFV	Semliki Forest virus
SMA	smooth muscle antibodies
SVR	sustained virologic response
TMA	thyroid microsomal antibodies
UTR	untranslated region

INTRODUCTION

Hepatitis C virus (HCV) was discovered in 1989 by the scientists from the Chiron Corporation by molecular cloning without the direct use of biologic or biophysical methods. This was accomplished by extracting, copying into cDNA, and cloning all nucleic acids from the plasma of a chimpanzee infected with non-A non-B hepatitis by contaminated factor XIII concentrate (Choo *et al*., 1989). Before then, it was classified as a non-A non-B hepatitis virus transmitted mainly through blood products, or also via other intravenous routes. With the introduction of blood and blood products screening for anti-HCV antibodies in 1990, new cases of posttransfusion hepatitis C have virtually disappeared and intravenous drug use has become the major identifiable mode of transmission in many countries. Sexual transmission is rare and correlates with high-risk sexual practices (Vandelli *et al*., 2004). Mother-to-infant transmission has been observed, but the risk is less than 5% (Ferrero *et al*., 2003). Household transmission is uncommon. In clinical practice no epidemiological risk factor can be identified in up to 40% of patients with hepatitis C.

The seroprevalence rate of HCV is about 1% in Western Europe and North America, 3–4% in some Mediterranean and Asian countries and up to 10–20% in parts of Central Africa and Egypt (Wasley and Alter, 2000). In Estonia, the routine measurement of anti-HCV antibodies in the donors' blood sera was started in 1991. The prevalence of anti-HCV antibodies among blood donors was investigated in 1995–1996 in the blood centers of Tartu and North-Estonia and was estimated to be about 1% (Tamme *et al*., 1997).

HCV infection is characterized by viral persistence and chronic liver disease in approximately 70–80% of cases (Alter, 1997; Global burden of disease for hepatitis C, 2004). Chronic hepatitis C is one of the main causes of chronic liver disease worldwide. There is a strong association between chronic hepatitis C, liver cirrhosis, and hepatocellular carcinoma (HCC) (Degos *et al*., 2000; Haydon *et al*., 1997). Liver cirrhosis develops in 5–20% of patients with chronic hepatitis C during approximately 20 years of disease duration (Di Bisceglie *et al*., 1991; Global burden of disease for hepatitis C, 2004). Cohort studies indicate that HCC is currently the major cause of liver-related death in patients with compensated cirrhosis. Hepatitis C virus infection is associated with the highest HCC incidence, occurring in 13–30% of patients with HCV-related cirrhosis (Degos *et al*., 2000; Fattovich *et al*., 2004). Hepatitis C-related end-stage liver disease is now the principal indication for liver transplantation in industrialized countries (Alter and Seeff, 2000). The development of a universally effective vaccine for prevention and treatment of HCV infection is greatly hampered by the significant heterogeneity of the genome of this virus.

An increasing number of registered HCV infection cases, growing clinical importance of chronic hepatitis C, initiation of interferon alpha and ribavirin treatment and the need for genotype identification of HCV infection in Estonia made the present study necessary.

REVIEW OF LITERATURE

1. HCV genome organization and functions of HCV proteins

HCV is classified as belonging to the distinct genus *Hepacivirus*, in the family *Flaviviridae*, along with the other members of this family, flavi- and pestiviruses with the prototype members Yellow fever virus and Bovine viral diarrhea virus, and hepatitis G virus (Miller and Purcell, 1990; Choo *et al*., 1991; Linnen *et al*., 1996). These viruses are characterized by enveloped particles, which contain a single RNA genome of positive polarity. The HCV genome encodes one large open reading frame, which is flanked with 5' and 3' untranslated regions (UTRs). Initiation of translation of the HCV genome is controlled by an internal ribosome entry site (IRES) located within the 5' UTR of the viral RNA. The length of the open reading frame of each genotype is characteristically different. While the open reading frame in genotype 1 isolates is approximately 9400 ribonucleotides, it is typically 9099 nucleotides in genotype 2 isolates and 9063 nucleotides in genotype 3 isolates (Bukh *et al*., 1995). These differences may potentially account for some of the phenotypic differences among the geno(sub)types discussed below.

A single polyprotein precursor of about 3000 amino acids (C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B) is processed co- and posttranslationally by a combination of host and viral proteases into ten discrete proteins (Tsukiyama-Kohara *et al*., 1992; Wang *et al*., 1993) (Fig. 1). The first cleavage product of the polyprotein is highly basic core (C) protein, forming the major constituent of the nucleocapsid (Yasui *et al*., 1998). In addition, a number of other functions like modulation of several cellular processes or induction of hepatocellular carcinoma in transgenic mice have been described for HCV core protein (Yamanaka *et al*., 2002; Moriya *et al*., 1998; Chen *et al*., 1997).

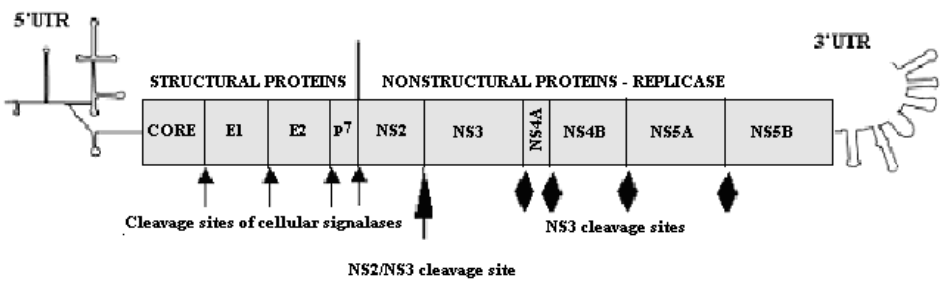

Figure 1. Genomic organization of HCV. Different types of the cleavage sites of polyprotein are indicated with different sets of arrows. Pavio and Lai, 2003, with modifications.

Envelope proteins (E1 and E2) are highly glycosylated transmembrane proteins, forming two types of stable heterodimeric complexes: a disulfide-linked form representing misfolded aggregates and a non-covalently linked heterodimer corresponding most likely to the pre-budding complex (Deleersnyder *et al*., 1997). Together, the structural proteins E1 and/or E2 in conjunction with the core were shown to have a stronger antiapoptotic effect than that of the core alone. As it was demonstrated recently, HCV core-E1-E2 transgenic mice developed significantly larger tumors than transgenic mice expressing the core alone, or nontransgenic mice (Kamegaya *et al*., 2005).

The short hydrophobic peptide, p7, which separates the structural and nonstructural (NS) proteins, may function as a membrane channel (Pavlovic *et al*., 2003). The structural proteins are cleaved from the polyprotein precursor by host signal peptidases. Generation of mature NS proteins is achieved by the action of viral proteases. The term "NS proteins" indicates that these proteins are not expected to be constituents of the virus particle. All NS proteins are thought to be required for viral replication.

The junction between NS2 and NS3 is processed by NS2/NS3 metallo-protease, consisting of NS2 and the amino-terminal NS3 protease domain flanking the cleavage site (Grakoui *et al*., 1993b; Reed *et al*., 1995; Hijikata *et al*., 1993a). The 180 amino-terminal residues constitute a chymotrypsin-like serine protease, which mediates the proteolytic release of mature NS4A, NS4B, NS5A, and NS5B (Bartenschlager *et al*., 1993; Grakoui *et al*., 1993a). In addition to its function as a protease, NS3, within its carboxy-terminal domain, also possesses helicase and nucleoside triphosphatase activities essential for translation and replication of the HCV genome (Kim *et al*., 1995; Suzich *et al*., 1993). The NS3 may have other properties involved in interference with host cell functions, like inhibition of protein kinase A-mediated signal transduction, cell transformation, or affecting of the functions of nuclear histones (Borowski *et al*., 1999; Borowski *et al*., 1996; Sakamuro *et al*., 1995). The NS4A polypeptide functions as an essential cofactor for the NS3 serine protease (Grakoui *et al*., 1993a). In addition to serving as a protease cofactor, NS4A has two further functions that might contribute to efficient polyprotein cleavage and replication. Firstly, it increases the metabolic stability of NS3 that in the absence of NS4A is degraded very rapidly. Secondly, it anchors NS3 to intracellular membranes, thereby increasing the local enzyme: substrate concentration and facilitating formation of a membrane-associated replicase complex (Wolk *et al*., 2000).

The function of NS4B is incompletely understood. The NS4B is a membrane-associated protein that co-localizes predominantly with endoplasmic reticulum (ER) markers and behaves as an integral membrane protein (Hugle *et al*., 2001). Recently, N-terminal amphipathic alpha helix was identified within the NS4B protein. It was shown that this helix in NS4B mediates membrane association, correct localization of replication complex proteins, playing an essential role in HCV RNA replication (Elazar *et al*., 2004).

The NS5A is a pleiotropic protein that plays key roles in both viral RNA replication and modulation of the physiology of the host cell. The NS5A is a highly phosphorylated polypeptide that associates with membranes via an amino-terminal amphipathic alpha helix (Brass *et al*., 2002). Apparently, NS5A, in parallel with NS4B, functions as an anchor, by means of which the HCV replication complex is bound to the host cell membranes. The NS5A is also involved in resistance of HCV-infected cells to the antiviral activity of interferon alpha through repression of the double stranded RNA-dependent protein kinase (Gale *et al*., 1997). In addition, NS5A was found to interact with the growth factor receptor-bound protein 2 adaptor protein (Tan *et al*., 1999), karyopherin ß3 (Chung *et al*., 2000), transcription factor SCRAP (Ghosh *et al*., 2000), p53 (Majumder *et al*., 2001), cyclin-dependent kinase 1 (Arima *et al*., 2001), and the tumor necrosis factor receptor-associated factor 2 (Park *et al*., 2003). These interactions may influence kinase-signaling cascades or disturb cell growth and cell signaling and thereby contribute to pathogenesis of hepatitis C.

The NS5B functions as RNA-dependent RNA polymerase (RdRp) – a key enzyme in the HCV replicase complex, responsible for the synthesis of new antigenomic and genomic RNAs. This protein has been extensively characterized biochemically (Behrens *et al*., 1996; Lohmann *et al*., 1997) and structurally (Ago *et al*., 1999; Lesburg *et al*., 1999). It contains structural motifs shared by all RdRps, and possesses classical fingers, thumb, and palm domains. The "fingertips" are in close contact with the thumb subdomain, thus forming a unique spherical shape of HCV RdRp, in contrast to other RNA polymerases that have a U-shape. The NS5B has an intrinsic ability to oligomerize or dimerize, which is necessary for its RdRp activity (Qin *et al*., 2002).

2. The HCV replication cycle and the role of protease interactions in viral replication

Recent studies have suggested that HCV infection is a highly dynamic process with a viral half-life of 3–5 hours and an average virion production and clearance rates of up to more than 10^{12} particles per day. Assuming that ~10% of the hepatocytes are infected and that the liver contains ~2×10^{11} hepatocytes, this would correspond to a virion production rate of 50 particles per hepatocyte per day (Neumann *et al*., 1998).

The presumed life cycle of HCV includes (1) penetration of the host cell and liberation of the genomic RNA from the virus particle into the cytoplasm, (2) IRES-mediated translation, polyprotein processing by cellular and viral proteases and formation of a membrane-bound replication complex, (3) synthesis of a minus-strand RNA intermediate on a plus-strand genomic RNA, (4) synthesis of new positive strands, which in turn can be used for polyprotein

production, synthesis of negative strands or packaging into the virion, (5) virion maturation and release from the host cell.

(1) The cell surface receptor, necessary for HCV virion binding and internalization into the host cell, is not clearly defined. A few candidate receptors have been proposed. The CD81 molecule was identified as a putative HCV receptor based on its strong interaction with E2 as well as with virus particles *in vitro* (Pileri *et al*., 1998). Apart from this route, HCV as well as the other members of the *Flaviviridae* family may enter the cell by binding to low-density lipoprotein receptors (Agnello *et al*., 1999). Recently, the human scavenger receptor class B type I (SR-BI) was identified as a receptor responsible for E2 binding to human hepatic cells. The E2 binding to human SR-BI was common to the viral isolates derived from subtypes 1a and 1b, was species specific and selective (Scarselli *et al*., 2002). Binding to the receptor activates conformational changes of the cell membrane and, as a result, internalization of the virion.

(2) Inside the cytoplasm the genomic RNA is released from the virion and directly translated on ribosomes. Translation of the viral RNA occurs through a cap-independent mechanism via an IRES located in the 5' UTR (Wang *et al*., 1993). This RNA element, residing approximately between nucleotides 40 and 355, forms four highly structured domains necessary for recognition and interaction with the ribosome 40S subunit (Kolupaeva *et al*., 2000). The activity of the IRES is influenced by viral and host cell factors. The X-tail at the 3' end of the HCV genome appears to enhance IRES-dependent translation by a yet unidentified mechanism (Ito *et al*., 1998). Several cellular factors have been demonstrated to bind to the HCV IRES and, in most cases, stimulate translation. The expression of HCV proteins was shown to be dependent on the cell cycle. IRES-dependent translation was the highest in mitotic cells and the lowest in quiescent cells. These findings suggest that HCV translation is regulated by cellular proteins that vary in abundance during the cell cycle and that viral translation may be enhanced by factors that stimulate the regeneration of hepatocytes in patients with chronic hepatitis C (Honda *et al*., 2000). Dependence of HCV translation and replication on cell proliferation was demonstrated in a recent study were viral RNA synthesis strongly decreased in poorly proliferating, confluent, or serum-starved cells and substantially increased in the S phase of the cell cycle (Scholle *et al*., 2004).

Directed by the IRES, the polyprotein is translated at the rough ER and cleaved co- and post-translationally by host cell signalases and two viral proteases into individual functional proteins. NS proteins NS3–5B form a replicase complex associated with intracellular membranes that most likely contain also cellular proteins. Formation of such a complex is a feature typical for positive-strand RNA viruses and allows the production of viral proteins and RNA in a distinct cellular compartment (Moradpour *et al*., 2004).

All NS proteins are thought to be required for viral replication. The junction between NS2 and NS3 is processed by the NS2/NS3 metalloprotease (Grakoui

et al., 1993b; Reed *et al*., 1995; Hijikata *et al*., 1993a). Although the NS2/NS3 protease is believed to be autocatalytic, bimolecular cleavage has been demonstrated (Grakoui *et al*., 1993b; Reed *et al*., 1995). The members of the HCV-related flavivirus genus form stable NS2B/NS3 heterodimers; in this protein complex, NS2B, in contrast to the HCV NS2, acts as a NS3 cofactor in all cleavages of the NS polyprotein. This interaction is essential for the function of the flavivirus NS3 serine protease and has additional functions during viral replication (Chambers *et al*., 1993; Arias *et al*., 1993; Westaway *et al*., 1997). Based on the genetic and functional similarity with flaviviruses, it was proposed that a stable heterodimer complex exists also between HCV NS2 and NS3 after cleavage at the 2/3 site.

Cleavages at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions are mediated by the serine protease domain located within the 180 amino acid residues of the N-terminus of the NS3 protein (Grakoui *et al*., 1993a). The active protease, however, is represented by the heterodimer consisting of NS3 and its cofactor NS4A (Lin *et al*., 1995). Besides acting as a cofactor of the serine protease, NS4A has also been shown to target NS3 to membranes and to increase its stability. All other NS proteins of HCV are also targeted to the ER membranes on their own (Wolk *et al*., 2000). It has been demonstrated that multiple interactions between the HCV proteins take place in the membrane-associated replication complexes and that NS3 co-immunoprecipitates with NS4A, NS4B, NS5A, and NS5B, and NS2 co-immunoprecipitates with NS5A and NS5B (Hijikata *et al*., 1993b). The interaction between NS2 and NS4A has been shown in the yeast two-hybrid system (Flajolet *et al*., 2000). The interaction between NS2 and NS3 by immunoprecipitation has been shown only recently in biochemical experiments with the use of a glutathione S-transferase pull-down assay with *in vitro* translated proteins, and *in vivo* experiments with the use of the yeast two-hybrid system (Dimitrova *et al*., 2003). Co-immuno-precipitation of NS2 and NS3 from the cells transfected by adenovirus expressing the HCV NS2-NS5B polyprotein was also demonstrated (Dimitrova *et al*., 2003). However, as in that study all six NS proteins were expressed in hepatic cells, it is difficult in this context to detect a particular association of two NS proteins since both of them possess multiple interaction partners. Thus, the existence of bimolecular complexes of NS2-NS3 in mammalian cells has not yet been directly demonstrated.

(3), (4) The individual steps of RNA replication process are largely unknown. The HCV NS5B RdRp plays a central role in catalyzing both negative and positive RNA strands. How template specificity is achieved remains an unresolved question, as in most studies NS5B was found to utilize and bind, although with different efficiencies, virtually every RNA template. It also remains unknown how switching from the synthesis of a RNA negative strand to the synthesis of a positive strand occurs.

(5) Particle formation is initiated by binding of the core protein to the genomic RNA. The HCV core protein interacts with the viral plus-stranded

RNA at specific regions in the 5' half of it to form nucleocapsids. Such binding not only ensures a selective packaging of the plus-stranded genome but also appears to repress translation from the IRES, suggesting a potential mechanism for switching from translation/replication to virus assembly (Shimoike *et al*., 1999). Conversely, in some studies the core protein-coding sequence but not the core protein itself was shown to modulate the efficiency of cap-independent translation directed by the HCV IRES (Wang *et al*., 2000; Kim *et al*., 2003). Positive-stranded viruses use the genomic RNA as a common template for translation and RNA replication, which proceed in the inverse direction; a certain regulatory mechanism for translation control is probably required to coordinate these two antagonistic processes. It is possible that this regulation is achieved by parallel independent mechanisms, as switching from replication to virion assembly and production of new particles is essential for completion of the viral life cycle. The viral life cycle is completed after assembly of viral particles and their release through the cell membrane. The mechanism of formation and release of HCV viral particles has not been well studied due to the lack of an effective model system.

Antiviral treatment with interferon alpha or interferon alpha and ribavirin for chronic hepatitis C has limited efficiency and is connected with several side effects, such as anemia, leukopenia, thrombocytopenia, influenza-like syndrome, thyroid dysfunction, positivity of serological markers of autoimmunity, depressive symptoms, etc. (Kraus *et al*., 2005; Wirth *et al*., 2005; Moncoucy *et al*., 2005; Toniutto *et al*., 2005). Development of new antiviral agents and strategies for interfering HCV replication is of great importance and one of the main priorities in HCV research. HCV proteases are essential for the replication of the virus and are attractive targets for the antiviral agents. Characterization of the interactions between viral proteins helps to understand better their role in viral replication and life cycle.

3. Genetic variability of HCV

Sequence diversity among different HCV isolates has been recognized since early studies and can reach as high as 35% of nucleotide sequence divergence over the entire genome. According to the commonly accepted Simmonds' system, HCV is classified, on the basis of the similarity of the nucleotide sequence, into major genetic groups designated as genotypes (sequence similarity between isolates 65–69%) (Simmonds *et al*., 1993). The HCV genotypes are designated by Arabic numerals in the order of discovery (genotypes 1, 2, 3, etc.). Closely related isolates within each of the major genotypes form subtypes with a genome similarity of 77–80% and are designated with lower case letters, also in the order of discovery (subtypes 1a, 1b, 2a, 2b, etc.). Quasispecies represent a

complex of genome variants within one host and are genetically very close (sequence similarity 91–99%).

The HCV RdRp, like other viral RNA polymerases, has high error rate, with misincorporation frequencies averaging about 10^{-3}–10^{-4} per base site, due to the absence of a proofreading mechanism. As a result, mutations accumulate in newly generated HCV genomes. During replication of HCV each new genome differs from the parental template by up to ten nucleotides. This high error rate is reflected by the high mutation rate observed in patients or in experimentally inoculated chimpanzees. Using comparative sequence analyses of the HCV genomes isolated over intervals of 8 or 13 years, a mutation rate of $1{\cdot}44 \times 10^{-3}$ or $1{\cdot}92 \times 10^{-3}$ base substitutions per site per year, respectively, was found (Ogata *et al*., 1991; Okamoto *et al*., 1992a). Most mutant viral particles are replication deficient, but some propagate efficiently. The fittest infectious particles are selected continuously on the basis of their replication capacities and environmental selective pressures (mainly host immune response). This explains why each infected individual harbors a pool of genetically distinct but closely related HCV variants referred to collectively as quasispecies (Martell *et al*., 1992; Herring *et al*., 2005).

Specific criteria have been established to standardize and facilitate subtype assignment once new sequences are obtained from HCV isolates across the world. Different HCV isolates cluster into a two-tiered classification, in which the major genetic groups, referred to as clades, are further divided into several subtypes (Robertson *et al*., 1998). According to these criteria, six clades are now recognized, which correspond to former genotypes 1–6. Genotypes 7–9 and 11 (Tokita *et al*., 1994; Tokita *et al*., 1995) have been reassigned to clade 6, and genotype 10 (Tokita *et al*., 1996) has been reassigned to clade 3, based on phylogenetic analysis rather than on sequence identity (Mizokami *et al*., 1996; Simmonds *et al*., 1996; de Lamballerie *et al*., 1997; Robertson *et al*., 1998). Although the classification of new HCV sequences should be preferably based on complete genome sequences, tentative clade/subtype assignments can be made on the basis of the phylogenetic analysis of the nucleotide sequences of at least two coding regions (Robertson *et al*., 1998). The precise subtype assignment of HCV isolates has not only taxonomic consequences, but plays an important role in the diagnosis of hepatitis C, therapeutic decision-making, and assessment of the sustained virologic response (SVR) to therapy (Poynard *et al*., 1998; McHutchison *et al*., 1998). Although the golden standard in type assignment is sequence analysis, the more widely used assays, currently available in the clinical setting, are a line-probe assay (INNO-LiPA; Innogenetics) (Pawlotsky *et al*., 1997), RFLP (restriction fragment length polymorphism) analysis of sequences in the 5' UTR of the HCV genome (Davidson *et al*., 1995), and RT–PCR (reverse transcription – polymerase chain reaction) assay amplifying the capsid-encoding region of HCV in a type-dependent manner (Okamoto *et al*., 1992b). These assays allow correct identification of the HCV genotype (and in some cases, the subtype) in more

than 90% of cases. Direct sequencing can solve controversies over clade determination in some cases.

4. Geographic distribution of HCV genotypes

Several authors have reported that the distribution of different HCV geno(sub)types varies geographically. Although HCV genotypes 1, 2, and 3 appear to have a worldwide distribution, their relative prevalence varies from one geographic area to another. HCV subtypes 1a and 1b are the most common genotypes in the United States and Europe. In Japan, subtype 1b is responsible for up to 73% of cases of HCV infection (Smuts and Kannemeyer, 1995). Generally, genotypes 1, 2 and 3 are predominantly found in Europe, Japan, Brazil, Australia and the United States (Bukh *et al*., 1995; Schreier *et al*., 1996; Busek and Oliveira, 2003; McCaw *et al*., 1997), genotype 4 in Central and North Africa and the Middle East (McOmish *et al*., 1994; Dusheiko *et al*., 1994), genotype 5 in South Africa (Smuts and Kannemeyer, 1995), genotype 6 in Hong Kong (McOmish *et al*., 1994), and the usually rare genotypes 7, 8 and 9, in Vietnam (Takada *et al*., 1993). Genotypes 10 and 11 were identified in patients from Indonesia (Tokita *et al*., 1996).

The geographic distribution and diversity of HCV genotypes provide clues about the historical origin of HCV (Smith and Simmonds, 1997). The high level of diversity (numerous subtypes) of the type distribution in some regions of the world, such as Africa and Southeastern Asia, may suggest that HCV has been endemic there for a long time. Conversely, the limited diversity of subtypes observed in the United States and Europe could be related to the recent introduction of these viruses from areas of endemic infection (Pybus *et al*., 2001; Simmonds, 1995). During recent years, new evidences of changes in the distribution of HCV subtypes and the emergence of new genome variants have been accumulated. A spontaneous recombinant isolate was reported from St. Petersburg, Russia (Kalinina *et al*., 2002). In Europe, dynamic changes over time have been reported in the prevalence of different HCV subtypes. For example, the prevalence of subtype 1b has decreased, while conversely, that of subtypes 1a and 3a has increased (Bourliere *et al*., 2002; Dal Molin *et al*., 2002; Ross *et al*., 2000; Nousbaum *et al*., 1995). Some rare genotypes have recently emerged in the Western world. Genotype 4 has been reported to exist in France (Morice *et al*., 2001), in the south of Spain (Sanchez-Quijano *et al*., 1997), Germany (Schroter *et al*., 2002) and in Italy (Matera *et al*., 2002). Recently, an unusually high prevalence of genotype 5 was reported for Southeastern Spain (Jover *et al*., 2001) and Central France (Henquell *et al*., 2004). Similar data about the changing epidemiology of HCV genotypes, in fact, about a significant decrease in the prevalence of subtype 1b, have been reported from the United States (Rosen *et al*., 1999).

Considering the growing evidence of the changing patterns of the HCV subtype distribution over the world and the emergence of new viral variants (Bourliere *et al*., 2002; Dal Molin *et al*., 2002; Ross *et al*., 2000; Kalinina *et al*., 2002; Morice *et al*., 2001; Rosen *et al*., 1999), there is a need for characterization of the HCV subtypes circulating in Estonia and for studies aiming to reveal epidemiological changes in the subtype distribution.

5. Clinical significance of HCV genotypes

5.1. HCV genotypes as epidemiological markers

The genetic heterogeneity and genotypes of HCV play an important role as epidemiological markers for HCV infection. Because of the geographic clustering of HCV genotypes, genotyping may be a useful tool for tracing the source of an HCV outbreak in a given population and for finding the directions of dissemination of infection. Examples include tracing the history of transmission in an outbreak of HCV infection among children attending a pediatric oncology-hematology outpatient ward in Italy (Spada *et al*., 2004). All children, but not the health care worker, showed subtype 3a. Sequence analysis and phylogenetic tree analysis revealed high identity among the isolates, which together with the clinical-epidemiological data allowed to identify the source of the outbreak and the most probable patient-to-patient chain of transmission. Recently, genotyping and molecular characterization of HCV isolates provided evidence of a nosocomial transmission of HCV in a liver unit in Spain (Forns *et al*., 2005). Phylogenetic analyses of recovered HCV sequences identified an HCV-infected roommate and a patient receiving care by one and the same nurse team as the source of infection. Several examples of identifying the transmission routes and sources of HCV infection, especially concerning nosocomial spread of infection, have been demonstrated in the literature, proving the importance of determination of HCV geno(sub)types in clinical practice (Massari *et al*., 2001; Krause *et al*., 2003; Furusyo *et al*., 2004).

Soon after the discovery of HCV, it was noted that different subtypes have different predominant transmission routes. Many epidemiological studies demonstrated evidence of an association between HCV subtypes and the mode of HCV acquisition (Berg *et al*., 1997; Pawlotsky *et al*., 1995; Watson *et al*., 1996). It was reported that patients who received blood transfusions and those with chronic hepatitis C with an unknown cause had similar age distributions, and HCV subtype distributions. Intravenous drug users (IVDUs) were significantly younger and had a different subtype distribution. Transmission of subtype 3a has only been observed over the past 20 years; the other subtypes were transmitted up to 40 years ago. These results suggest that during about 20 years there have been two independent ongoing hepatitis C epidemics. One

affects persons who received blood transfusions or whose source of infection is unknown. These persons are older and are mainly infected by subtype 1b. The second type of infection occurs in IVDUs and infects younger persons, mainly with subtypes 3a or 1a (Berg *et al*., 1997; Pawlotsky *et al*., 1995; Watson *et al*., 1996).

5.2. Progression of liver disease

The role of HCV genotypes in the progression of liver disease is one of the controversial areas of HCV research. Due to overall slow progression of liver disease in HCV-infected patients and presence of many viral and host factors that may influence the natural course of the disease (virus genotype, viral load, alcohol intake, iron overload, duration of hepatitis, co-infection with other hepatotropic viruses, etc.), prospective studies are difficult to perform. Despite controversies, general associations can be outlined. In patients with chronic hepatitis C, subtype 1b is reportedly associated with a more severe liver disease and a more aggressive course than is infection with the other HCV subtypes (Nousbaum *et al*., 1995; Silini *et al*., 1995). Furthermore, a possible link to HCC has been proposed for subtype 1b. Taking into account that subtype 1b is prevailing among the Japanese population, there is convincing evidence that HCC occurs more frequently or emerges earlier among chronic hepatitis C Japanese patients (Higuchi *et al*., 2002; Chayama, 2002) than among patients in Western countries (Di Bisceglie *et al*., 1991; Tong *et al*., 1995). Some reports refute the associations mentioned above (Benvegnu *et al*., 1997; Brechot, 1997; Lau *et al*., 1996; Yamada *et al*., 1994). A possible explanation for these reported discrepancies could be the following. It was found in most studies that patients infected with subtype 1b were older than those infected with the other subtypes and that subtype 1b may have been present before the other subtypes (Zein *et al*., 1996; Higuchi *et al*., 2002). Thus, patients infected with subtype 1b may have been infected for a longer time. According to this explanation, subtype 1b is a marker for a more severe HCV-associated liver disease, because it reflects a longer time of infection rather than a more aggressive form of chronic hepatitis C.

5.3. Response to antiviral treatment

Determination of HCV subtypes has a high clinical value in management of chronic hepatitis C patients, especially concerning decision making about antiviral treatment. Administration of interferon alpha, or interferon alpha and ribavirin are the basic treatment strategies for chronic hepatitis C. Many viral and host factors may influence the efficacy of antiviral treatment. The duration of treatment, viral RNA level and its dynamics during treatment (Castro *et al*.,

2002; Tsubota *et al*., 2005; Fried, 2004), and liver histology (Myers *et al*., 2003) – all seem to play a role in predicting response. Genotype has been shown to be the most important factor in predicting response to antiviral therapy. Studies of interferon-based regimens alone or in combination with ribavirin have consistently demonstrated a significant association between the HCV genotype and SVR, while genotype 1 (especially subtype 1b) is relatively resistant to therapy compared to genotypes 2 and 3 (Fried *et al*., 2002b; Fried *et al*., 2002a; Manns *et al*., 2001; Hadziyannis *et al*., 2004).

In a multicenter trial, Fried and colleagues demonstrated, among patients with HCV genotype 1, a SVR rate of 46% in those treated with pegylated interferon alpha-2a and ribavirin, versus 36% in those treated with standard interferon and ribavirin and 21% in those receiving pegylated interferon alfa-2a alone. In contrast, patients with genotype 2 or 3 had SVR rates that were significantly higher: 76%, 61%, and 45%, respectively (Fried *et al*., 2002b). In a similar study of pegylated interferon alpha-2b and ribavirin (Manns *et al*., 2001) SVR 41% was achieved in patients with genotype 1 treated with this combination, compared to 34% in patients treated with standard interferon and ribavirin, and 33% in those receiving pegylated interferon alpha-2b alone. SVR rates for patients with genotypes 2 or 3 were approximately 80% for all treatment regimens. In multivariate analyses, both of these studies showed that genotype was the strongest predictor of SVR (Fried *et al*., 2002b; Manns *et al*., 2001). In a study of Hadziyannis *et al.* (Hadziyannis *et al*., 2004), patients with genotype 1 had the best chance of SVR (52%) when treated with a higher dose of ribavirin for 48 weeks. In contrast, patients with genotypes 2 or 3 had similar SVR rates, approaching 80%, regardless of the duration of therapy or ribavirin dosing, indicating that these patients could be treated successfully with less aggressive regimens.

HCV genotype 4 appears to be difficult to treat, the SVR rates range from 40% to 68% for pegylated interferon plus ribavirin, and from 16% to 39% for interferon plus ribavirin (Hasan *et al*., 2004). HCV genotype 5 appears to be sensitive to therapy and, at least when treated for 48 weeks, the SVR may resemble those observed for genotypes 2 and 3 (Nguyen and Keeffe, 2004). Only a few studies to date have examined treatment outcomes in patients with chronic hepatitis C infected with genotypes 6 through 9. The results of these small studies suggest that the SVR to combination therapy may be somewhat higher than that seen in patients with genotype 1 but lower than that seen in patients with genotypes 2 and 3 (Dev *et al*., 2002; Hui *et al*., 2003). Based on the data presented above, determination of the HCV genotype prior to initiation of antiviral therapy is needed to prescribe optimal treatment regiments.

6. Clinical manifestations of chronic hepatitis C

The human hepatocyte is the primary locus of HCV infection, and chronic hepatitis, cirrhosis, and HCC are the major clinical sequelae (Hoofnagle, 1997). Chronic hepatitis C is characterized by presence of serum HCV-RNA and anti-HCV antibodies for more than 6 months after the onset of illness (Hoofnagle, 1997). Clinical symptoms are generally quite mild and non-specific. The overwhelming complaint of patients with chronic hepatitis C is fatigue.

However, HCV infection is a systemic illness and extrahepatic manifestations are common. Negative-strand HCV RNA (a marker for ongoing viral replication) has been detected in various cells and tissues supporting HCV replication, such as hematopoietic (Lerat *et al*., 1998) and dendritic cells (Goutagny *et al*., 2003), salivary and sweat glands (Ortiz-Movilla *et al*., 2002), as well as osteoprogenitors and osteoblasts (Kluger *et al*., 2005), myocardial cells, epithelial cells of the intestine, interstitial and epithelial cells of the kidney, acinar cells and epithelial cells of the pancreatic duct and the cortex and medulla cells of the adrenal gland, and epithelial cells of the gallbladder (Yan *et al*., 2000). The infected extrahepatic tissues are likely to act as a reservoir for HCV, and play a role in both HCV persistence and reactivation of the infection.

There is strong evidence that HCV can also replicate in peripheral blood mononuclear cells (PBMC) and in experimentally infected B- and T-cell lines (Lerat *et al*., 1996; Cheng *et al*., 2001). Such lymphotropism may account for immunological, autoimmune, and lymphoproliferative disorders. These can include positivity of serological markers of autoimmunity (organ- and nonorgan-specific autoantibodies – NOSA), autoimmune hepatitis (AIH), renal manifestations (membranoproliferative glomerulonephritis), essential mixed cryoglobulinemia, non-Hodgkin lymphoma (Dammacco *et al*., 1998; De Vita *et al*., 1997; Paoletti *et al*., 2002; di Belgiojoso *et al*., 2002), various dermatological diseases (porphyria cutanea tarda, psoriasis, oral lichen planus) (Gisbert *et al*., 2003; Paoletti *et al*., 2002). Recent reports have led to the suggestion that HCV may infect the central nervous system (Forton *et al*., 2004b; Forton *et al*., 2004a). Although autoimmune manifestations in chronic hepatitis C patients are present quite often, evident association with HCV infection has been reported for only some of them. It seems that there are no significant differences in the clinical and biochemical parameters between chronic hepatitis C patients with and without autoimmune features (Sachithanandan and Fielding, 1997). In many cases these relationships remain controversial or restricted to case reports. All these associations may be interpreted as being the result of immune modulation induced by the lymphotropism of HCV itself or a manifestation secondary to the hepatocellular damage favored by the genetic background of the host.

In chronic hepatitis C, the clinical significance of the serological markers of autoimmunity is still an object of discussion. Being among autoimmune manifestations, many organ- and nonorgan-specific autoantibodies are commonly found in the HCV-infected patients' sera. Smooth muscle antibodies

(SMA) are a heterogeneous group of antibodies of different specificity, which react with cytosceleton antigens of smooth muscle cells. In chronic HCV infection, SMA are found in 10–66% of cases. The prevalence of antinuclear antibodies (ANA) ranges between 6 and 22%, they are usually presented at low titers. Liver-kidney microsomal antibodies (LKMA) are found in the cytoplasm of hepatocytes and in proximal renal tubes. They are directed against different epitopes on cytochrome P450. LKMA type 1 are detected at low titers in up to 10% of chronic hepatitis C patients (Bortolotti *et al*., 1996; Drygiannakis *et al*., 2001; Luo *et al*., 1998; Lenzi *et al*., 1999; Kammer *et al*., 1999; Meyer zum Buschenfelde *et al*., 1995). Anti-asialoglycoprotein receptor, anti-liver membrane antigen, anti-liver cytosol antigen, anti-hepatocyte plasma membrane, anti-thyroglobulin, anti-thyroid peroxydase, anti-phospholipid, anti-neutrophil cytoplasmic, anti-GOR and many other autoantibodies have also been described in patients with HCV infection (Monti *et al*., 2005; di Belgiojoso *et al*., 2002; Bortolotti *et al*., 1996). Each antibody is directed against a particular intracellular antigen released during cell death and presented to the immune system. A positive correlation has been demonstrated between presence of NOSAs and the biochemical and histological activity of underlying liver disease (Cassani *et al*., 1997; Lenzi *et al*., 1999).

The persistence of HCV in PBMCs, preferentially in B-cells (Zehender *et al*., 1997), results in chronic stimulation of B-cells, leading to their polyclonal and later to monoclonal proliferation, which may result eventually in malignant transformation and development of overt lymphoma (Franzin *et al*., 1995; Zuckerman *et al*., 2003). It was found that the HCV envelope protein E2 binds to the CD81 receptor, which is expressed in various cell types including hepatocytes and B-lymphocytes (Pileri *et al*., 1998). Binding of HCV particles to a CD81-containing complex lowers the threshold for B-cell activation and proliferation, facilitating B-cell activation. It has been shown that the peripheral blood $CD5^+$ B-cell subpopulation is expanded in patients with chronic HCV infection. These cells are characterized by the production of low-affinity IgM with rheumatoid factor activity, arise early in ontogeny, and are considered to represent the bridge linking innate and acquired immune responses, as well as are implicated in development of autoimmune diseases (Curry *et al*., 2000). Recently, it has been demonstrated that combined antiviral treatment leads to a significant decrease in peripheral B-cell CD81 expression and the disappearance of $CD5^+$ B-cell expansion in all patients in whom SVR was achieved. The decrease in CD81 overexpression and $CD5^+$ B-cells expansion in these patients was strongly associated with the decrease or disappearance of autoimmune markers (cryoglobulin, autoantibodies), whereas in nonresponders the overexpression of CD81 and the expansion of the $CD5^+$ B-cell subpopulation did not significantly change and were comparable to the corresponding phenomena in untreated patients (Zuckerman *et al*., 2003). These observations confirm the role of HCV in induction of autoimmune disturbances, in fact, serological markers of autoimmunity (presence of autoantibodies).

As different HCV genotypes have been associated with different courses and outcomes of liver disease, it was hypothesized that various amino acid sequences of each genotype may elicit different autoantibodies or other immune reactions (or both) (Zein *et al*., 1999). The results of the studies performed to clarify the relationship between the HCV genotype and autoimmune manifestations are controversial. Numerous studies have reported that mixed cryoglobulinemia may be related, at least in part, to the HCV geno(sub)type infecting the host. Gad and colleagues (Gad *et al*., 2003) have shown that cryoglobulinemia was prevalent in Japanese patients with chronic hepatitis C infected with subtype 1b, but was not common in Egyptians with HCV genotype 4. Although it was not possible to evaluate ethnicity and the HCV genotype separately in that study, HCV subtype 1b appeared to predispose more to cryoglobulinemia than genotype 4. In contrast, in an investigation of Zignego *et al*., subtype 2a has been demonstrated to be more prevalent in patients with mixed cryoglobulinemia compared to subtypes 1a and 1b (Zignego *et al*., 1996). It was shown that anti-GOR autoantibody positivity correlates with HCV subtypes 1a and 1b (Quiroga *et al*., 1996). Presence of LKMA-1 antibodies has also been shown to associate with HCV genotype 1 (Gerotto *et al*., 1994). More recent studies, however, tend to demonstrate that the serological markers of autoimmunity are not related to the HCV genotype (Luo *et al*., 1998; Rostaing *et al*., 1998; Weiner *et al*., 1998; Zein *et al*., 1999).

7. Genotyping techniques

Reliable methods for determining the genotype of the HCV isolates are essential for diagnostic and epidemiological studies. The European Association for the Study of the Liver (EASL) has recommended determination of HCV genotype before initiation of antiviral treatment (EASL International Consensus Conference on hepatitis C, 1999). In general, genotyping assays could be divided into two groups: assays for serologic typing and assays for molecular typing.

7.1. Serologic typing

Genotype-specific antibodies could be used as indirect markers for the HCV genotype (serotyping or serologic genotyping). Serologic genotyping has several advantages that make it suitable for large epidemiological studies. These advantages include the low risk for contamination and the simplicity of the assay. However, serologic typing seems to have low specificity and sensitivity, which limits its usefulness. Two commercially available serologic genotyping assays have been introduced into routine use. The recombinant immunoblot assay (RIBA) was introduced by the Chiron Corporation and it contained five

different serotype-specific peptide sequences taken from the NS4 region and two serotype-specific peptide sequences taken from the core region of the HCV genomes for genotypes 1, 2 and 3 (Dixit *et al*., 1995). Another serologic genotyping assay is the Murex HCV serotyping enzyme immune assay (Murex Diagnostics Ltd), which is based on the detection of genotype-specific antibodies directed to the epitopes encoded by the NS4 region of the genomes for genotypes 1 through 6 (van Doorn *et al*., 1996). These two assays have been compared and have shown a concordance rate of higher than 96% for genotypes 1, 2, and 3. Despite the relative simplicity of serologic genotyping, the greatest shortcoming of this method is determination of HCV isolates only at the level of genotypes. It is not possible to discriminate between different subtypes by this method.

7.2. Molecular typing

A definitive determination of genotypes and subtypes can be obtained by sequence analysis of PCR-amplified genomic fragments. For example, in the study of Bukh and colleagues, it was possible to distinguish the isolates representing genotypes 1 to 6, and 12 subtypes by sequencing a small fragment of 100 nucleotides within the E1 region (Bukh *et al*., 1993). However, direct sequencing on a large scale is impractical because of the complexity of the procedure. Additionally, sequencing of amplified DNA does not usually identify mixed infections with two different HCV genotypes.

Other typing methods that are not based on DNA sequencing have been developed. Although faster and less expensive, these methods usually address only a limited number of the existing genotypes because they depend on distinguishing of genotypes through a few specific nucleotide changes. Among the different HCV genotypes, the sequence of the 5' UTR region is relatively well conserved and is therefore most often applied for diagnosis of HCV infection by PCR (determination of HCV RNA in serum). In contrast, the sequences of NS3, NS5, and the core regions are more variable and are therefore often used to define HCV genotypes and to distinguish them.

Genotyping methods that have been reported consist mainly of the amplification of HCV RNA from clinical specimens, followed by either reamplification with type-specific primers or hybridization with type-specific probes, or by digestion of PCR products with restriction endonucleases that recognize a genotype-specific cleavage site. HCV genotyping by using subtype-specific primers was first introduced by Okamoto *et al*. (Okamoto *et al*., 1992b) who used primers specific for the core region. This method had poor sensitivity and specificity: without modification, this method was able to detect only subtypes 1a, 1b, 1c, 2a, 2b, and 3a. Later, the subtype-specific PCR method was improved whereafter it allowed for identification of isolates from the other genotypes (Ohno *et al*., 1997; Spada *et al*., 1998). Several DNA hybridization

assays for HCV genotyping have been described. In this method, PCR amplification products are hybridized to nitrocellulose paper embedded with subtype-specific probes. A commercial kit (INNO-LiPA) for HCV genotyping is based on hybridization of 5' UTR amplification products with subtype-specific probes (Stuyver *et al*., 1993). Although the initial version of INNO-LiPA had lower sensitivity, the newer version is capable of discriminating among HCV subtypes 1a, 1b, 2a to 2c, 3a to 3c, 4a to 4h, 5a, and 6a (Stuyver *et al*., 1996).

In the RFLP method, a PCR-amplified DNA fragment is digested into fragments with different lengths by enzymes (restriction endonucleases) that recognize cleavage sites specific for each subtype. Investigators have used different regions of the HCV genome for restriction fragment length polymorphism, including NS5 and the 5' UTR (Stuyver *et al*., 1993; Pohjanpelto *et al*., 1996). Additional methods include heteroduplex analysis (White *et al*., 2000), probe melting curve analysis (Bullock *et al*., 2002), and denaturing high-performance liquid chromatography (Liew *et al*., 2004). The genome regions used in the high throughput assays are usually 5’ UTR, core, NS5B, NS3, and E1.

Although these methods are sensitive for identification of genotypes, they can fail in some cases in discriminating between subtypes, or result in subtyping misclassification (in particular, subtypes 1b and 1a), or yield doubtful results (in particular subtypes 2a/2c and 4a/4c). For example, genotyping methods using 5' UTR, including INNO-LiPA, may not distinguish subtype 1a from 1b in 5 to 10% of cases and they may not distinguish between subtypes 2a and 2c (Chen and Weck, 2002). It was postulated that phylogenetic analysis using both the 5' UTR and the NS5B regions is reliable and convenient for HCV typing in clinical practice. However, analysis of the NS5B region may be more useful for tracing the source of HCV infection (Sandres-Saune *et al*., 2003).

OBJECTIVES OF THE STUDY

The overall goal of the research was to characterize HCV infection and the virus itself from different points of view: to give an overview of the distribution pattern of the HCV subtypes circulating in Estonia, to investigate the presence and distribution of autoantibodies in patients with chronic hepatitis C, and to provide an insight into the molecular mechanism of viral replication by studying the interactions between HCV proteases.

The aims of the study were:

1. To identify the HCV subtypes circulating among the Estonian population (I);
2. To compare the distribution pattern of HCV subtypes in Estonia in 1997–1998 and in 2000–2004 (I, III);
3. To compare two genotyping techniques: multiplex PCR with subtype-specific primers from the core region and restriction fragment length polymorphism analysis of amplified 5' UTR of the HCV genome (I);
4. To determine the prevalence of ANA, AMA, SMA, LKMA, PCA, TMA, and ARA autoantibodies in drug-naïve patients with chronic hepatitis C and to establish whether HCV subtypes are associated with presence of auto-antibodies (II);
5. To characterize the complex formation between the HCV NS2 and NS3 proteins (IV);
6. To assess the possibility of using E2 epitope tagging technique for the study of interactions between the HCV replicase proteins (IV).

MATERIALS AND METHODS

1. Patients and sera

In the presented studies altogether 765 serum samples from patients with hepatitis C with or without liver cirrhosis were investigated. Serum samples collected from Estonian hospitals, or the sera, sent to diagnostic laboratory for HCV RNA detection and genotype identification, were used in the studies (Table 1). After collection, all sera were frozen and stored at -20°C until use.

Table 1. Study objects

Study	Number of samples	Source of serum samples	Goals of the study	Publications
Genotyping 1997–1998	215	Tartu University Hospital, hospitals of Tallinn, Kohtla-Järve, Narva, and Pärnu	1) HCV subtype distribution in Estonia 2) Comparison of two genotyping methods	APMIS 2000 (I)
Genotyping 2000–2004	459	Laboratory "Quattromed AS", Tartu	Revealing the changes over the time in the HCV subtype distribution	Eesti Arst 2005 (III)
HCV genotypes and auto-antibodies	90	East-Tallinn Central Hospital and Tartu University Hospital	1) Prevalence of auto-antibodies in chronic hepatitis C patients 2) Association between HCV subtype and the presence of autoantibodies	World J Gastoenterol 2005 (II)
Interactions between HCV proteases	1	Tartu University Hospital	Characterization of inter-action between the NS2 and NS3 proteases of HCV	Virus Res in press, 2005 (IV)

1.1. HCV genotypes in Estonia 1997–1998

A total of 215 serum samples were collected from consecutive patients with acute hepatitis C and chronic hepatitis C with or without liver cirrhosis from different hospitals of Estonia (Tallinn, Tartu, Kohtla-Järve, Narva, and Pärnu). The presence of the HCV RNA in the sera was previously determined by RT-PCR based on the highly conserved 5' UTR primer sequences (Amplicor™ Hepatitis C Virus Test). Acute cases were characterized by the presence of

HCV RNA and elevated aminotransferases detected in the serum for the first time. Cases were determined as chronic, if the patients' sera were positive for HCV RNA and the patients had had elevated serum aminotransferases' values for at least six months. Eighty-nine patients with chronic hepatitis C had undergone a liver biopsy whose results were consistent with the diagnosis of chronic hepatitis alone, or with cirrhosis. The patients' age ranged from 9 to 77 years (median 33 years).

In order to carry out an epidemiological analysis of the data, special questionnaires were sent to doctors. According to the returned questionnaires, the patients were divided into the following groups: age (under 30 years, over 30 years), gender (male, female), stage of liver disease (acute hepatitis, chronic hepatitis), and transmission route (blood transfusion or surgery, IVDU). Differences (in comparison to the total population of 215 patients: subtype 1b – 146 patients, and subtype 3a – 50 patients, other genotypes – 19 patients) in the prevalence of subtypes 1b and 3a were evaluated for these groups of patients. This study was approved by the Ethics Committee of the University of Tartu.

1.2. HCV genotypes in Estonia 2000–2004

To evaluate the appearance of HCV subtypes during the following years, the data from the diagnostic laboratory "Quattromed AS" were analyzed. A total of 459 serum samples sent to this laboratory for determination of HCV RNA and the genotype between the second half of 2000 and the beginning of 2004, were analyzed.

1.3. HCV genotypes and autoantibodies in chronic hepatitis C patients

In this study, sera from 90 consecutive patients (male 58–64%, female 32–36%) with established chronic hepatitis C from two hospitals of Estonia (East-Tallinn Central Hospital and Tartu University Hospital) were investigated. All patients were drug-naïve (not treated with interferon alpha or interferon alpha and ribavirin). The study was approved by the Ethics Committee of the University of Tartu.

1.4. Interactions between HCV proteases

Serum for HCV RNA isolation was collected from a chronic hepatitis C patient (Tartu University Hospital).

2. RNA isolation and reverse transcription

RNA was extracted from 100 µl of the serum with RNA Zol™ B (WAK-CHEMIE MEDICAL GmbH). Isolated RNA was dissolved in 10 µl of diethylpyrocarbonate treated (DEPC) water. For studying the interactions between HCV proteins, viral RNA was isolated from 500 µl of serum, dissolved in DEPC treated water and stored at –70°C. A copy DNA synthesis was performed in 20 µl reaction mixture using the Moloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase. For every reaction up to 5 µl of HCV RNA isolated from the patients' sera were used.

3. HCV genotyping methods

Genotyping was performed with two independent methods (PCR with subtype-specific primers from the core region of the HCV genome and RFLP analysis of the amplified 5' UTR region) in order to enhance the sensitivity of detection and to compare these techniques for further routine diagnostic laboratory use. First, the subtype-specific multiplex PCR for the core region (Ohno *et al*., 1997) was selected because this method allowed higher specificity of detection of the viral genotypes compared to the previously described method of Okamoto *et al.* (Okamoto *et al*., 1992b). Second, the RFLP method with the restriction endonucleases digestion of amplified 5'UTR according to Pohjanpelto *et al.* (Pohjanpelto *et al*., 1996) allowed to perform successfully one-tube assay of several stages of RT-PCR.

3.1. Subtype-specific PCR

For subtype-specific multiplex PCR, two rounds of PCRs following RT product preparation, as described by Ohno *et al*. (Ohno *et al*., 1997), were used. In the first round of PCR, a larger fragment was amplified by using conservative Ac2 and Sc2 "outer" primers. In the second round of PCR, either conservative S7 and A5 "inner" primers (amplification control), or S7 separately in combination with two different mixtures of antisense subtype-specific primers (genotyping) were used (Fig. 2). The amplification products of the second PCR round were subjected to agarose gel electrophoresis, and the HCV genotype was determined according to the length of the PCR fragments.

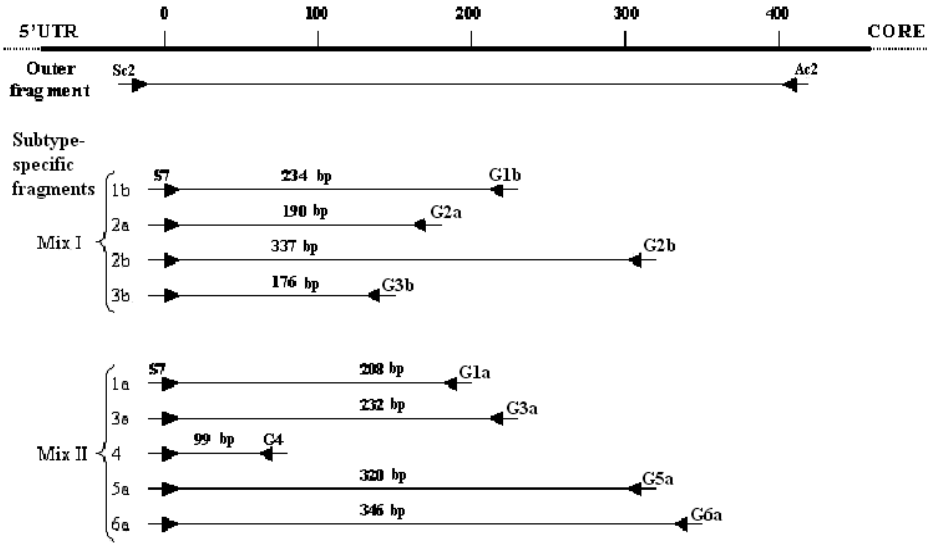

Figure 2. Schematic presentation of genotyping with subtype-specific primers (Ohno *et al*., 1997). The genetic scale is presented in hundreds of nucleotides, counting is started from the core region. Primers are shown as arrows at the edges of the lanes – PCR products. The outer fragment: a conservative fragment of PCR amplification with Sc2 and Ac2 primers, a template for subsequent nested genotype-specific PCR with S7 and the corresponding G primers. The potential resulting subtype-specific fragments are presented in two groups: mix I and mix II, as they were used in the study. The subtypes were divided into groups taking into account the length of the PCR fragments – they should be sufficiently different (at least 20 bp) to allow discrimination between subtypes within one mixture.

3.2. RFLP

RFLP analysis was carried out in three variants in order to simplify this genotyping method. The traditional variant a (Fig. 3) consisted of 3 separate stages: RT, followed by two rounds of PCR with two sets of primers (OUT primer pair for the first PCR, and IN pair for the second nested PCR). In variant b (2 stages in one tube), 5 µl of the RT product were used for the first PCR amplification with a pair of OUT primers in 20 µl of the reaction mixture. Then, 40 µl of the second round PCR mixture, containing a pair of IN primers, were added to the same tube to generate the nested PCR product. In the case of variant c, all 3 stages of RT-PCR were performed in one tube. RT and the first round PCR with a pair of OUT primers were performed in the same reaction mixture by incubation first under RT conditions, and then under PCR cycling conditions. Finally, 40 µl of the second PCR mixture with a pair of IN primers were added as in variant b; amplification was performed to generate the nested PCR product.

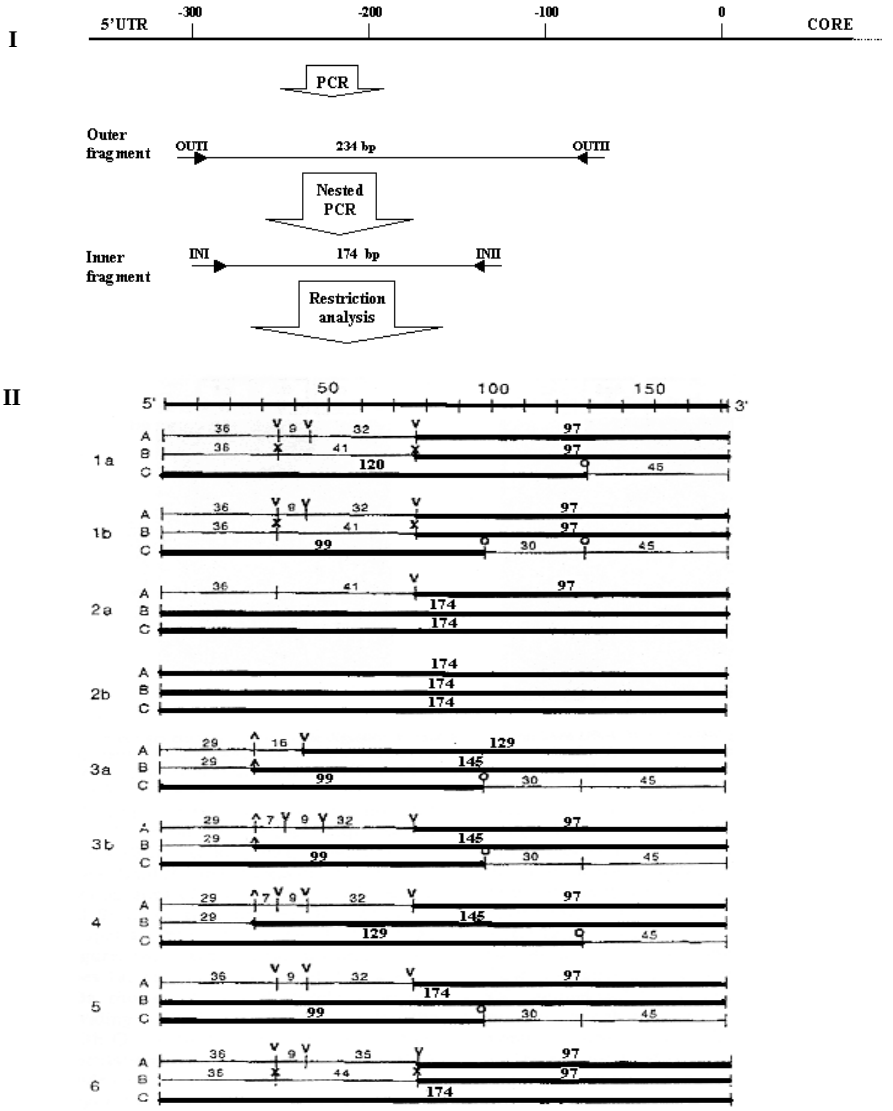

Figure 3. Schematic presentation of genotyping by restriction fragment length polymorphism (RFLP) (Pohjanpelto *et al*., 1996), traditional variant a. **I** – nested PCR from the 5' UTR. The genetic scale is shown in hundreds of nucleotides, point 0 corresponds to the beginning of the core region. Primers are shown as arrows at the edges of the lanes – PCR products. The outer fragment: a conservative fragment of PCR amplification with OUTI and OUTII primers, a template for subsequent nested PCR with INI and INII primers. The resulting inner fragment is subjected to digestion with three different mixtures of restriction endonucleases. **II** – RFLP analysis of the amplified 174 bp PCR fragment from the 5' UTR of different HCV subtypes: A – *Scr*FI+*Hin*fI; B – *Mva*I+*Hin*fI; C – *Bsh*1236I. The restriction sites are designated: ˅ – *Scr*FI; ^ – *Hin*fI; x – *Mva*I; ○ – *Bsh*1236I.

The amplification products of the second PCR (RFLP) were subjected to treatment with three mixtures of restriction endonucleases. The restriction fragments were visualized by gel electrophoresis, and the HCV subtype was determined by recognizing a specific pattern of restriction fragments.

4. Autoantibodies

ANA (antinuclear), AMA (antimitochondrial), SMA (anti-smooth muscle), LKMA (anti-liver-kidney microsomal), PCA (anti-parietal cell), TMA (anti-thyroid microsomal), and ARA (antireticulin) antibodies were investigated. Standard indirect immunofluorescence tests on unfixed 4 μm cryostate sections from a composite block of a mouse stomach, and a rat kidney and liver as well as from a hyperplastic human (blood group 0) thyroid gland were used. The sera were diluted to 1:10 and 1:100. The rabbit anti-human IgG (FITC – fluorescein isothiocyonate – conjugated) secondary antibody was used (Uibo *et al*., 1998).

5. Molecular biological methods

5.1. Plasmid construction and generation of recombinant SFV

The full-length NS2, NS3, and NS4A coding regions were amplified by RT-PCR from isolated viral RNA. For the expression of HCV NS2, NS3, and NS4A proteins in Cos7 cells, the fragments were ligated into eukaryotic expression vectors pCG3F12 or pCG1E2 containing sequences for BPV-1 E2 3F12 or 1E2 epitope-tags, respectively. For the expression of NS2, NS3 proteins as well as NS23 polyprotein in Huh7 cells, the Semliki Forest virus (SFV) replicon vector pSFV2gen was used (Lundstrom *et al*., 2001). The 3F12 epitope tag was introduced into the N-terminus of the NS2 protein by use of the PCR oligonucleotide.

The constructed pSFV-3F12NS2, pSFV-NS3, and pSFV-3F12NS23, as well as the pHelper1 (Lundstrom *et al*., 2001) (containing structural genes for production of SFV particles) vectors were linearized and used for *in vitro* RNA synthesis. The RNA transcripts were co-electroporated into BHK21 cells. After incubation at 28ºC for 72 hours (to reduce the cytotoxic effect of SFV), the cells were collected and the viruses were purified through a sucrose cushion.

5.2. Cells

African green monkey kidney (Cos7) and human hepatoma (Huh7) cell lines were grown in Iscove's modified Dulbecco's medium; Glasgow modified Eagle's medium was used for growing Baby Hamster Kidney (BHK21) cells (used for recombinant SFV particle production). Cos7 and Huh7 cells were cultured under standard conditions (37ºC, 5% CO_2). For the production of recombinant viruses, the BHK21 cells were incubated at 28ºC, 5% CO_2.

For the investigation of the interactions of HCV proteases in the Cos7 cell line, the expression plasmids, containing the respective HCV genes with fused tags, were transfected into the cells by electroporation. For the expression of HCV NS2, NS3 proteins and NS23 polyprotein in the Huh7 cell line, the cells were infected with recombinant SFV "suicide" particles expressing the respective proteins. After the procedure, cells were grown for 24–48 hours and were then collected for subsequent protein studies.

5.3. Antibodies

Mouse monoclonal antibodies against BPV-1 E2 tags were used for the detection of tagged HCV proteins. The monoclonal antibody anti-1E2 tag recognizes the epitope RFSTTGHYSVRD (amino acids 179–190 of the BPV-1 E2 protein); the monoclonal antibody anti-3F12 tag recognizes the epitope GVSSTSSDFRDR (amino acids 197–208 of BPV-1 E2) (Kurg *et al*., 1999). Rabbit polyclonal serum against full-length HCV NS3 was used to detect the non-tagged NS3 protein.

5.4. Immunofluorescence microscopy

The Cos7 cells were transfected with pCG-3F12NS2, pCG-3F12NS3, or pCG-3F12NS4A plasmids and seeded on cover slips in 35 mm dishes. The Huh7 cells were grown on cover slips to the confluent state and infected with recombinant SFV-3F12NS2, SFV-NS3, SFV-3F12NS23 or double infected with SFV-3F12NS2 and SFV-NS3 at 10 infectious units/cell. The transfected or infected cells were incubated for 24 h in growth chamber conditions, fixed with 4% paraformaldehyde and permeabilized with cold methanol. The cells were treated with primary mouse anti-3F12 tag (Kurg *et al*., 1999) and secondary goat anti-mouse FITC or Texas red-conjugated (Santa Cruz Biotechnologies) antibodies. For double immunostaining, the infected Huh7 cells were treated with primary mouse anti-3F12 tag and anti-NS3 rabbit polyclonal antibodies. Treatments with secondary, species-specific antibodies conjugated with FITC, and Texas red or rhodamine red (Santa Cruz Biotechnologies) were subsequently performed. The preparates were analyzed by fluorescent microscopy using the Olympus U-RFL-TX microscope or the Bio-Rad MRC-1024 confocal microscope.

5.5. Co-immunoprecipitation

The pCG-1E2NS3 and pCG-3F12NS2 or pCG-1E2NS3 and pCG-3F12NS4A plasmids were co-electroporated into the Cos7 cells. The transfected Cos7 cells were lysed under nondenaturing conditions on ice and the immune complexes were precipitated by the use of the 1E2 tag specific mouse monoclonal antibody bound to agarose beads. Immunoprecipitated proteins were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blot. For the detection of proteins, co-precipitated with the 1E2-tagged NS3, the mouse anti-3F12 tag monoclonal antibody was used.

For the characterization of the interaction between the HCV NS2 and NS3 proteins in Huh7 cells, the infected cells were lysed under nondenaturing conditions and sonicated. The membranous fraction of the cells was lysed under more stringent lysis conditions to allow solubilization of membrane-bound proteins. The NS2 protein with 3F12 tag at the N-terminus was precipitated with the 3F12 tag specific mouse monoclonal antibody; in the reciprocal experiment the HCV NS3 protein was precipitated with respective rabbit polyclonal serum by the use of Protein A Sepharose^{TM} CL-4B (Amersham Biosciences). The immunoprecipitated proteins were subjected to SDS-PAGE and western blot analysis with appropriate antibodies.

6. Statistical analysis

Statistical analysis (χ^2-test) was performed to determine the statistical significance of the differences: 1) in the percentages of subtypes 1b and 3a between different groups of patients; 2) in the prevalence of the HCV subtypes for the study periods (1997–1998 and 2000–2004); 3) in the distribution of autoantibodies in patients infected with different HCV subtype and between men and women. Differences were considered statistically significant for p value less than 0.05.

RESULTS AND DISCUSSION

1. HCV genotypes in Estonia (Papers I and III)

1.1. Genotyping 1997–1998

The results of subtype-specific multiplex PCR analysis are presented in Fig. 4A. The characteristic electrophoretic bands of subtypes 1a, 1b, 2a, 3a, and mixed subtype 1b+2a are shown, together with the band of 355 bp, as the PCR product of the conservative "inner" primer pair. The results of the traditional variant a of RFLP are shown in Fig. 4B. The corresponding bands for subtypes 3a, 2a, and 1b are shown together with those of the untreated controls.

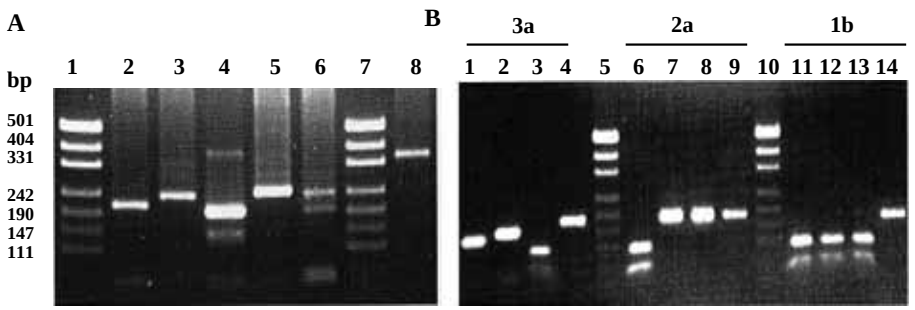

Figure 4. HCV genotyping. **A** – genotyping with subtype-specific PCR. Lanes 1 and 7 – molecular size marker pUC19 DNA/*Msp*I (MBI Fermentas), lane 2 – subtype 1a (208 bp), lane 3 – subtype 1b (234 bp), lane 4 – subtupe 2a (190 bp and 139 bp), lane 5 – subtype 3a (232 bp), lane 6 – mixed subtype 1b + 2a (234 bp and 190 bp), lane 8 – PCR product of "inner" primers S7-A5 (355 bp). **B** – genotyping with RFLP. Lanes 5 and 10 – molecular size marker pUC19 DNA/*Msp*I (MBI Fermentas). Lanes 4, 9, 14 – undigested PCR products (174 bp). Lanes 1, 6, 11 – digestion with *Scr*FI+*Hin*fI; lanes 2, 7, 12 – *Mva*I+*Hin*fI; lanes 3, 8, 13 – *Bsh*1236I. All restrictions were carried out with 10 U of the corresponding enzyme for 2 hours at 37°C. Characteristic restriction patterns for subtype 3a: lanes 1, 2, and 3 (fragments 129 bp, 145 bp, and 99 bp, respectively); subtype 2a: lanes 6, 7, and 8 (fragments 97 bp, 174 bp, and 174 bp, respectively); subtype 1b: lanes 11, 12, and 13 (fragments 97 bp, 97 bp, and 99 bp, respectively).

The subtype-specific PCR and RFLP methods used in this study revealed 200 and 202 (93% and 94%) HCV RNA positive samples of 215 sera, respectively. The results of genotyping are summarized in Table 2. The prevailing HCV subtype in Estonia is 1b. Subtypes 3a and 2a are found in lower amounts, and some representatives of subtypes 1a and 4 are found as well.

In determination of the geno(sub)type of 215 HCV RNA positive samples, both methods yielded completely or partially concordant results in the majority

of cases (85%). In the remaining 15% of the cases only one of the methods yielded a positive result. No diverse results were found.

Table 2. Distribution of HCV genotypes by subtype-specific PCR and RFLP.

HCV subtype	Subtype-specific PCR	RFLP-analysis
1a	2 (1%)	2 (<1%)
1b	121 (56%)	138 (64%)
3a	30 (14%)	48 (22%)
2a	14 (7%)	12 (6%)
4	1 (<%)	0
Mixed infections	29 (14%)	0
Untypable	3 (1%)	2 (<1%)

Despite the generally concordant genotyping results, obtained with subtype-specific PCR and RFLP analysis, these two methods differed significantly regarding detection of cases of mixed infection. An unusually high number of mixed infections (14%, 29 cases) were detected by multiplex subtype-specific PCR, whereas none was detected by RFLP. Comparison of the genotyping results for every single sample revealed that RFLP determined only one of the subtypes, present in subtype-specific PCR-determined samples of mixed infection (mainly 1b or 3a). This can reflect either the non-specific annealing of primers or real occurence of sequence heterogeneity in serum samples. These data show that RFLP analysis is more reliable and produces fewer artifacts in the genotyping of HCV.

For epidemiological analysis, the data obtained from special questionnaires were analyzed. Not all returned questionnaires were complete. The prevalences of HCV subtypes 1b and 3a in different groups of patients were compared with the corresponding prevalence in the total study population (Table 3). The results of genotyping by RFLP were used for epidemiological analysis. The significance level $p<0.05$ was considered statistically significant. The distribution of HCV subtypes 1b and 3a was not different in either sex or in the groups of acute and chronic hepatitis. Nevertheless, the relative presentation of subtype 3a in young (under 30 years) people and in drug addicts was significantly higher than in the total population, while the relative presentation of subtype 1b in these groups was accordingly significantly lower. The other subtypes (1a, 2a, and 4) were found in only a few patients; they were not prevalent in any of the groups. These data might indicate the increasing importance of subtype 3a of HCV infection compared with subtype 1b in Estonia.

Table 3. Distribution of subtypes 1b and 3a in different groups of patients. Statistically significant *p* values are shown in bold. In brackets – percentage.

Group	Number of patients	1b – number of patients (%)	Significance level (*p*) of the differences in the presentation of subtype 1b	3a – number of patients (%)	Significance level (*p*) of the differences in the presentation of subtype 3a	Other subtypes (%)
Total population	215	146 (67.9%)		50 (23.3%)		19 (8.8%)
Age under 30 years	73	35 (47.9%)	**0.0026**	31 (42.5%)	**0.0016**	7 (9.6%)
Age over 30 years	92	78 (84.8%)	0.11	8 (8.7%)	**0.0028**	6 (6.5%)
Age unknown	50					
Acute hepatitis	32	18 (56.3%)	0.19	12 (37.5%)	0.084	2 (6.2%)
Chronic hepatitis	117	86 (73.5%)	0.29	22 (18.8%)	0.76	9 (7.7%)
Stage unknown	66					
Drug addicts	40	17 (42.5%)	**0.0021**	20 (50.0%)	**0.0005**	3 (7.5%)
Blood transfusions and surgical interventions	36	30 (83.3%)	0.062	2 (5.5%)	**0.015**	4 (11.2%)
Transmission route unknown	139					
Men	130	85 (65.4%)	0.63	35 (26.9%)	0.45	10 (7.7%)
Women	70	50 (71.4%)	0.58	12 (17.1%)	0.28	8 (11.5%)
Gender unknown	15					

1.2. Genotyping 2000–2004

HCV subtypes were determined by RFLP analysis of the amplified 5' UTR. The distribution of HCV subtypes detected in 459 investigated sera was as follows: 1a – 9 (2%), 1b – 319 (70%), 2a – 32 (7%), 3a – 86 (19%), mixed subtype – 12 samples (3%), and unidentified subtype – 1 sample (<1%). The data obtained from HCV genotyping in the years 2000–2004 were compared with the genotyping results from the years 1997–1998. Only the genotyping data of RFLP analysis (in 1997–1998) were used for comparison.

The distribution pattern of HCV subtypes was not changed (Fig. 5). Differences in the prevalence of HCV subtypes were statistically nonsignificant ($p>0.05$). There was no increase in the occurrence of HCV subtype 3a or decrease in the occurrence of subtype 1b as was expected in the previous study.

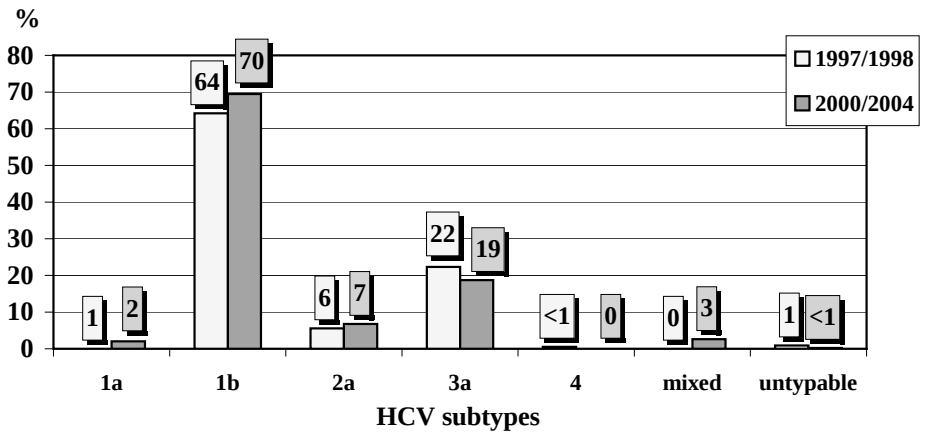

Fig 5. Occurrence of HCV subtypes in different periods of time in Estonia (%).

There was found a remarkable increase in mixed HCV infections detected by the RFLP method. In 1997–1998, there were no mixed infections detected by RFLP. Nonspecific annealing of primers is one of the shortcomings of the subtype-specific PCR method. It yieldes more nonspecific genotyping results, particularly in the form of mixed subtypes (14% in 1997–1998). This was the reason for regarding the RFLP genotyping method as more reliable compared to the subtype-specific PCR. In 2000–2004 the number of mixed subtypes detected by RFLP rose to 3% (12 cases of total 459 serum samples tested). This difference was found to be statistically significant ($p=0.025$). The more frequent detection of mixed HCV infection in this case could indicate the real presence of diverse HCV sequences in the serum samples and reflect infection with more than one HCV strains. For example, in high-risk population (e.g. IVDU), the probability to acquire HCV from multiple sources is very high.

2. HCV genotypes and autoantibodies in chronic hepatitis C patients (Paper II)

Among the 90 patients 46 (51%) were positive for at least one autoantibody. SMA and ANA were detected in the observed sera most frequently. SMA were present in 39 (43%) and ANA in 13 (14%) sera. SMA 1:100 were detected in 4 male patients and 1 female patient, and SMA at 1:10 were detected in 25 male and 9 female patients. ANA at 1:100 were positive in 1 male patient and ANA at 1:10 in 8 male and 4 female patients. In 9 cases the sera were positive for two autoantibodies (ANA and SMA at different dilutions). TMA (1:10 and 1:100) were found in 2 female patients. ARA at 1:100 were found in 1 male patient. AMA, PCA, and LKMA were not detected in the observed sera. The data about the prevalence of different autoantibodies in chronic hepatitis C patients are presented in Table 4.

Table 4. Distribution of autoantibodies in chronic hepatitis C patients. N – number of patients, in brackets – percentage.

AUTOANTIBODIES	PATIENTS, total N=90	MALE N=58	FEMALE N=32
ANA 1:10	12 (13%)	8 (14%)	4 (12%)
ANA 1:100	1 (1%)	1 (2%)	0
AMA 1:10	0	0	0
SMA 1:10	34 (38%)	25 (43%)	9 (28%)
SMA 1:100	5 (6%)	4 (7%)	1 (3%)
ANA + SMA	9 (10%)	7 (12%)	2 (6%)
PCA 1:10	0	0	0
ARA 1:100	1 (1%)	1 (2%)	0
LKMA 1:10	0	0	0
TMA 1:10	1 (1%)	0	1 (3%)
TMA 1:100	1 (1%)	0	1 (3%)
TOTAL	**46 (51%)**	**32 (55%)**	**14 (44%)**

HCV subtypes were determined by the RFLP analysis Table 5 presents the prevalence of HCV subtypes in the study population. HCV subtypes were distributed as follows: 1b – 66 (73%), 3a – 18 (20%), and 2a – 6 patients (7%). In this study, there were no differences in the distribution of different auto-antibodies between the patients with different HCV subtypes or between the male and female patients ($p>0.05$).

Table 5. Distribution of HCV subtypes in male and female patients with chronic hepatitis C. N – number of patients, in brackets – percentage.

HCV SUBTYPE	**PATIENTS**, total N=90	**MALE** N=58	**FEMALE** N=32
1b	66 (73%)	42 (72%)	24 (75%)
2a	6 (7%)	3 (5%)	3 (9%)
3a	18 (20%)	13 (22%)	5 (16%)

Our study demonstrated the high prevalence of the serological markers of autoimmunity among patients with chronic hepatitis C. In other similar studies such a serological feature has been found in chronic hepatitis C patients irrespective of their clinical status, i.e. patients with chronic hepatitis C, patients with mixed cryoglobulinemia or other autoimmune manifestations or symptom free HCV-infected individuals (Valentini *et al*., 1999). In a majority of patients different autoantibodies were found at low titers (1:10).

The presence of LKMA, ANA or SMA autoantibodies is a marker for AIH. Chronic hepatitis C with positive serological markers of autoimmunity may, on one hand, mimic and, on the other hand, hide underlying AIH (Hano *et al*., 2000). These two alternative diagnoses must be distinguished because of different treatment strategies. Taking into account the high prevalence of autoantibodies in chronic hepatitis C in Estonia, these patients should be carefully and thoroughly investigated to exclude classical AIH.

In Estonia, autoantibody studies have been conducted in unselected adult populations (Turchany *et al*., 1997; Uibo *et al*., 1998). According to Turchany *et al*., the prevalence of AMA (when tested by immunoblotting against beef heart mitochondria) was <1% (13 positive sera out of 1461 samples), this is in agreement with the reported incidence of less than 1% AMA in a mixed hospital population (Turchany *et al*., 1997). In our study, AMA were not detected in chronic hepatitis C patients. In another investigation in Estonia the prevalence of common tissue autoantibodies was studied in 448 healthy adult persons (Uibo *et al*., 1998). According to that paper, ANA were found in 3% of male and in 11% of female persons, so the prevalence of ANA was significantly higher among the latter. The other autoantibodies were presented as follows (male/female): SMA 11%/10%, ARA <1%, PCA 2%/6%, TMA 2%/4%, total 19%/25% (22%). None of the serum samples was positive for LKMA in either study. Compared to the findings, the presence of different autoantibodies in chronic hepatitis C patients in our study is significantly higher (total prevalence 52.2% *vs.* 22%). An exception was the presence of ANA autoantibodies in female patients; their prevalence did not differ significantly (12% *vs*. 11%). The differences in the distribution of different autoantibodies between men and women in our study were also nonsignificant, although in the total population

the prevalence of autoantibodies usually tends to be higher in women. The absence of LKMA antibodies in the investigated hepatitis C patients group, as well as in the study of Uibo *et al*. (Uibo *et al*., 1998), could be a reflection of the real low prevalence of these autoantibodies in the Estonian population.

The genome of HCV is very variable, having an extremely high spontaneous mutation rate. Different HCV subtypes have been shown to have a varying impact on severity of chronic disease, effectiveness of interferon alpha treatment or interferon alpha and ribavirin treatment, consequences of liver transplantation and diagnostic procedures. In Estonia, the most prevalent (determined by the RFLP method) HCV subtype is 1b (64%), subtypes 3a, 2a, and other subtypes (among them undetermined results) were presented in 22%, 6%, and 8% of the cases, respectively (Zusinaite *et al*., 2000). The distribution of HCV subtypes in the present study group (1b – 73%; 3a – 20%; 2a – 7%) is very similar to that in a previous study, which may indicate independent selection of patients. It was hypothesized that the viral antigens of different subtypes may elicit different autoantibodies or other immunological reactions in a particular host. Several studies performed in this field have shown that the serological pattern of autoantibodies does not correlate with a particular subtype of HCV (Luo *et al*., 1998; Zein *et al*., 1999). Our study also failed to find any association of the presence of different autoantibodies with the HCV genotype. One of the reasons for this could be the real absence of such an association that could indicate generalized nonspecific activation and alteration of the reactivity of the host immune system during chronic HCV infection. Another reason is the relatively small study group, which does not allow making statistical analysis (e.g. there was only one patient with HCV subtype 2a who was positive for autoantibodies).

3. Interactions between HCV proteases (Paper IV)

The use of epitope tagging and epitope-specific antibodies is a reasonable alternative for and/or supplement to protein-specific antibodies. The short epitope-peptide introduced in the N- or C-terminus, or in-frame into the coding sequence of the protein, should have minimal effects on its conformation and biological activity. If this is the case, it simplifies detection, characterization and purification of proteins. In the present study, two Bovine Papillomavirus type 1 (BPV-1) E2 protein-derived epitope tags and respective monoclonal antibodies (Kurg *et al*., 1999; Kaldalu *et al*., 2000), together with the protein-specific polyclonal antibody, were used to study the interactions between the HCV proteases.

To obtain a specific evidence of complex formation between HCV proteases, the experiments confirming the co-localization of proteins in distinct cellular compartments and coimmunoprecipitation experiments were performed.

3.1. Subcellular localization and co-localization of epitope-tagged HCV proteases

The localization patterns of individually expressed HCV proteins in the transfected Cos7 cells and in the recombinant SFV infected Huh7 cells were found to be highly similar. In both cases localization in perinuclear network-like structures was detected for NS2 (Fig 6A, B). The localization of NS4A was analyzed only in the Cos7 cells where the distribution over the cell cytoplasm in the form of a clear dot-like pattern was detected (Fig. 6E). Individually expressed HCV NS3 protease was distributed diffusely through the cell cytoplasm and, to a less extent, in the nucleus in both the Cos7 and Huh7 cell lines (Fig. 6C, D). In Cos7 cells NS3 showed some preference for the perinuclear region.

The co-expression of NS3 and NS4A in Cos7 cells, as well as the co-expression of NS2 and NS3 in Cos7 or Huh7 cells led to distinct changes in NS3 localization in favor of membranous structures. Immunofluorescence analysis of the cells expressing NS2 and NS3 in the form of a polyprotein precursor revealed extensive co-localization of dotted perinuclear NS2 and NS3 signals against the background of diffuse cytoplasmic localization of a small fraction of NS3 (Fig. 7B). The localization pattern in cells double-infected with SFV-3F12NS2 and SFV-NS3 (Fig. 7A) was largely identical to the localization pattern in case of NS23 expression indicating that co-localization of NS2 and NS3 is not strictly dependent on their expression in the form of a single polyprotein precursor.

3.2. Co-immunoprecipitation of NS2-NS3 and NS3-NS4A proteins

Interactions between NS2 and NS3 in transfected or infected cells were studied by the use of co-immunoprecipitation technique. The complexes of proteins, formed in the cells transfected with pCG-1E2NS3 and pCG-3F12NS2 or pCG-1E2NS3 and pCG-3F12NS4A, were precipitated with the 1E2 antibody bound to agarose beads, and the co-precipitated proteins were detected with antibodies against the second tag. First, NS3 and NS4A dimer formation was analyzed by this method. Since this complex has been previously described (Failla *et al*., 1995; Wolk *et al*., 2000), the analysis served as positive control for the approach used. The interaction between NS3 and NS4A proteins was readily detected (Fig. 8A, B). Similarly, the interaction between NS2 and NS3 proteins was detected by the same approach (Fig. 8C, D). These results demonstrated complex formation between NS2 and NS3 in Cos7 cells in the absence of other HCV NS-proteins.

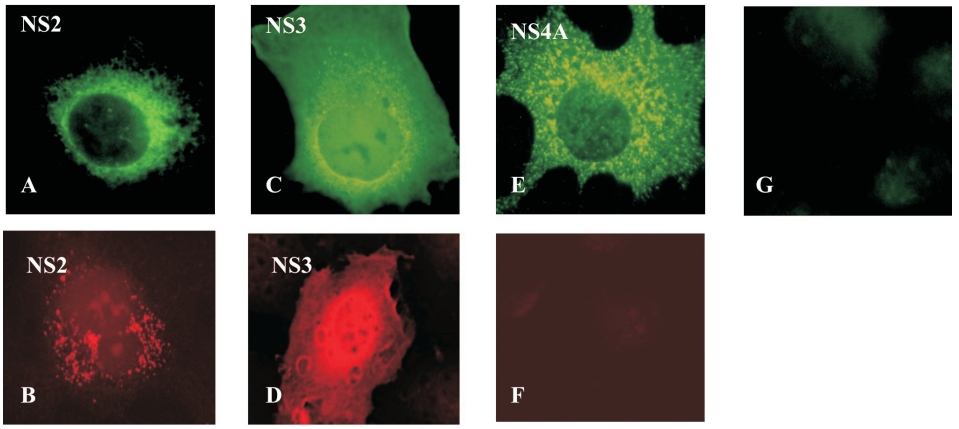

Figure 6. Subcellular localization of NS2, NS3 and NS4A proteins. Cos7 cells were analyzed 48 h post-transfection and Huh7 cells analyzed 24 h post-infection with recombinant SFV particles at 10 infectious units/cell. The names of expressed proteins are shown on each panel. Mock-transfected Cos7 cells (G) and mock-infected Huh7 cells (F) were used as negative controls. Recombinant 3F12-tagged NS2 (A), NS3 (C), and NS4A (E) proteins in Cos7 cells were detected with mouse monoclonal anti-3F12 tag and goat anti-mouse FITC-conjugated antibodies. Monoclonal mouse anti-3F12 tag and goat anti-mouse Texas red-conjugated antibodies were used for detection of recombinant NS2 protein (B), and polyclonal rabbit anti-NS3 and goat anti-rabbit rhodamine red-conjugated antibodies were used for detection of NS3 protein (D) in Huh7 cells. Images A, C, E and G were generated by Olympus U-RFL-TX microscope; images B, D and F by confocal Bio-Rad MRC-1024 microscope.

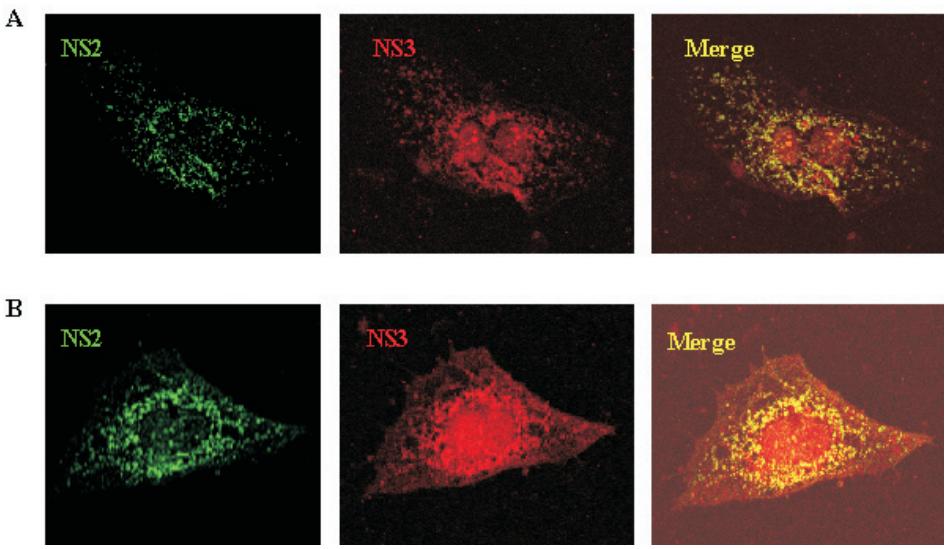

Figure 7. Subcellular localization of NS2 and NS3 in Huh7 cells infected with recombinant SFV 24 h post-infection. **A** – Huh7 cells, double infected with SFV-3F12NS2 and SFV-NS3 at 10 infectious units/cell. **B** – Huh7 cells, infected with SFV-3F12NS23 at 10 infectious units/cell. Cells were fixed with 4% paraformaldehyde and NS2 was detected by anti-3F12 tag monoclonal and anti-mouse FITC-conjugated antibodies, NS3 was detected by anti-NS3 rabbit polyclonal and anti-rabbit rhodamine red-conjugated antibodies. Names of proteins are indicated at the each panel. Images were generated by confocal BioRad MRC-1024 microscope.

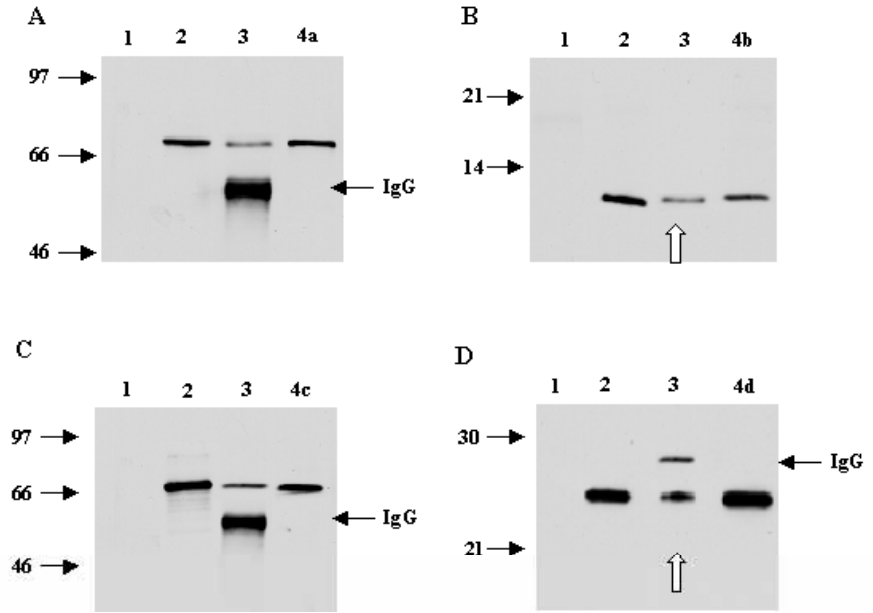

Figure 8. Immunoprecipitation of the NS3/4A and NS3/NS2 complexes from Cos7 cells co-transfected with pCG-1E2NS3 and pCG-3F12NS4A or pCG-1E2NS3 and pCG-3F12NS2. Immunoprecipitation was performed by the use of agarose beads conjugated with the 1E2 antibody; the precipitated samples were subjected to SDS-PAGE and western blotting. Lane 1 – mock-transfected Cos7 cell lysate; lane 2 – lysate from Cos7 cells co-transfected with pCG-1E2NS3 and pCG-3F12NS4A (A, B) or pCG-1E2NS3 and pCG-3F12NS2 (C, D); lane 3 – immunoprecipitation fraction; lane 4 – positive controls – lysates from Cos7 cells transfected with pCG-1E2NS3 (A, C), pCG-3F12NS4A (B) or pCG-3F12NS2 (D). Arrows on the left of each panel indicate the positions of molecular mass standards (kiloDaltons). Panels **A** and **C –** Western blots with the anti-1E2 tag antibody. Panels **B** and **D –** Western blots with the anti-3F12 tag antibody. The open arrows on panels B and D indicate co-immunoprecipitated NS4A and NS2 proteins, respectively.

The interactions between HCV NS2 and NS3 were confirmed by co-immuno-precipitation of the NS2/NS3 complexes from both Cos7 and Huh7 cell lines. Co-immunoprecipitation of the NS2/3 complex from the infected Huh7 cells was found to require more stringent buffer conditions than co-immuno-precipitation of the same complex from Cos7 cells, probably due to a much closer association of the NS2 protein with cellular membranes. Complex formation between the NS2 and NS3 in Huh7 cells infected with SFV-3F12NS23 was confirmed by a reciprocal co-immunoprecipitation procedure: NS3 was found to co-precipitate with 3F12-tagged NS2 by the use of the 3F12

tag-specific antibody (Fig. 9B, C), and NS2 was found to co-precipitate with NS3 by the use of the polyclonal anti-NS3 antibody (Fig. 9D, E).

Similarly, NS2/NS3 complex formation was demonstrated also in Huh7 cells double infected with SFV-3F12NS2 and SFV-NS3. These interactions as well as co-localization were found to be not dependent on the cell line used, nor did they depend on the fact whether NS2 and NS3 were expressed as individual proteins or as a NS23 polyprotein precursor. This data is in good agreement with the results obtained in co-localization experiments (Fig. 7).

An understanding of the molecular mechanisms of viral replication and the life cycle constitutes a basic step towards management of viral infection in clinical practice, treatment of patients and development of antiviral drugs. In case of HCV, interactions between viral-encoded proteins are crucial links for formation of the functional membrane-bound replicase complex. The cleavage of NS2/3 junction represents the first event in the proteolytic processing of the nonstructural region of the HCV polyprotein. Although it has been demonstrated that NS2 is not directly needed for HCV replication (Pietschmann *et al*., 2001), the possibility that this protein has accessory functions in formation of the HCV replication complex cannot be excluded, e.g. favouring the retention of yet unprocessed polyprotein in the membrane compartments before complex formation between NS3 and its cofactor NS4A.

The interactions as well as the enzymatic activities of individual HCV proteins represent very attractive targets for developing antiviral drugs. Agents affecting interactions between the components of viral replicase, thus inhibiting the enzymatic activities of virus-encoded proteins, represent one of the reasonable alternatives for antiviral drugs. The interaction between HCV NS2 and NS3 proteins represents a promising potential target for this kind of inhibitors. The fact that the *in vivo* interactions of NS2 and NS3 were not found to be dependent on the mode of their expression and/or the cell line used for their expression can be used for development of a test system for this kind of therapeutics.

High specificity of the epitope-antibody interaction allowed to study the localization and co-localization of proteins in the cells as well as to perform the immunoprecipitation procedure without any significant non-specific background. In all cases the subcellular localization of tagged proteins was similar to that of non-tagged HCV NS2, NS3 and NS4A. Importantly, the presence of the epitope tag at the N-terminus of HCV NS2 did not affect the activity of NS2/NS3 protease, since in all experiments where NS2 and NS3 were initially expressed as one polyprotein precursor, only fully processed forms were detected by immunoblotting. These findings confirm the high value of epitope-tagging technique for studies of HCV replicase proteins. On the basis of these findings, *in vivo* systems for analysis of NS2/NS3 interactions can be constructed and used for high throughput screening of potential inhibitors of HCV replication.

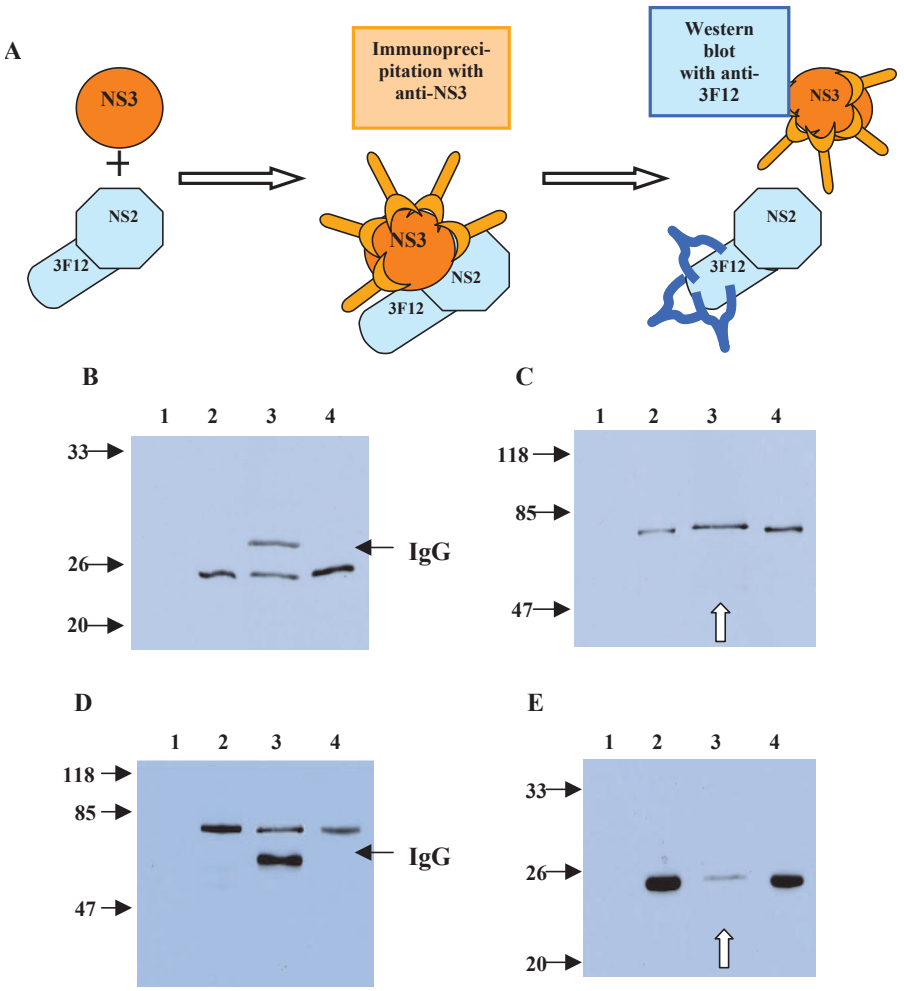

Figure 9. Immunoprecipitation of the NS2/3 complex from Huh7 cells infected with SFV-3F12NS23. Lane 1 – mock-infected Huh7 cell lysate; lane 2 – total lysate of Huh7 cells infected with SFV-3F12NS23; lane 3 – immunoprecipitation fraction; lane 4 – positive control (total lysate of BHK cells infected with SFV-3F12NS23). Immunoprecipitation was performed either with the anti-3F12 tag antibody (**B**, **C**) or with the anti-NS3 antibody (**D**, **E**). Arrows on the left of each panel indicate the positions of molecular mass standards (kiloDaltons). Panel **A** – schematic overview of the technique used. **B** – western blot with the anti-3F12 tag antibody. **C** – western blot with the rabbit anti-NS3 antibody. **D** – western blot with the anti-NS3 antibody. **E** – western blot with the anti-3F12 tag antibody. Open arrows indicate co-immunoprecipitated NS3 (**C**) or NS2 (**E**) proteins, respectively.

CONCLUSIONS

1. The prevailing HCV subtype in Estonia is 1b (64% by RFLP), while subtype 3a (22%), as well as minute amounts of subtypes 2a, 1a and 4 are also found. In groups of young people under 30 years of age and IVDUs, the relative presence of subtype 3a is significantly higher compared to the total population of HCV-infected patients.
2. The distribution pattern of HCV subtypes has not changed during the study period (1997–1998 in comparison to 2000–2004). Detection of mixed HCV infection in 2000–2004 (2.6% compared to 0% in 1997–1998) by RFLP could indicate the real presence of diverse HCV sequences in serum samples and reflect infection with multiple HCV isolates.
3. Subtype-specific PCR and RFLP analysis yielded high resolution of the investigated HCV RNA-containing serum samples (positive results 93.0% and 93.9%, respectively). Compared to subtype-specific PCR, RFLP is a more reliable method in the genotyping of HCV. While multiplex PCR gave ambiguous results (detection of mixed infections 14%), the genotyping with RFLP was more definite and easier to interpret.
4. A high prevalence of ANA (14%) and SMA (43%) was found in drug-naïve chronic hepatitis C patients. Autoantibodies were present in the sera at low titers in most cases. Compared to total Estonian population, the presence of different autoantibodies in chronic hepatitis C patients was significantly higher. The distribution of the autoantibodies showed no differences in either sex group or between the patients infected with different HCV subtypes.
5. The existence of the protein-protein complex between HCV NS2 and NS3 proteases in two different cell lines (Cos7 and Huh7) has been confirmed. Complex formation of NS2 and NS3 *in vivo* was found not to depend on the mode of their expression and/or the cell line used for their expression, which indicates a direct interaction of these proteins.
6. E2 epitope tagging is a valuable technique for the study of HCV replicase proteins, owing high specificity and a significant signal-to-noise ratio. The use of BPV-1 E2-protein derived epitope tags with respective monoclonal antibodies allowed to identify tagged HCV proteins on immunoblots and to study the localization of proteins in the cells as well as to perform immuno-precipitation procedure with high efficiency. Addition of the E2 epitope to the N-terminus of the HCV NS2 protein did not affect the activity of NS2/NS3 protease.

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SUMMARY IN ESTONIAN

C-HEPATIIDI VIIRUS: GENOTÜÜPIDE MÄÄRAMINE JA VIIRUSE PROTEAASIDE-VAHELISTE INTERAKTSIOONIDE ISELOOMUSTAMINE

C-hepatiidi viirus (HCV) on sagedasemaks kroonilise maksakahjustuse (hepatiidi ja tsirroosi) tekke põhjuseks. Enne HCV identifitseerimist klassifitseeriti seda mitte-A-mitte-B-hepatiidi viiruseks, mis kandus üle peamiselt saastunud vereproduktide või teiste veenisiseste teede kaudu. Pärast HCV-vastaste antikehade rutiinse skriinimise kasutusele võtmist veredoonoritel aastal 1990 jäi HCV peamiseks ülekandeteeks veenisisene narkootikumide kasutamine.

HCV-infektsiooni on ligi 1%-l populatsioonist arenenud riikides ning ulatub kuni 10–20%-ni arengumaades. Eestis alustati veredoonorite seerumite skriinimist aastal 1991. Aastail 1995–1996 tehtud uuringutel on leitud HCV-vastaseid antikehi ligi 1% veredoonorite seerumis. Äge C-hepatiit muutub 70–80%-l juhtudest krooniliseks. Maksatsirroos areneb 20 aasta jooksul 5–20%-l kroonilise C-hepatiidiga patsientidest, nendest 13–30%-l võib tekkida tsirroosi foonil maksarakuline vähk. Kroonilisest HCV-infektsioonist põhjustatud lõpp-staadiumis maksahaigus on praegu arenenud maades peamiseks maksasiirdamise näidustuseks. Efektiivse ennetava ja ravivaktsiini väljatöötamine on takistatud viiruse genoomi suure varieeruvuse tõttu.

HCV-infektsiooni registreeritud juhtumite arvu tõus, kasvav kroonilise C-hepatiidi kliiniline tähendus, interferoon-alfa- ja ribaviriinravi alustamine Eestis ning HCV genotüüpide määramise vajadus tegidki käesoleva uurimuse vajalikuks.

Töö eesmärgid

Töö üldiseks eesmärgiks oli iseloomustada HCV-infektsiooni ja viirust ennast mitmest küljest: anda ülevaade Eestis ringlevate HCV genotüüpide jaotumusest, uurida autoantikehade esinemist kroonilise C-hepatiidi haigetel ja nende seost HCV alatüübiga ning iseloomustada viiruse replikatsioonis osalevate valkude interaktsiooni. Uurimuse konkreetsed eesmärgid olid järgmised.

1. Identifitseerida Eesti populatsioonis ringlevaid HCV alatüüpe (I).
2. Võrrelda HCV alatüüpide jaotumust Eestis aastail 1997–1998 ja 2000–2004 (I, III).
3. Võrrelda kahte genotüpeerimise meetodit: PCR-meetodit alatüübispetsiifiliste praimeritega ja HCV genoomi 5’ UTR osa PCR amplifikatsiooni produkti RFLP (restriktsioonifragmentide pikkuse polümorfismi) analüüsi (I).

4. Määrata autoantikehade (ANA, AMA, SMA, LKMA, PCA, TMA ja ARA) esinemist kroonilise C-hepatiidiga viirusevastast ravi mittesaanud haigetel ning uurida, kas HCV alatüübid on seotud autoantikehade olemasoluga (II).
5. Iseloomustada kompleksi moodustumist HCV NS2- ja NS3-valkude vahel (IV).
6. Hinnata E2 epitoopliidese kasutamise võimalust HCV replikaasivalkude interaktsioonide uurimiseks (IV).

Uuritav materjal ja meetodid

Käesolevas töös uuriti kokku 765 C-hepatiidi patsiendi seerumit. Seerumid koguti Eesti haiglatest või kasutati seerumiproove, mis olid saadetud "AS Quattromed" diagnostikalaborisse HCV RNA ja viiruse genotüübi määramiseks. Kogumise järel seerumid külmutati ja säilitati –20°C juures.

Geno(ala)tüüpide esinemist uuriti 215 järjestikuse HCV-infektsiooniga patsiendi seerumis (seerumid koguti 1997.–1998. a). HCV genotüüpe määrati kahe meetodiga: PCR alatüüp-spetsiifiliste praimeritega genoomi kapsiidiosast ja 5' mittetransleeritava ala amplifikatsiooniprodukti RFLP analüüs. Epidemioloogiliseks analüüsiks saadeti arstidele küsimustikud. Viimastest saadud andmete põhjal jaotati patsiendid soo (mehed, naised), viiruse edasikandmise tee (vereülekanded ja kirurgilised operatsioonid, veenisisene narkootikumide süstimine), hepatiidi staadiumi (äge, krooniline) ja vanuse (alla 30 aasta, üle 30 aasta) alusel mitmeks rühmaks ning võrreldi HCV genotüüpide jaotumust nendes rühmades.

HCV alatüüpide jaotuse muutuste jälgimiseks uuriti 459 järjestikuse HCV-infektsiooniga haige seerumit (seerumid koguti "AS Quattromed" laboris 2000.–2004. a). Selles uuringus kasutati HCV alatüüpide määramiseks RFLP meetodit.

Järgnevaks uuringuks koguti 90 järjestikuse kroonilise C-hepatiidiga patsiendi seerumit (patsiendid ei olnud viirusevastast ravi varem saanud). Kaudsel immunofluorestsentsmeetodil määrati IgG tüüpi tuumavastaste (ANA), mitokondritevastaste (AMA), silelihaskoevastaste (SMA), maksa-neeru mikrosoomide vastaste (LKMA), parietaalrakkudevastaste (PCA), kilpnäärme mikrosoomide vastaste (TMA) autoantikehade esinemist. HCV alatüüp määrati RFLP-analüüsiga.

HCV mittestruktuursete NS2- ja NS3-valkude vahelise interaktsiooni iseloomustamiseks kasutati algmaterjalina kroonilise C-hepatiidiga patsiendi seerumist (alatüüp 1b RFLP meetodil määratuna) isoleeritud HCV RNA-d, mille pealt pöördtranskriptsioon-PCR meetodil amplifitseeriti uuritavad geenijärjestused. Töös kasutati BPV-1 E2-valgust pärinevaid epitoopliideseid 1E2 ja 3F12 ning vastavaid hiire monoklonaalseid antikehi. NS3-valgu detekteerimiseks Huh7-rakkudes kasutati NS3-vastast küüliku polüklonaalset seerumit.

HCV proteaaside ekspresseerimiseks Cos7 rakuliinis elektroporeeriti koos NS2 ja NS3 või NS3 ja NS4A järjestusi sisaldavad vektorid erinevate E2 liidestega. NS2 ja NS3 valkude ekspresseerimiseks inimese maksarakuliinis Huh7 kasutati rekombinantseid Semliki Forest viiruse partikleid. Varem hästi iseloomustatud interaktsiooni NS3 ja NS4A proteaaside vahel kasutati sisemiseks kontrolliks. Komplekside moodustamist iseloomustati valkude kolokaliseerumisega immunofluorestsentsmeetodil ja koimmunosadestamisega epitoobi- ja valguspetsiifiliste antikehadega.

Uurimuse peamised tulemused

HCV genotüübid Eestis (artiklid I ja III). HCV alatüübid esinesid (vastavalt alatüübi-spetsiifilise PCR-iga ja RFLP-analüüsiga) järgmiselt: 1a – 1% ja <1%, 1b – 56% ja 64%, 2a – 7% ja 6%, 3a – 14% ja 22%, 4 – <1% ja 0%, segainfektsioon – 14% ja 0%, tüpiseerimata – 1% ja <1%. Alatüüpide 1b ja 3a esinemises ei olnud erinevust meeste ja naiste ega maksahaiguse eri staadiumite vahel. Samas oli 3a alatüübi osakaal uuringupopulatsiooniga võrreldes suurem alla 30-aastaste ja narkootikume süstivate patsientide rühmas ning 1b alltüübi osakaal nendes rühmades oluliselt väiksem.

Kasutatud genotüpeerimise meetodid erinesid oluliselt segainfektsioonide määramisel. Segainfektsioone esines 14% alatüübispetsiiflise PCR ja 0% RFLP analüüsi puhul. Alatüüpspetsiifilise PCR-iga määratud kahest alatüübist oli RFLP-analüüsiga detekteeritud neist vaid üks. Kuna PCR-meetodi puuduseks on praimerite mittespetsiifiline seondumine, siis RFLP-meetod osutus genotüpeerimisel usaldusväärsemaks.

Aastail 2000–2004 tehtud uurimuses oli HCV alatüüpide jaotus järgmine: 1a – 2%, 1b – 70%, 2a – 7%, 3a – 19%, segainfektsioon – 3%, tüpiseerimata – <1%. Seega, võrreldes eelneva uurimisajaga (1997–1998), polnud üldine alatüüpide jaotus muutunud. Ilmnes statistiliselt oluline (p=0,025) segainfektsioonide arvu tõus RFLP-meetodiga määratult. Selline segainfektsioonide sageduse tõus viitab mitme viiruse alatüübi olemasolule seerumis, mis on väga tõenäoline kõrge riskiga populatsioonides (näiteks süstivate narkomaanide seas).

HCV alatüübid ja autoantikehad kroonilise C-hepatiidiga patsientidel (artikkel II). Vähemalt üks autoantikeha leiti 46 (51%) patsiendil. SMA leiti 39 (43%) ja ANA 13 (14%) seerumis. SMA lahjenduses 1:100 oli 4 mees- ja 1 naispatsiendil, SMA lahjenduses 1:10 – 25 mees- ja 9 naispatsiendil. ANA 1:100 oli positiivne 1 meespatsiendil ja ANA 1:10 – 8 mees- ja 4 naispatsiendil. 9 juhul olid seerumid positiivsed kahe antikeha suhtes (ANA ja SMA erinevatel lahjendustel). TMA (vastavalt lahjendustes 1:10 ja 1:100) leiti 2 naispatsiendil. ARA 1:100 oli 1 meespatsiendil. AMA-t, PCA-d ja LKMA-d ei leidunud. Võrreldes Eesti üldpopulatsiooniga oli kroonilise C-hepatiidiga patsientidel autoantikehi sagedamini. HCV alatüüpe oli uuritavail järgnevalt: 1b – 66 (73%),

2a – 6 (7%) ja 3a – 18 patsienti (20%). Statistiliselt ei erinenud autoantikehade jaotus meeste ja naiste ning erinevate HCV alatüüpidega kroonilise C-hepatiidiga patsientide vahel.

HCV proteaasidevahelised interaktsioonid (artikkel IV). HCV NS2- ja NS3-valkude lokalisatsioon oli väga sarnane Cos7- ja Huh7-rakkudes. NS3 lokaliseerus difuusselt üle tsütoplasma ja raku tuumas, NS2-l oli perinukleaarne võrgutaoline muster ning NS4A lokaliseerus (Cos7 rakkudes) tsütoplasmas täpilise mustrina. NS3 koekspressioon NS4A-ga Cos7-rakkudes ja NS3 koekspressioon NS2-ga Cos7- ja Huh7-rakkudes andis tulemusena NS3 lokalisatsiooni märkimisväärse muutuse membraanistruktuuride kasuks perinukleaarregioonis. NS2 ja NS3 ekspressioon eraldi või ühe polüproteiinina ei mõjutanud valkude lokalisatsiooni rakus. NS2 ja NS3 interaktsioon tõestati NS2/NS3 kompleksi immunosadestamisega nii Cos7- kui Huh7-rakkudest.

Järeldused

1. Levinum HCV alatüüp Eestis on 1b (64% RFLP-analüüsiga); on ka 3a (22%) alatüüpi; vähesel määral leidub 2a, 1a ja 4 geno(ala)tüüpide esindajaid. 3a alatüübi osakaal alla 30-aastaste ja süstivate narkomaanide seas oli suurem, võrreldes HCV-infitseeritute üldpopulatsiooniga.
2. HCV alatüüpide üldine jaotumus pole kahel uuritud ajavahemikul (1997–1998 ja 2000–2004) muutunud. Sagedasem segainfektsioonide detekteerimine (2,6% aastail 2000–2004, võrreldes 0%-ga aastail 1997–1998) võib peegeldada viiruse mitme alatüübi esinemist uuritud seerumites ning viitab mitmest allikast saadud HCV-infektsioonile.
3. Alatüübispetsiifiline PCR- ja RFLP-analüüs andsid kõrge detekteerimisresolutsiooni uuritavatest HCV RNA-d sisaldavatest seerumitest (positiivne tulemus vastavalt 93% ja 94%). Võrreldes alatüübispetsiifilise PCR-meetodiga, on RFLP-analüüs HCV genotüpeerimiseks usaldusväärsem. Et PCR alatüübispetsiifiliste praimeritega annab kaheldava tähendusega tulemusi segainfektsioonide näol (14%), on RFLP-meetodiga saadud tulemus kindlam ja kergemini interpreteeritav.
4. Kroonilise C-hepatiidiga viirusevastast ravi mittesaanud patsientidel oli sageli ANA (14%) ja SMA (43%). Enamusjuhtudel esinesid autoantikehad madalas tiitris. Võrreldes Eesti üldpopulatsiooniga, oli erinevate autoantikehade esinemissagedus kroonilise C-hepatiidiga haigetel oluliselt kõrgem. Autoantikehade jaotumus meestel ja naistel ning erinevate HCV genotüüpidega infitseeritud patsientidel ei erinenud.
5. Valk-valk-interaktsiooni olemasolu HCV NS2- ja NS3-proteaaside vahel tõestati kahes rakuliinis (Cos7 ja Huh7). Kompleksi moodustumine *in vivo*

ei sõltunund valkude ekspressiooni viisist ja/või ekspressiooniks kasutatud rakuliinist, mis viitab NS2 ja NS3 otsesele interaktsioonile.

6. HCV replikaasivalkude uurimisel on väärtuslikuks meetodiks E2 epitoopliidese lisamine, sel on kõrge spetsiifilisus ja märkimisväärne signaal/foonsuhe. BPV-1 E2- valgust pärinevad epitoopliidesed koos vastavate monoklonaalsete antikehadega lubasid identifitseerida HCV valke immunoblottidel, uurida valkude rakulist lokalisatsiooni ning teostada immunosadestamist kõrge efektiivsusega. E2 epitoobi lisamine HCV NS2-valgu N-terminusesse ei mõjutanud NS2/NS3 proteaasi aktiivsust.

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Teadustöö

Peamiseks uurimisvaldkonnaks on positiivsete RNA-viiruste (Semliki Forest-viirus, C-hepatiidi viirus) replikatsioonimehhanismid.

Kutseorganisatsioonid

Eesti Gastroenteroloogide Seltsi liige