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Study of Resistance in Hepatitis C Virus Prior to Treatment with Direct Acting Antivirals

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Abstract

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The rapid advancement of Hepatitis C (HCV) treatment presents a great challenge to clinicians in optimising therapy for their patients. Genotype (GT), efficacy, side-effects, drug combinations and treatment durations must be tailored to individual patients, considering comorbidities, degree of fibrosis, adherence and antiviral resistance.

Resistance associated substitutions (RASs) may impair treatment response to direct-acting antiviral agents (DAA). Almost all patients who fail treatment acquire RASs that may persist for years. Even treatment-naïve patients can harbour naturally occurring RASs against currently approved DAAs, i.e. resistance at baseline. Prevalence of key NS3 and NS5A-RASs is relatively high (3-9%) at baseline for DAA-treatment-naïve GT1a and 3a patients with population sequencing at 20% cut-off in Sweden and Norway.

The studies in this thesis comprise investigations on the prevalence and the effects of baseline RASs on treatment outcome in patients with HCV GT1 and GT3 receiving personalised treatment based on results from NS3 and NS5A resistance testing. We developed a pan-genotypic population sequencing method for detecting NS5A RASs (Paper I), which is certified and used in routine diagnostics at our laboratory together with our previously developed NS3 RAS sequencing method. We acquired data on RAS prevalence and treatment outcome from the early DAA management and carried out a non-randomised, prospective real-life study seeking to examine the impact on treatment outcome in patients receiving treatment tailored to baseline resistance testing.

The studies were carried out between 2011 and 2017, one retrospective study comprising patients in the Uppsala region (Paper II) and two prospective studies with patients in a multicentre study involving sites in both in Sweden and Norway (Paper III and IV).

RAS prevalence data from the prospective studies was obtained from a total of 401 patients and was shown to be slightly lower than reported from previous studies. Still, although not statistically significant due to the low prevalence of RASs in the cohort, we could show that there was a trend toward tailoring treatment to baseline RAS testing has a favourable impact on treatment outcome over treatment according to standard recommendations, especially in patients with cirrhosis. The economical and best practise objectives were important factors to consider when treatment costs were high and adverse effects were challenging at the initiation of the studies.

In summary, this doctoral thesis presents results from real-life studies that indicate that tailoring treatment based on baseline RAS-testing have beneficial impact on patients that are treatment experienced and/or patients with cirrhosis.

Keywords: Hepatitis C, HCV, DAA, RAS, resistance-associated substitution, baseline resistance, NS5A, NS3, SVR

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To my surprise

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I Lindstrom I, Kjellin M, Palanisamy N, Bondeson K, Wesslen L, Lannergård A, Lennerstrand J. (2015) Prevalence of polymorphisms with significant resistance to NS5A inhibitors in treatment-naive patients with Hepatitis C virus genotype 1a and 3a in Sweden. *Infectious Disease*, 2015 Aug;47(8):555-62.
- II Kjellin M, Wesslén T, Löfblad E, Lennerstrand J, Lannergård A. (2018) The effect of the first generation HCV-protease inhibitors boceprevir and telaprevir in relation to baseline NS3 resistance mutations in a small Swedish-cohort. *Ups J Med Sci*. 2018 Mar 14:1-7.
- III Kileng H, Kjellin M, Akaberi D, Bergfors A, Duberg A.S., Wesslén L, Danielsson A, Gangsoy M, Gutteberg T, Goll R, Lannergård A, Lennerstrand J. (2018) Personalized treatment of hepatitis C genotype 1a in Norway and Sweden 2014-2016: a study of treatment outcome in patients with or without resistance-based DAA-therapy. *Scand J Gastroenterol*. 2018 Nov 5:1-7.
- IV Kjellin M, Kileng H, Akaberi D, Palanisamy N, Duberg A.S., Danielsson A, Gangsoy M, Nöjd J, Aleman S, Gutteberg T, Goll R, Lannergård A, Lennerstrand J. Effect of the baseline Y93H resistance-associated substitution in HCV genotype 3 for direct-acting antiviral treatment: real-life experience from a multicenter study in Sweden and Norway. *Scand J Gastroenterol*. 2019 Aug 19:1-9.

Reprints were made for Paper I with permission from the publisher.

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Abbreviations

ALT	alanine aminotransferase
BOC	boceprevir
cDNA	complementary DNA
DAA	direct-acting antiviral
DCV	daclatasvir
DNA	deoxyribonucleic acid
EBR	elbasvir
GLE	glecaprevir
GT	genotype
GZR	grazoprevir
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	Hepatitis C virus
HVR	hypervariable region
HIV	human immunodeficiency virus
IFN	interferon
IL	interleukin
kPa	kilopascal
LDV	ledipasvir
LloD	lower limit of detection
LOQ	limit of quantification
MD	medical doctor
NGS	next generation sequencing
NI	nucleos(t)ide inhibitors
NNI	non-nucleoside inhibitors

NS	non-structural
OBV	ombitasvir
PCR	polymerase chain reaction
PI	protease inhibitor
PIB	pibrentasvir
PTV	paritaprevir
PWID	people who inject drugs
Q	quarter
RAS	resistance-associated substitution
RBV	ribavirin
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RT	reverse transcriptase
SIM	simeprevir
SOC	standard of care
SOF	sofosbuvir
SVR	sustained virologic response
SVR12	sustained virologic response 12 weeks after end of treatment
TVR	telaprevir
VEL	velpatasvir
VOX	voxilaprevir
WHO	World Health Organization
WT	wildtype

Introduction

Background

Hepatitis C virus (HCV) is a major health concern worldwide with estimated 71 million people chronically infected. Of these, 20% may subsequently develop liver cirrhosis and a further 5% of the cirrhotic patients will develop hepatocellular carcinoma. Every year, almost four hundred thousand deaths are attributed to the consequences of HCV infection and it is one of the primary causes of liver transplantation (1).

While there is still no vaccine against HCV, the introduction of direct acting antivirals (DAAs) in the HCV field, has recently made cure feasible. However, cure rates vary by geography, genotype as well as individual host treatment response. Also, as with most other antimicrobial agents, DAA efficiency may be compromised due to development of drug resistance amid several other factors affecting treatment outcome.

In a global perspective, treatment is largely still only granted to a minority of patients in the western industrialised world. For more efficient treatment and possible eradication of HCV (in the lack of vaccine development) better HCV-screening strategies must be performed in combination with a wide distribution of generic drugs that are easily accessible and cost-effective to treat the currently infected.

Epidemiology of HCV Infection

Dissemination and transmission

HCV is a bloodborne RNA virus which causes both acute and chronic infection with symptoms ranging from asymptomatic in the acute phase to severe liver disease and cancer in the chronic phase, decades after infection. Three major transmission routes can be discerned for HCV dissemination. The most common route world-wide is through exposure to blood or blood contaminated products such as unsafe drug injection practices in intravenous drug users and occupational exposure. In the lower income countries, HCV is mainly transmitted by transfusion of unscreened blood or blood products and through unsterilised needles and syringes for health care purposes. A third major route is sexual transmission in HIV-positive men who have sex with men (MSM).

Heterosexual transmission and maternal-foetus transmission are much less common (2,3).

Following the acute infection, approximately 55-85% of HCV infected individuals will fail to clear the virus and progress to chronic disease with elevated risk of developing cirrhosis and hepatocellular carcinoma (HCC), several decades post-infection. The prevalence numbers of chronically infected are however likely to be underestimated since both the acute phase and the first decades of chronic infection is often asymptomatic, increasing the risk of serving continuous dispersion of new infections.

Global prevalence and distribution of HCV genotypes

HCV is divided into eight distinct genotypes (GT1-8) and >90 subtypes (4,5). The 71 million chronically infected translates to around 1% of the total population with approximately 23.7 new infections per 100 000 which give around three million new infections every year (1).

Due to varying screening and reporting practices, the number of unrecorded infections and the variable proportion of treated versus newly infected, deducting HCV prevalence and genotype distribution worldwide is a daunting task and the figures should be interpreted as rough estimates of a dynamic situation. Studies show that distribution of the different genotypes varies greatly with considerable differences between regions and countries (6,7). Furthermore, epidemic HCV strains can be distinguished from the more endemic strains in epidemiology and prevalence. The epidemic strains are typically spreading through contaminated blood-products, medical equipment or intravenous drug use, such as the most prevalent GTs 1a, 1b, 2 and 3a seen in the industrialised world. Contrarily, the endemic strains are associated with locally transmitted, rare genotypes, primarily seen in Africa and South Asia (7,8).

The overall prevalence is lowest in the Northern European countries (except Russia) and in the North Americas with around 1% prevalence. Higher rates are reported in South and East Asian countries, except for Japan with similar rates as in the North Americas. In contrast, higher rates have been recorded in Russia, Pakistan, Mongolia and in many African nations with the highest prevalence of, as high as 15% in Egypt (4,6,8,9).

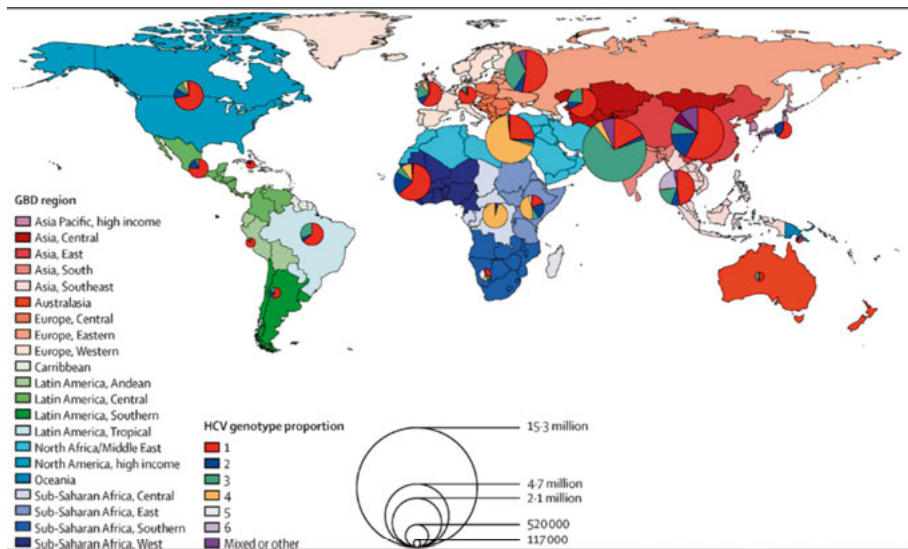


Figure 1. Worldwide distribution of HCV genotypes. <https://www.ncbi.nlm.nih.gov/books/NBK531718/figure/ch2f1/> The Polaris Observatory HCV Collaborators. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study (9).

GT1a (~50%) and 3a (~30%) are by far the two most widespread genotypes, followed by genotype 2 (~10%) (8). In Europe, nearly 90% of all infections are with GT 1, 2 and 3. GT1a and 3a are widespread in North Western Europe and in the United States and are mostly associated with intravenous drug use. As such, GT3a once originated from the Indian subcontinent where it still is the predominant genotype in India, Pakistan and Malaysia but in later decades is thought to have spread from this region to now being one of the most prevalent amongst people who inject drugs (PWID) in the industrialised world (6,10). As a matter of fact, the major transmission route of all HCV in the industrialised countries is attributed to intravenous drug abuse (11).

GT1b with unknown etiology is mostly found in elderly people in the Mid- and South Europe and Japan. GT2 and its several subtypes are also mostly found in an older population in the Mediterranean region and East Asia. The most common genotypes in Japan are 1b and 2a and b with unclear mode of dissemination (4,12). However, the prevalence of HCV antibodies in the Japanese population is increasing with age from zero in the younger population to more than 30% in elderly >60 years, leading to a speculation that HCV was once spread through anti-Shistosomal injections with non-sterile equipment, which was common practice in Japan from 1920-ies to 1970-ies, as well as treatment of traumatized soldiers with intravenously administered methamphetamine after World War II (13,14).

GT4 and its subtypes are mostly spread in the Middle East and Northern Africa with GT4a having its main source in Egypt, transmitted also there with

mass administration of anti-Shistosomal injections from 1950-ies through 1980-ies (15). GT5 with only one recognized subtype, 5a is found in South Africa whilst only a few cases of genotype 7a has been documented in Central Africa. GT6 is prevalent in East and Southeast Asia with a frequency as high as 10-20% in some areas (4,12). Finally, a recent report described a novel GT8 in patients of Indian origin (5). Undoubtedly, genotype distribution worldwide and in specified areas is a dynamic issue due to migration, treatment availability and unrecorded statistics. For example, it is apparent that there is information about genotype frequency only in about 60% of the world's countries (7). Furthermore, in recent years, in high income countries with readily available treatment, HCV prevalence has decreased whereas parts of Central Africa and Central Asia has seen a significant increase in HCV infections (6).

Prevalence of HCV infection in Norway and Sweden

Spread of HCV in Sweden began in the 1970-ies with the increasing use of intravenous drugs. Studies have estimated the prevalence of HCV in Sweden to 0.3 – 0.5 %, which would mean from about 33000 or up to about 45000 infected, also accounting for the unrecognised cases (16,17). HCV infection in PWID in Sweden are among the highest in Europe with up to 81-90% infected and with predominantly GT1a and GT3a, in that order of frequency (18–20).

In Norway, there is no collected nation-wide account for the prevalence of HCV. One population-based report from 2003 estimated the prevalence of viraemic HCV infected patients to 0.5% of the adult population in Oslo (21) and a similar report from the Tromsø area reported 0.2% prevalence (22). The most prevalent GT in Norway is GT3a and some reports have deduced that GT3a is prevalent in up to 50% of all the HCV positive patients in Norway (23–25).

The HCV genome and its genetic heterogeneity

The HCV genome

HCV is a small single-stranded, enveloped RNA virus belonging to the *Hepacivirus* genus within the *Flaviviridae* family where HCV is the only known member infecting humans.

The 9.6 kb long positive stranded RNA genome contains one single ORF flanked at the 5' and 3' termini by untranslated regions (UTR) vital in viral replication and translation. The internal ribosome entry site (IRES)-mediated translation produces one polyprotein of approximately 3000 amino acids which is cleaved by both viral and host proteases resulting in three structural proteins that make up the viral particle, core and the envelope proteins E1 and

E2, and seven non-structural proteins that are involved in replication and virion assembly, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Figure 2a) (26).

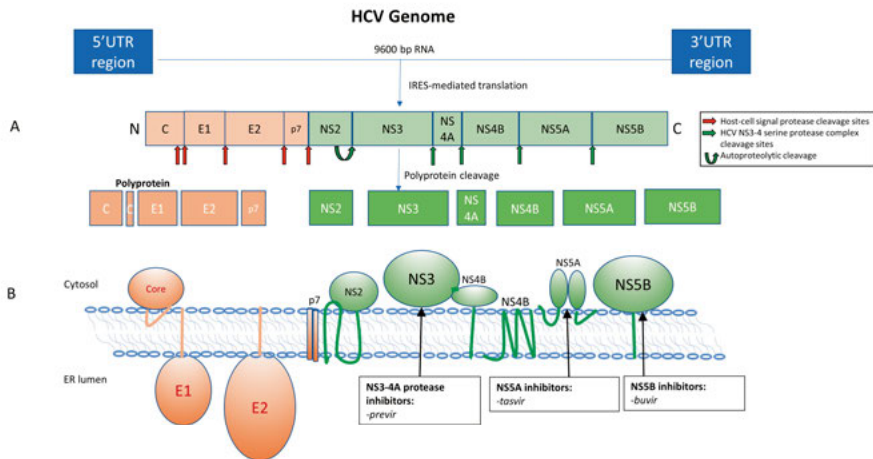


Figure 2. HCV genome organization. *A.* The 9600 bp long (+)-sense RNA HCV genome is flanked by internal ribosome entry site (IRES) which mediates translation into one single polyprotein of about 3000 amino acids. This polyprotein is cleaved by both cellular (red arrows) and viral (green arrows) proteases. The viral particle is made up by three structural proteins, core, surface proteins E1, E2 and the p7 protein which acts like an ion channel (orange) which are cleaved by host cell endoplasmic reticulum signal proteases. The non-structural proteins (green) are cleaved by the viral NS2-protein, which also cleaves itself, and the NS3-4A proteases. *B.* Schematic representation of viral proteins in relation to the host cell membranous web and the targets of the direct acting antivirals and their generic suffixes. Adapted from Lindenbach et al Nature 2005 (27) and Preciado et al World Journal of Gastroenterology 2014 (28).

The 30 nm nucleocapsid is enveloped by a lipid membrane derived from host endoplasmic reticulum (ER) and is studded by the viral glycosylated surface proteins E1 and E2 which contain host cell receptors and are thus involved in viral entry. E1 and E2 are the two most variable proteins and contribute in HCV immune evasion. P7 functions as an ion channel involved in translocation of the proteolytic NS2, to the cellular membranous web surface. NS2 is a protease cleaving itself from NS3 which in formation with cofactor NS4A functions as a protease complex, catalysing the cleavage of the polyprotein into the non-structural proteins. NS3 also exerts helicase activity. NS4B is a small hydrophobic protein that is involved in forming and anchoring the viral replication complex to the surface of the ER. Still to be completely elucidated, is the role of NS5A which is known to be essential for viral replication and assembly. The NS5B

is the HCV RNA dependent RNA polymerase (RdRp) which lacks proofreading and error correcting functions, resulting in highly error prone replication of the HCV genome to which the high mutation rate and heterogeneity of HCV can be attributed (28–34).

HCV replication has been shown to have a rapid turn-over rate to approximately 10^{12} virions per day, a half-life of two to five hours and a mutation rate due to replication errors of 10^{-3} to 10^{-5} per nucleotide per genomic replication (35–37). Like most RNA viruses, mutations arise mostly by chance due to an error prone RdRp which in addition lacks proofreading functions. This ultimately contributes to how the virus evade host immune response.

Even by RNA virus standards, HCV has an extremely high mutation rate. This may explain the large variability of HCV and the classification of it into eight distinct genotypes as per 2019, each of which diverges about 30% from another at the nucleotide level. The number of genotypes is likely to increase as novel genotypes are continuing to be described. Within each genotype, HCV is classified into subtypes diverging about 15% and there are now more than 90 subtypes identified (4,5,38).

Furthermore, it has been observed that even within one individual patient, multiple genetically distinct but closely related viral populations exist and is described as the *HCV quasispecies*. The populations making up the quasispecies are continuously evolving and may diverge up to 10%, helping the virus evade host (humoral) immune response, leading to persistency (4,39,40).

In addition, it has been suggested that different modes of transmission may differ in both the composition of virus inoculum at the moment of infection and subsequent evolution of HCV quasispecies. Risk group populations e.g. PWIDs and MSMs more often may transmit HCV while still in the acute phase, possibly spreading more virulent virus, while other transmission events, e.g. transfusion with contaminated blood, assuming from a chronically infected donor, may give rise to less virulent HCV variants (41).

Not surprisingly, the HCV genetic variability is heterogeneously spread along the genome, the most prominent sites being in the region coding for the membrane surface glycoproteins E1 and E2 with only up to 50% identity between isolates within the same GT in especially hypervariable parts (39,42). In contrast, the non-structural region and regions coding for the major structural domains in the 5'- and 3'-NCR regions are the most conserved with up to 90% identity between isolates (42,43).

The HCV infection

HCV life cycle and acute HCV infection

HCV entrance occurs primarily parenterally. Following entrance into the bloodstream, HCV binds to the receptor complex on the surface of the hepatocytes, where subsequently much of the HCV lifecycle depends on interactions with host lipid metabolism. Unique to HCV, the highly complex entry into hepatocytes involves binding to Low-density Lipoproteins (LDL) (44). Although the mechanism by which the virion fuses with the lipid to *lipovirion particles* is still not completely understood, nor the exact function for this fusion, it has been shown that the HCV virion consists of up to 30% of lipoprotein and that this plays a central role in its infectivity (44,45).

Decapsidation of viral nucleocapsids releases the positive strand genomic RNAs into the cell cytoplasm. Translation occurs at the membranes of the ER and requires host proteins to interact with HCV core and non-structural proteins. The resulting HCV polyprotein is cleaved by both host cell proteases and HCV NS2 and NS3/4A serine protease to produce three structural and seven non-structural proteins (Figure 2) (28,45).

Viral clearance or progression to chronic infection

In 50-85 % of the patients, HCV infection leads to chronic, persistent infection. The outcome between clearance and progression of chronic infection is not completely understood but it involves a complex matrix of multiple factors consisting of viral characteristics, host genetics and immune response. Other factors that have been shown to be associated with affecting outcome of HCV infection includes gender, age at infection, co-morbidity with certain metabolic disorders, diabetes, co-infection with HBV or HIV (46,47). Evidences indicate that patients that have a fast decline in serum HCV-RNA in the acute phase and an icteric, symptomatic course have an increased chance to spontaneously clear the infection (48–52).

It has been shown that acute HCV infection is distinguished by a sharp peak in viral load followed by a rapid decline owing to initial host immune response. In spontaneous clearance, this decline propels the events leading to viral clearance. In cases that progress to chronicity a slower decrease is seen, leading to a plateau in HCV viral load, followed by a renewed increase in viral load (49).

HCV induces a relatively ineffective adaptive immune response. Because of HCV hypervariability, variants can persist by evading host cytotoxic T lymphocytes (CTL) and antibodies targeting regions of the viral envelope. Logically, it has been postulated that large numbers of HCV variants inoculated at the moment of infection would contribute to a higher risk of chronicity due to the probability that more variants evade the host immune response. In contrast,

a narrower range of variants make immune control easier and thus the chance of clearing the infection is higher. Moreover, the transmission route may also have implications, where transmission not involving direct blood to blood contact, and thus smaller virus inoculum would work in a more favourable way in terms of clearance (48,53).

Another outcome of the acute infection may also occur when HCV replicates only in the liver or peripheral blood mononuclear cells (PBMCs), but without detectable serum HCV RNA, so called occult HCV. Moreover, some of these patients may even be anti-HCV seronegative. This condition is also associated with chronic infection but with lower risk of developing liver deterioration (54).

HCV chronic infection and liver disease

Among the chronically infected patients, approximately 20% will develop cirrhosis. Because of constant inflammation due to the persistent infection, fibrosis of the liver may develop and slowly progress into cirrhosis, typically in a time range of 10-40 years. Liver cirrhosis is in turn a major risk factor of developing hepatocellular carcinoma (HCC), occurring in approximately 2-5% of the patients with cirrhosis. Globally, HCV is the second leading cause of all HCC cases (31%), only surpassed by chronic HBV infection (54%) (55-57).

Co-morbidities such as metabolic disorders, insulin resistance, obesity, male gender and older age at time of infection may accelerate progression and is further deteriorated by large alcohol consumption (56,58). Moreover, infection with GT3a is associated with development of steatosis and more rapid progression of the liver fibrosis (47,59-61).

Co-infection with HIV and/or HBV also increases the progression rate to fibrosis and cirrhosis. In the Western world, the population with highest transmission rates of HCV are PWIDs. This patient category is frequently infected with both HCV and HIV or HBV. They are also more vulnerable to abuse of alcohol as well as substance abuse and are therefore at greater risk of progressing to more rapid and severe course of disease by the cumulative effect of these cofactors (62).

Antiviral therapy

A historical overview

Since the first full genome sequence of HCV in 1989, research has progressed from blood donor screening programs but without means of treatment to interferon monotherapy with very low cure rates and severe side effects to where more than 95% are cured with today's modern direct acting antivirals (DAAs)

(Figure 3). This has incited World Health Organization (WHO) to set an ambitious goal to eradicate HCV by the year 2030.

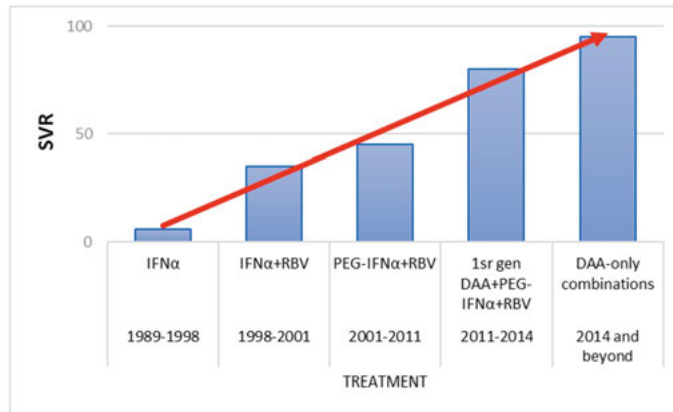


Figure 3. Progress in the treatment of HCV since the discovery of the virus in 1989., Rate of sustained virologic response (SVR), a term used to describe non-detectable viral RNA in blood, is set on the Y-axis.

Host interferons (IFN) are naturally occurring proteins, cytokines, that function as antivirals in two main ways; as inhibitors of virus replication by blocking cell surface receptors that are essential for viral replication and by regulating host immune response by activating immune cells and rendering already infected cells more susceptible to the activated immune system (63).

Even before HCV was first acknowledged 1989, it was shown that treatment with interferon-injections when administering synthetically produced interferon-alpha (IFN α) had an effect in normalisation of transaminases and improvement of liver histology (62). After the identification of HCV, the effect could also be shown in serum HCV RNA load (35,63).

In the beginning, IFN-injections had to be repeated with very short intervals, due to its short half-life, to obtain an even serum drug concentration. However, when IFN was coupled to polyethylene glycol (PEG) in 1998, which made its half-life longer PEG-IFN injections together with the use of ribavirin (RBV), a nucleoside analogue that is another non-specific antiviral, SVR rates increased to 45-55% (64,65). The PEG-IFN+RBV treatment administered 24 to 48 weeks became the standard of care (SOC) in treating HCV GT1a during 2001-2013. However, this regimen proved to provide a relatively low cure rate and was accompanied with several adverse side effects.

The first attempts in development of the first generation NS3 protease inhibitors resulted from large amount of work carried out by several multidisci-

plinary research groups over a period spanning more than a decade. A breakthrough in 2011 with the approval of the first-generation direct acting antiviral compounds granted a paradigm shift in HCV treatment.

Direct acting antivirals (DAAs)

Protease inhibitors

NS3-4A is a coupled protein-protein-complex with several functions essential for HCV replication in cleaving the HCV polyprotein and host proteins involved in the cellular immune response (28,66).

Boceprevir (BOC) and telaprevir (TVR) were the first two DAAs to be approved. Both bind to the active site of the targeted the NS3 (67–69). Both were administered as a triple combination regimen with PEG-IFN+RBV but had low barrier to resistance and were accompanied by serious adverse side effects. SVR rates, although higher than with the interferon-only based therapy, ranged from 40-75% depending on other factors affecting treatment outcome such as previous treatment history, cirrhosis and high viral load. Other limitations to these first-generation drugs were that the treatment was restricted to comprise only GT1 patients and at extremely high cost at around 0.5-1M SEK per treatment.

The second-wave drug simeprevir (SIM), differed from the first wave of the first-generation drugs in that it belonged to another class of protease inhibitors, being macrocyclic in structure as opposed to linear as BOC and TVR. SIM had an improved affinity and specificity for the target protein and had fewer side effects. Although, initially used in combination with PEG-IFN+RBV and only in GT1 patients, it wasn't until the first nucleoside analogue sofosbuvir (SOF) (discussed below) was introduced to the regimen and the PEG-IFN was abandoned, that the SVR rate and drug tolerability were really increased (70,71).

Other compounds of both the second and third generation NS3 inhibitors, e.g. paritaprevir (PTV), grazoprevir (GZR), pibrentasvir (PTV), voxilaprevir (VOX) respectively, exhibit similar properties, although compounds of the third generation inhibitors have been optimised to exert pan-genotypic activity, show decreased toxicity and are more unaffected by RASs (72).

NS5A inhibitors

The NS5A protein is comprised of approximately 447 amino acids forming three distinct domains. The N-terminal of domain I is the most conserved and best characterized and towards which the NS5A inhibitors are targeted (30,73,74).

However, enigmatic in function and seemingly lacking enzymatic activity, progress towards suitable NS5A inhibitors was hampered for a long time. Instead, a group of small molecule inhibitors were identified by an approach

based on chemical genetics, using a cell-based random high-throughput HCV replicon screening method by Lemm and colleagues (74). This method would subsequently identify daclatasvir (DCV) as the first direct acting NS5A inhibitor, after screening more than a million compounds against HCV replicons.

This method also offered suggestions on which mechanism of action NS5A inhibitors work by. They reduce the hyperphosphorylation and self-dimerization of NS5A, evidently inhibiting its function. Furthermore, analysing mutations which render the virus resistant to NS5A inhibitors, it could be shown that the first part of NS5A amino acid compositions 28-93 were important determinants for HCV replication susceptibility to NS5A inhibitors (74–77).

Other compounds of both the first and second generation NS5A inhibitors, e.g. ledipasvir (LDV), ombitasvir (OMB), elbasvir (ELB) and velpatasvir (VLP), respectively, exhibit similar properties, although compounds of the second-generation inhibitors have been optimised to exert pan-genotypic activity, show decreased toxicity and be more unaffected to RASs. (78).

NS5B inhibitors

The function of the HCV NS5B protein was relatively easily deciphered since its typical secondary structure is similar to that of other known viral RdRp (vRdRp) with a hand-like appearance with thumb, fingers and palm domains, as well as its enzymatic activity as a replicase (79).

There are two classes of NS5B inhibitors: Nucleos(t)ide and non-nucleoside inhibitors. Nucleos(t)ide inhibitors (NI) are also called chain terminators because by mimicking a natural nucleotide, they are incorporated into the growing RNA chain during replication and thereby effectively terminate the chain progress. The non-nucleoside inhibitors (NNI) unlike the NIs bind to typical right hand motif of the protein to inhibit polymerase activity with some kind of conformational mode of action (79,80).

The first and only approved NI, and still in use in some combination regimens is sofosbuvir, a uridine analogue. It has been established that the catalytic site of the vRdRp is highly conserved between genotypes. This explains sofosbuvir's pan-genotypic profile and with high genetic barrier to resistance with only rare occurrences of baseline and emerging RASs and which also renders the virus relatively unfit and soon replaced by more fit variants

The second class, the non-nucleoside inhibitors were discovered by the replicon assay method described previously. Dasabuvir (DSV) and beclabuvir (BSV), are approved for treating primarily GT1a and 1b (BSV only in Japan), have lower barrier to resistance due to the variability of the binding sites, and consequently do poorly as pan-genotypic inhibitors (79,80).

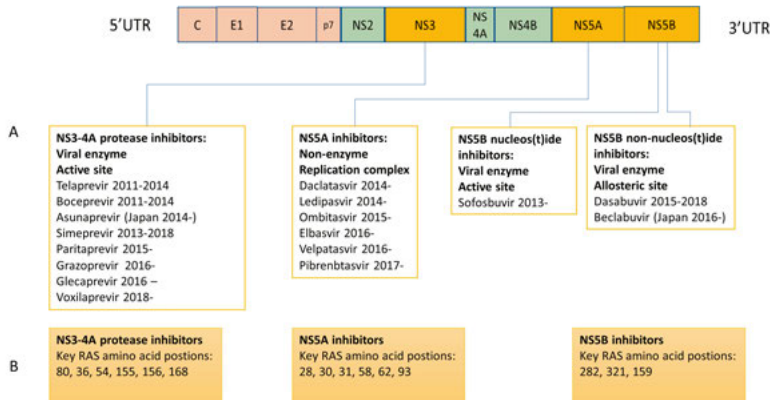


Figure 4. Previous and current DAAs and their key RASs.

Treatment recommendations

HCV genotyping

A crucial prerequisite tailoring treatment against HCV with DAAs is that the correct genotype, including subtype, is determined. Many treatment failures have occurred due to inaccurate genotyping and subtyping (81,82).

Numerous methods, both commercial and in-house, to determine HCV genotype in clinical samples are available. The most common methods, which also have the highest specificity and sensitivity, albeit more laborious and/or are more resource-demanding, are based on amplification and sequencing of either the whole genome or a conserved part of a suitable gene, e.g. NS5B, and subsequent phylogenetic analysis.

Other approaches, many of which intended to streamline the typing procedure, uses detection of genotype-specific markers, including restriction fragment length polymorphisms where enzymes are used to recognize genotypic cleavage sites in PCR-amplified products as well as line probe assays (LiPa), where PCR-products are hybridised to genotype-specific probes fixed on nitrocellulose strips (38,83,84).

Certainly, the heterogeneity of the HCV genome poses a challenge to any method intended to type HCV variants. While sequencing of HCV remains the golden standard for genotyping, it may not be readily available to all settings where HCV treatment is in demand and therefore novel, simple and cost-effective, yet accurate genotyping methods are still very much needed.

Negative predictive factors

Despite as high as 95% SVR rates with present day modern DAAs reported in clinical trials and real-life studies, the remaining ~5% of the patients who fail

treatment translates to millions of patients. Most failures today are observed in cirrhotic patients and treatment experienced GT3 patients.

With the early DAA regimens however, other factors were observed as unfavourable, including high viral load, HIV coinfection, and the presence of RAS, with the latter particularly true when multiple unfavourable factors were present concomitantly (85,86).

During the interferon-based treatment era, GT1a was considered the most difficult genotype to treat. However, since the introduction of DAAs, even if they are efficient towards most genotypes, infection with GT3 has the lowest SVR rates. GT3 infection is also associated with steatosis and more rapid liver disease progression (61,87,88).

The identification and understanding of predictive factors enable tailoring and individualising therapy with the objective to increase response rates in optimising treatment duration to minimise failure due to non-adherence and ensuing resistance-development.

HCV resistance to DAAs

Resistance-associated substitutions (RASs)

Resistance to DAAs is driven by selection of mutations at different positions in the gene targets, i.e. NS3, NS5A and NS5B, that renders the mutant more viable under drug pressure. The factors that determines resistance to any given DAA depends primarily on the genetic barrier to resistance, fitness of the resistant viral population, the potency of the drug and host response. Because of the quasispecies nature of HCV, and the rate at which HCV replicates, some of the polymorphisms that arises with every replication cycle will induce amino acid changes that also changes the phenotype conferring resistance at some level against a given DAA even without exposure, just by chance. Virus populations harbouring one or more of these RASs have fitness levels close to the wildtype virus and may accumulate through selection or emerge during treatment, especially under suboptimal conditions (89,90).

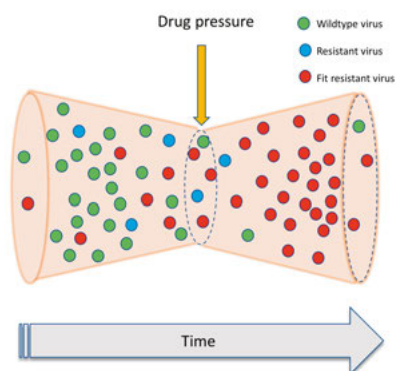


Figure 5. Development of drug resistance. Without drug pressure, wildtype susceptible virus variants are usually the fittest. During selection pressure exerted by a drug, variants harbouring resistant associated substitutions are selected. Their survival and growth depend more on their fitness in the presence of the drug than the level of resistance of the substitution(s), so that when the selective pressure of the drug is ceased, the remaining dominant population is the fittest, but not necessarily the population harbouring the highest level of resistance (89).

Viral factors

HCV genotypes and subtypes have been established to be a key determinant of DAA efficacy (38,91). This is partly due to certain geno- and subtypes are harbouring naturally occurring polymorphisms in greater frequency that confer resistance toward DAAs. Furthermore, different genotypes exhibit various predisposition to the number and type of substitutions necessary to induce resistance associated substitutions at a locus, which is defined as the genetic barrier (92). One single nucleotide substitution can be sufficient to change the amino acid at one codon to another in one genotype (low genetic barrier) while it may require two or three nucleotide changes in another genotype to generate a resistant associated amino acid (high genetic barrier) (93). Another factor defining the genetic barrier is the type of substitution that may occur. The NS5B RNA dependent RNA polymerase has been shown to make nucleotide transitions more frequently than transversions, a feature that may explain why some RASs are rarer than others (36,89). The S282T is an example of a substitution with high genetic barrier to resistance due to the type of substitution it must make in order to become a RAS. This change also entails a severe decrease in fitness from 100% in wt to only 2-5% in the variant harbouring the substitution. As a consequence, this decrease in fitness leads to a short half-life of about three days (94).

Additionally, RASs in the different genes confer resistance at different levels and remain in various extent in the host due to their corresponding fitness.

Studies have shown that RASs in the NS5A gene are highly stable and retain fitness level close to wt, rendering the variants persistent for long time, perhaps forever while NS3 RASs are relatively short-lived and due to reduced fitness lost within months (95).

Variants harbouring multiple RASs and in more than one gene have frequently been detected in DAA treatment-failing patients and displays a complex pattern involving genotype, subtype and the DAAs. In addition, many combinations of multiple RASs seem to add a cumulative resistance and confer higher level of resistance compared to their corresponding single-RASs in separate (82,96–98).

Host factors

Factors influencing treatment outcome and emerging resistance has been revised along with the development of new DAAs. Still valid and possibly the most important factors are patient adherence and severity of liver disease.

In the pre-DAA era, and with the first generation DAAs and PEG-IFN+RBV triple combination treatment, host genotype in the interleukin 28 beta (IL28B) gene was considered an important factor in patient responsiveness to IFN treatment (99–101). However, with the later generations of DAAs, the impact of host IL28B genotype on treatment outcome has been moderated although its predictive value is still elusive.

Other host factors that have been associated with poorer treatment outcome with DAAs are male gender, high BMI, comorbidities including diabetes mellitus, HIV coinfection, and non-responders to PEG-IFN+RBV treatment (91).

Prevalence of naturally occurring RASs

The prevalence of pre-existing RAS among patients with chronic HCV varies considerably depending on genotype, target gene and geographic variation (37,102). The actual prevalence of naturally occurring RASs is difficult to establish due to several concurring factors that are yet to be solved.

The lack of practice to test for baseline resistance is one factor. Apart from one instance regarding GT1a patients planning to be treated with elbasvir/grazoprevir (EBR/GZR) who are recommended to perform NS5A resistance analysis to deduce whether to prolong treatment and or add RBV, there is no consensus to test if testing facility is provided (95). However, the lack of standardisation of RAS detection methods has led to biased and arbitrary information about the prevalence of pre-existing RASs as the detection sensitivity (with different cut-off levels) and specificity depends on the performance of used method. Other factors affecting data are timing of the sampling, varying reporting customs and lack of an international consensus of clinically relevant RASs (103–106).

Since 2018, there has been an international collaborative effort of setting up a complete database of HCV sequences, where both pre-existing and treatment-acquired RASs, treatment histories and health outcome related to HCV is under construction in Surveillance of Hepatitis-C Antiviral Resistance, Epidemiology and methodologies, SHARED. The objective is to use this database to characterise resistance patterns in DAA failures and rare genotypes, to develop a consensus interpretation algorithm and provide a comprehensive and standardised collection of protocols, software and literature (107).

Clinical relevance of RASs

A paradoxical consequence of the high efficacy of the antiviral treatment, is the limited data from treatment failures. Indeed, repeated observations have been made where presence of RASs are involved in treatment failures, but most often in concert with other negative predictive factors. Yet, treatment failures also occur in the absence of neither detectable RASs nor known negative factors. Thus, it poses a challenge to assess the true clinical impact of RASs in treatment failure. Furthermore, RASs are defined as RASs in a pattern depending on in-vitro fold resistance of the RAS which in turn depend on DAA and genotype (Figure 6 and 7).

RASs are detected in nearly 100% of patients experiencing viral breakthrough, but in patients who relapses, RASs are detected in approximately 50-90% depending on genotype, DAA regimen and with which method RAS analyses has been used. Moreover, the quasispecies dynamics in the patient at the time of sampling presents only a snapshot of a rapidly revolving setting in terms of RASs, where low frequency RASs, e.g. S282T are quickly disappearing and are replaced by wt or other variants (104,108).

Currently, there are no concluding studies that indicate the necessity of baseline RAS testing, except in the case of GT1a for baseline Q80K in simeprevir-based regimes and certain NS5A RASs in elbasvir-based regimens. However, simeprevir is no longer used in today's treatment regimes. Furthermore, the baseline Y93H is considered important on treatment outcome of VEL/SOF in GT3 patients. Nonetheless, the presence of Y93H does not appear to have significant impact on VEL-based treatment outcome except for cirrhotic patients (109,110)

Class	Antiviral Potency	Genotype Activity	Resistance Barrier	EMA/FDA Approvals
NS3 Protease Inhibitors	Intermediate to High	1, 4 (± 2, 3, 6)	Low to High ↓	Simeprevir (2013) Paritaprevir (2014) Grazoprevir (2016) Voxilaprevir (2017) Glecaprevir (2017) Voxilaprevir (2018)
N55B Nucleotide	High	1-6	Very High	Sofosbuvir (2013)
N55B Nonnucleoside	Low	1	Low	Dasabuvir (2014)
N55A Inhibitors	High	1, 4, 6 (± 2, 3)	Low To High ↓	Ledipasvir (2014) Daclatasvir (2015) Ombitasvir (2014) Elbasvir (2016) Velpatasvir (2016) Pibrentasvir (2017)

Figure 6. Resistance characteristics of HCV antiviral classes (clinicaloptions.com)

RASs in NS3

The NS3 inhibitors are relatively susceptible to resistance and cross-resistance is common. Key NS3 inhibitor RASs with fold resistance are shown in Figure 7 A. These resistant variants have usually reduced fitness in an inverse correlation between fold resistance and fitness. This may explain why they are rarely detected as they are rapidly outnumbered by the wt virus when drug pressure is retracted (111). The resistant variants, with the exception of R155, are thus lost in relatively short time period in the order of magnitude of months. Their natural prevalence in GT1 patients is relatively low, 0.1 to 3.1% (92,112,113). One exception is RAS Q80K, which doesn't render much reduction in fitness but was associated with only a moderate 10-fold level resistance toward simeprevir in GT1a patients. On the contrary to other NS3 RASs at baseline, Q80K has high prevalence in North America and North Europe with up to 48% and 19%, respectively (114). Furthermore, key RAS D168Q, which confers high level resistance toward many NS3 inhibitors is the wt amino acid phenotype in the GT3a, which is why many of the first generation NS3 inhibitors were not suitable for treatment of GT3 patients.

In contrast, it has been shown that relative fitness increased in presence of two RAS, i.e., secondary mutations have been shown to restore fitness thereby compensate for the impairment on HCV replication by the primary RAS (115). Although the resistance barrier has improved in the second generation NS3 inhibitors, some of the RASs at amino acid positions 155, 156 and 168 still confer significant resistance (92,112,113,116).

RASs in NS5A

As with the NS3 inhibitors, first generation NS5A inhibitors too came with relatively low genetic barrier to resistance and extensive cross-resistance pattern (117). In the second-generation NS5A inhibitors, this have been amended to some extent and the genetic barrier to resistance have been increased. The prevalence of naturally occurring RASs in NS5A has been reported to range from 0.3 to 18% (86,102,118). It was shown that DCV had low barrier to resistance in mutations at amino acids at certain positions at the N-terminal of domain 1 in the protein; M28, Q/A30, L31 and Y93, depending on genotype (Figure 7 B) (118–120).

A notable feature in NS5A RASs is the relatively low effect on virus fitness thus prevalence of naturally occurring RAS are widespread and emerging RASs due to treatment failure may persist for years, perhaps forever in the majority of patients (89,121).

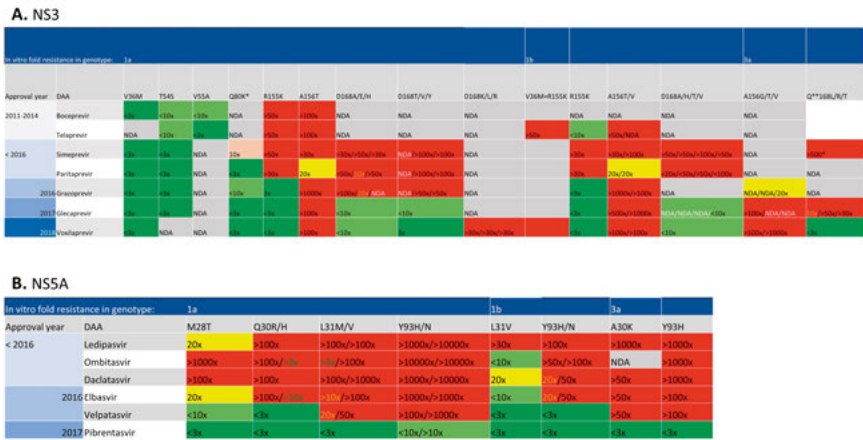


Figure 7. In vitro fold resistance of RASs of toward DAAs in EC₅₀ determined using replicon assays. Since propagating HCV in vitro is difficult and inefficient, sub-genomic replicons that efficiently replicate in a human hepatoma cell lines, was first constructed in 1999 (122). Using these, preclinical evaluation of potent drugs in development and measuring viral fitness is feasible and identification of mutations associated with drug susceptibility and resistance is possible in vitro (111).

NDA: No data available

*Q80K: Baseline prevalence of 5-10% at baseline for DAA treatment naive patients in Sweden and Norway, when using Sanger sequencing method (20% cut-off).

** Q168: Natural NS3 polymorphism of 100% at baseline for genotype 3a.

RASs in NS5B

Treatment failure from combinations containing sofosbuvir (SOF) is relatively rare since there are no or few naturally occurring RAS in NS5B. The reason for this is that substitutions conferring resistance to SOF also leads to loss of fitness. It has nevertheless been shown in replicon assays that some virions indeed exist with the S282T being the most important conferring resistance up to 20-fold depending on genotype (123). However, the S282T leads to a severe decrease of fitness from 100% in wt to only 2-5% in the variant harbouring the substitution (124). Therefore, this decrease in fitness leads to a very short half-life of a few days, which may make it almost impossible to detect with Sanger sequencing due to difficulties in the timing of the sampling. Consequently, this substitution has never been detected at baseline and only rarely in patients failing regimens which included SOF except in GT4r patients where according to a report up to 20% selected S282T after failing SOF-based treatment (125). Even so, variants harbouring this substitution have a severe decline in replicative fitness and thereby these RASs are eliminated within a week, making re-treatment with SOF based regimes a very good option (94,107).

Aims and hypothesis of the thesis

Treatment of Hepatitis C infection has undergone a paradigm shift with the introduction of direct acting antivirals (DAAs) leading to treatment efficacy of more than 95% cure rate. Although the market has seen a significant price reduction since the first DAA-based regimens in 2011 the cost of the new treatment is still high, and the relatively long treatment duration may jeopardise compliance.

Accordingly, development of resistance poses problems when inhibitors of especially NS3-protease and NS5A are used in sub-optimal conditions. HCV populations with naturally occurring and pre-existing RASs against DAAs may exist in treatment-naïve patients. Such NS3 and NS5A RASs with high fold resistance may predict, together with other negative factors (such as cirrhosis, high fibrosis stage), the efficacy of a treatment. It is therefore important to find the most cost-efficient combination of DAA treatment and reduce the treatment duration for an HCV-infected individual.

This thesis is a compilation of real-life studies conducted early in the DAA era. Many examples of discrepant data between registry studies and real-life studies have been reported. Hence real-life studies are still valuable in learning about the clinical relevance of drug resistance, knowledge to be used in future treatment strategies, particularly in less resourceful areas where these older but still useful generic drugs are more readily available.

Paper I

The aim of Paper I was firstly to develop a pan-genotypic NS5A resistance analysis method suitable for routine diagnostics use. Secondly, using this method, we sought to analyse the prevalence of baseline NS5A RASs in HCV-infected treatment-naïve patients in the Uppsala region of Sweden.

Paper II

The aims of papers II to IV comprises evaluations on the impact of baseline resistance on treatment outcome in real-world settings. Paper II is an early retrospective study on a small local cohort receiving triple therapy containing the first-generation NS3 protease inhibitors Boceprevir or Telaprevir, PEG-IFN and RBV between 2011-2013. Adverse effects and workload were two parameters also evaluated.

Paper III

We conducted a prospective, real-life, non-randomised, open-label multicentre study on the impact on treatment outcome when tailoring treatment according to the results from baseline RAS testing. In the presence of RAS, treatment is either prolonged and/or RBV is added at the discretion of the treating physician (MD).

The collaboration on interventional resistance testing was initiated by the boards in the Department of Infectious diseases in Uppsala and Gävle, Sweden and the Department of Gastroenterology in Tromsø, Norway. Thus, group affiliation was determined by site. Control groups were chosen by lead researchers at Uppsala and Tromsø and comprised patients from Bodø, Falun, Stockholm and Örebro.

The aim of paper III was to evaluate the impact of tailoring treatment according to presence of baseline NS3 RASs, especially RAS Q80K in a Nordic multicentre HCV genotype 1 cohort during 2014-2016. *Hypothesis:* The presence of baseline RASs may impair treatment response to DAAs in chronic HCV infection in GT1a.

Paper IV

The aim of paper IV was to evaluate the impact of tailoring treatment according to presence of baseline NS5A resistance, especially RAS Y93H in the Nordic multicentre HCV GT3 cohort during 2014-2017.

It was of particular interest to assess negative factors for treatment of GT3 since the global prevalence of GT3 is between 20 to 50%, and its association with more rapid progression of liver fibrosis, and subsequent increase of HCC.

Study population and methods

Samples

Paper I

A total of 127 plasma and serum samples used in this study are clinical specimens previously genotyped and subsequently stored in the Biobank in department of Clinical Microbiology, Akademiska Hospital, Uppsala. The samples had the following distribution GT1a = 55 (43%), GT1b = 14 (11%), GT2b = 23 (18%) and GT3a = 35 (28%) (which is in agreement to the normal distribution in the Uppsala region) (18).

Paper II

This retrospective study was conducted on 36 clinical samples from patients previously analysed for HCV RNA load and genotyped 1a and 1b and stored in the Biobank in department of Clinical Microbiology, Akademiska Hospital, Uppsala.

Paper III

In paper III, the study was conducted on HCV GT1a samples collected from sites in a joint Nordic multicentre study in Sweden and Norway between 2014 and 2016. In total, 95 eligible patient samples from Uppsala, Gävle and Tromsø in the intervention cohort, and 105 eligible samples in the control group from Bodø, Falun and Örebro were collected.

Paper IV

Paper IV was conducted on HCV GT3a patients and correspondingly to paper III, collected from sites in a joint Nordic multicentre study in Sweden and Norway between 2014 and 2017. Assessed for eligibility in the intervention group from Uppsala and Tromsø were 141 patients, while the control group were comprised of 85 patient samples from Bodø, Falun, Stockholm and Örebro.

Resistance analysis based on nested PCR and Sanger sequencing

Nucleic acid extraction

Total nucleic acid was extracted from 0.5mL of plasma or serum samples collected from HCV infected patients using the BioMérieux NucliSense®easyMAG® (BioMérieux, Marcy-l'Étoile, France). This method is based on the protocol first described by Boom et al (126). Lysis of the cells is accomplished by chaotropic agent guanidinium thiocyanate (GuSCN). In this environment the released nucleic acid binds to magnetic silica beads whereby the nucleic acid is purified in a series of washing steps. Elution is mediated by heat-deactivating the magnetic energy, releasing the purified nucleic acid to elution buffer.

Nucleic acid amplification and sequencing

The NS3 and NS5A amplification steps follow the same protocol, differing only in primer sequences, which are specified in Table 2. Parts of the HCV NS3 and NS5A genes, respectively, were amplified in two steps in nested PCRs, using outer and inner primer pairs.

In papers I to III, the synthesis of complementary DNA (cDNA) from purified HCV RNA was performed using SuperScript™ III Reverse Transcriptase (Invitrogen™, Thermo Fisher, Waltham, MA; USA) and random hexamers. First round PCR and nested PCR were performed with in-house primers targeting parts of the NS5A-regions using the *Taq* PCR Master Mix (QIAGEN, Hilden, Germany). The amplicons were verified by agarose-gel electrophoresis. PCR-positive samples were purified using QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany) before they were sent to Uppsala Genome Centre for sequencing by capillary electrophoresis (Sanger) method using the same 2nd primer pair used in the nested PCR.

Table 1. *Primer sequences for HCV NS3 and NS5A genes used for amplification in first and second rounds of nested PCR.*

Primer	Sequence 5' to 3'	Nucleotide position in HCV genome
NS3		
1 st Forward	AATAAATCATAAATCACSTGGGGRGCRGAYAC	3238-3258
1 st Reverse	AATAAATCATAAAAYTTGCCRTAKGTGGAG-TAYGT	4162-4185
2 nd Forward	AATAAATCATAAACSGCRGCRTGYGGGGACAT	3257-3276
2 nd Reverse	AATAAATCATAAGTGCTCTTRCCGCTRCCRG	3983-4004
NS5A		
1 st Forward	AATAAATCATAAGGGCDGTRCARTGGATGAAC	6073-6092
1 st Reverse	AATAAATCATAAGGMTCGAADGAG-TCMAGAAT	7095-7114
2 nd Forward	AATAAATCATAAGATGAACMGGCTSATMGC-STTCG	6086-6108
2 nd Reverse	AA-TAAATCATAACCCRTCCAMTTCWGTGAARAA YTC	6699-6722

The position in the HCV genome is based on the GT 1a reference sequence H77 (accession no. AF011751). The second-round primers are also used in the sequencing step. Amplification enhancer attached to the 5' of each primer is indicated with green letters: AATAAATCATAA.

In paper IV, the PCR amplification step preceding the sequencing as well as the PCR purification method was revised as of Q1 2017 in order to optimise the method. Synthesis of cDNA and first round of PCR was hence done using Takara PrimeScript™ One Step RT-PCR Kit Ver.2 (Takara BIO Inc, Kusatsu, Shiga prefecture, Japan). Nested PCR was performed as previously. The nested PCR products were verified by e-Gel® 2% agarose electrophoresis (Invitrogen, ThermoFischer Scientific, Waltham, MA, USA). Samples were purified using ExoSAP-IT™ (Applied Biosystems™, ThermoFischer Scientific, Waltham, MA, USA).

For samples analysed from Q3 2017 onward, the sequencing was switched to a certified facility at EurofinsGenomics, Ebersberg, Germany for capillary electrophoresis (Sanger) sequencing on 3730xl DNA Analyzer (Applied Biosystems™, ThermoFischer Scientific, Waltham, MA, USA).

Lower limit of detection (LloD) was determined to around 2-3000 IU/mL for both the NS3 and NS5A genes. As patients eligible for resistance testing are undeniably positive for HCV RNA and typically have intermediate to higher viral loads, this limit is regarded as satisfactory for this purpose. A situation when a patient sample has HCV RNA level below this limit could occur when a relapsing patient is sampled too soon after viral reappearance measured by more sensitive viral load assays. This is however easily amended by delaying sampling for another week since HCV viral load typically increases rapidly after relapse.

Sequence analysis and interpretation of RASs

Using SeqScape® Software v2.6 (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA), the forward and reverse nucleotide sequences of all samples, regardless of genotype were aligned to reference strain GT1a H77 (Accession number NC_004102.1) to generate consensus sequences of the viral quasispecies with a sensitivity of approximately 20% for (minority) variants, recognized as mixed peaks in the resulting electropherogram.

We used H77 as a generic reference strain to simplify the method as we considered this template suffice also for aligning other genotypes to yield consensus sequences as described in a previous report (18).

To distinguish clinically relevant substitutions and evaluate their implications, the consensus sequences were enquired in the web-based mutation detection algorithm, Geno2Pheno [hcv] 0.92 (G2P) (127). Substitutions scored by G2P were further interpreted as clinically relevant RASs by relating scores with current European Association for the Study of the Liver (EASL) guidelines 2016 (128) and 2018 (91), in addition to RASs reported to bear impact on DAA treatment outcome *in vitro* and/or *in vivo* in reference literatures (98,110,129).

In the papers included in this thesis, the NS3 RASs V36M, T54S, T55A, Q80K, R155K and D168G/A and NS5A RASs M/V28A, Q/A30H/K/R, L31M/I and Y93H/N, respectively were defined as clinically relevant to the specific drugs or drug combinations included in the papers.

Results and discussion

Prevalence of baseline NS5A RAS in GT1a, 1b and 3a in Sweden (Study I)

We successfully developed a multi-genotypic population-sequencing (Sanger) method with degenerated primers targeting the NS5A region.

Using this method, we investigated the prevalence of baseline RASs in 127 treatment-naïve patients of GT1a, 1b, 2b and 3a in the Uppsala region.

Our method successfully amplified and sequenced 107 (84%) of the tested samples, more specifically 96% of the GT1a, 100% of GT1b and 97% of GT3a samples but only 30% of GT2b emphasising the nucleotide sequence differences between genotypes and the challenges in developing a truly pan-genotypic sequencing method for this region. GT2b on the other hand, historically and currently, is not really associated with having problems with treatment failures attributed to the presence of RAS. Thus, RAS testing GT2b patients may be superfluous in most cases, provided that prevailing treatment recommendations are observed.

With nucleotide sequence variations up to 30% between genotypes, it is inevitable that some RASs almost always exists in majority in some genotypes thus defines the wildtype variants. Accordingly, we found that 100% (14/14) of the GT1b had Arginine at aa position 30 (R30) and five out of seven (71%) of the GT2b had Lysine (K30), both of which conferring medium to high level resistance to DCV, LDV, OBV, ELB and/or VLP (Figure 7). Furthermore, the majority of GT1b and GT2b were found to be H58P. In our study, all but one of the GT3a 33/34 (97%) were H58P and all (100%) were Q30A, which is the wildtype in GT3.

Interpretation of fold-resistance data against the contemporary NS5A inhibitors were done with the help of earlier published phenotypic data (Figure 7B) (90,98,110). Baseline NS5A RASs associated with high resistance (1 000 - 50 000-fold) to LDV and DCV was found in three patients: Q30H and Y93N in two GT1a patients, respectively and Y93H in one GT3a patient. These findings are in line with other prevalence reports.

Impact of baseline RAS testing on treatment outcome

First generation HCV PI BOC and TVR and NS3 mutations in GT1 patients (Study II)

For patients with GT1a and 1b first generation PI in combination with previous SOC treatment PEG/IFN + RBV was introduced as a new triple therapy against chronic HCV infection in Q3 2011. Both treatment-naïve and treatment-experienced patients were approved to attempt this new regimen. However, a number of factors intervening with treatment efficacy had already been described; patients with, HCV GT1a, liver cirrhosis, IL28B non-C/C genotype and previous non-response to SOC treatment (130). Nevertheless, treatment efficacy with the new triple therapy were promising and a two-fold increase of SVR rate was predicted. Moreover, adverse effects were at the time of approval expected to be acceptable but turned out to be significantly worsened in regard to severe rash and allergic reactions with TVR and anaemia and thrombocytopenia with BOC, hence a thorough assessment of such factors before start and during treatment was essential (131).

Furthermore, the resistance profiles of BOC and TVR were known and accounted for when monitoring viral load during treatment duration every four weeks. If viral RNA level increased, RAS could often be detected. In case RAS was found treatment was aborted. The incidence of emerging RAS in treatment failures was lower and half-life shorter in in GT1b than in GT1a with undetectable RAS at six months and 18 months in GT1b and GT1a, respectively (132).

In our study, a total of 36 patients; 25 GT1a, 8 GT1b and 3 with unclear GT1 subtype, were treated according to Swedish recommendations during 2011 through 2013 (Q2) using triple therapy with either BOC or TVR. Patient data were retrospectively extracted from patient registry and evaluated regarding treatment outcome in the event of baseline NS3 RAS findings, adverse effects and workload.

Baseline NS3 RAS, and IL28B genotyping if not previously done, were analysed on the patient samples previously collected and stored in the Biobank at Clinical microbiology department, Akademiska Hospital.

Overall, a little more than half of the patients, 56%, achieved SVR and the SVR rate was comparable between those who received BOC regimen and those who received TVR although slightly higher with BOC.

As predicted and reported by the registry studies conducted by the drug manufacturers, SVR rate was higher in treatment-naïve patients, and in the absence of negative predictive factors such as old age, cirrhosis and high viral load at baseline, with 70% achieving SVR. In particular, patients without cirrhosis achieved the highest SVR rate at 89.5%. However, in cirrhotic and treatment-experienced patients the SVR only reached 17.6 and 37.5%, respectively, which was lower than reported in the registry studies. These real-world

data show similar SVR rates in treatment-naïve and non-cirrhotic patients as the registry studies but lower SVR in cirrhotic and treatment experienced patients (133–136).

The reason for this discrepancy is not fully clear but high degree of comorbidities may be one explanation. At the time of the start with triple therapy in Sweden, it was restricted to and prioritised to the most ill patients. The combination of a relatively weak PI and cirrhosis Child-Pugh A or B would be another explanation for the many failures.

Adverse effects and healthcare workload

Of the non-SVR patients, nine had viral breakthroughs, six relapsed and one died before SVR24 although of these, eight of the cases can be attributed to premature discontinuation of treatment due to adverse effects experienced by the patients.

In the follow-up of treatment, the mean number of contacts with nurses was higher than expected and higher than the SOC protocol with 20.4 times versus 11.3 times. The mean number of contacts with doctor was comparable. Among the adverse effects reported, anaemia was common in both BOC- and TVR-receiving groups and measures to deal with the affliction was comparable between the SVR and non-SVR group. Eight patients suffered from side effects to the extent that they discontinued treatment prematurely, which led to emerging RASs. Side effects caused by BOC treatment included besides anaemia, autoimmune haemolysis, septic shock or worsened psoriasis in three of the patients. Four patients in the TVR-receiving group experienced severe rash covering more than 50% of the body surface while a fifth patient deceased from massive pulmonary embolism not due to TVR side effect but from HCC.

Baseline and emerging RASs

Baseline NS3 RASs were detected in both SVR and non-SVR groups. However, only low-level resistant RASs were found and only in seven cases. Interestingly, 75% were found in the SVR group and only one in the non-SVRs group. This should be explained by that the RASs found were of low resistance types. T54S and T55A giving 2-20-fold resistance and a natural prevalence of approximately 3%. Although not significant at the time of this study, the signature RAS regarding simeprevir, Q80K, was observed in two of the SVR-patients, and another patient was found to harbour D168G, both of which are not classified as RAS in regard to BOC or TVR and simeprevir was yet to be certified. It is not surprising to find patients harbouring Q80K at baseline since the prevalence of this RAS is high in GT1a with as much as 48% in the US, while in Sweden it is estimated to be 15.2% (37).

In the non-SVR group, emergent RAS was detected in 57%. Key RAS R155K with high fold resistance (>50-fold) toward BOC and TVR was detected either singularly or together with V36M or with T54S, each of which also appeared singularly. At viral breakthrough, emerging low-level V36M

seems to be more frequent with four out of eight having this substitution while only one of the patients with viral relapse was found with V36M.

Testing for baseline IL28B and HCV RAS was shown not to have any significant impact on treatment outcome. Only few RASs were found and were of low-level type at 2- to 20-fold. While baseline RASs were rare, emergent high-level resistant RASs were more frequent. The reason for emergence is attributable mainly to treatment discontinuation and thus allowing resistant variants inevitably present to overgrow wt variant.

Indeed, this study was conducted on a small cohort and the risk of type II error is impending where the null hypothesis is not rejected because of too weak power. However, although BOC and TVR no longer are in question, as descriptive data there are lessons to be learnt in terms of future perspectives.

Tailoring treatment based on baseline RAS testing and the impact on SVR (Study III and IV)

A total of 200 GT1a and 226 GT3a patients were assessed to be eligible in the joint Nordic multicentre studies.

GT1a and RAS NS3 Q80K and R155K (Study III)

In the GT1a arm of the study, results from baseline RAS testing were successfully obtained from 92 patients in the intervention group and 101 in the control group. The samples in the control group were tested retrospectively.

The prevalence of baseline RASs in the NS3 region were low with Q80K in 7.1% and R155K in 5.2% of the patients. Nation-wise, the prevalence of both Q80K and R155K was slightly higher in the Norwegian cohort than in the Swedish. The figures are, nevertheless in line with prevalence accounted for by previous studies with Q80K (18,114). Prevalence of NS5A RASs were not assessed for all patient samples since mandatory screening for NS5A RAS was not implemented until Q1 2015, thereby not covering the whole duration of this study which was conducted in Q2 2014 to Q1 2016.

Overall SVR rate was high, as was anticipated with this second wave of DAA-based regimen in their registry studies. Furthermore, a somewhat higher SVR rate was discerned in the intervention group with 97.8% compared to 93.1% in the control group. In the intervention group, all patients harbouring NS3 RAS (n=11) were treated with a regimen that contained a NS5A inhibitor and all of them achieved SVR. In the control group however, only five out of 13 patients with NS3 RAS were given a regimen containing NS5A inhibitor. Control group SVR rates were 89% and 75%, respectively with patients harbouring Q80K and R155K, i.e. were treated with prevailing standard recommendation. Nevertheless, only 30% of the patients in the control group were treated with SIM+SOF compared to patients in the intervention group where

50% were given this regimen. This was probably due to new guidelines introduced when simeprevir was superseded to the advantage of NS5A-based regimen with ledipasvir plus sofosbuvir, and especially the recommendation was that this regimen was to be given to previous failures with BOC/TVR without the requirement of prior resistance testing. It is noteworthy that the only two patients in the control group who had the clinically relevant baseline NS3 RAS Q80K and R155K, respectively, and not achieving SVR had been given SIM+SOF (without RBV) for 12 weeks.

Most pronounced difference in SVR rate was observed in cirrhotic patients. Although we could not statistically prove it due to low prevalence of RASs, our results indicate that this is the patient group that would benefit the most by having a RAS test prior to determining the best treatment regime.

GT3a and NS5A Y93H (Study IV)

In the GT3a study, 130 and 78 patient samples in the intervention and control groups, respectively were sequenced for baseline RAS during Q2 2014 to Q4 2017. Patient characteristics in both intervention and control groups were similar to the GT1a patients; the majority were male, median age 52 and 55, respectively, median viral load 6 log₁₀ IU/ml and treatment naïve. The study was somewhat skewed regarding the proportion of cirrhotic patients was three times larger in the control group compared to the intervention group. It should be noted that the inclusion period differed between intervention and control group, as the inclusion period of the intervention group continued for a year longer (due to new resistance guidelines in autumn 2016 that affected enrollment of patients in the control group).

The patients enrolled in the early part of the study had more pronounced disease because treatment at the initiation of this study was provisioned patients with more severe liver disease and deteriorated general condition. But even adjusted for the inclusion timing, the proportion of cirrhotic patients in the control group remain higher than in the intervention group. What is important in this context is that the proportion of SVR rate in the cirrhotic patients in the two groups remains similar even after adjusting for inclusion period with 91.8% versus 90.2% in the intervention group compared to 85.4% SVR rate in cirrhotic patients in the control group.

We found baseline RAS Y93H at 3.8% and 5.1%, in the intervention and control groups, respectively. Moreover, although at the initiation of this study in 2014, A30K was not considered significant in terms of resistance, we recorded the frequency in our cohorts. We found this RAS in a lower frequency, 3.8% and 2.6% in the intervention and control groups, respectively. The prevalence of baseline NS5A RASs was lower than the prevalence of NS3 RASs but then, this is in line with previously published data as well as the latest data compiled by SHARED (137). This report included a compilation of GT3a NS3 and NS5A sequences from 315 treatment-naïve patient's samples from five

continents in which prevalence of 8.9 % for NS3 RASs (e.g. Q80K, Q168Q) and 4.4% and 6.0% for NS5A A30K and Y93H, respectively was reported.

In the perspective of nation-wise prevalence, the Y93H was found at similar frequency in Sweden and Norway, but a significant difference was noted for A30K which was only found in one Swedish patient (0.8%) but found in six Norwegian patients (7.0%). Undeniably, this is a comparatively small-sized study-population but even so, the prevalence numbers are representative of other published data (90,118,138).

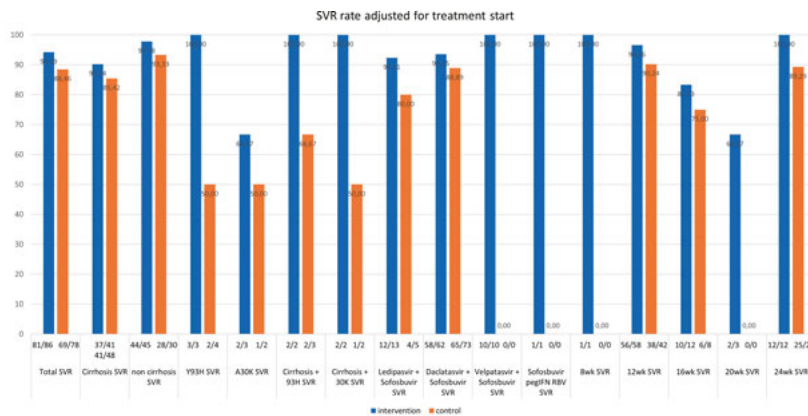


Figure 8. SVR rate, adjusted for inclusion period, in intervention (blue bars) and control (orange bars) groups of GT3a patients. SVR on Y-axis in percent (%).

Considering the early scheduling of this study and to the extent that some of the regimens are no longer in use i.e. DCV/SOF (and LDV/SOF for GT3), the overall SVR rate was high at 95.4% (or 94.2 adjusted for matching inclusion period Q2 2014- Q4 2016) and 88.5%, in intervention and control groups, respectively. Nonetheless, SVR rate in the intervention group was consistently higher in all aspects considered, than in the control group, even after adjusting for inclusion period (Figure 8).

All five patients with baseline Y93H in the intervention group achieved SVR with treatment tailored based on results from the resistance testing. These patients were treated with either 24 weeks DCV/SOF or LDV/SOF treatment without RBV, 12 weeks VEL/SOF with RBV, or 12 weeks SOF plus PEG-IFN with RBV.

Noteworthy is that one out of five patients with A30K in the intervention group subsequently relapsed. This patient was treated according to standard treatment recommendation of 12 weeks with DCV/SOF and was the only one of the five that did not receive RBV. At the beginning of our study during 2014-2015, the RAS A30K was not considered a clinically relevant NS5A

RAS but during 2016 Q2 and treatment in the intervention group patients was not tailored based on this RAS. According to replicon assay data, A30K in GT3a confers resistance level of around 50-fold to DCV and VEL (Figure 7) (96,118).

Even though these resistance levels are lower than those for Y93H, it could have an accumulative negative effect in patients who already are difficult to treat, for instance in patients with cirrhosis. This patient had a viral load at >2 million IU/mL and thus the high viral load together with the presence of A30K may have contributed to the non-SVR. The other four in the intervention group with A30K were having RBV in their regimen primarily because of either pronounced cirrhosis or fibroscan measurement bordering to cirrhosis (>12.5 kPa) or that treatment was started after 2016 Q2.

In the control group, two out of four of the patients with Y93H at baseline failed treatment and relapsed. These two patients were treated with LDV/SOF plus RBV for 16 weeks and DCV/SOF without RBV for 24 weeks, respectively. The reason of non-SVR for the first patient should mainly be that LDV is useless for treatment of GT3. Whereas for the second patient, it could be that treatment although long duration lacked RBV. Furthermore, one of two patients with A30K relapsed. The one achieving SVR received 24 weeks treatment with added RBV and was treatment-naïve whilst the patient who relapsed was treatment experienced but only had 12 weeks treatment and low dose RBV.

As RASs were only detected in a small number of patients, these results may be interpreted with caution but still, they are in line with what we observed in Paper I and other previously published observations (23,90,102,110).

The patients in the control group were more frequently given prolonged treatment (>12 weeks), addition of RBV or both compared to the patients in the intervention group. Quite remarkably, 32.1% in the control group received both 24 weeks and the addition of RBV but only 3.8% in the intervention group ($p<0.05$). This indicates an extensive and unproductive overmedication with all that it entails regarding cost, health care burden and patients suffering adverse effects.

On the other hand, only one of the five patients in the intervention group that received both 24 weeks of treatment with the added RBV harboured baseline 93H indicating that baseline RAS was not the major factor that determined the choice of treatment strategy. Factors such as cirrhosis and previous treatment response also play key roles. Indeed, as reported by both registry studies and real-world studies, as well as our study on GT1, the most predominant factor affecting treatment outcome seem to be attributed to cirrhosis, and consideration of the combination of confounding factors is a key to treatment success.

Economic implications

The Nordic multicentre study comprises real-world data from DAA treatment of GT1 and GT3 at sites in Sweden and Norway from April 2014 to December 2017. During this time, treatment options for HCV was experiencing a paradigm shift, data on optimal regimens were still emerging and guidelines were rapidly changing.

For GT1 treatment, the EASL guidelines recommended LDV/SOF or SIM/SOF and for GT3, DCV/SOF during 2014– 2015 and a change to VEL/SOF in 2016– 2017. The cost of treating a patient with either of these regimens for 12 weeks was 0.5 to 1.0 M NOK or SEK with a slight yearly reduction during 2015 to 2017.

At the time of initiating the study, knowledge on baseline NS3 and NS5A RASs affecting treatment was limited. However, according to published *in vitro* data, it was conceived that some key pre-existing RASs, such as NS3 R155K and NS5A Y93H, could confer some adverse effect on treatment outcome and this could subsequently be confirmed in clinical trials where SVR rates were indeed lower in patients with baseline RASs (96,118).

While all patients in the GT1 intervention group harbouring baseline RASs Q80K or R155K, achieved SVR with treatment adjusted to the findings, two out of five patients in the control group harbouring baseline Q80K or R155K failed treatment with SIM/SOF. Furthermore, in one of three patients failing the NS5A-inhibitor-based regimen LDV/SOF, NS5A RASs were detected when analysed retrospectively. In the GT3 arm of the study, baseline Y93H RAS was detected retrospectively in two patients in the control group who experienced virologic relapse after treatment with DCV/SOF and LED/SOF.

Adjusting these patients accordingly to either prolonged treatment periods, an NS5A inhibitor-based regimen (or in the case of LDV to prolonged DCV) and/or addition of RBV could possibly have reduced treatment costs, and in addition, contributed to a “best practice” approach. Thus, in GT1 and GT3 studies together, the control group there was an economical loss of >1 M NOK or SEK compared to the intervention group in which no patients with RASs at baseline experienced treatment failure. In comparison, the baseline analysis costs (2000 NOK or SEK per analysis) for the 95 GT1 and 130 GT3 patients in the intervention groups were less than 0.5 M NOK or SEK.

Clearly, currently approved (in Sweden Jan 2018 and in Norway February 2018) DAAs (glecaprevir (GLE)/pibrentasvir (PIB) and VEL/SOF plus voxilaprevir (VOX)), have greatly improved the SVR rates, even for GT3 and in the presence of NS5A RASs. This is mainly attributable to the inclusion of GLE or VOX, which are effective NS3 protease inhibitors against both GT1 and GT3. Still, the current Swedish and Norwegian recommendations for the first-line treatment of treatment-naïve GT3 patients is, due to cost-effective

considerations, VEL/SOF (139,140). Ensuring that the cheaper first-line treatment is adequate by resistance testing at baseline to avoid the high cost of retreatment and the emergence of RASs should therefore be justifiable.

Conclusions

Study I

We have shown that our pan-genotypic NS5A sequencing method for detecting NS5A RASs can successfully detect relevant RASs in most common genotypes, which may be useful in RAS testing aiding in tailoring treatment. Furthermore, with this method we determined the baseline prevalence of NS5A RASs in genotypes 1a, 1b and 3a in the Uppsala region. The resulting prevalence data were in concordance with previous estimates.

Study II

The real-world data in this small study showed higher SVR-rates in the non-cirrhotic, treatment-naïve patients, but significantly lower SVR-rates in treatment-experienced and cirrhotic patients. Prevalence of baseline RASs was low and could not be shown to impact treatment outcome, but RASs were frequently detected in the non-SVR patients, disclosing a liability to re-treatment options and future drug development approaches.

Also, the adverse effects associated with these early DAAs, raises the question whether exposure to these first-generation protease inhibitors were appropriate for the most severely ill patients and the consequences of accompanying RASs.

Study III

In line with the registry studies for NS3 RAS Q80K and the EASL guidelines 2016 for NS5A-RASs, baseline genotype 1a RASs appear to have an impact on treatment outcome. Personalised treatment tailored to baseline resistance analysis could thereby be of importance to guide the selection of cost-effective treatment strategies, both in a perspective of evidence-based healthcare delivery and in the case of the individual patient to avoid treatment failure.

Study IV

Similar to the study III of the Nordic multicentre study, even if prevalence is low, presence of baseline genotype 3 NS5A RASs seems to bear impact on treatment outcome primarily in cirrhotic patients. Although statistical significance could not be proved, a trend in overall higher SVR-rate could be observed when treatment was personalised.

In addition, an extensive over-treatment could be perceived in the control group patients who could not recognisably be benefitted from prolonged and/or administered also with RBV. Thereby, tailoring for the presence or absence of RAS may be as useful in optimising treatment.

Concluding remarks

The importance of real-world studies such as these presented in this thesis are clear when the results are compared with those of the registry studies and the discrepancies become apparent. In many cases the SVR data from clinical trials are based on replicon assay data and well-ordered patient groups. Patients included in clinical trials are often extracted from large groups allowing easier selection of patient with certain traits, which could induce a bias in the patient material. Timing of the study may also be a factor of bias as cohorts included in clinical trials are recruited when a drug is to be approved or new guidelines are to be published. Additionally, health care staff at clinical trials may be more focused on the task than in a real world-setting where a multitude of patients are cared for in parallel.

Therefore, knowledge gained from real-life studies are useful as complement to clinical trials and registry studies in guiding future treatment strategies and preparedness regarding for example adverse effects and health care burden. At the time of our BOC/TVR studies, the cost of every treatment was exceedingly high, more than one million SEK per treatment, and even if treatment costs since has decreased considerably, the cost of resistance testing remains very low in proportion.

It should be important to use the new DAA-based treatments in an optimal way in order to avoid emerging and non-amendable resistance, of which we still know little. Although current modern DAAs in most cases surmount the problem with baseline and emerging RASs, there will still be some difficult, especially re-treatment cases where RAS testing could be of great value. Numerous reports have been published pointing out that predominantly GT3-patients, with cirrhosis and a history of treatment failure, are at risk of being difficult to cure and would need carefully tailored treatment strategies. This is in stark contrast to a trending opinion towards uniform treatment and there are even opinions proposing genotyping redundant.

We consider tailored treatment important, also independent of baseline RAS found, both in terms of cost-efficiency with the regimen per se and in terms of total health care burden. The well-being of the patient should also be a strong factor in avoiding suffering from re-treatment and unnecessary adverse effects. Treatments void of adverse effects would also encourage adherence and thus preventing emerging resistant variants and the need to re-treat.

In addition, the best treatment options, e.g. GLE+PIB and SOF+VEL+VOX (and even SOF+GLE+PIB) are still costly and are therefore

saved for salvage treatments. Accordingly, to be able to optimise treatment and reduce overtreatment when negative factors are absent, standardised RAS testing could be included in assessing best treatment strategy, i.e. cheapest and shortest alternative. At least where modern treatment as well as resistance testing is readily available, the lowest dose and shortest treatment duration must be the goal in controlling transmission and eradication of HCV. However, standardisation of RAS detection and testing guidelines are still lacking and many in-house protocols using Sanger- or deep sequencing have varying degree of specificity and sensitivity. Thus, there is currently a debate on consensus for clinically relevant cut-off sensitivities (106). However, there are global efforts, where e.g. *SHARED* recently published a collaborative database of HCV sequences and clinical data collected from five continents, for the monitoring of DAA RASs (107). This will provide further information on the clinical relevance of RASs and RAS testing.

Finally, in order to be able to meet the WHO global HCV eradication proposal, readily available treatment options must be standardised, potent enough yet non-toxic and that can be mass-administered to less resourceful areas around the world.

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To all my wonderful friends! Thank you for listening, understanding and encouraging! Thank you for forgiving me for the parties I didn't throw. I will make up for it now! (*Addendum*: – or perhaps not. At the last minute of writing this thesis, we are amid a pandemic with prompted social distancing...)

To my mother who sadly will never know that I achieved this, I believe you would have been proud although probably as surprised as I am!

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Sammanfattning på Svenska

Hepatit C är en infektionssjukdom orsakat av Hepatit C virus (HCV) som i de flesta fall orsakar kronisk infektion och inflammation av levern. Mer än 71 miljoner individer är drabbade runt hela världen och infektionen utgör en tung börda för hälso- och sjukvården. HCV är en blodsmitta och vanliga smittvägar tidigare i västvärlden, och än idag i tredje världen, är via icke-steril sjukvårdsutrustning eller blodkomponenter kontaminerade med smittat blod. I västvärlden är numera den vanligaste smittvägen genom sprutdelning hos personer som injicerar droger.

Viruset finns i många olika men närbesläktade varianter, s.k. genotyper. Det finns idag åtta identifierade genotyper med olika utbredningar i världen. Totalt sett är de vanligaste genotyperna 1a, 2 och 3a. För många infekterade leder sjukdomen så småningom till levercirrhos och även levercancer och den är idag den ledande orsaken till behov av levertransplantation.

Viruset är ett RNA-virus som förökar sig snabbt och har en hög mutationsfrekvens, vilket är förklaringen till att det existerar så många olika varianter. Till och med hos en enda infekterad individ uppstår hela tiden nya varianter som på så sätt undkommer immunförsvaret och kan fortleva och samexistera i kroppen. Detta orsakar också problem med att hitta effektiva läkemedel (antiviraler) och försvårar vaccintvecklingen även om det har skett stora framgångar, framförallt inom antiviralutvecklingen.

Dagens antiviraler mot HCV är direktverkande, d.v.s. de är specifikt mål-inriktade mot viruset i sig och mycket effektiva, och de allra flesta patienter botas med en tremånaders behandling med två eller tre direktverkande läkemedel som angriper viruset från olika håll. För att säkerställa eliminering av alla samexisterande virusvarianter och därmed att sjukdomen botas undviker man att behandla med endast en typ av läkemedel. Då det alltid finns en andel virus med en naturlig mutation, d.v.s. pre-existerande resistens eller baseline-resistens, som gör det resistent mot en viss given antiviral redan innan behandlingen. De flesta av dessa naturliga resistent varianter har reducerande överlevnadsförmåga och försvinner därför spontant, men vissa andra har tillräcklig fitness för att överleva och under vissa ogynnsamma omständigheter kan dessa resistent viruspopulationer sållas fram vilket leder till fortsatt infektion trots behandlingen. När detta sker bidrar det i likhet med antibiotikaresistens, till att även nya förvärvade resistensmutationer uppstår och har patienten otur kvarstår både de pre-existerande och förvärvade resistensmutationerna så att det blir svårare att återbehandla, även med nya preparat.

I denna avhandling har studerats vilken roll pre-existerande mutationer kan ha vid behandling med direktverkande antiviraler och om man kan effektivisera HCV-behandlingen genom att skraddarsy den bl.a. utifrån att testa för resistensmutationer för att vid eventuella fynd justera behandlingen jämfört med standardbehandling enligt rådande rekommendationer. Studierna har gjorts i samarbete med flera nordiska centra i *real-life*-studier där vi har delat in patienterna i interventionsgrupper och kontrollgrupper. Patienterna i interventionsgruppen har testats för relevanta resistensmutationer och fått justerad behandling vid fynd av resistens, vanligtvis med tillägg av ribavirin och/eller förlängd behandlingstid eller att man ordinerar ett annat läkemedelsalternativ som är dyrare och förbehållet främst för svårbehandlade fall.

För att hitta resistensmutationerna har vi utvecklat en sekvenseringsmetod, som bygger på att dechiffrera de direktverkande antiviralernas målgener hos viruset för att därmed upptäcka resistensmutationerna. Med metoden kan man analysera förekomst av resistensmutationer i flera HCV genotyper, och den är framförallt optimerad för de i Norden vanligaste förekommande genotyperna 1a och 3a.

När studierna i denna avhandlings inleddes uppgick kostnaden för en behandling uppemot en miljon kronor medan analyskostnaden för resistensbestämning var ca 2000 kronor, en liten kostnad i sammanhanget för att säkerställa att rätt behandling sätts in och undvika återbehandling. Därigenom minskas också onödigt lidande i form av biverkningar under en upprepad och flera månader lång behandlingsperiod. En annan aspekt är att undvika överbehandling genom att skraddarsy dosering och behandlingstid.

Även om resultaten inte alltid kunde med signifikans säkerställa att resistensmutationer spelar roll, visades ändå att andelen som kunde botas konsekvent var större i interventionsgruppen med skraddarsydd behandling jämfört med de som fick standardbehandling. Därmed kan, förutom pre-existerande resistens, andra negativa faktorer också spela in, där de viktigaste är graden av leverskada samt infektion med genotyp 3a.

Även om de antiviraler som nu används för att behandla HCV är mycket effektiva, och mer än 95% botas, återstår ca fem procent som inte gör det. Det betyder globalt att uppemot fyra miljoner inte botas och många av de misslyckandena kan tillskrivas pre-existerande resistensmutationer. De av misslyckade behandlingar förvärvade resistensmutationerna riskerar att kvarstå hos den infekterade individen och till och med spridas till andra. Därför är det av största vikt att kartläggning och forskning kring resistenser mot antiviraler fortskrider så att HCV kan elimineras globalt.

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