Hepatitis C virus: risk factors and disease progression
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Chapter 3.3

BMI, male sex and IL28B genotype associated with persistently high Hepatitis C Virus RNA levels among chronically infected drug users up to 23 years following seroconversion
BMI, male sex and Il28B genotype associated with persistently high Hepatitis C Virus RNA levels among chronically infected drug users up to 23 years following seroconversion

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Abstract
Background: The natural course of serum HCV RNA levels during chronic infection remains unclear. We investigated HCV RNA levels and factors associated with HCV RNA levels for the entire course from HCV seroconversion.

Methods: We measured HCV RNA levels of 54 HCV seroconverters from the Amsterdam Cohort Studies among drug users at yearly intervals up to 23 years using bDNA (VERSANT 3.0, lower limit of detection 615 IU/mL). Samples below the cut-off of the assay were tested by TMA (Siemens VERSANT, detection limit 5 IU/ml). We used a latent class linear mixed model (LCLMM) to examine the HCV RNA patterns and factors associated with HCV RNA levels.

Results: The median follow-up time was 10.8 years (IQR 6.5-14.9). We found two distinct HCV RNA patterns characterized by 45/54 cases and 9/54 cases. In multivariable analyses, HCV RNA levels were 0.41 log10 IU/mL (95% confidence interval (CI) 0.06-0.75) higher for males as compared to females. Individuals with the IL28B CC genotype had 0.40 log10 IU/mL (95% 0.08-0.73) higher HCV RNA levels than individuals with IL28B CT/TT genotypes. Body-mass index (BMI) was associated with higher HCV RNA levels, 0.055 (95% CI 0.027-0.083).

Conclusion: In this unique study, which examines the HCV RNA patterns over an extended period and following seroconversion, male sex, IL28B CC genotype, and BMI were independently associated with higher average HCV RNA levels. These results contribute to defining the natural history of HCV infection and could play an important part in clinical decision-making.
Introduction
Infection with hepatitis C virus (HCV) is a global health problem, with an estimated 185 million individuals infected [1]. Following acute infection, about 75% of cases progress to chronic infection with detectable HCV RNA levels [2], putting them at risk for progressive liver disease, including liver cirrhosis and hepatocellular carcinoma [3]. An improved understanding of the long-term HCV RNA levels and factors associated with HCV RNA levels during well-defined chronic infection is important to better understand the pathogenesis of HCV infection and could play an important part in clinical decision-making.

Quantification of viral load has proven to be useful in chronic viral infections. It is widely accepted that human immune deficiency virus (HIV) RNA levels are associated with HIV disease progression [4]. In addition, monitoring HIV RNA levels is critical to assess the efficacy of combined anti-retroviral therapy (cART) [5]. To date, there is no evidence that HCV RNA levels are associated with HCV disease progression [6,7] or that HCV RNA levels might reflect the level of immune suppression, as is the case for HBV [8]. In HCV treatment however, assessing HCV RNA levels has important clinical consequences. Under treatment with pegylated interferon (PEG IFN) and ribavirine (RBV), higher baseline RNA levels are associated with reduced sustained virological response (SVR) rates [9,10]. Furthermore, HCV RNA levels are used to guide the duration and outcome of interferon–containing treatment regimens including direct-acting antiviral agents (DAA) [11,12].

However, in contrast to HIV infection, knowledge about the natural course of HCV RNA levels following HCV seroconversion is limited. Evidence for factors associated with HCV RNA levels is mainly obtained through cross-sectional studies: increasing age [13,14], favorable interleukin 28 B (Interferon-λ3) genotype [14-16], male gender [14,17], HCV genotype 1 [14,17], high body-mass index (BMI) [18] and HIV coinfection [13,14,18] are associated with higher HCV RNA levels. In addition these studies were biased by the unknown duration of infection. The few studies that had a longitudinal study design and that were not biased by unknown duration, found that HIV coinfection was associated with increased HCV RNA levels [19,20]. However, these studies were limited by a relatively short follow-up period, the use of first and second-generation quantitative assays, and did not adjust for potential confounders.

The open and ongoing Amsterdam Cohort Studies (ACS) among drug users (DU) started in 1985, and HCV antibodies and HCV RNA were retrospectively tested. Therefore we had the unique opportunity to evaluate HCV RNA levels for the entire course from HCV seroconversion and to identify factors associated with RNA levels in 54 identified HCV seroconverters or recently infected individuals who developed a chronic infection.

Methods
Study population
The ACS among DU is an open, prospective cohort study initiated in 1985 [21]. Aims are to investigate the epidemiology, natural history, and pathogenesis of HIV infections and other blood-borne and/or sexually transmitted diseases, as well as the effects of interventions. Enrollment is voluntary, anonymous, and written informed consent is obtained from each participant at the intake visit. The medical ethics committee of the Academic Medical Center approved this observational study. ACS participants visit the Amsterdam Public Health Service every 4-6 months; they complete a standardized questionnaire about their health, risk behavior, and sociodemographic situation. Questions at ACS entry refer to the 6 months preceding the visit; questions at follow-up refer to the interim since the preceding visit. Blood is drawn each visit for laboratory testing and storage. All samples are processed within 24 hours and serum/plasma is stored at -80°C.

Screening for HCV
To identify HCV seroconverters, we retrospectively determined the presence of anti-HCV antibodies in stored serum from all participants with at least two visits between December 1985 and November
Individuals who were anti-HCV-negative at ACS entry were tested at their most recent ACS visit. Upon seroconversion, we tested samples taken between these two visits to determine the precise interval of seroconversion. Third-generation commercial microparticle EIA system tests (AxSym HCV version 3.0; Abbott, Wiesbaden, Germany) were used. All seroconverters for HCV during follow-up (n=55) and DU who were anti-HCV-positive at ACS entry and had started injecting drug use within 2 years before entry (n=51) were longitudinally tested for HCV RNA. Since we have shown that approximately 50% of DU acquire HCV infection within 2 years after starting injecting drug use [26], the latter group most likely represent recent infections.

**HCV RNA level and genotyping**

HCV RNA levels were measured by branched–chain DNA (bDNA) assay (VERSANT HCV RNA 3.0, lower limit of 615 IU/mL) at yearly intervals and more frequently around seroconversion. Samples below the cut-off were tested qualitatively for HCV RNA using transcription-mediated amplification (TMA) with a lower detection limit of 5-10 IU/mL (Versant; Siemens Medical Solutions Diagnostics, Munich, Germany). Viral genotyping of HCV was performed on the NS5B region using primers and conditions as described elsewhere [22].

**IL-28B genotyping**

Single nucleotide polymorphism genotyping was performed using Allelic Discrimination assays from Applied Biosystems for rs12979860 following the instructions of the manufacturer. Custom-designed primers and probes have been described previously [23].

**Definitions and statistical analyses**

For prospectively identified seroconverters, we estimated the date of HCV seroconversion as the midpoint between the last anti-HCV-negative visit and the first anti-HCV-positive visit. For HCV cases who started injecting <2 years before cohort entry, the date of HCV seroconversion was estimated as the midpoint between the start of injecting drug use and cohort entry. For inclusion in this study HCV seroconverters had to meet the following inclusion criteria: no evidence of HCV clearance (defined as two consecutive negative quantitative or qualitative RNA tests following HCV seroconversion); having at least one quantitative RNA test result; and having a follow-up greater than 2 years. If HCV RNA was below the detection level but qualitative testing was positive for HCV RNA, the quantitative level was set on the detection limit (615 IU/mL). Follow-up was calculated from the estimated date of seroconversion and individuals were censored at start of HCV treatment or last HCV RNA measurement, whichever occurred first.

To examine the HCV RNA patterns we used a latent class linear mixed model (LCLMM) [24]. A mixed model includes individual specific random effects that describe deviations around the average trajectory. It corrects for correlated data from the same individual due to unobserved characteristics. A latent class model additionally allows average patterns to differ according to unobserved group characteristics; individuals are allocated to these groups with a certain probability depending on their individual profile. To determine the number of classes for the model with the best fit, we used the bayesian information criterion (BIC). Within each class we modeled a biphasic pattern with a linear trend during the first 2 years and a smoothly varying trend thereafter, which was modeled by the use of restricted cubic splines [25]. We allowed for individual random intercepts and slopes. The limited number of cases allowed us to investigate covariables that were most strongly associated with HCV RNA in previous studies: age (time-updated), BMI (time-updated), IL28B genotype, sex, HCV genotype at baseline, and HIV coinfection as a time-updated variable. BMI was calculated as the weight (in kg) divided by height (in m²). Data on weight and length were missing in 80 of 654 visits of 54 participants. For 4 participants BMI data were missing at all visits. Therefore, we used multivariable imputation of length an weight (time-updated) by chained equations and created 10 imputed datasets [26]. The covariables were allowed to affect the average level only. For inclusion
in the multivariable model a univariable p-value < 0.05 was set as a threshold. Statistical analyses were performed using SPSS software (version 19.0; SPSS Inc.) and the R statistical computing environment (version 2.14.2; http://www.R-project.org/), together with the LCMM package [27].

Results

Baseline characteristics

Of the total of 106 seroconverters, we included 54 chronic cases that met the inclusion criteria. Twenty-eight out of 54 (52%) DU were observed HCV seroconverters and 26 (48%) started injecting drug use less than two years before cohort entry. The median age at HCV seroconversion was 27.9 years (interquartile range (IQR) 25.2-34.6), the majority were men and had a Western European ethnicity (table 1). HIV coinfection was present at the first HCV-positive visit in 12 out of 54 cases (22%) and an additional 10 cases seroconverted for HIV during follow-up. HCV genotype 1 (n=29) and 3 (n=16) were most prevalent at baseline. IL28B TT/CT genotypes were most abundant, 33 out of 54 (61.1%) cases. The median follow-up was 10.8 years (IQR 6.5-14.9), and the total person years (PY) of follow-up was 589.86 years. In total, 645 HCV RNA tests among 54 cases were evaluated in this analysis, with a median of 11 HCV RNA tests (IQR 7-15) per case. In the latent class analysis, we compared the performance of models including 2, 3, and 4 classes. The 2-class model showed the best fit with the lowest BIC value. Nine out of 54 cases (16.7%) had an estimated probability of more than 50% to belong to class 1, and 45 out of 54 (83.3%) to belong to class 2. Most of the individuals were assigned to a class with high probability: the average probabilities to belong to the assigned class were 92% and 97% for class 1 and 2 respectively. This means that the class assignment was quite unequivocal. The individual RNA patterns stratified for both classes are demonstrated in figure 1. Being in class 1 mainly depends on the slow increase to reach peak HCV RNA levels and the decline in HCV RNA of three cases. The slow increase is partly due to a lack of sufficient HCV RNA measurements during the first year and the decline of HCV RNA at the end of follow-up in three cases. We observed no clear differences between the characteristics of both classes, although HIV at baseline was borderline significant (p=0.07 Fisher-exact test).

<table>
<thead>
<tr>
<th>Overall Class 1</th>
<th>Class 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number 54</td>
<td>9</td>
</tr>
<tr>
<td>Age, median (IQR) 27.9 (25.2-34.6)</td>
<td>27.1 (25.6-28.1)</td>
</tr>
<tr>
<td>Male sex, n (%) 33 (61)</td>
<td>6 (67)</td>
</tr>
<tr>
<td>Western European ethnicity, n(%) 45 (83)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>BMI, median (IQR) 20.5 (18.6-22.4)</td>
<td>20.2 (17.7-21.3)</td>
</tr>
<tr>
<td>IL28 B, n (%) 21 (39)</td>
<td>5 (56)</td>
</tr>
<tr>
<td>CC 33 (61)</td>
<td>4 (44)</td>
</tr>
<tr>
<td>Baseline HCV genotype, n (%) 29 (54)</td>
<td>6 (67)</td>
</tr>
<tr>
<td>1 6 (11)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2 16 (30)</td>
<td>3 (33)</td>
</tr>
<tr>
<td>3 3 (5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>4 12 (22)</td>
<td>4 (44)</td>
</tr>
<tr>
<td>HIV at baseline, n (%) 2 (4)</td>
<td>1 (11)</td>
</tr>
<tr>
<td>Active HBV (HBsAg+ anti-HBc), n(%) Follow-up 0</td>
<td>0</td>
</tr>
<tr>
<td>HIV sc, n 10</td>
<td>3</td>
</tr>
<tr>
<td>Follow-up, median (IQR) 10.8 (6.5-14.9)</td>
<td>8.3 (5.2-11.3)</td>
</tr>
</tbody>
</table>

BMI, body-mass index; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; anti-HBc, hepatitis B core antibody; HCV, hepatitis C virus; IL28B, interleukin 28 B (interferon λ-3) rs12979860; IQR, interquartile range.
Figure 1. Individual HCV RNA patterns of 54 chronic HCV-infected individuals with a documented date of seroconversion from the Amsterdam Cohort Studies among drug users, 1985-2013. Data is stratified by 2 classes of HCV RNA patterns identified by latent class linear mixed model analysis. This model allows average patterns to differ according to unobserved group characteristics; individuals are allocated to these groups with a certain probability depending on their individual profile. Dashed horizontal line represents the detection limit (615 IU/mL).

Viral dynamics and determinants of HCV RNA levels
Using LCLMM we were able to investigate the impact of HIV, sex, age, BMI, HCV genotype, and IL28B on the HCV RNA levels over time (table 2). In univariable analysis, age was not associated with high HCV RNA levels, p=0.56. We found no significant association between HCV genotype
Figure 2. Fitted HCV RNA levels of individuals who have the covariable combination with the lowest mean HCV RNA levels (A) and highest mean HCV RNA levels (B). Data is derived from 54 chronic HCV-infected individuals with a documented date of seroconversion from the Amsterdam Cohort studies among drug users, 1985-2013. A: Female, HIV-negative, IL28B CT/TT genotype. B: Male, HIV-positive, and Il28B CC genotype. Dashed horizontal line represents the detection limit (615 IU/mL).

HCV, Hepatitis C virus; HIV, Human immunodeficiency virus; IL28B, Interleukin 28B (rs12979860).

at baseline and HCV RNA levels, $p=0.45$. HIV coinfection was significantly associated with an increase in HCV RNA levels, $0.42 \log_{10}$ IU/mL (95% confidence interval (CI) 0.18-0.67) as compared to HCV mono-infected individuals, $p<0.001$. In multivariable analysis, HCV RNA levels were $0.41 \log_{10}$ IU/mL (95% CI 0.27-0.65) higher for males as compared to females, $p=0.017$. Individuals with the favorable II28B CC genotype had $0.40 \log_{10}$ IU/mL (95% CI 0.20-0.73) higher HCV RNA levels than individuals with the II28B CT/TT genotype, $p=0.012$. BMI was significantly associated with an increase in HCV RNA levels, $0.055 \log_{10}$ IU/mL per BMI point increase (95% CI 0.027-0.078), $p<0.001$. Figure 2 shows the covariable combinations that had the highest and lowest mean longitudinal HCV RNA levels in class 2 ($n=45$): males with the II28B CC genotype and a high BMI (highest quartile) (panel A) have markedly higher levels than females who have the II28B CT/TT genotype and low BMI (panel B).

HCV RNA drops below the detection limit

We expected HCV RNA levels to be relatively stable during chronic infection. Although this was observed in the majority of cases ($n=46/54$), we observed drops in HCV RNA levels below the detection limit in 8 out of 54 individuals. In 4 out of 8 individuals HCV RNA levels declined at the end of follow-up, which occurred just before death (supplement figure A). All four cases were HIV-positive and CD4 T cell numbers at the last visit ranged from 4 to 150 cells/mL. Death occurred within 6 months after the last HCV RNA test in 3 out of 4 individuals and the primary causes were: AIDS wasting, progressive multifocal encephalopathy, and natural death. Two cases had persistently low HCV RNA levels during the early course of infection (supplement figure B).
Although the patterns might reflect consecutive reinfections we did not observe any HCV RNA-negative visits. In addition, in one case (ID19674) the same HCV genotype 1a strain was found based on NS5B sequencing during follow-up, suggesting persistent infection. Two individuals experienced one (ID18783) or multiple (ID18898) HCV RNA levels below the detection limit (supplement figure C). Case ID18783 was HIV-positive from HCV seroconversion and had the same genotype 1a infection at multiple visits throughout follow-up. We examined his medical records but were unable to find any explanation for this single HCV RNA drop. The HCV RNA pattern of case ID18898 was unexpected: this female was HIV-negative and she did not receive any HCV treatment during follow-up. Ten years following seroconversion the HCV RNA levels became quantitatively undetectable, suggesting late clearance, but qualitative tests were positive through the remainder of follow-up.

**Discussion**

This is the first time a study has described HCV RNA dynamics in a unique cohort of HCV seroconverters for a period of up to 20 years. BMI, male sex and IL28B CC genotype were independently associated with increased average HCV RNA levels over the entire course of infection. Here we show that increased BMI is associated with increased HCV RNA levels. Only two previous cross-sectional studies found an association between high BMI and high HCV RNA levels [18,28]. BMI has been reported as an independent negative predictor for HCV treatment outcome in both the RBV/PEG-IFN regimen as the boceprevir regimen [29,30]. Moreover, weight greater than 75 kilogram was associated with relapse in a sofosbuvir-based regimen [31]. A study investigating liver samples from obese and lean individuals with chronic HCV found increased intrahepatic levels of IFN-gamma-inducible protein 10 (IP-10) and monocyte chemotactic protein-1 (MCP-1) among obese individuals [38]. Increased expression of MCP-1 and IP-10 has been associated with hepatic inflammation and fibrosis. Although high IP-10 level has been correlated with increased HCV RNA levels [32], a direct relation between HCV RNA and liver disease has not been shown [6,7]. Likely there is a complex and multifactorial interaction between etiologies of liver disease, including drug-associated hepatotoxicity, non-alcoholic steatohepatitis, and alcohol [33].

To the best of our knowledge this is the first longitudinal study that demonstrates that females have lower average HCV RNA levels than men. It has been reported that females have a decreased risk of liver disease progression [34] and that this could be due to a protective effect of estrogens [35]. This hypothesis is further supported by a faster progression of hepatic fibrosis in postmenopausal versus premenopausal women [36]. Whether estrogens have a direct effect on HCV RNA levels remains unclear. However, evidence pointing in this direction comes from an in vitro study demonstrating that estrogens (17β-estradiol) inhibit the production of HCV virions [37]. Sex-based differences in the immune response to other viral infections have been described previously [38]. For instance, HIV-infected women clearly have lower HIV RNA levels than men, especially when CD4+ cell counts are high [39,40]. However, a clear underlying mechanism has not yet been defined.

Female sex is associated with increased spontaneous clearance, decreased fibrosis progression and lower HCV RNA levels. Likewise, IL28B CC genotype is associated with spontaneous clearance, but counter intuitively IL28B CC genotype is also independently associated with increased HCV RNA levels in our study and others [14-16]. Individuals with the unfavorable CC/TT genotypes generally have higher (interferon stimulating gene) ISG expression than individuals with the favorable CC genotype [41]. Higher ISG expression leads to increased pressure on HCV and should result in lower levels of HCV RNA.

This study was unable to confirm that HCV RNA levels are higher among HIV-coinfected individuals [19,20]. Although a univariable association was found, we did not have the power to demonstrate this association in multivariable analysis.
In 4 HIV-coinfected individuals the HCV RNA levels dropped below the detection limit shortly before death with low CD4 counts. Unfortunately, no clinical data were available regarding the presence of liver disease, nor was any liver disease reported as a contributing cause of death. We assume that these individuals experienced very rapid liver disease progression as has been observed among HIV-coinfected patients [42]. Low HCV RNA levels have been associated with decompensated liver disease and death can follow rapidly after the first hepatic decompensation in HIV-infected individuals [43].

By using a latent class model, we identified two patterns in HCV RNA trends. There may be unobserved characteristics that are responsible for a slower increase to peak HCV RNA levels during the first two years. In our approach this resulted in two subgroups with different patterns. However, it may also be a gradual difference, which gives a better fit in a model with two classes than in a model with only one class.

There were several limitations in this study that need to be addressed. Due to the retrospective testing in this study, specimens had been stored up to 23 years at -80°C. Subsequently, these samples might have been subject to degradation due to multiple freeze-thaw cycles and natural degradation over time during storage. However, specimens can be thawed up to eight times without affecting the accuracy of the bDNA assay, and thus we believe the results of this study are not seriously affected by its retrospective character [44]. Furthermore, we did not have sufficient power to investigate interactions between variables on the level and slopes of HCV RNA levels, although we used a novel statistical approach in analyzing HCV RNA levels. To examine the association of HCV genotypes on HCV RNA levels we used the baseline HCV genotype. We acknowledge that HCV genotype switches can occur following reinfection after spontaneous clearance and that multiple infections may occur in chronically infected individuals [45]. However, in concordance with other studies [14,46] we did find a decrease in HCV RNA levels for those with HCV genotype 3 as compared to those with HCV genotype 1. Unfortunately these results did not reach statistical significance and could have been biased by genotype switches or multiple infection in chronically infected individuals. In conclusion, in this longitudinal study with more than 20 years of follow-up, we found that male sex, IL28B CC genotype, and BMI are independently associated with increased HCV RNA levels. These results contribute to our understanding of defining the natural history of HCV infection and could play an important part in clinical decision-making.

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References


Supplementary figure. HCV RNA drops below the detection limit during follow-up. Out of 54 chronic HCV-infected individuals with a documented date of seroconversion, some individuals showed marked decreases in HCV RNA levels before death ("X") occurred (A), early during infection (B), or when they had an established chronic infection (C). Dashed line represents an HIV-negative status and the solid line represents an HIV-positive status. HCV, Hepatitis C virus; HIV, Human immunodeficiency virus; CC, IL28B, interleukin 28B (rs12979860)