Factors associated with resolution and progression of HIV/hepatitis C virus infection

Louise Nygaard Clausen

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Tutors: Thomas Benfield, Jens Bukh and Nina Weis

Official opponents: Peter Garred, Court Pedersen and Martin Lagging

Correspondence: Department of Infectious Diseases and the Clinical Research Centre, Hvidovre Hospital, Kettegårds Allè 30, 2650 Hvidovre, Denmark.

E-mail: louisenygaardclausen@gmail.com

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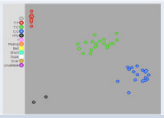
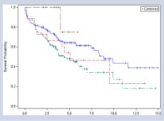
THE THREE ORIGINAL PAPERS ARE

I Clausen LN, Weis N, Schonning K, Fenger M, Krarup H, Bukh J, et al. Correlates of spontaneous clearance of hepatitis C virus in a Danish human immunodeficiency virus type 1 cohort. Scand J Infect Dis 2011 Oct;43(10):798-803.

II Clausen LN, Weis N, Astvad K, Schonning K, Fenger M, Krarup H, et al. Interleukin-28B polymorphisms are associated with hepatitis C virus clearance and viral load in a HIV-1-infected cohort. J Viral Hepat 2011 Apr;18(4):e66-e74.

III Clausen LN, Astvad K, Ladelund S, Larsen MV, Schonning K, Benfield T. Hepatitis C viral load, genotype 3 and interleukin-28B CC genotype predict mortality in HIV and hepatitis C-coinfected individuals. AIDS 2012 Jul 31;26(12):1509-16.

THESIS AT A GLANCE

Study	Aim	Method	Results	Conclusion
I	To identify demographic factors associated with spontaneous hepatitis C virus (HCV) resolution	Multivariate logistic regression analysis adjusted for sex, race, hepatitis B virus coinfection, AIDS and HIV therapy		Female sex, route of exposure through injecting drug use or men-who-have-sex-with-men and hepatitis B co-infection were associated with higher resolution rate
II	To identify genetic factors associated with spontaneous HCV resolution and viral kinetics	Multiple logistic regression adjusted for sex, HIV exposure group, hepatitis B virus coinfection, AIDS and antiretroviral therapy. Haplotype inference		Interleukin 28B CC genotype associated with spontaneous HCV resolution and increased HCV viral load
III	To identify genetic and viral predictors of all-cause mortality	Time-updated Poisssion regression adjusted for age, sex, HIV exposure group, CD4 count and HIV viral load		HCV genotype 3 and viral load were associated with increased rate of all-cause mortality

INTRODUCTION

Hepatitis C virus infection

A hallmark feature of infection with hepatitis C virus (HCV) is its propensity for lifelong chronic infection. Three-quarter of all infected individuals remain chronically infected and at risk of severe liver disease (cirrhosis, end-stage liver disease and liver cancer), the remaining 25% spontaneously resolve their infection [1]. Worldwide this has resulted in 130 to 180 million chronically infected individuals of whom 90 million are believed to have been infected through unsafe health practices in third world countries. In Denmark approximately 15000 or more individuals are estimated to be infected with HCV, primarily through injecting drug use (IDU).

Because of shared routes of transmission, HCV and human immunodeficiency virus (HIV) coinfection is frequent with an estimated 5 million coinfected individuals [2]. HIV and HCV coinfection differs from HCV monoinfection in several aspects. Coinfected individuals have higher HCV viral load and lower rates of spontaneous and treatment-induced resolution [3] and are associated with more rapid progression of liver-related disease [4-6].

FACTORS ASSOCIATED WITH HCV RESOLUTION

Demographics and clinical characteristics

Several demographic and clinical factors affect the rate of resolution of HCV. There is overall consensus that female sex, younger age at infection, Caucasian race [7], coinfection with hepatitis B virus (HBV) [8-10] and symptomatic acute hepatitis [11-14] are associated with resolution. Reversely, male sex, older age at infection, black race and coinfection with HIV are associated with chronicity.

Host immunology

Innate immune response

The innate immune response is induced upon recognition of viral pathogen-associated molecule patterns sensed by pathogen-recognition receptors. The sensing of HCV, which occurs through interaction with toll-like-receptors (TLR) and retinoic acid-inducible gene-I (RIG-I), leads to an intracellular signalling cascade activating interferon (IFN) regulatory factors and ultimately induces production of IFNs. The production of IFNs leads to transcription of hundreds of IFN- stimulated genes (ISG) through the JAK-STAT signal pathway resulting in an antiviral state in the liver.

In addition to the rapid induction of IFNs and ISGs, first line defences against HCV include natural killer (NK) cells, which are activated by IFN-α released from infected hepatocytes. Simplified,

NK cells produce IFN-γ and induce cytotoxic killing of infected hepatocytes. The IFN-γ production leads to release of proinflammatory cytokines including TNF-α and interleukin(IL)-18, which are known to induce maturation of dendritic cells (DC) [15]. DCs bridge the innate and adaptive immune response by both producing IFN-α and priming naïve T-cells in lymphoid tissue.

Adaptive immune response
Adaptive immunity is believed to make an important contribution to spontaneous resolution. HCV resolution has been associated with a rapid production of broadly neutralizing antibodies and a strong, broadly-targeted T cell response.

With regard to the impact of neutralizing antibodies in HCV resolution, results are conflicting. Over the last decade, our understanding of viral entry and antibody neutralization has advanced with the development of cell culture-derived HCV (HCVcc) and retroviral particles pseudotyped with HCV (HCVpp) envelope proteins [16]. A number of studies have shown high neutralization titers in patient-derived chronic phase sera by HCVcc and HCVpp [17-20]. However, spontaneous resolution of HCV infection can occur without the induction of neutralizing antibodies [21,22], and generally, humoral antibody responses are believed to play a marginal role in HCV resolution[23].

Individuals who resolve HCV infection have a broader and more sustained CD4 and CD8 T-cell response (reviewed in [24]) than individuals with chronic HCV. The detection of fully functional HCV-specific CD4 cells during acute infection is associated with subsequent HCV resolution. Further, a temporal association between the detection of HCV-specific CD8 T-cells responses indicates that CD8 cells are also important in HCV resolution. Hence, both CD4 and CD8 T-cell responses are required to gain HCV resolution. The importance of CD4 cells in resolution is further underscored by the lower resolution rate found in HIV coinfection which may in part be due to depletion of CD4 cells. CD4 cells recognize their target by binding to antigen associated with human leukocyte antigen (HLA) class II and CD8 cells by binding to HLA class I.

In summary, the protective effect of host immunity is complex and likely to be multifactorial, comprising both adaptive and innate components.

Host genetics
Genetic associations with spontaneous resolution of HCV have been investigated in many different parts of the human immune response to HCV. Knapp et al. found genetic associations in three main effector pathways of the IFN-mediated antiviral response, MxA, 2'5'-oligoadenylate-synthetase-directed ribonuclease L (OAS-1) and protein kinase R (PKR) [25]. Schott et al. reported an association of polymorphisms in TLR-7 and chronic HCV [26]. Khakoo et al. showed that a weaker inhibition of NK by their ligand killer immunoglobulin-like receptors (KIR) appeared to confer protection against chronic HCV [27].

Many studies have investigated genetic differences in HLA class I and II (reviewed in Rauch28). The HLA class II loci DQ and DRB1 are the loci with the most consistent association with spontaneous HCV resolution [13,29-36] . Further, in HLA class I, HLA-B57 and B27 have been associated with resolution [37-39]. The association with B57 is interesting because B57 has also been reported to be associated with progression of HIV disease [40-43].

Cytokines and chemokines, which coordinate both the innate and the adaptive immune response, have been associated with HCV resolution. The cytokines most consistently associated with resolution have been the proinflammatory cytokines, IL10 and IL12B and the chemotactic cytokine, IFN-γ-inducible protein-10 (IP-10, CXCL10). IP-10 is produced by hepatocytes upon HCV infection [44]. Individuals with spontaneous resolution have lower IP10 levels than those with persistent infection [45].

However, many of the genetic associations identified have not been replicated and others again have been observed to have the opposite effect. The limitations of many genetic studies are that the studies are underpowered, they lack validation cohorts and correction for multiple testing and population stratification. Further, comparison between cohorts is difficult because of diverse populations and varying definitions of controls.

A strong genetic association which has been consistently replicated in several independent studies is the association with spontaneous HCV resolution of a single nucleotide polymorphism (SNP) located app. 3 kb upstream of interleukin 28B (IL28B; rs12979860) [46-51]. A recent genome-wide association study (GWAS) evaluated the host genetic influence on HCV resolution in multiple cohorts. The authors replicated the known association with rs12979860 and identified a new SNP (rs4272729) near the HLA class II gene on chromosome 6 which was associated with spontaneous HCV resolution at genome-wide statistical significance level (p = 10-8). Further, they performed classical HLA typing and found that HLA-DQ-0301 attributed to spontaneous resolution of HCV at a significance level of p = 10-5. They concluded that the genetic variations in SNPs marking IL28B and HLA-DQ-0301 explained app. 15% of spontaneous HCV resolution [52].

PROGRESSION AND MORTALITY IN HIV/HCV COINFECTION

The improvement of HIV treatment during the 1990s led to greatly improved survival from HIV infection. However, with the decline in AIDS-related deaths, non-AIDS causes of morbidity and mortality have become prevalent [53]. For HIV/HCV-coinfected individuals, the burden of disease is largely related to their HCV disease [54-58]. Individuals infected with both viruses have a faster progression to liver fibrosis [59], cirrhosis [60] and liver-related deaths [61,62]. Moreover, CD4 cell counts have been inversely associated with liver inflammation in some studies [3,63]. Recently, Kirk et al. reported that even after adjustment for HCV viral load (VL) levels, HBV chronicity, sex, race and alcohol use, HIV/HCV coinfection was associated with liver fibrosis as advanced as those without HIV who were 10 years older [64].

Antiviral and antiretroviral therapy
Although antiretroviral therapy (ART) has been strongly associated with decreased risk of liver-related outcome or death from HIV/HCV coinfection [59,65-69], the adverse impact of HIV coinfection on HCV disease is not entirely ameliorated by ART [62,70]. Therefore, treatment aimed at eradication of chronic HCV infection may also be required despite the many barriers (including decompensated liver disease, substance abuse, socioeconomic condition, and compliance) in this patient group often comprising a large numbers of IDUs.

Guidelines recommending initiation of HCV therapy prior to ART if CD4 cell counts are > 500/uL are based on the evidence that HCV

therapy is associated with lower responses in individuals with CD cell counts < 500/uL [71,72]. The use of the protease inhibitors, telaprevir and boceprevir plus pegylated IFN and ribavirin (peg IFN/RBV) for treatment of individuals with HIV/HCV coinfection and HCV genotype 1 has been described in two small phase IIa studies demonstrating a high sustained virological response (SVR) [73]. It is clinical practice to offer triple therapy to HIV-HCV coinfected patients with HCV genotype 1. With triple therapy adding telaprevir to pegIFN/RBV, HIV/HCV-coinfected individuals have an SVR of 74% compared with 45% if telapravir is not added, and 61% for boceprevir compared to 26.5% without the addition of boceprevir [73]. In individuals infected with HCV genotype 2 or 3, the standard of care is still peg IFN/RBV, which has proven less successful than in patients without HIV with SVR rates of 62% [74].

Progression of liver disease

How HIV and HCV contribute to disease progression and mortality in HIV/HCV-coinfected individuals has been reported with conflicting conclusions. HCV viral load has been reported to predict development of fibrosis [64,70], end-stage liver disease (ESLD) and death in some studies [70,75-77] but others observed no effect [7,78-82]. HCV genotype 3 has been associated with steatosis, inflammation and mortality [77,83-86]. The differences reported may be attributed to different study designs, varying study cohorts and controls (haemophiliacs, HCV-mono-infected), different definitions of levels of HCV VL (viremia vs. non-viremia, varying categories of HCV VL), varying laboratory assays for HCV VL measurements and genotypes, over representation of HCV genotype 1 vs. 3.

RATIONALE AND OBJECTIVES

The main purpose of the present thesis was to identify and characterize a cohort of individuals with HIV/HCV coinfection in order to get a better understanding of the demographic, viral and host genetic determinants of the natural control of HCV infection. Better understanding of such determinants may reveal novel therapeutic and preventive strategies.

In study I, the objective was to identify demographic factors associated with spontaneous HCV resolution.

In study II, the objective was to investigate the role of IL28B in viral control of HCV infection.

In study III, the objective was to investigate the effect of viral factors and IL28B on all-cause mortality in individuals chronically infected with HCV.

METHODOLOGICAL CONSIDERATIONS

Study design

We identified an observational prospective cohort by consecutively including anti-HCV-positive HIV-1-infected patients that attended the outpatient clinic at the Department of Infectious Diseases, Hvidovre Hospital, University of Copenhagen, between January 1, 1995, and January 1, 2008. Individuals were considered to have HCV infection if HCV antibodies were detected in a serum or plasma sample by 3rd generation enzyme-linked immunosorbent assay (ELISA) and confirmed by recombinant immunoblot assay (RIBA), ELISA or HCV VL PCR. HCV resolution was defined as the presence of confirmed anti-HCV with undetectable HCV VL in serum or plasma specimens from at least two measurements more than 6 months apart. Figure 1 presents the flow of the included individuals through studies I – III.

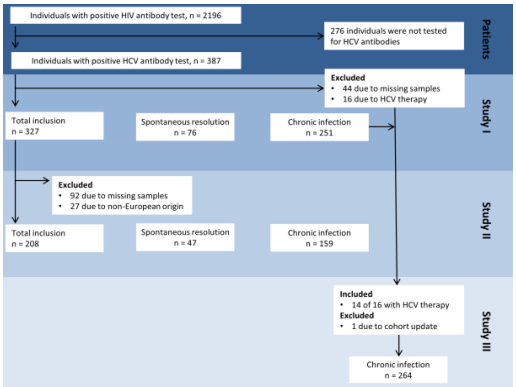


Figure 1. Overview of individuals included in study I - III.

Study II was a genetic association study aiming to detect associations between genetic variations and outcome of HCV infection. Genetic association studies can be approached as either a candidate gene study similar to our study or as a GWAS. Both types of studies have advantages and disadvantages. A candidate association study evaluates biologically plausible genes and tests genetic variation against an outcome. An advantage is the ability to study specific genes with a high coverage, another that it is less expensive than a GWAS. A GWAS tests common variation across the human genome for association with an outcome and uses 100 000 to 5 million SNPs. Unlike the more traditional candidate gene studies, a GWAS does not have a priori hypotheses in which genes may be involved and evaluates common SNPs across the genome. A complete GWAS relies on large sample sizes and replication studies to confirm the initial results and to identify important genetic regions or genes, but on its own it does not identify causal alleles. The coverage of genes is less accurate.

HCV Outcome

Spontaneous resolution: Individuals were classified in this group if they had detectable HCV antibodies by third-generation ELISA and no detectable HCV viremia on a minimum of 2 occasions at least 6 months apart.

Chronic HCV infection: Individuals had detectable HCV antibodies by third-generation ELISA and positive HCV VL measurements on a minimum of 2 occasions at least 6 months apart or a positive HCV VL measurement more than 12 months after a positive HCV antibody test (n = 12).

We hoped to limit the risk of spontaneous resolvers actually developing chronic infection and vice versa during our study period by this classification of outcome. However, we were not able to investigate the risk of HCV reinfections, which may have been a potential confounder in our study, considering some individuals could have had several resolved HCV infections before the one we observed as chronic. Further, cases of resolution after several years of chronic infection have been reported [87] and we could not rule out that some of the individuals classified with chronic HCV infection actually resolved HCV spontaneous later in the study period. In long-term follow-up studies, a proportion of patients who recovered from hepatitis C lost anti-HCV reactivity and were left with no serologic markers of previous infection

[88,89]. Also, loss of anti-HCV reactivity has been reported in cases of HIV infection [90].

Diagnostics

HCV antibody measurements were evaluated by 3rd generation ELISA. Assessment of HCV viral load was done by either a commercially available assay according to the manufacturers' specification or by one of two in-house assays. The limit of detection (LOD) for the three assays during the study period was 15, 20 and 500 IU/mL, respectively. HCV genotype was determined with genotype specific primers from the 5' noncoding region of the virus by RT-PCR or by one-step RT-PCR using C/E1 and NS5B-specific primers. SNP genotyping was performed by LGC genomics using a KASP competitive allele-specific PCR [91]. Information regarding dates of first positive HIV test and ART, HIV exposure group, CD4 counts and HIV RNA was retrieved from the Danish HIV Cohort Study [92]. Quantification of HIV RNA was performed with different assays with detection limit of 20, 40 or 400 copies/mL over time, and for statistical analyses we used 400 copies/mL as the lower detection limit. Causes of death were obtained from the Danish Registry of Causes of Death (DRCD), which is a non-blinded recording of death causes performed by a physician [93]. Causes of death during the study period were coded using ICD-10.

Statistical methods

We applied logistic regression to identify variables associated with HCV resolution. To investigate viral and immunogenetic factors associated with mortality we applied Poisson regression in a time-updated model. The time-updated modeling allowed us to assess the most recent values at any time as potential predictors of mortality. CD4 counts were fitted as a linear spline with a knot at 200 counts/uL. HIV VL was fitted as either undetectable for samples ≤ 400 copies/mL or with a knot in the detection limit for HIV RNA at 400 copies/mL. The fit of continuous variables in linear splines divided the variable into different categories with a specific slope of each category. Hence, each variable had a more flexible effect size on the outcome according to which category you were in, and the categories were fitted continuously in such a way that there were no sudden jumps across category boundaries [94]. Continuous variables were compared using Kruskal-Wallis test and categorical variable using Fisher's exact test or chi-square statistics as appropriate. To account for multiple testing in the genetic association study we applied Bonferroni correction.

Bias and confounding

Missing samples

In order to classify resolved or persistent infection we performed additional testing (described below in measurements of HCV viral load). A subset of individuals had no samples for additional testing and was excluded due to missing samples. Hypothetically, the individuals with missing samples could be too sick – or even dead – to provide additional sampling. Further, some samples were missing because they were used in other studies.

In study I, 44 individuals out of 387 were excluded due to missing samples. The 44 excluded individuals did not differ significantly from the individuals included with respect to sex, race or mode of HIV exposure. Neither did they differ significantly by age at first positive HIV or HCV testing, nor did they differ with respect to mortality rate (MR) or mortality causes. Further, we excluded 16 individuals due to HCV therapy. They differed with respect to route of HIV exposure ($p = 0.005$) in such a way that more IDUs were included than excluded. They were comparable with respect to sex, race, MR and mortality causes.

We acknowledge that we risked introducing selection bias in the cohort by excluding individuals with missing samples and thereby selected individuals more likely to be sicker than the ones included. However, due to the above mentioned similarities, we believe that the risk of selection bias is low.

In study II, SNP genotyping was performed in genomic DNA extracted from whole blood. Ninety-two individuals lacked frozen whole blood. We found no differences with respect to race, sex, HIV exposure route, HCV genotype or HCV viral load between individuals with or without blood samples available. Further, we excluded 27 individuals due to non-Caucasian origin to avoid population stratification [95].

Study III only concerned the individuals with persistent infection from study I and we additionally included 14 out of 16 individuals who had received HCV therapy. The two remaining individuals were not included due to treatment during their acute HCV infection. SNP genotyping was done from DNA extracted from either blood or plasma samples. Genotypes were identical whether performed on blood or plasma.

Measurements of HCV viral load

HCV viral load (VL) measurements were performed with three different assays. HCV VL measurements in the outpatient HIV clinic were done with a quantitative reverse transcription polymerase chain reaction (RT-PCR) (hereafter referred to as in-house quantitative RT-PCR, Aalborg) [96], which was the assay available at the time the testing was done. The limit of detection (LOD) was 20 IU/mL. Additional quantification of HCV VL for individuals who did not fulfil our outcome definition of either HCV resolution or persistence was performed on stored plasma samples with one of two assays. The HCV VL of samples from individuals with a presumed positive HCV VL was determined by an in-house real-time PCR using TaqMan EZ reverse-transcription PCR Kit (Applied Biosystems, Forster City, California, USA) (hereafter referred to as in-house real-time PCR, Hvidovre)[97]. LOD was 500 IU/mL. For individuals with a presumed negative HCV VL or HCV VL at values < 500 IU/mL in the in-house real-time PCR, Hvidovre, we measured HCV VL with the commercially available quantitative real-time PCR system COBAS AmpliPrep/COBAS TaqMan HCV version 1 (CAP/CTM HCV) (hereafter referred to as commercial, CAP/CTM) [98]. LOD was 15 IU/mL.

There have been concerns that the commercial CAP/CTM assay version 1 overestimates HCV VL for HCV genotype 1 [99,100] and underestimates certain genotype 2 and 4 samples [99].

Whether the association detected in our study of HCV VL and mortality was related to the wider linear range of newer HCV VL real-time assays compared with former assays is not evident. We observed a statistically significant interaction between the assays used and HCV VL ($p = 0.02$), which probably reflects that we determined which assay to be used according to former HCV VL level, as previously described. The vast majority of HCV VL measurements (n = 671, 81%) were performed using the in-house RT-PCR, Aalborg [96]. We observed a uniform distribution of HCV VL measurements and HCV assay during our study period, as illustrated in figure 2.

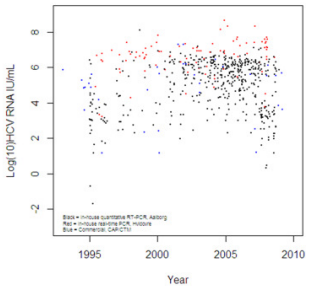


Figure 2. Distribution and quantification of HCV VL measurements according to assay used.

Ideally, all HCV VL measurements were performed with the same assay; however, this was not possible with respect to available samples. Further, we chose the commercial CAP/CTM, which has a low detection limit, to determine the spontaneous resolvers with certainty. One of the lessons learned from the present thesis was that had we conducted a pilot study, measuring a subset of samples with all three assays, it would have enabled us to correlate a possible difference according to assay used.

Timeframe between HIV and HCV
We were not able to determine the timeframe between HIV and HCV diagnosis and as such not able to clarify whether this cohort was co-infected with both viruses at time of resolution of HCV. One-hundred-and-twenty-seven individuals had a positive HCV test around the time they tested positive for HIV and it was impossible to determine if they had first been infected with HIV or with HCV or with both concomitantly. For the remaining individuals, we established that 25 had acquired HCV before HIV, 13 had acquired HIV before HCV, and 162 had unknown time of diagnosis. We were able to establish a time of infection for 38 individuals because samples were available for repeated testing back in time till the appearance of the first negative HCV antibody test.

Other studies report that most IDUs acquire HCV before HIV and are unlikely to be severely immunodeficient during the first years of HCV when HCV resolution occurs [101-103]. The majority of our cohort is probably not coinfected with HIV at time of HCV infection. The high rate of resolution (23%) in our cohort is suggestive of this.

Alcohol and drug use
A possible confounder in our studies may be the inadequate reporting of the actual drug use. However, as more detailed data on alcohol consumption and drug using habits are not available in our cohort, this cannot be further clarified.

Multiple testing
In genetic association studies there is an issue with respect to multiple testing and a controversy exists in determining significance levels for candidate gene studies. Regardless of whether each SNP is analysed one at a time or as part of a haplotype, the number of individual tests can become very large and can lead to an inflated type 1 error rate [104].

Bonferroni correction is the traditional way of correcting for multiple testing. However, the Bonferroni approach is probably not appropriate because it is not the number of tests in any one investigation that is important. Rather, what is important is that the vast majority of loci tested will not be associated, so that even a small false positive probability will mean that most positive results will turn out to be false. Thus, it is the a-priori probability of association that needs to be accounted for, rather than the number of tests.

Another strategy is controlling the false discovery rate (FDR), which controls the expected proportion of errors committed by falsely rejecting the null hypotheses. For instance, using Bonferroni correction, testing 15 hypotheses would yield a significance level of 0.05/15 = 0.003 for all 15 hypotheses. Thereby we risk falsely rejecting the null hypothesis. With FDR, the significance level would differ from each hypothesis. We rank the tests in ascending order of p-values and compare: the smallest p-value with α/N (as in Bonferroni correction), with α being the significance level. The second smallest p-value with $2\alpha/N$, the third smallest p-value with $3\alpha/N$, and so on N times. The correction will be lesser and lesser for each hypothesis tested [105].

A third strategy is permutation testing. Permutation testing calculates an empirical p-value by determining how frequently the identified association would occur by chance. In each permutation, the case-control status is shuffled, and the maximum likelihood test statistic that is observed is compared with the experimental test statistics for each SNP. An empirical p-value is calculated that provides a point wise estimate for the significance of each SNP [106]. Permutation testing does not provide p-value correction.

RESULTS

Study I

Main finding
We identified a cohort of 327 individuals with HIV-HCV coinfection and a rate of spontaneous HCV resolution of 23%. The majority were exposed to HCV through IDU (79%) whereas 8% and 10% were exposed through homosexual contact (men who have sex with men (MSM)) and heterosexual (HSX) contact, respectively.

The main finding was that individuals exposed through IDU or MSM had a higher rate of spontaneous HCV resolution compared with individuals exposed through HSX. This association was robust when adjusted for demographic (sex, race), clinical (HBV surface antigen (HBsAg), AIDS) and therapeutic factors (ART). We also confirmed a known association between females and spontaneous HCV resolution and between individuals coinfected with HBV and spontaneous HCV resolution.

Study II

Main finding
We genotyped 3 SNPs (rs12979860, rs8103142, rs11881222) in the IL28B gene of which rs8103142 was a non-synonymous coding SNP and we showed that all three SNPs were associated with spontaneous HCV resolution and HCV VL in HIV-HCV-coinfected individuals. The major allele homozygote genotype was associated with resolution and VL for all three SNPs. In fact, none of the individuals who were carriers of the minor allele homozygote genotype for any of the SNPs studied had resolved HCV.

IL28B
Below, I report the results of rs12979860, the results were similar for rs8103142 and rs11881222. The major allele homozygote

genotype (hereafter referred to as CC) was associated with 4.3 (95% CI; 2.0, 9.3, p = 0.0002) fold increased odds for HCV resolution compared with the combined TC and TT genotype (hereafter referred to as non-CC) in multivariate logistic regression.

HCV viral load

HCV VL was significantly higher for carriers of IL28B CC genotype for both HCV genotype 1 and 3. In individuals infected with HCV genotype 1, HCV VL was increased in carriers of the IL28B CC genotype (median, 6.3; IQR, 5.9, 7.3) compared with the non-CC (median, 6.1; IQR, 5.6, 6.4, p = 0.028). For HCV genotype 3 HCV VL was increased in carriers of the CC or CT genotypes (median, 5.8; IQR, 5.4, 6.3) compared with the TT genotype (median, 5.1; IQR, 4.8, 5.5; p = 0.01) (figure 3).

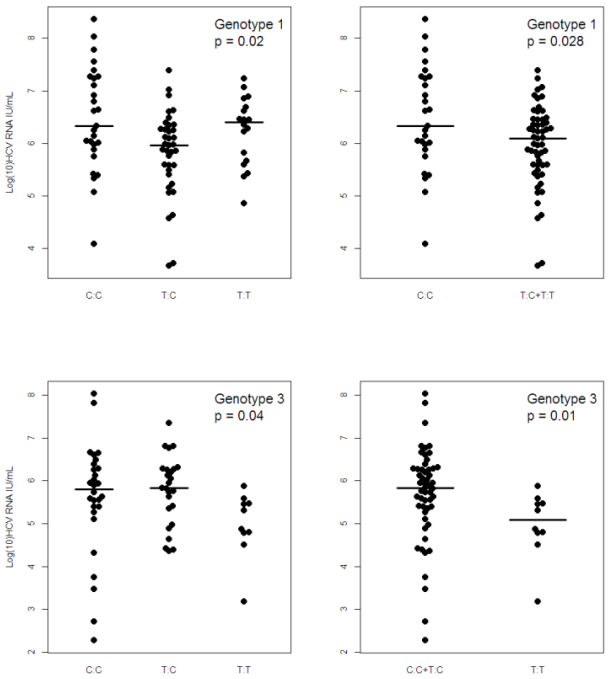


Figure 3. Plot of medians of HCV VL measurements distributed on HCV and IL28B genotypes. Medians were significantly higher for IL28B CC genotypes in HCV genotypes 1 and 3.

Haplotypes

The three SNPs were in full linkage disequilibrium with each other. We inferred haplotypes and identified 4 haplotypes. Not surprisingly, the haplotype consisting of the major allele of the 3 SNPs were associated with HCV resolution and VL.

Study III

The examination of this large group of longitudinally followed HIV-HCV-coinfected individuals provided a unique opportunity to investigate the impact of HCV VL, IL28B genotypes and HCV genotypes on mortality in a time-updated model.

Main findings

In a subgroup analysis of the individuals with chronic infection, we found 118 deaths during 1142 person years at risk (PYR) corresponding to a mortality rate ratio (MRR) of 10/100 (95% CI; 8, 12). HCV VL and HCV genotype were associated with all-cause mortality independently of other important predictors such as age, sex, HIV exposure group as well as HIV-related factors such as CD4 count and HIV VL (figure 4).

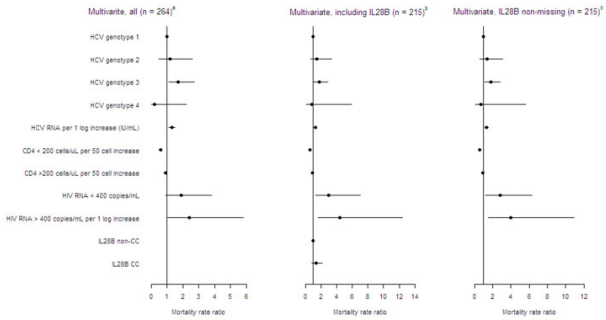


Figure 4. Plot of mortality rate ratios and 95% confidence interval illustrating factors associated with all-cause mortality. a Adjusted for age, sex, HIV exposure group, HCV genotype, HCV viral load, HIV VL and CD4 count. b Also adjusted for known IL28B genotypes. c Sensitivity analysis showing the model adjusted as in a, excluding individuals with unknown IL28B genotypes.

For HCV VL we found a 30% increased MR per log increment in HCV VL (MRR 1.3 (95%CI; 1.1,1.5)) in adjusted analysis. HCV genotype 3 was associated with higher MR than genotype 1 with an MRR of 1.8 (95% CI; 1.1, 3.0) in adjusted analysis. Figure 5 illustrates the increased MR for HCV genotype 3 compared with HCV genotype 1 in univariate analysis.

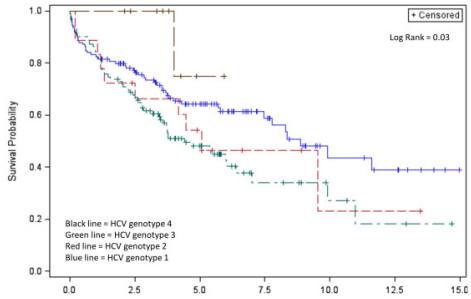


Figure 5. Kaplan Meier plot illustrating survival probability for HCV genotypes.

IL28B

DNA for genotyping of the IL28B SNP, rs12979860 was available for 215 (87%) of 247 individuals with Caucasian origin of whom 60 had died. The IL28B genotypes were distributed as follows: CC, 40%, TC, 41% and TT 19%. There was an MR of 13/100, 9/100 and 6/100 PYR among the individuals with the CC, TC and TT genotype, respectively.

In univariate analysis, the IL28B CC genotype was associated with a 2.2-fold increased risk of death compared with the TT genotype (MRR, 2.2; 95% CI, 1.2, 4.1; p = 0.02). The TC genotype was associated with a modestly increased risk compared with the TT genotype that did not reach statistical significance (MRR, 1.5, 95% CI, 0.8, 2.8; p = 0.2). Figure 6 illustrates the increased mortality among IL28B CC genotypes in unadjusted analysis.

Genotype CC vs. the non-CC was associated with an MRR of 1.7 (95% CI 1.1, 2.5; p = 0.01) in univariate analysis. In adjusted analysis IL28B genotype CC was associated with an MRR of 1.4 (95% CI; 0.8, 2.2; p = 0.2) compared with the non-CC genotypes but the effect of IL28B lost statistical significance.

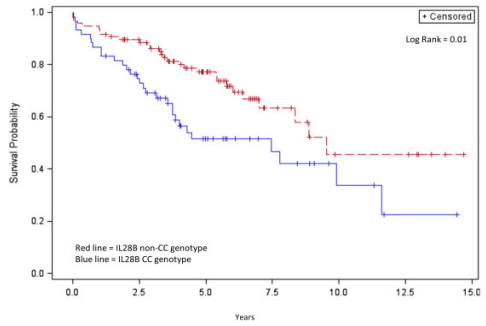


Figure 6. Kaplan Meier plot illustrating survival probability for IL28B genotypes.

Hepatitis C viral load.
For both non-survivors and survivors, a median of 2 (range, 1-12) measurements were done. At the first measurement non-survivors and survivors had similar HCV viral load (log10 5.9 (4.2, 6.5) vs. log10 5.6 (4.4, 6.3) IU/mL, p = 0.8), respectively. Over time HCV viral load increased in non-survivors and the latest available measurement prior to death was higher than the latest available measurement in survivors (log10 6.1 (5.1, 6.8) vs. log10 5.7 (3.6, 6.3) IU/mL, p = 0.002).

Inflammation
We used the alanine transaminase (ALT)/platelet ratio 107 as a surrogate marker of hepatic inflammation because aspartate aminotransferase levels were unavailable for the AST/platelet ratio index (APRI) and liver biopsy or transient elastography had only been performed infrequently. At baseline, ALT/platelets ratios were comparable (0.3 (0.1, 0.7) vs. 0.3 (0.1, 0.6), p = 0.2) for non-survivors vs. survivors. Over time the ratios increased in individuals who died while they were stable in individuals who survived (ALT/ratio: 0.4 (0.2, 1.1) vs. 0.3 (0.2, 0.5), p = 0.006).

HIV
Non-survivors had lower CD4 counts at baseline than survivors (238 (133, 407) cells/µl vs. 363 (238, 554) cells/µl, p < 0.0001) whereas HIV RNA viral loads were comparable at 1070 (< 400, 44300) copies/mL for non-survivors vs. < 400 copies/mL (< 400, 24050) copies/mL for survivors (p = 0.2). Over time, CD4 counts decreased in non-survivors while they increased in survivors (203 (93,380) cells/µl vs. 475 (284,628) cells/µl, p < 0.0001). HIV RNA viral loads were higher at latest measurement before death (< 400 (< 400, 40200) copies/mL vs. < 400 (< 400, 3040) copies/mL, p < 0.001) for non-survivors vs. survivors, respectively. Fifty-one individuals had a diagnosis of AIDS prior to entering the cohort and another 38 developed AIDS during follow up. The majority (73%) of individuals were treated with ART and of these, 127 individuals were treated with ART at entry and 65 individuals initiated ART during follow-up.

	Alive	Dead	p - value *
CD4+ count/uL			
CHC	363 (238-554)	238 (133-407)	< 0.0001
Latest	475 (284-628)	203 (93-380)	< 0.0001
HIV RNA (copies/mL)			
CHC	<400 (<400-24050)	1070 (<400-44300)	0.6
Latest	<400 (<400-3040)	<400 (<400-40200)	0.5
HCV VL (log 10 IU/mL)			
CHC	5.6 (4.4-6.3)	5.9 (4.2-6.5)	0.8
Latest	5.7 (3.6-6.3)	6.1 (5.1-6.8)	0.002
ALT/platelets			
CHC	0.3 (0.1-0.6)	0.3 (0.1-0.7)	0.2
Latest	0.3 (0.2-0.5)	0.4 (0.2-1.1)	0.0006

Table 1. Measurements of virological and immunological markers at time of fulfillment of our definition of chronic HCV infection (CHC) and at latest visit in our department. All values are medians and interquartile range. *Kruskal-Wallis.

DISCUSSION

Study I

The main finding in study I was that IDUs and MSMs have a higher resolution rate than individuals reporting HSX contact as HIV exposure route, which may indicate some degree of protective immunity against HCV.

Even though the existence of protective immunity in humans is controversial, others have also found higher resolution rates among IDUs [27,108]. A possible explanation was given by Khakoo et al. when they identified a weaker inhibition of NK-cells by the receptor-ligand pairing (KIR2-DL3-HLAC1) favorable of spontaneous HCV resolution among IDUs. They suggested that this inhibition was only favorable among IDUs compared with haemophiliacs because of the low-dose inoculum, IDUs received in contrast to the high-dose inoculum received through clotting factor substitution or blood transfusion. Thus, the immune response may easier overcome the low-dose inoculum resulting in resolved HCV infection. Individuals with negative HCV antibody test but likely repeatedly exposed to HCV through IDU (exposed uninfected, EU) have been demonstrated to have a frequency of KIR2-DL3-HLAC1 similar to that of IDUs with spontaneous resolution, implying that the low-dose inoculum may contribute to the protective role of KIR2-DL3-HLAC1.

Another explanation for the higher resolution rate in IDUs may be that IDUs were left with an immunological memory because of repeatedly low-dose HCV inoculum, as indicated in two studies reporting detectable HCV-specific T-cell responses in EU individuals [109,110]. A recent study of reinfections in IDUs showed that viral kinetics during primary infection and during reinfection differed from each other by lower levels and shorter duration of viremia during reinfection [111]. Further, reinfections were associated with a significant increase in the breadth of T-cell responses and cross reactivity of neutralizing HCV antibodies. The authors found an incident reinfection rate of 30 per 100 person-years and an 83% resolution rate in reinfected individuals. These findings were in accordance with chimpanzee studies where it had been suggested that immunity could be generated by initial infection and vaccination [112-117].

Together these studies supported our findings of a higher resolution rate in IDUs and MSMs. However, we acknowledge that the comparison group composed of HSXs may have comprised some

undisclosed IDUs and MSMs and that this group had a remarkably low resolution rate.

The study of reinfections is associated with several difficulties. Instances of reinfection are easily missed by infrequent sampling, and viral sequencing is necessary to distinguish a new infection from a reinfection. Our study was not designed to assess the grade of reinfection and we were as such not able to consider this as an explanatory factor for the increased resolution rate we observed among IDUs. Despite this, we believe that our associations were true and together with other studies provide evidence for an acquired protective immunity against HCV, which provides hope for the development of a therapeutic or protective vaccine.

Studies II and III

IL28B

Since 2009, when the first studies of IL28B were published and a strong association with both spontaneous [49,50] and treatment-[49,118-120] induced resolution was established, host genetics have been a great focus of interest. Of note, the IL28B SNP rs12979860 is an SNP located near the IL28B gene and the precise interaction between the rs12979860 SNP and IL28B gene is not clarified. There are three IFN-λ genes within the region, IFNL1, IFNL2 and IFNL3 (IL29, IL28A, IL28B, respectively) which encode type III IFNs 121. Type I IFNs (α, β) and type III IFN (λ) induce antiviral activity and suppress HCV replication through activation of the JAK-STAT pathway and up- regulation of ISGs [122] whereby the host immune system is activated.

IL28B and HCV viral load

The influence of IL28B on HCV VL is still unclear. Rallon et al. found no association of rs12979860 with VL [123], Ge et al. found lower VL in carriers of rs12979860 CC genotype [118] in contrast to Labarga et al. and Rembeck et al. who found higher levels of VL for rs12979860 CC [124,125]. Further, Bucci et al. found higher VL in the IL28B SNP, rs8099917 TT genotype [126], which is in strong linkage with rs12979860 CC [127].

We noticed that levels of viral load differed according to HCV genotype and that the effect in HCV genotype 3 was only statistically significant when collapsing CC with TC using a dominant genetic model. On the contrary, the effect seemed to follow the CC genotype in a recessive model for HCV genotype 1. Thus, the difference between HCV genotypes may be reflected in the allelic effect, thus in HCV genotype 1 two C alleles were needed for an effect on HCV VL, whereas one C allele was sufficient to cause an effect in HCV genotype 3. This is in line with other studies reporting that the effect of IL28B is different according to HCV genotype [49,123,125,128]. Further, several studies observed a higher prevalence of HCV genotype 3 among IL28B CC carriers which may reflect that HCV genotype 3 preferentially infects subjects with IL28B CC genotype or that HCV genotype 1 is more efficiently resolved in IL28B CC. Others have suggested that the higher viral load in individuals with chronic HCV infection may be attributable to higher resolution rate among carriers of the CC genotype with lower viral load, resulting in a higher proportion of individuals with CC genotype and high VL in the chronically infected population [129].

Although the differences in viral load between the respective IL28B genotypes were small and perhaps of limited clinical significance, it seems odd that the favorable IL28B CC genotype was associated with higher HCV VL, given that most therapeutic studies have noted an association between high viral load and reduced responses to treatment. This paradox may be explained by a pre-activated, perhaps maximally or perhaps inappropriately stimulated intrahepatic ISG expression for carriers of the unfavorable TT genotype resulting in lower HCV VL, but not sufficient antiviral response to resolve the infection. This preactivation may also have resulted in reduced responsiveness to type I and type III IFNs [130].

HCV viral load and mortality

Previous studies have produced conflicting results, most reported no predictive value of HCV VL on progression of liver disease or mortality [78,80,81,83,131-133], others that viremia per se was associated with mortality [75,134] and others again that the size of HCV VL was predictive of mortality [76,134,135]. The apparent conflict may in part reflect: 1) the heterogeneity of patient populations with respect to age, gender, duration of disease, viral type/subtype and route of exposure; 2) the use of different quantitative methods to assess serum HCV VL at unspecified time spans between serology and death; 3) the use of different study design including time-updated, cross-sectional and longitudinal studies.

Even though HCV VL is not generally considered to have a direct effect on liver cell damage, our findings may reflect an increased immune reactivity correlated with increased HCV VL. We assessed levels of inflammation and immunological activity by ALT/platelet ratio and CD4 cell count at first and last visit in lack of better markers. We noticed increasing ALT/platelet ratio and decreasing CD4 cell count for non-survivors, which may indicate increased immune activity or dysfunction resulting in liver fibrosis. Although we could not assess liver disease progression, clinical outcomes may be better endpoints of disease progression than e.g. fibrosis staging, and therefore may be the more biologically relevant measure of phenotype-genotype correlation.

We could not identify a direct causality between HCV VL and all-cause mortality. However, we speculate whether the progression to death occurs through higher VL causing more inflammation, leading to liver fibrosis and ESLD and ultimately resulting in death as depicted in figure 7. A potential explanation for the relation between HCV VL and inflammation may be that HCV indirectly or directly causes hepatic inflammation by inducing immune activation and thereby hepatocyte damage. In favor of this, Daar et al. found in a large longitudinal study that increasing HCV VL correlated with increasing ALT [3].

HIV-induced chronic immune activation and inflammation very likely also contributed to the high HCV VLs through depletion of CD4 cells and “over-activated” CD8 T cells (figure 7) [136]. Individuals infected with HIV experience immunological changes similar to those associated with normal ageing (reduction of T-cell renewal, shortened telomeres in terminally differentiated T-cells) [136]. This was illustrated by Kirk et al. who observed that HCV-infected individuals with HIV developed liver fibrosis app. 10 years earlier than individuals without HIV [64]. Moreover, the role of immune activation is further underscored by the ability of ART to normalize ALT levels and halt liver fibrosis development.

In summary, we speculate that our findings indicate that both HCV and HIV play a role in disease progression and mortality in HIV-HCV-coinfected individuals. The interaction between HIV and HCV could not be separately investigated in a study like ours.

However, we believe that it is too simple to consider the increased mortality to be due to behavioral conditions of IDUs combined with HIV-induced immune suppression.

IL28B and HCV genotype and mortality

The distribution of IL28B genotypes has been shown to vary across HCV genotypes. Others found that HCV genotype 3 was more prevalent among the IL28B CC genotype [123,128,137], which may indicate an interaction between IL28B CC genotype and infection with HCV genotype 3 influencing the MR we observed. Additionally, HCV genotype 3 has been shown to be the most prevalent genotype among IDUs [138], which may also confound our mortality analysis. Neither for HCV genotypes and route of exposure nor for HCV genotypes and IL28B did we find any statistically significant interactions, though.

The increased MR in HCV genotype 3 may reflect a higher grade of steatosis, inflammation and fibrosis linking the excess mortality with liver disease progression (figure 7). This may also be underscored by other studies confirming a higher mortality [137,139] and higher grade of inflammatory markers [140,141] in individuals carrying the "beneficial" IL28B CC genotype without HIV [123]. Hypothetically, carriers of the IL28B CC genotype had a vigorous antiviral immune response which in the case of persistent HCV infection was associated with a state of enhanced antiviral immune response promoting inflammation and more severe clinical outcomes such as death that we used as an outcome event. Others observed an association with mortality in HIV-HCV-coinfected carriers of the IL28B CC genotype [139], which together with our study may be an encouragement for lowering or eradicating HCV VL levels by pegIFN/RBV [142].

We acknowledge that our adjusted analysis of IL28B lost statistical significance. However, we noticed that the MRRs in the univariate analysis are comparable with the ones in the adjusted analysis and that the confidence interval is narrow and close to 0. We therefore believe that this association warrants further investigations.

Function of IL28B

In the search of a causal variant to explain the IL28B genotype effect on HCV, we and others have looked for a non-synonymous variant linked to the rs12979860 SNP. We genotyped rs8103142 which causes an ARG79LYS change and identified a strong linkage to rs12979860, but no causal explanation was found [143-145].

Recently, new insight into the biological role of IL28B in HCV was revealed. The IL28B rs12979860 single nucleotide polymorphism (SNP) was shown to be strongly linked to a genetic variant encoding a new IFN protein (IFN lambda 4), which was suggested to lower the responsiveness to treatment with IFN-α [146]. The authors discovered a new transiently induced region that harbors a dinucleotide variant, ss46915590, which was in high linkage disequilibrium with rs12979860. ss46915590[ΔG] is a frameshift variant that created a novel gene encoding the IFN-λ4 protein which was moderately similar to IFN-λ3. Overexpression of IFN-λ4 induced expression of IFN-stimulated genes unfavorable for HCV resolution.

In conclusion, the discovery of IL28B's association with both spontaneous HCV resolution and response to pegIFN/RBV therapy has brought HCV into an era of host genetics. Even though the causality of the association is weakly elucidated, IL28B is now a tool among others used in the clinical setting to assess chances of spontaneous resolution and beneficial outcome of treatment. IL28B is also likely to play a role in the coming era of direct-acting antivirals and maybe even in IFN free regimens [147-151]. Further, with the discovery of the IFN-λ4, there may be room for new therapies inhibiting the IFN-λ4 in the individuals with the appropriate IL28B genotype. Lastly, several clinical studies are on their way or ongoing (primarily in phase 2) to study the effect of pegylated interferon lambda in combination with DAAs compared with pegIFN/RBV (www.clinicaltrials.gov).

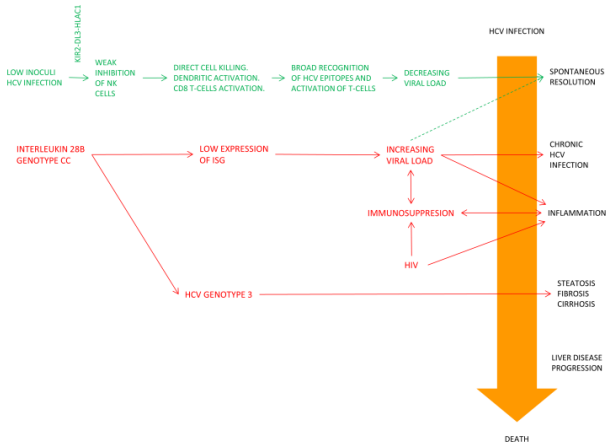


Figure 7. Model illustrating in green a suggested way to spontaneous resolution, when HCV infection is caused by low-dose inoculum. In red, factors hypothetically involved in progression of liver disease toward death. The different factors involved in the figure are described in more details in the discussion section.

CONCLUSION

In the present thesis, we identified a cohort of 330 HIV-HCV-coinfected individuals and investigated factors associated with spontaneous resolution and progression of HCV disease.

We showed that route of exposure through IDU and MSM was associated with a higher rate of spontaneous resolution compared with HSX, which may suggest that repeated low-dose HCV inoculum is easier overcome by the immune response and may leave a degree of immunological memory in favor of HCV resolution.

We found IL28B CC genotype to be associated with HCV resolution and higher levels of HCV VL. This finding is explained by a higher level of intrahepatic ISGs in non-CC genotypes resulting in lower HCV VL but not sufficient antiviral response to resolve the infection. This is in contrast to the CC genotype, where no preactivation of ISGs is present which hypothetically explains the more profound effect of the anti-viral immune response's production of IFN and consequently, spontaneous resolution.

We observed that HCV VL and HCV genotype 3 were independently associated with all-cause mortality, a finding that may reflect a complex interplay between chronic infection, chronic inflammation and behavioral conditions associated with intravenous drug use, which was the mode of HIV infection for the majority of study participants. Further we found a non-significant association between IL28B and mortality, which may warrant further investigation.

PERSPECTIVES

During the past decade GWAS studies have become more and more common, due to fewer expenses and facilitated laboratory work. Further, the GWAS technology has advanced and the numbers of known SNPs have increased and it is now possible to genotype more than 4 million SNPs in one chip.

The IL28B gene's association with HCV outcome was identified in a GWAS study. However, interferon lambda is part of the innate immunity and it would be an obvious choice to conduct a candidate gene association study to evaluate upstream or downstream genes from interferon lambda to obtain more knowledge of the function of IL28B.

Candidate gene association studies may also be used in identifying other markers of HCV outcome which could be combined in a score of genetic, virological and immunological factors used to predict HCV outcome. We have studied genes in the TLR/RIG-I pathway and identified genetic variations in the nuclear factor kappa B complex associated with spontaneous resolution.

Another developing area is the next generation sequencing. Next generation sequencing will hopefully enable us to identify changes in the viral genome possibly explaining the difference in HCV outcome which we have not been able to understand so far. Further, next generation sequencing may be promising when it comes to identifying resistance patterns in HCV treatment. Also, discovery of new viruses will be possible by next generation sequencing. However, to gain power enough to detect significant changes using next generation sequencing, large study cohorts will be needed.

SUMMARY

Coinfection with Hepatitis C virus (HCV) is common in human immunodeficiency virus (HIV) - infected individuals as a result of shared routes of transmission, and this coinfection represents a special challenge. For HIV-HCV-coinfected individuals, the burden of disease is largely related to their HCV diseases, including a faster progression to liver fibrosis, cirrhosis and liver-related deaths. In the present thesis we investigated factors associated with spontaneous resolution and progression of HIV-HCV coinfection.

In study I, we identified the study cohort of 327 individuals with HIV-HCV coinfection and a rate of spontaneous HCV resolution of 23%. We showed that female sex, coinfection with hepatitis B virus and individuals exposed through injecting drug use (IDU) or homosexual contact (MSM) had an increased rate of spontaneous HCV resolution. We speculate that differences in resolution rate may be caused by immunological memory induced by repeatedly being exposed to low-dose inoculum of HCV.

In study II, we found 3 single-nucleotide-polymorphisms (SNPs) in the interleukin 28B (IL28B) gene associated with spontaneous HCV resolution in 208 Europeans of Caucasian origin with HIV-HCV coinfection. Further, we showed that the IL28B CC genotype favourable of HCV resolution was associated with a higher HCV viral load (VL) than non-CC genotypes. These results may indicate an influence of IL28B in viral control.

In study III, we conducted a survival analysis in the 264 HIV-HCV-coinfected individuals with chronic infection. We showed in a time-updated Poisson regression that HCV VL, HCV genotype 3 and IL28B CC genotype were predictors of increased mortality. This may indicate a need for closer observation in HIV-HCV-coinfected individuals with HCV genotype 3 and maybe even initiation of antiviral therapy.

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