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## Very low hepatitis C viral loads in treatment-naïve persons: do they compromise hepatitis C virus antigen testing?

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**Abstract:** BACKGROUND Hepatitis C virus (HCV) antigen testing is less expensive than quantitative RT-PCR but has lower sensitivity for very low viral loads (VLVL; HCV RNA 3,000 IU/ml). Currently the benefits of antigen testing for screening are discussed, but data on prevalence and outcomes of persons with VLVL are scarce. METHODS We assessed prevalence and predictors of VLVL by logistic regression in treatment-naïve participants in the Swiss Hepatitis C Cohort Study. We analyzed if the last viral load after VLVL was low, compared cirrhosis and mortality in persons with and without VLVL, and evaluated the number of samples with VLVL that were reactive by antigen testing. RESULTS We included 2,533 treatment-naïve persons with available quantitative HCV RNA testing results. Overall, 133 persons (5.3%) had a VLVL. Age 18-40 years, female gender and HIV coinfection were associated with VLVL. Of 72 persons with a viral load available after VLVL, 14% had a VLVL and 17% had spontaneous viral clearance. The prevalence and incidence of cirrhosis and mortality were comparable in persons with and without VLVL; all 24 persons with VLVL and cirrhosis had excessive alcohol consumption or immunosuppression. Overall 33% of samples with VLVL were reactive by antigen testing. CONCLUSIONS The frequency of VLVL was low. Among the persons who would probably be missed by antigen screening, some had a favorable disease course, but some had immunosuppression and liver cirrhosis. The benefit of HCV antigen testing for screening may be limited by the risk of missing patients with severe liver disease.

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# Very low hepatitis C viral loads in treatment-naïve persons: do they compromise hepatitis C virus antigen testing?

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## Background

Hepatitis C virus (HCV) antigen testing is less expensive than quantitative RT-PCR but has lower sensitivity for very low viral loads (VLVL; HCV RNA  $\leq$  3,000 IU/ml). Currently the benefits of antigen testing for screening are discussed, but data on prevalence and outcomes of persons with VLVL are scarce.

## Methods

We assessed prevalence and predictors of VLVL by logistic regression in treatment-naïve participants in the Swiss Hepatitis C Cohort Study. We analyzed if the last viral load after VLVL was low, compared cirrhosis and mortality in persons with and without VLVL and evaluated the number of samples with VLVL that were reactive by antigen testing.

## Results

We included 2,533 treatment-naïve persons with available quantitative HCV RNA testing results. Overall, 133 persons (5.3%) had a VLVL. Age 18-40 years, female gender and HIV coinfection were associated with VLVL. Of 72 persons with a viral load available after VLVL, 14% had a VLVL and 17% had spontaneous viral clearance. The prevalence and incidence of cirrhosis and mortality were comparable in persons with and without VLVL; all 24 persons with VLVL and cirrhosis had excessive alcohol consumption or immunosuppression. Overall 33% of samples with VLVL were reactive by antigen testing.

## Conclusions

The frequency of VLVL was low. Among the persons who would probably be missed by antigen screening, some had a favorable disease course, but some had immunosuppression and liver cirrhosis. The benefits of HCV antigen testing for screening may be limited by the risk of missing patients with severe liver disease.

## Background

Treatment-naïve persons with hepatitis C virus (HCV) infection usually have viral loads around  $10^5$ - $10^6$  IU/ml [1-3]. Viral loads < 400,000-800,000 IU/ml have been referred to as “low viral loads” [4-7] and have been associated with higher proportions of spontaneous viral clearance [7,8], slower liver disease progression [9-11], improved response to interferon-based treatment [4,6], and more successful treatment of liver cancer [12]. In contrast, other studies have found no association between low viral loads and liver disease progression [2,13,14].

Viral loads < 600 IU/ml [1] and < 5,000 IU/ml [15] have been referred to as “very low viral loads” (VLVL). Recently, the frequency and prognostic value of VLVL have been discussed controversially in the context of HCV antigen testing [16,17]. HCV antigen testing can be a less expensive alternative to quantitative RT-PCR [14,16,18-21]. In resource-limited settings with high HCV prevalence, point-of-care antigen testing is discussed as a one-step procedure, without HCV antibody screening [21]. The sensitivity of HCV antigen testing to detect VLVL is low: in most studies, the only commercially available HCV antigen test had a detection limit of 3,000 IU/ml [17,22-27]; in a few studies the detection limit was 1,000 IU/ml [28-30].

Little is known about the prevalence of VLVL among treatment-naïve persons. A study on laboratory samples with first-time HCV antibody positivity found viral loads  $\leq 3,000$  IU/ml in 3% (6/221) [24]. A meta-analysis on viral loads in untreated persons had exclusion criteria such as viral loads < 2,000 IU/ml and genotypes other than one; the results are therefore not relevant in our context [31]. Other studies were limited to settings where the majority of persons were treatment-experienced [1,15,29]; results are therefore not applicable to screening, where persons are treatment-naïve. More data are needed to better estimate the probability of missing HCV-positive persons with a VLVL at the time of screening, and to obtain information on their disease course. We therefore assessed the prevalence of VLVL in treatment-naïve persons, compared the characteristics and outcomes of persons with and without VLVL and analyzed the number of VLVL detected by antigen testing.

## Methods

We considered all persons enrolled in the Swiss Hepatitis C Cohort Study (SCCS) until September 10, 2017. The SCCS is a multicentre cohort study with continuous enrolment of anti-HCV positive persons aged > 18 years [32]. Study sites include five university hospitals, three additional large hospitals, and some affiliated centres. Enrolment started in the year 2000, and persons in any stage of disease are included. The standardized questionnaire includes data on demographic characteristics, HCV risk factors, genotype, HIV coinfection, organ transplantation and alcohol consumption. Clinical information is collected at enrolment and during yearly follow-up visits; at the same time plasma samples are collected and stored. The ethics committees of all eight participating hospitals granted ethical approval and all participants gave written informed consent.

To avoid any treatment effects, we excluded all participants having received antiviral treatment before enrolment into the SCCS. For participants starting HCV treatment in the SCCS, we only considered viral loads measured before treatment start. In treatment-naïve participants, we considered all viral loads. We defined a VLVL as HCV RNA  $\leq$  3,000 IU/ml because most studies on HCV antigen testing had used this detection limit [17,22-27].

We performed five different analyses. First, we calculated the proportion of persons who ever had a VLVL among all persons with at least one available viral load. We evaluated if this proportion changed over time, considering the following time intervals after SCCS enrolment:  $\geq$  6 months to < 3 years,  $\geq$  3 to < 5.5 and  $\geq$  5.5 to < 8 years. We performed a chi-squared test for trend in proportions, and excluded the first six months from the analysis because spontaneous viral clearance is more frequent during this time period [7,33,34]. We compared the number of viral load tests in persons with and without a VLVL. Since a VLVL may trigger more frequent testing, we compared the mean time between viral load tests before and after the first documented VLVL. We compared the proportion of people with a VLVL (among all patients with an available viral load) by type of quantitative RT-PCR assay used.

Second, we assessed possible predictors of VLVL. We used univariable and multivariable logistic regression and included the following explanatory variables at cohort enrolment: gender, age (18-40, > 40 years old), HCV genotype (1, 2, 3, 4; other genotypes were very rare), history of intravenous drug use (IVDU, yes/no), time elapsed between first documented HCV infection and enrolment (< 2 years, 2 to < 6, 6-10 and > 10 years), alcohol consumption (no or light: < 20 grams of alcohol per day; moderate: 20-40; excessive: > 40; former: > 40 before enrolment), HIV coinfection, HBV coinfection, and presence of liver cirrhosis at enrolment.

Third, we compared the prevalence and incidence of liver cirrhosis and all-cause mortality in persons with and without VLVL. We used univariable and multivariable logistic regression for prevalent cirrhosis, and Cox

regression for incident cirrhosis and mortality. We included the same covariates as in the analysis of predictors of VLVL, but excluded “presence of liver cirrhosis at enrolment”. In persons with VLVL and cirrhosis, we extracted information from the patient charts on additional immunosuppressive conditions and treatments.

We used multiple imputation by chained equations (MICE package for R; Van Buuren & Groothuis-Oudshoorn 2011 [35]) to impute missing values in the covariates. Because the quality of the imputation can be improved by adding variables outside the analysis [36], we included if HCV treatment was started within the SCCS, if spontaneous HCV clearance occurred, the SCCS center enrolling the participant, and the respective outcome variable. We ran the model on 20 imputed datasets for each analysis, using Rubin’s rule to pool the estimates.

Fourth, we analyzed the viral load trajectories after VLVL in persons with at least one viral load available  $\geq 6$  months after the first VLVL. Depending on the last viral load value (negative,  $> 0$  to  $\leq 3,000$  IU/ml or above), we classified the persons as “undetectable”, “continuous VLVL” or “rebound after VLVL”. We further classified persons with an undetectable last viral load as “confirmed spontaneous clearers” if they had two undetectable viral loads  $\geq 6$  months apart, otherwise as “possible spontaneous clearers” [7].

We repeated the analyses with a viral load detection limit of 1,000 IU/ml instead of 3,000 IU/ml; for the fourth analysis, the sample size was too small.

Fifth, in all participants with a frozen plasma sample available at the time of the VLVL, we performed antigen testing. We analyzed the antigen test results separately for VLVL  $\leq 3,000$  and VLVL  $\leq 1,000$  IU/ml, classifying them as reactive ( $> 10$  fmol/l), borderline reactive (3-10 fmol/l) and negative ( $< 3$  fmol/l).

As controls, we performed HCV antigen testing in 18 plasma samples with viral loads  $> 3,000 - 50,000$  IU/ml, and in two plasma samples with undetectable HCV RNA.

All the analyses were performed with R version 3.4.3 (R Core Team 2017, <https://www.r-project.org>).

## Results

We included 2,533 persons (46.8 % of 5,409 persons enrolled in the SCCS) who had HCV viral loads measured in the absence of therapy or before treatment start (Figure 1 and Table 1). Overall, 5.3% (133/2,533) of persons ever had a VLVL (Figure 1, Table 1), and 26 persons had a VLVL at least twice. Acute HCV infection before documentation of VLVL was excluded in 125 of the 133 persons (94%; HCV diagnosis  $\geq$  6 months before VLVL), was unlikely in seven persons (5%; probable HCV infection  $\geq$  5 years before VLVL), and could not be excluded in one person (1%; active IVDU with negative HCV test 1.5 years before VLVL).

The percentage of persons with a VLVL remained stable over time, from 3.0 to 4.1% for the time intervals of 2.5 years (Table 1). Viral loads were measured more frequently in persons with than without a VLVL (mean number of measurements 4.2 vs. 2.3,  $p$ -value  $< 0.001$ ); the time interval between measurements remained similar before and after a VLVL (mean of 671 days before and 723 days after a VLVL,  $p = 0.703$ ). For different quantitative RT-PCR assays, the proportion of people with a VLVL (among all persons with a detectable viral load) was similar.

Older age ( $> 40$  years), male gender, and being HIV-negative were associated with a lower probability of a VLVL (adjusted odds ratios 1.79, 95% CI 1.77-2.73; 1.50, 95% CI 1.00-2.24; and 2.20, 95% CI 1.24-3.92), while HCV genotype, history of IVDU, time between documented HCV infection and cohort enrolment, alcohol consumption, HBV coinfection and cirrhosis at enrolment were not. Results were similar in the univariable analysis and if missing explanatory values were imputed by multiple imputation (Table 2), except for gender that was no longer associated. Viral load levels before the VLVL were comparable for persons with and without subsequent spontaneous clearance (median 233,615 vs 117,253 IU/ml,  $p=0.91$ ;  $n= 35$  persons).

Among the 133 persons with a VLVL, 18% (24 persons) had liver cirrhosis. Cirrhosis and mortality were similar in persons with and without VLVL (Table 3). All 24 persons with a VLVL and liver cirrhosis had at least one of the following conditions: HIV coinfection, organ transplantation, (history of) excessive alcohol consumption, immunosuppressive treatment, diabetes mellitus, active IVDU with opioids, biliary cirrhosis or hepatitis B/D virus coinfection.

Seventy-two of the 133 persons with a VLVL had at least one viral load available  $\geq 6$  months after their first VLVL. Of these, 14% (10/72) had a VLVL as their last viral load, 17% (12/72) spontaneously cleared the virus (eight with confirmed, four with possible clearance), and 69.4% (50/72) had a last viral load  $> 3,000$  IU/ml. Four of the 72 persons, all with active IVDU, were probably reinfected after viral clearance; however a detailed sequence analysis was not available. Among the 24 persons with a VLVL and liver cirrhosis, 12 persons had at least one viral load available  $\geq 6$  months after their first VLVL. Of these, 42% (5/12) spontaneously cleared the virus or had a VLVL as last result (four after stop of excessive alcohol use, one

after steroid treatment); 58% (7/12) had a viral load > 3,000 as last result (six being immunosuppressed, one with excessive alcohol consumption and hepatitis B/D virus coinfection).

When we repeated the analyses using  $\leq 1,000$  IU/ml to define a VLVL, results were similar. Overall 3.5% (88/2,533) of the participants ever had a VLVL  $\leq 1,000$  IU/ml, with stable percentages over time (2.1% to 2.8% for the time intervals of 2.5 years). Young age (18-40 years), excessive alcohol consumption and absence of IVDU were associated with VLVL  $\leq 1,000$  (adjusted odds ratios 2.15, 95% CI 1.26-3.67; 1.92, 95% CI 1.03-3.57; and 1.94, 95% CI 1.10-3.42), while the other covariates were not. In the univariable analysis, the covariates associated with VLVL  $\leq 1,000$  were young age and excessive alcohol use (compared to no or light drinking). There were no differences between persons with and without a VLVL  $\leq 1,000$  for prevalence of cirrhosis at enrolment, incidence of cirrhosis and mortality.

Among the 133 persons with a VLVL, 90 persons had the VLVL documented on the occasion of the yearly SCCS visit and 43 on another visit when no plasma sample was stored. For one person, the frozen plasma sample was unavailable. For the remaining 89 persons, HCV antigen testing for VLVL  $\leq 3,000$  IU/ml was reactive in 24% (21/89), borderline reactive in 9% (8/89) and negative in 67% (60/89) persons. Among the 89 persons, 54 persons had a VLVL  $\leq 1,000$  IU/ml, with HCV antigen testing reactive in 20% (11/54), borderline reactive in 4% (2/54) and negative in 76% (41/54) persons. For the 24 persons with a VLVL and cirrhosis, plasma samples were available in 13 persons; HCV antigen testing was reactive in four, borderline reactive in one and negative in eight persons.

The hepatitis C antigen test was reactive in all control samples with viral loads > 3,000 – 50,000 IU/ml, and negative in those with undetectable viral loads.

## Discussion

Using real-life data from the SCCS, we could show that the proportion of treatment-naïve persons with a VLVL was low. Overall 5.3% of persons ever had a VLVL  $\leq 3,000$  IU/ml, and during shorter time intervals of 2.5 years this proportion dropped to 3.0-4.3%. This is in line with a proportion of 3% VLVL in a HCV screening study in Switzerland [24] and equivalent to a proportion of 1.2% viral loads below 1,500 IU/ml in data from the Georgian HCV Elimination Program [37]. In other studies, the prevalence of VLVL had been evaluated among persons who were mostly treatment-experienced. Despite these differences in study design, the frequency of VLVL was often similar. For example, in two studies including persons with a relapse after HCV treatment, a VLVL  $\leq 1,000$  IU/ml occurred in 4% [29], and a VLVL  $< 5,000$  IU/ml in 16% of persons [15]. In evaluations of blood samples of mostly treatment-experienced persons, a VLVL  $< 600$  IU/ml occurred in 2% [1], and a VLVL  $< 1,000$  in 3.5% of persons [29].

In our study, some aspects were favorable in persons who had a VLVL. Firstly, while HCV viral load values usually increase with longer follow-up duration [38,39], the percentage of persons with a VLVL remained constant over a period of  $> 8$  years (Table 1). Second, although spontaneous clearance is rare during chronic infection [7,34], we found a relatively large proportion of spontaneous clearance: among the 72 persons with a viral load determination performed after their first VLVL, eight (11%) were confirmed and four (5.5%) were possible viral clearers. Four active IVDU with confirmed or possible viral clearance and a subsequent detectable viral load were probably reinfected. It is possible that more persons cleared the virus among the 61 persons without viral load tests after the VLVL. Interestingly, young age, which was associated with VLVL  $\leq 3,000$  and VLVL  $\leq 1,000$  in our study, had previously been described as a predictor of spontaneous clearance [7,8,34], and low viral loads have been reported to precede spontaneous clearance [7,8].

Although the prevalence and incidence of cirrhosis were comparable in persons with and without VLVL in our study, a proportion of 18% (24/133) cirrhosis in persons with VLVL is worrisome; all of these had excessive alcohol consumption or immunosuppression. If population-based screening for HCV is done with an HCV antibody test followed by HCV antigen testing, or with HCV antigen testing alone, the chronic HCV infection of these persons may be missed. This problem would not occur in the case of etiological screening of an otherwise unexplained liver disease, where persons with HCV antibody positivity would in general undergo diagnostic workup. However, the likelihood and negative consequences of missing a person with VLVL and cirrhosis during a general population-based screening may be reduced for two reasons: First, point-of-care assays for HCV core antigen are in development, and they may have a lower limit of detection compared to the currently available assay [18]. Second, in some persons with a VLVL and cirrhosis, factors other than HCV were likely predominant in the pathogenesis of the cirrhosis. Indeed, some persons spontaneously cleared their HCV after excessive alcohol consumption or after steroid therapy had been

stopped. The frequency of alcohol consumption and immunosuppression including HIV coinfection in our study may mirror the HCV epidemic in Switzerland, characterized by a large proportion of IVDU with increased HIV coinfection and potentially increased alcohol consumption [40], and by an ageing population with age-related comorbidities [41,42,43]. This setting may differ for other countries, affecting the frequency of VLVL and cirrhosis. We had too few cases of HBV coinfection and only incomplete information on host genetics for meaningful analyses. Similar to previous studies [17,22-24,27,28], we found no difference in VLVL prevalence by HCV genotype.

Usually persons with immunosuppression have high HCV viral loads [13,36,39]. The occurrence of a VLVL in immunosuppressed or HIV coinfecting patients is therefore surprising. We have at present no explanations for this counterintuitive observation.

Our study has a number of strengths and limitations. All persons came from the multicentric SCCS, which includes a large number of patients, some followed up for 16 years [32]. Despite its large size, the SCCS only includes about one tenth of the estimated 40,000 HCV-infected persons in Switzerland [40], and persons in the SCCS may be in a more advanced liver disease stage. Nevertheless, patient characteristics are similar to the national notification data from the Federal Office of Public Health [32,44]. The high frequency of viral load tests in some persons may have led to a higher probability of detecting a VLVL. Indeed, persons with a VLVL had more viral loads recorded than those without. However, the detection of a VLVL did not lead to an increase in testing. For persons with HIV coinfection, the degree of immunosuppression and the effect of antiretroviral treatment could not be taken into account. It was not always clear whether immunosuppressive treatment was still ongoing. The number of deaths was likely underestimated because some of the patients lost to follow-up may have died. Some of the covariates had missing values that reduced the number of people who could be included in the multivariable analysis, but results were similar when we imputed the missing values by multiple imputation. Information on IFNL3/4 polymorphisms was available for too few persons with VLVL to allow for meaningful analyses.

HCV antigen testing has been shown to perform well in different geographic locations [21,37], but the low sensitivity for VLVL remains problematic. We show that numbers of VLVL that might not be detected by antigen testing are low: VLVL ever occurred in 5.3% of treatment-naïve persons, and numbers will likely be lower for screening, which is based on a single test. In addition, some persons with VLVL had favourable disease courses. On the other hand we found VLVL among persons with immunosuppression and liver cirrhosis. Thus, the benefits of HCV antigen testing for screening may be limited by the risk of missing patients with severe liver disease.

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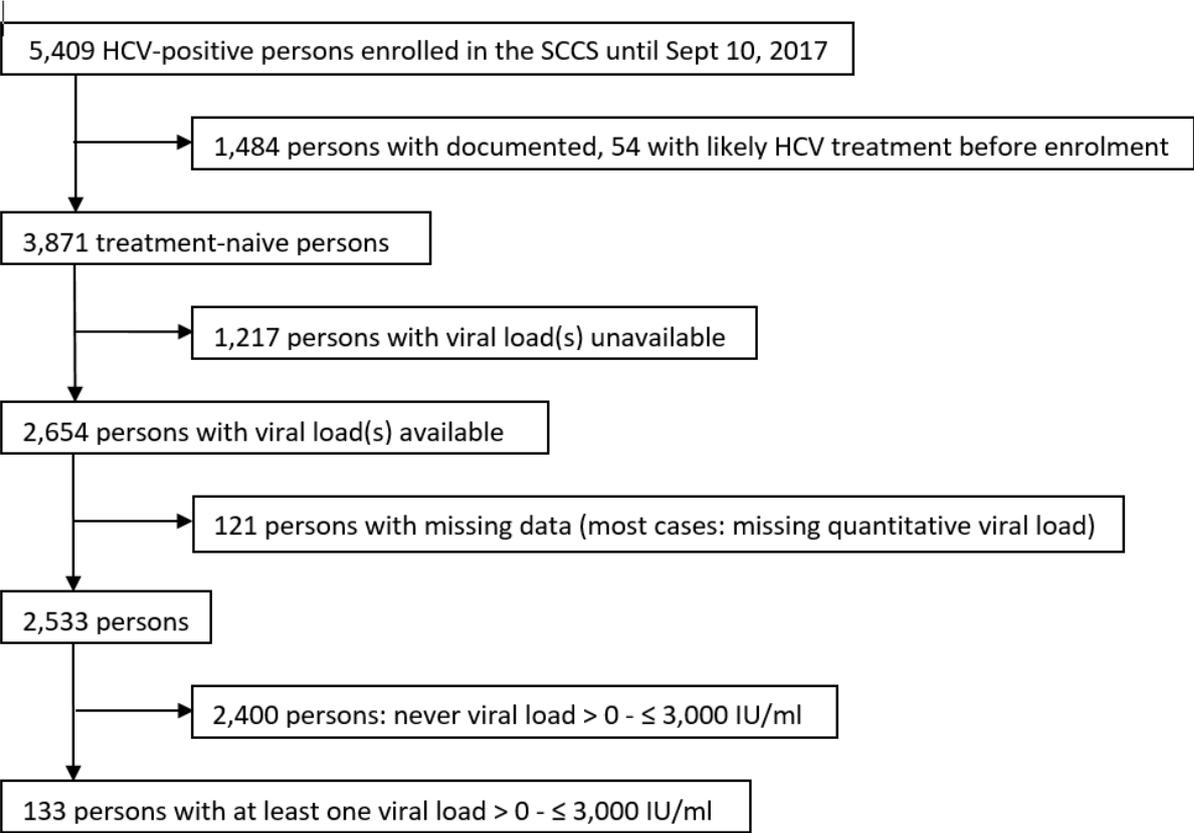
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## Notes

Matteo Brezzi deceased

**Figure 1: Flowchart of the patient selection process in the Swiss Hepatitis C Cohort Study (SCCS)**



**Table 1: Frequency of very low viral loads (VLVL) among persons in the Swiss Hepatitis C Cohort Study**

	<b>Persons with at least one VLVL value, among all participants with <math>\geq 1</math> HCV RNA value(s)</b>
<b>Total</b>	133/2,533 (5.3 %)
<b>Time since enrolment</b>	
< 6 months	50/1,760 (2.8 %)
$\geq 6$ months to < 3 years	37/1,237 (3.0 %)
$\geq 3$ years to < 5,5 years	27/662 (4.1 %)
$\geq 5,5$ years to < 8 years	17/399 (4.3 %)
$\geq 8$ years	18/442 (4.1 %)

VLVL = detectable HCV RNA  $\leq 3,000$  IU/ml

**Table 2: Predictors of very low viral loads (VLVL) in the Swiss Hepatitis C Cohort Study**

	No of persons (N=2,533)	Ever VLVL (N=133)	Univariable OR for VLVL	Multivariable OR for VLVL (N = 2,166)	Multiple imputation OR
<b>Gender</b>			(N = 2,532)		
Female	1,016	61	1	1	1
Male	1,516	72	0.78 [0.55;1.11]	0.67 [0.45;1.00]	0.72 [0.50;1.04]
<b>Age (years)</b>			(N = 2,527)		
18-≤40	1,064	71	1	1	1
≥41	1,463	62	0.62 [0.44;0.88]	0.56 [0.37;0.85]	0.58 [0.40;0.86]
<b>Genotype</b>			(N = 2,244)		
1	1,185	61	1	1	1
2	181	11	1.19 [0.62;2.31]	1.33 [0.66;2.69]	1.22 [0.61;2.45]
3	604	25	0.80 [0.49;1.28]	0.77 [0.47;1.27]	0.76 [0.46;1.25]
4	274	18	1.30 [0.75;2.23]	1.20 [0.68;2.12]	1.29 [0.74;2.26]
<b>IVDU</b>			(N = 2,508)		
No	972	53	1	1	1
Yes	1,536	79	0.94 [0.66;1.34]	0.70 [0.44;1.12]	0.77 [0.51;1.18]
<b>Time (years)</b>			(N = 2,485)		
0-2	1,124	67	1	1	1
>2-6	581	27	0.77 [0.49;1.22]	0.74 [0.45;1.23]	0.69 [0.43;1.11]
>6-10	389	16	0.68 [0.39;1.18]	0.75 [0.41;1.36]	0.61 [0.34;1.08]
>10	391	19	0.81 [0.48;1.36]	1.10 [0.62;1.95]	0.88 [0.52;1.50]
<b>Alcohol consumption</b>			(N = 2,520)		
No or light	1,323	64	1	1	1
Moderate	398	20	1.04 [0.62;1.74]	1.25 [0.71;2.18]	1.18 [0.69;1.99]
Excessive	633	40	1.33 [0.88;1.99]	1.46 [0.89;2.38]	1.48 [0.95;2.31]
Former excess.	166	9	1.13 [0.55;2.31]	0.85 [0.33;2.21]	1.32 [0.62;2.77]
<b>HIV coinfection</b>			(N = 2,533)		
Yes	212	20	1	1	1
No	2,321	113	0.49 [0.30;0.81]	0.45 [0.25;0.81]	0.47 [0.28;0.81]
<b>HBV coinfection</b>			(N = 2,533)		
Yes	47	5	1		1
No	2,486	128	0.46 [0.18;1.17]	0.94 [0.22;4.06]	0.50 [0.19;1.33]
<b>Cirrhosis</b>			(N = 2,506)		
Yes	236	13	1		1

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No	2,270	119	0.95 [0.53;1.71]	0.99 [0.48;2.06]	0.87 [0.47;1.63]
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VLVL = detectable HCV RNA  $\leq$  3,000 IU/ml. IVDU = (history of) intravenous drug use. Time (years) = time elapsed between first documentation of HCV infection and enrollment. Alcohol consumption: no or light: 0- <20 grams of alcohol per day, moderate: 20-40, excessive: >40, former: >40 before enrolment.

Numbers do not always sum up to the total number of persons, due to missing values in covariates.

Odds ratios (OR) with 95% confidence intervals, based on logistic regression.

**Table 3: Cirrhosis at enrolment, incident cirrhosis and mortality in persons with compared to persons without a very low viral load value, in the Swiss Hepatitis C Cohort Study**

	Univariable	Multivariable*
Cirrhosis at enrolment	OR 0.95 [0.53;1.71]	OR 0.99 [0.47;2.08]
Incident cirrhosis	HR 0.77 [0.38;1.56]	HR 0.63 [0.28;1.44]
Mortality	HR 0.73 [0.39;1.37]	HR 0.46 [0.20;1.06]

Very low viral load = detectable HCV RNA  $\leq$  3,000 IU/ml.

OR: odds ratio from logistic regression; HR: hazard ratio from Cox regression.

\* The inclusion of covariates is explained in the methods section.